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Viral and Host Determinants of Dengue Susceptibility and Severity in Endemic Southeast Asia

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Abbreviation list
ADE: Antibody-dependent enhancement
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
BASO: Basophils count
Beta-NGF: Beta nerve growth factor
CTACK: Cutaneous T-cell attracting chemokine
DC: Dendritic cells
DENV: Dengue virus
DENV-1,2,3,4: Dengue virus serotypes 1,2,3,4
DF: Dengue without warning signs
DHF: Dengue haemorrhagic fever
DNA: Deoxyribonucleic acid
DSS: Dengue Shock Syndrome
DWS: Dengue with warning signs
EOS: Eosinophils count
Eotaxin: Eotaxin (CCL11)
FGF-basic: Fibroblast growth factor basic
G-CSF: Granulocyte colony stimulating factor
GM-CSF: Granulocyte macrophage colony stimulating factor
GRO-alpha: Growth regulated oncogene alpha
HCT: Haematocrit
HGF: Hepatocyte growth factor
IFN-alpha: Interferon alpha
IFN-gamma: Interferon gamma
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IL-10: Interleukin 10
IL-10: Interleukin-10
IL-12p40: Interleukin 12 subunit p40
IL-12p70: Interleukin 12 subunit p70
IL-13: Interleukin 13

IL-15: Interleukin 15
IL-16: Interleukin 16
IL-17: Interleukin 17
IL-18: Interleukin 18
IL-1-alpha: Interleukin 1 alpha
IL-1-beta: Interleukin 1 beta
IL-1RA: Interleukin 1 receptor antagonist
IL-2: Interleukin 2
IL-2R-alpha: Interleukin 2 receptor alpha
IL-3: Interleukin 3
IL-4: Interleukin 4
IL-5: Interleukin 5
IL-6: Interleukin 6
IL-7: Interleukin 7
IL-8: Interleukin 8
IL-9: Interleukin 9
IP10: Interferon gamma-induced protein 10
LIF: Leukaemia inhibitory factor
LYM: Total lymphocytes count
MCP1/MCAF: Monocyte chemoattractant protein1/monocyte chemotactic and activating factor
MCP3: Monocyte chemoattractant protein 3
M-CSF: Macrophage colony stimulating factor
MIF: Macrophage migration inhibitory factor
MIG: Monokine induced by gamma interferon
MIP1-alpha: Macrophage inflammatory protein 1 alpha
MIP1-beta: Macrophage inflammatory protein 1 beta
MONO: Monocytes count
NEU: Neutrophils count,
NK: Natural killer cells
NS1: Non-structural protein 1
PCR: Polymerase chain reaction

PDGF-BB: Platelet derived growth factor BB
PLT: Platelet count
RANTES: Regulated on activation, normal T-cell expressed and secreted
RBC: Erythrocytes count
RNA: Ribonucleic acid
RT-PCR: Reverse transcription - polymerase chain reaction
SCF: Stem cell factor
SCGF-beta: Stem cell growth factor beta
SD: Severe dengue
SDF-1-alpha: Stromal cell derived factor 1 alpha
sHLA-G: Soluble human leukocyte antigen-G
TNF-beta: Tumour necrosis factor beta
TNF-alpha: Tumour necrosis factor alpha
TRAIL: Tumour necrosis factor related apoptosis inducing ligand
VEGF: Vascular endothelial growth factor
WBC: Total leucocytes count
WHO: The World Health Organisation
ELISA: Enzyme-linked immunosorbent assay
RDTs: Rapid diagnostic tests

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1. INTRODUCTION

1.1 Dengue Virology and Genetic diversity

Dengue is the most widespread mosquito-borne viral disease, caused by the Dengue virus (DENV), which belongs to the *Flavivirus* genus of the Flaviviridae family. This genus also includes other significant viral pathogens responsible for haemorrhagic fever and neurological diseases, such as Japanese encephalitis virus (JEV), Tick-borne encephalitis virus (TBEV), Yellow Fever virus (YFV), and West Nile virus (WNV). The incidence and geographic spread of these viruses are increasingly influenced by climate change and rapid urbanization, impacting both developed and developing regions worldwide (Pierson & Diamond, 2020).

DENV is a spherical virion with a diameter of approximately 50 nm and a 10.7 kb positive RNA genome encoding ten genes (Nature Education, 2014). The genome is translated into a single polypeptide, which is subsequently cleaved by host and viral enzymes into three structural (capsid, pre-membrane/membrane, and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Nature Education, 2014). These non-structural proteins are essential for viral replication, especially the NS1 protein.

The first DENV was isolated in the middle of the 20th century, and soon afterwards four antigenically different types of DENV, or four serotypes: DENV-1, DENV-2, DENV-3 and DENV-4 were fully characterised (Nature Education, 2014). DENV serotypes differ from each other in 35% of their genomes and show different interactions with antibodies in human serum. The term "serotype" refers to a specific variant within a virus, classified based on differences in surface antigens, particularly those that are recognized differently by host antibodies.

DENV replication is mediated by RNA-dependent RNA polymerase; however, due to the lack of proofreading activity in this enzyme, mutations frequently occur during the process. Consequently, DENV has high genetic variability, giving rise to distinct genotypes and lineages within each serotype (Suppiah et al., 2018). This genetic diversity potentially allows DENV to adapt to different environments, evade host immune responses, and enhance its fitness in both human and mosquito (Lee et al., 2022). The virus can present as a diverse population of closely related variants, exhibiting a quasispecies dynamic that enhances its adaptability and survival (Bifani et al., 2022). Nonetheless, the virus has several conserved regions (NS5, NS3) in its genome that have been utilized for diagnostic purposes and as targets for drug development (Bollati et al., 2010).

1.2 Global Epidemiology and Geographic Expansion of Dengue

Dengue has spread rapidly around the world, exposing millions of people to the risk of infection. The number of reported cases has increased significantly over the last two decades, from 500,000 in 2000 to 5.2 million in 2019, marking a serious concern for public health in many regions (Americas, 2023). In 2024, dengue cases are reported across 90 countries, with the highest prevalence in Southeast Asia, the Pacific Islands and Latin America.

In Asia, particularly in Southeast Asia, dengue is hyperendemic, with all four serotypes of the virus co-circulating (Alied et al., 2023). Large dengue outbreaks frequently occur in countries such as India, Thailand, Vietnam and the Philippines (ECDC, 2025). By 2023, Asia recorded the highest cumulative number of dengue-related deaths, with 3,637 deaths out of 1.6 million reported cases (Haider et al., 2024). During the same period, South America documented the highest number of infections, totalling 3.9 million cases, with Brazil experiencing the highest incidence (Alisson, 2025). DENV has also caused recurrent outbreaks in the Caribbean, particularly in countries such as Puerto Rico and the Dominican Republic, where all four serotypes frequently co-circulate (Harris, 2024).

Compared to Asia and the Americas, Africa appears to bear a lower burden of dengue. Over the past decade, dengue outbreaks have been reported in 13 African countries, with more than half occurring in East Africa. DENV-1 and DENV-2 being the commonly detected serotypes in these regions (Mwanyika et al., 2021). Additionally, although dengue remains rare in Europe, locally transmitted (autochthonous) cases were reported in countries such as France, Italy, and Spain due to the expansion of *Aedes* mosquitoes, sporadically with different serotypes (ECDC, 2025; Pozzetto et al., 2015).

The substantial expansion of dengue across spatial and temporal scales is influenced by factors such as climate change, increased international travel, and ineffective vector control. The World Health Organisation (WHO) has classified dengue as one of the neglected tropical diseases requiring urgent global attention due to its increasing incidence and significant public health and economic burden (World Health Organization, 2025). Therefore, continuous surveillance of dengue and other arboviruses in the context of a changing climate is essential for effective disease control and the protection of global health.

1.3 Transmission Dynamics and Vector Ecology of Dengue Virus

While dengue is primarily transmitted to humans through mosquito bites, cases of vertical transmission, as well as transmission through organ transplantation and blood transfusion, have also been reported (Pozzetto et al., 2015). The vectors that transmit dengue are *Aedes* mosquitoes, especially *Aedes aegypti*, *Aedes albopictus*, and *Aedes polynesiensis*. These mosquitoes live mostly in tropical and subtropical regions, where the temperature, humidity and rainy seasons are favourable for their reproduction and propagation. Nonetheless, *Aedes* mosquitoes have been detected in temperate regions, largely due to climate change in recent decades, which has led to increased rainfall, more breeding sites, and prolonged transmission periods for the vectors (Feng et al., 2024). These mosquitoes transmit diseases not only between humans but also from infected animals to humans (Gwee et al., 2021), facilitating the spread of dengue and other viruses such as Zika (ZIKV), chikungunya (CHIKV), and YFV. This transmission contributes to the enzootic, zoonotic, and anthroponotic cycles of arboviruses (Hanley et al., 2024).

The life cycle of DENV in mosquitoes begins after a blood meal from an infected host. The virus then infects the mosquitoes midgut, and disseminates to other tissues, ultimately reaching the salivary glands. In the glands, DENV viral particles are released into other hosts through its saliva during another blood meal. Previous studies have shown that DENV infection not only enhances the mosquitoes blood-feeding behaviour, but also suppresses the mosquitoes immune response, facilitating viral replication and persistence within the vector (Mukherjee et al., 2019; Wei Xiang et al., 2022). Compared to other arboviruses such as ZIKV and CHIKV, DENV is more successful in infecting *Aedes* mosquitoes (González-Flores et al., 2023). In cases of co-infection within a single mosquito, DENV can suppress the replication of other viruses, enabling it to become the dominant virus (González-Flores et al., 2023). This competitive advantage may thus explain predominance of dengue in regions, where multiple arboviruses co-circulate.

1.4 Immunopathogenesis of Dengue Virus Infection

The pathogenesis of dengue is a multi-stage process initiated following the bite of an infected mosquito. Upon entry, DENV targets dendritic cells (DCs) in the skin, where the virus begins its replication cycle. These infected DCs then circulate towards the lymph nodes, promoting systemic viral dissemination and facilitating the infection of other immune cells, including monocytes and macrophages, which act as major viral reservoirs. In parallel, DENV infection activates pattern recognition receptors in DCs, triggering the production of interferons and other antiviral responses as part of the host innate immune defense (Khanam et al., 2022). These early responses contribute to the onset of systemic symptoms like fever and headache.

As DENV infection progresses to the critical phase, typically occurring between days 4-6 of illness, viral replication and dissemination in the bloodstream can lead to endothelial damage and plasma leakage, potentially resulting in hypovolemic shock in affected patients (World Health Organization, 2009). Multiple mechanisms contribute to these vascular alterations, including direct infection of endothelial cells by DENV, excessive production of proinflammatory cytokines and reactive oxygen species. Notably, the NS1 protein secreted by DENV during viral replication plays a central role in disrupting the endothelial glycocalyx, leading to vascular permeability, immune evasion, and inflammation (Bhatt et al., 2021).

During this critical phase, vital organs including the liver, spleen, bone marrow are frequently affected. Hepatocytes are damaged through both direct viral infection and immune-mediated effects, resulting in elevated liver enzymes and impaired liver function, which are indicators of disease severity. The spleen and bone marrow are sites of intense immune activation and hematopoietic suppression, contributing to leukopenia and thrombocytopenia in dengue patients (Khanam et al., 2022; World Health Organization, 2009). These pathophysiological changes account for the commonly observed laboratory abnormalities at this stage, including elevated liver enzymes, thrombocytopenia, and altered haematological profiles.

As previously mentioned, the four serotypes of DENV have distinct immunogenic characteristics (Nature Education, 2014). Subsequently, an individual can be infected with DENV up to four times, with each infection caused by a different serotype. A primary DENV infection typically induces long-term immunity against the infecting serotype, while providing only short-term, partial cross-protection against the other serotypes, usually lasting 2-3 months (World Health Organization, 2009).

Notably, secondary infections involving a different (heterologous) serotype are associated with higher levels of severity compared to primary infections (Soo et al., 2016). This phenomenon is primarily explained by antibody-dependent enhancement (ADE), wherein non-neutralizing antibodies generated during a previous DENV infection facilitate the uptake of a heterologous serotype into monocytes and macrophages via Fc gamma receptors (World Health Organization, 2009). This enhances viral replication and triggers an exaggerated immune response, often culminating in a "cytokine storm" and systemic inflammation (Khanam et al., 2022).

After a primary infection, an effective immune response is triggered, characterised by the formation of neutralising antibodies and memory T cells for a specific serotype, providing lasting protection against that serotype. However, secondary infections with heterologous DENV serotype may result in hyperactivation of cross-reactive T cells, which can produce excessive amounts of proinflammatory cytokines, contributing to host tissues damage and increased disease severity, while failing to effectively eliminate the virus (Soo et al., 2016). Although a strong initial immune response is beneficial for viral clearance, its dysregulation may drive severe manifestations (Halstead & Crowe, 2014). Therefore, understanding the balance between protective immunity and immunopathology is essential for guiding the development of effective treatments.

1.5 Viral Genetics and Host Immune Responses

1.5.1 Viral Genetic Determinants in Dengue Severity

The infection susceptibility and clinical course of dengue are influenced by both the viral and host factors. Among viral determinants, genomic changes in the viral genome have been shown to influence viral load, protein interactions, host immunomodulation, and thus overall virulence. It has been postulated that four DENV serotypes have different immunomodulatory effects, which are known to influence clinical course and severity (Suchita Chaudhry, 2006). Of the four serotypes, DENV-2 has been most frequently associated with severe clinical manifestations (Suppiah et al., 2018; Vicente et al., 2016). A 19-year longitudinal study in Nicaragua reported that both DENV-2 and DENV-3 were associated with severe outcomes (Narvaez et al., 2024), while another study in Brazil suggested that DENV-4 infection may have a less complicated clinical course (Hernández Bautista et al., 2024). Yet another study demonstrated that DENV-2 and DENV-1 exhibit higher replication rates in mosquito cell cultures compared to other serotypes, suggesting that these two serotypes may be associated with increased transmissibility (Quintero-Gil et al., 2018). Also, DENV-2 infections were shown to increase viral load in the liver in mouse models compared to other serotypes, confirming previous observations on the DENV-2 serotype (Rathore et al., 2021).

Beyond serotype-level differences, genetic variability within each DENV serotype also influences disease severity (Suppiah et al., 2018). Distinct genotypes within a single serotype have been shown to modulate clinical outcomes and outbreak intensities (Phadungsombat et al., 2018; Rahim et al., 2023) and also been shown to influence viral infectivity and virulence, both in vitro and in vivo (Zou et al., 2019). In regions where multiple DENV serotypes or genotypes circulate simultaneously, genetic recombination between different types can lead to the emergence of more virulent DENV strains (Senaratne et al., 2016). This underscores the critical importance of continuous molecular surveillance, which is essential for understanding virus evolution and supporting effective outbreak control and public health measures.

1.5.2 Host Factors and Immune Responses in Dengue Severity

In addition to viral determinants, host factors such as genetic predisposition, immune status of the individual, and underlying health conditions significantly influence disease progression and clinical outcomes (Lan & Hirayama, 2011). Demographic factors such as male gender and advanced age were reported to associate with an increased risk of severe dengue and mortality (Huang et al., 2023; Zohra et al., 2024). People with underlying conditions are also associated with more severe dengue, including higher hospitalisation rates and significantly increased mortality (Lien et al., 2021).

The host immune response plays a major role in the pathogenesis of dengue fever, as an unbalanced or excessive response can worsen the severity of the disease (Khanam et al., 2022). For instance, dysregulated cytokine production can lead to excessive release of pro-inflammatory mediators, causing endothelial damage and contributing to clinical complications such as plasma leakage, haemorrhage, shock, and multi-organ dysfunction

(Khanam et al., 2022; Patro et al., 2019). Particularly, high levels of pro-inflammatory cytokines, including TNF-alpha, IL-1, and IL-6, alongside reduced expression of anti-inflammatory cytokines, such as TGF-beta, have been associated with systemic inflammation and endothelial injury in severe dengue cases (World Health Organization, 2009; Patro et al., 2019; Puc et al., 2021). However, many of these findings are based on studies that analysed only a limited number of cytokines and chemokines, potentially overlooking important mediators involved in the immune cascade. A more comprehensive and simultaneous assessment of a broader range of immune markers could improve our understanding of the host immune response in dengue fever.

Immune checkpoint mechanisms also play a crucial role in regulating immune responses during infectious diseases by maintaining immune homeostasis. These molecules, known for their role in inducing T cell dysfunction and modulating the activity of other immune cells, have been recognised for their involvement in impaired host defense responses during infections (He & Xu, 2020; Loacker et al., 2023; Sarkar et al., 2024). One such molecule is human leukocyte antigen G (HLA-G), a ligand that interacts with receptors on B cells, dendritic cells, monocytes, T cells and natural killer cells (NK cells) (Carosella et al., 2015). HLA-G has been implicated in the inhibition of NK cell activity, suppression of CD4+/CD8+ T cell maturation, and promotion of regulatory T cell (Treg) development (Carosella et al., 2015). In particular, it has been shown that DENV infection upregulates the expression of HLA-G and its soluble isoforms, suggesting a role for this molecule in the immunopathogenesis of dengue fever (Almeida et al., 2021; McKechnie et al., 2019). However, the mechanisms by which DENV interacts with immune checkpoints are still largely unknown and need to be further investigated.

Genetic predisposition is another important host factor that influences susceptibility and severity of dengue. Variations in host genes involved in immune signalling, cytokine production and pathogen recognition can modulate the immune response and thus influence virus clearance and disease progression (Lan & Hirayama, 2011). Also, the distribution of certain genetic polymorphisms varies according to population and ethnicity and may contribute to geographical differences in dengue exposure.

Several polymorphisms have been associated with dengue outcomes, including those in genes encoding cytokines, HLA alleles, and components of the complement system

(Xavier-Carvalho et al., 2017). For example, HLA-A*0203 and HLA-B*51 are associated with increased risk of developing dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), whereas HLA-DRB1*0901 appears to confer protection (Xavier-Carvalho et al., 2017). Similarly, the TNF-alpha -308A allele has been associated with severe dengue, and polymorphisms in Fc-gamma RIIa receptors can potentially influence the risk of ADE (Xavier-Carvalho et al., 2017).

Another important example is interleukin-10 (IL-10), a key anti-inflammatory cytokine that regulates immune activity associated with DENV infection. Studies have shown that DENV infection induces the production of IL-10 by monocytes, which can suppress the antiviral immune response and impair virus clearance (Adikari et al., 2016; Tian et al., 2019). An increase in IL-10 levels has been associated with severe dengue fever (G. N. Malavige et al., 2013) and recognized as a possible biomarker for predicting secondary DENV infections (Bhatt et al., 2024). In addition, it was proposed that polymorphisms in the IL-10 promoter region, such as rs1800872 (-592C/A) and rs1800871 (-819C/T), influence IL-10 expression levels (Iyer & Cheng, 2012) and thus influence disease susceptibility and clinical severity.

1.6 Clinical Features and WHO Classification of Dengue

Dengue fever has a wide range of clinical features and non-specific symptoms. While most infected people are asymptomatic or only show mild symptoms, severely ill patients can develop life-threatening conditions that require hospitalisation and intensive medical care (World Health Organization, 2009). The illness begins with high fever, pain behind the eyes, headache, aching limbs, skin rash and possibly slight bleeding such as petechiae or nosebleeds (World Health Organization, 2009). As the disease progresses after 4-6 days, warning signs such as mucosal bleeding, persistent vomiting, hepatomegaly, low platelet counts and elevated liver enzymes may occur, requiring close medical monitoring of the patient (World Health Organization, 2009). However, the clinical signs and symptoms of dengue fever are non-specific throughout the course of the disease and often cannot be distinguished from other viral diseases (World Health Organization, 2009).

Patients with the warning signs mentioned above have a poorer prognosis, which can lead to life-threatening complications such as hypovolemic shock, severe bleeding and multiple organ failure. However, early detection and timely treatment can reduce the overall mortality rate of dengue fever to less than 1% (Macias et al., 2021; Salles et al., 2018). To improve diagnosis and treatment, dengue is divided into three clinical categories in the 2009 WHO guidelines: Dengue without warning signs (DF), Dengue with warning signs (DWS) and severe dengue (SD) (World Health Organization, 2009). This classification is more practical and comprehensive than the previous classification (DHF/DSS) introduced by the WHO in 1997 (World Health Organization, 1997). It facilitates early detection of patients at risk of serious complications, minimises misdiagnosis and improves treatment outcomes (Bandyopadhyay et al., 2006).

1.7 Diagnostic Approaches and Challenges in Dengue Fever

The diagnosis of dengue is based on a combination of clinical presentation, laboratory findings and epidemiological characteristics (World Health Organization, 2009). A suspected case is defined as a person with dengue-like symptoms who has had epidemiological exposure, e.g. through living in or recent travel to dengue-endemic areas. In these cases, laboratory tests are important to confirm the infection and rule out other febrile illnesses.

Laboratory diagnosis of dengue can be done using various methods. The most reliable is the detection of DENV RNA using PCR-based assays, which is effective during the early phase of infection (World Health Organization, 2009). Another commonly employed approach involves serological testing for dengue-specific antibodies, including immunoglobulin M (IgM) and immunoglobulin G (IgG), using ELISA or rapid diagnostic tests (RDTs) employing lateral flow immunoassays. IgM antibodies generally appear 3-5 days after symptom onset and indicate a recent infection, while IgG antibodies develop later, typically after the seventh day of illness (World Health Organization, 2009). However, in secondary infections, IgG antibodies may appear earlier and can even be detected before the onset of IgM. Evaluating the IgM/IgG ratio can support in distinguishing between primary and secondary infections, thereby informing case management strategies (Kalra et al., 2024). Additionally, the NS1 protein serves as an important marker for DENV detection (Adikari et al., 2016). NS1 is released into the bloodstream in substantial quantities during the acute phase, usually within the first five days of illness, prior to the appearance of detectable antibodies (World Health Organization, 2009).

However, it is noteworthy that the clinical diagnosis of dengue is complicated by the presence of other arboviruses. Among them are ZIKV, JEV, WNV, TBEV, and CHIKV, all of which have been reported in both human and animal populations in Vietnam (Bayandin et al., 2022; Quyen et al., 2017; Van Cuong et al., 2015; Yen et al., 2010). These viruses may circulate transiently and cause infections with non-specific symptoms that overlap with dengue, complicating clinical differentiation (Quyen et al., 2017). Additionally, a major challenge in laboratory diagnosis is serological cross-reactivity among these viruses, especially among flaviviruses, which may cause false-positive results and misdiagnosis (Chan et al., 2022; Dejnirattisai et al., 2010). Some flaviviruses also share immunological features that contribute to ADE, enhancing viral replication and inflammation in dengue (Dejnirattisai et al., 2010; Halstead & Crowe, 2014). Subsequently, improving accurate diagnostic methods is essential, particularly in resource-limited settings where multiple arboviruses co-circulate.

1.8 Dengue Management: Current Strategies and Future Directions

Dengue treatment remains principally symptomatic and supportive, since specific antiviral treatments are not yet available. Management focuses on maintaining adequate hydration, relieving symptoms, and monitoring for clinical deterioration (World Health Organization, 2009). Patients with mild dengue are advised to increase fluid intake and use antipyretic medications, such as paracetamol, for fever and body pain. Non-steroidal anti-inflammatory substances should be avoided due to their association with increased bleeding risk.

For patients exhibiting warning signs or severe manifestations, hospitalization is recommended for intravenous fluid therapy. Continuous monitoring of vital signs and timely intervention in cases of shock or organ failure are essential (World Health Organization, 2009). Blood and other derivatives should be readily available for transfusion according to local treatment protocols. A platelet transfusion may also be given as a preventive measure when counts fall under 20,000/µL (Kaur & Kaur, 2014). Importantly, early and accurate diagnosis of severe cases plays a critical role in ensuring timely medical intervention and significantly reducing the risk of death (Wong et al., 2020).

Despite long-standing eradication efforts, the most promising vaccine currently available is Qdenga, a live-attenuated vaccine that has been licensed in some endemic countries only (Angelin et al., 2023). In contrast, the earlier vaccine Dengvaxia demonstrated limited efficacy in dengue-naïve individuals and children and was associated with higher risk of severe disease in those populations (Huang et al., 2021). As a result, the optimal approach to dengue control rests on three key pillars: vector control, infection prevention, and early detection and management of severe cases.

Effective vector control strategies include eliminating mosquito breeding sites, applying insecticides, and using biological methods to suppress *Aedes* mosquito populations. An innovative approach involves releasing mosquitoes infected with Wolbachia, which suppresses DENV replication within the mosquito and, as a result, lowers its capacity to transmit the virus to humans (Utarini et al., 2021). Infection prevention also depends on public awareness campaigns, community engagement, and personal protective measures including insect repellents, mosquito nets, and window screens (World Health Organization, 2009).

As mentioned earlier, early detection and proper treatment of severe dengue cases are critical to preventing deaths. During outbreaks, the surge in patient numbers can overwhelm healthcare systems, highlighting the urgent need for faster, more accurate, and cost-effective triage methods. The development of alternative diagnostic strategies, such as the use of key biomarkers to predict disease severity and guide clinical monitoring, is critical for optimizing patient care and prioritizing treatment.

1.9 Aims and Objectives of The Thesis

The pathogenesis of dengue is a multifactorial process governed by complex interactions between host and viral factors. This doctoral thesis aims to identify the key determinants of susceptibility to dengue virus infection and the progression to severe disease, with a particular focus on Vietnam, a dengue-endemic country in Southeast Asia and a recognised hotspot for emerging and re-emerging infectious diseases.

Viral factors: A central objective of this work is to characterise the genetic diversity and spatiotemporal distribution of DENV in endemic regions and to investigate associations between specific viral variants and clinical disease severity. In addition, the study explores the incidence and co-circulation of other arboviruses, including Zika virus

(ZIKV), chikungunya virus (CHIKV), and several flaviviruses (Japanese encephalitis virus [JEV], West Nile virus [WNV], and tick-borne encephalitis virus [TBEV]), as well as their potential for serological cross-reactivity, which complicates diagnosis in clinical settings. Collectively, these studies aim to provide new insights into the molecular epidemiology of dengue and related arboviruses and to contribute evidence for improving diagnostic and surveillance strategies, particularly in resource-limited, high-transmission regions. To address these aims, three complementary studies were conducted:

First, a prospective study in Northern Vietnam was conducted over three consecutive dengue seasons (2020-2022). A total of 426 patients with clinically suspected dengue were enrolled to assess the circulation of DENV, ZIKV, and CHIKV. This study aimed to characterise the circulating DENV serotypes and genotypes and to examine potential associations between viral variants and clinical disease severity.

Second, a retrospective study analysed a cohort of 146 hospitalised patients from the 2016 dengue outbreak in Binh Dinh Province, Central Vietnam. The objective was to determine the predominant circulating DENV serotypes and genotypes and to evaluate possible serological cross-reactivity with other flaviviruses (JEV, WNV, TBEV, ZIKV) and CHIKV.

Third, a prospective study conducted in 2023 involved 108 febrile patients admitted to hospitals in Jember, East Java, Indonesia. This study aimed to reassess the incidence of DENV, ZIKV, and CHIKV infections among clinically diagnosed dengue patients, to characterise circulating DENV serotypes, and to investigate the diagnostic challenges posed by arboviral co-circulation in this endemic setting.

Host factors: This component of the thesis aims to investigate host immunological and genetic determinants that influence susceptibility to DENV infection and progression to severe disease. The focus is on profiling key immune mediators and regulatory molecules associated with disease severity during acute infection in Vietnamese dengue patients.

First, a prospective study in Vietnam enrolled 306 dengue patients during the 2021 and 2022 outbreaks. Patients were clinically stratified into dengue without warning signs, dengue with warning signs, and severe dengue. The objective was to examine clinical, virological, and immunological profiles across severity groups, with a specific focus on interleukin-10 (IL-10) levels as a potential marker of disease progression.

Second, a prospective study involving 238 hospitalised dengue patients and 118 healthy controls assessed the association between plasma levels of soluble human leukocyte antigen G (sHLA-G), a known immunoregulatory molecule and dengue severity. This study explored the potential utility of sHLA-G as a biomarker of immune modulation during acute infection.

Third, using the cohort of 306 dengue patients from the 2021 and 2022 outbreaks, a broad panel of 48 inflammatory mediators, including cytokines, chemokines, and growth factors, was quantified from plasma samples collected at hospital admission. A supervised machine learning approach was applied to identify a subset of immune mediators capable of reliably predicting clinical severity. These findings offer promising tools for early triage and clinical decision-making in dengue-endemic regions.

2. RESULTS

2.1 Chapter 1: Epidemiology and Evolutionary Dynamics of Dengue and Co-Circulating Arboviruses

Publication No.1

Epidemiology and Genotype Dynamics of Dengue in Hospitalized Patients in Northern Vietnam Between 2020 and 2022

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MAJOR ARTICLE

Epidemiology and Genotype Dynamics of Dengue in Hospitalized Patients in Northern Vietnam Between 2020 and 2022

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Background. Arboviruses, including Dengue (DENV), Zika, and chikungunya, cause recurrent outbreaks of varying intensity in tropical countries. This study aimed to investigate other arboviruses, including Zika and chikungunya, in patients clinically suspected of Dengue and to characterize the circulating Dengue serotypes and genotypes in Northern Vietnam from 2020 to 2022. To date, information on this topic in the region has been limited.

Methods. Multiplex real-time polymerase chain reaction (PCR) was used to detect Dengue, Zika, and chikungunya RNA, and DENV serotypes were identified via real-time reverse transcriptase PCR from 426 clinically Dengue suspected patients. Patients were screened for NS1 antigen and anti-DENV immunoglobulin (Ig) G/IgM antibodies. Phylogenetic analysis of DENV Capsid-premembrane gene sequences was performed to investigate genotype distribution.

Results. Dengue was confirmed in 95% of cases, with no Zika or chikungunya RNA detected. DENV-2 was the predominant serotype (61%), followed by DENV-1 (31%) and DENV-4 (7%). Coinfections were observed, with DENV-1 and DENV-2 being the most common. In 2022, a high incidence of Dengue cases with warning signs and severe Dengue was observed, accompanied by elevated liver enzyme levels and reduced platelet counts. Phylogenetic analysis revealed that the DENV-1 and DENV-4 serotypes clustered with previously reported regional virus, while DENV-2 showed a shift from genotype Asian I to Cosmopolitan over the study period.

Conclusions. This study underscores a significant rise in Dengue severity and shifts in DENV genotypes in recent years in Northern Vietnam, emphasizing the importance of understanding genotype dynamics and clinical dynamics for improving outbreak preparedness and response strategies.

Keywords. arboviruses; chikungunya; Dengue; Vietnam; Zika.

Arboviruses, primarily transmitted by arthropods like *Aedes* mosquitoes, present a significant public health challenge, particularly in tropical regions [1]. These viruses, including Dengue, Zika, and chikungunya, are known for causing outbreaks and inducing severe health and economic impacts globally.

Dengue virus (DENV), with its 4 distinct serotypes, is known for causing cyclical outbreaks that burden populations across continents [2]. Dengue fever is a constant problem in Vietnam, with the risk generally being highest during and after the rainy season. Vietnam has entered its peak season for Dengue fever in October and November in recent years [3, 4]. Current data from the Vietnamese Ministry of Health revealed a staggering 5-fold increase in Dengue cases in 2022 compared with 2021 [5]. Despite predictive models, accurately forecasting and preparing for Dengue remain problematic [6], partly due to the high genetic variability of the virus and the emergence of different genotypes and lineages within each serotype, which are each associated with varying disease severities [7].

Zika virus, which gained significant attention during outbreaks in the Americas (2015–2016) and India (2018) [8], is notably linked to severe neurological complications in neonates. In Vietnam, a significant Zika outbreak occurred in the southern region between 2016 and 2017 [9]. Chikungunya virus, known for its debilitating joint pain, has also spread to new areas, resulting in localized epidemics across Africa, Asia, and the Indian subcontinent [10]. In Vietnam, the presence of chikungunya has been detected through chikungunya antibodies in febrile patients and viral RNA in mosquitoes across several provinces [11, 12].

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The clinical manifestations of chikungunya and Zika closely mimic those of Dengue, particularly in the early stages of infection [13], complicating differential diagnoses during outbreaks. Serological diagnostics often face limitations due to cross-reactivity among the Flaviviridae family, which can obscure the detection of Zika and even chikungunya [14, 15]. Furthermore, most epidemiological research on these viruses has been concentrated in Southern Vietnam, leaving a significant gap in understanding for the northern regions [12, 13, 16].

In this context, the current study seeks to enhance the understanding of arbovirus epidemiology in Vietnam by utilizing a range of serological and molecular techniques. This longitudinal analysis, conducted over 3 years from 2020 to 2022 (during the coronavirus disease 2019 [COVID-19] outbreak), aims to assess the prevalence and circulation of these arboviruses among individuals suspected to have Dengue in the northern region of Vietnam.

METHODS

Ethical Approval Statement

All study participants provided signed informed consent before enrollment. The Institutional Review Board of the 108 Military Hospital and the University of Tübingen approved the study, titled "Host and Viral Factors Influencing Dengue Severity and Susceptibility" (Ethics Approval No. 274/2022B02). The study complies with the Nagoya Protocol, and authorization for the use of genetic resources in Germany was obtained from the Vietnamese Ministry of Natural Resources and Environment (Reference No. 2995/QĐ-BTNMT). All procedures followed the Good Clinical Practice (GCP) and Good Clinical Laboratory Practice (GCLP) guidelines.

Study Population

Samples were collected during 3 consecutive seasonal outbreaks in Vietnam, spanning September to November in 2020, 2021, and 2022. The study population consisted of 426 civilian patients suspected of having Dengue, who were admitted to the 108 Military Central Hospital in Hanoi. The Dengue diagnoses followed the World Health Organization diagnostic's criteria (https://apps.who.int/iris/handle/10665/44188) [17], as adopted by the Vietnamese Ministry of Health. The inclusion criteria are patients presenting fever with at least 2 clinical sign/symptoms suggesting Dengue (Nausea/Vomiting, Rash, Aches and Pains, Tourniquet test positive) and/or positive for at least 1 of the indirect diagnostic methods (serological rapid test), as recommended and detailed in the 2009 World Health Organization (WHO) guidelines [17]. Patients with bacterial or other viral infections, chronic diseases, or hematological disorders were excluded. Blood samples were collected upon admission, and plasma was separated and stored at −70°C until further use.

Dengue Diagnosis and Dengue NS1/IgG-IgM Testing

All collected plasma samples were analyzed for nonstructural protein 1 (NS1) DENV antigen and anti-DENV immunoglobulin M (IgM) and G (IgG) antibodies using the Bioline Dengue Duo kit (Abbott, Santa Clara, CA, USA; formerly Alere Inc., Waltham, MA, USA), following the manufacturer's instructions. Febrile patients presenting with symptoms consistent with Dengue and/or testing positive for at least 1 of the NS1, IgM, or IgG assays were diagnosed with Dengue. Among confirmed Dengue patients, those who tested IgG-positive within 8 days of fever onset were classified as having secondary infections, while cases that tested positive only for NS1 or IgM were considered primary infections. Tertiary and quaternary infections could not be distinguished from secondary infections in this study.

Dengue Severity Classification and Laboratory Assessment

In Vietnam, admitted patients were clinically classified into 3 severity levels according to WHO guidelines: Dengue without warning signs (DF; $n = 275$), Dengue with warning signs (DWS; $n = 138$), and severe Dengue (SD; $n = 13$). Clinical presentations were documented upon admission. Laboratory parameters assessed during admission included aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, white blood cell (WBC) count, red blood cell (RBC) count, hematocrit (HCT), and platelet count (PLT).

Polymerase Chain Reaction Detection of Dengue, Zika, and Chikungunya

Total genomic RNA was isolated from 140 µL of patient plasma utilizing the QIAmp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. All samples ($n = 426$) underwent multiplex real-time polymerase chain reaction (PCR) analysis for Dengue, Zika, and chikungunya viral RNA using the Fast Track Diagnostics Kit (Siemens Healthcare G+mbH, Erlangen, Germany) on a LightCycler480-II (Roche, Mannheim, Germany), following the manufacturer's guidelines. The testing was performed using internal controls and standards. Each sample was run in duplicate for each multiplex reverse transcriptase PCR (RT-PCR) analysis, minimizing the risk of technical errors. Confirmed Dengue cases were identified by the presence of Dengue viral RNA through real-time RT-PCR and the absence of Zika and chikungunya viral RNA. Other arboviruses including yellow fever, Japanese encephalitis, West Nile, Tick-borne encephalitis, Rift valley fever, and Sindbis virus were not screened.

Dengue Serotype Detection by Real-time PCR

DENV serotypes in confirmed Dengue cases were identified using the RealStar Dengue Type RT-PCR kit 1.0 (Altona Diagnostics GmbH, Hamburg, Germany) on a LightCycler480-II (Roche, Mannheim, Germany), following the manufacturer's instructions. All samples were tested in duplicate, minimizing the risk of technical errors.

Amplification and Sequencing of the Dengue Capsid-Premembrane

cDNA was synthesized from viral RNA using the LunaScript RT-SuperMix, following the manufacturer's protocol. The primers for capsid-premembrane (CprM) region amplification were those described by Lanciotti et al. [18]. The PCR product from the first round of amplification (PCR-outer), which produced an amplicon of ~511 bp, was used for phylogenetic analysis. In brief: PCR reactions were performed in a 20-µL reaction volume with 3 µL of synthesized cDNA (~5 ng cDNA), 1× buffer (Qiagen GmbH, Hilden, Germany), 0.5 µM of each primer, 200 µM of dNTPs, and 1 U (unit) of Taq DNA polymerase (Qiagen GmbH, Hilden, Germany). The thermal cycling parameters consist of an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation (30 seconds at 94°C), annealing (60 seconds at 55°C), and extension (60 seconds at 72°C), followed by a final extension at 72°C for 10 minutes. PCR amplicons were stained with SYBR green and visualized on a 1.2% gel electrophoresis.

PCR products were purified using Exo-SAP-IT (Applied Biosystems, Beverly, MA, USA) and used for sequencing reaction using the BigDye Terminator, version 1.1, Cycle Sequencing Kit on an ABI 3130XL DNA sequencer (Applied Biosystems, Beverly, MA, USA). Sequencing reactions were done for both strands using forward and reverse primers. The sequences were assembled and checked for nucleotide ambiguities manually using Seqman, version 6.1 (DNASTAR, Lasergene, Wisconsin, USA). The consensus sequences were verified using National Center for Biotechnology Information (NCBI) BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

DENV Phylogenetic Analysis

The sequences were aligned using ClustalW in MEGA, version 11 [19], with respective reference sequences for each DENV serotype (DENV-1: NC_001477; DENV-2: NC_001474; DENV-4: NC_002640). The phylogenetic analysis was performed using MEGA. The phylogenetic tree was reconstructed with the maximum likelihood method based on the Tamura-Nei model with 1000 bootstrap iterations. Reference sequences from various geographical regions were obtained from the NCBI genotyping tool, with accession numbers provided for each serotype. The sequences generated from this study were submitted to GenBank and were assigned the accession numbers PP957471–PP957572 (n = 102).

Statistical Analysis

Data were analyzed and visualized using R, version 4.3.2 (http://www.r-project.org). Clinical and demographic data were presented as median values (with ranges) or mean values (with SDs) for quantitative variables and absolute numbers with percentages for categorical variables. The normality of the distribution in the quantitative variables was tested using the Shapiro-Wilk test. Categorical data were compared using the chi-square or Fisher exact test, while continuous variables were compared using analysis of variance, the Kruskal-Wallis test, or the Wilcoxon test, as appropriate. Patient characteristics over the 3-year period were compared, with a *P* value of <.05 considered statistically significant.

RESULTS

Demographic and Clinical Characteristics of Dengue Patients

All patients were of Kinh ethnicity and residents of the Hanoi metropolitan area or neighboring municipalities. Detailed demographic and clinical data are presented in Table 1 and Supplementary Figure 1. In 2022, patients admitted had a higher median age (52 years) compared with 2021 (42 years) and 2020 (44 years) and were admitted later, a median of 5 days after fever onset, vs 4 days in 2020 and 3 days in 2021.

The number of Dengue cases with DWS and SD increased significantly in 2022 (DWS: n = 83; SD: n = 11) compared with 2021 (DWS: n = 32; SD: n = 2) and 2020 (DWS: n = 23; SD: n = 0). Bleeding manifestations were more common in 2022 (65% of cases) than in 2021 (29%) and 2020 (15%), predominantly as subcutaneous or mucosal bleeding (Table 1). Additionally, in the year 2022 patients exhibited more severe clinical symptoms, including retroocular pain, myalgia, arthralgia, rash, and edema, as detailed in Table 1. They also showed significantly higher levels of liver enzymes (AST, ALT) and HCT, along with lower PLT compared with patients from 2021 and 2020 (Table 1).

Dengue NS1, IgM, and IgG Positivity

In 2020, 79% of patients (95/120) tested positive for NS1, but this percentage gradually decreased to 56% in 2021 and 54% in 2022. In contrast, IgM and IgG positivity rates were higher in 2022, with 23% (33/161) testing positive for IgM and 28% (40/161) for IgG, compared with the previous 2 years (Table 1). However, the year-to-year differences in IgM and IgG positivity did not reach statistical significance. Additionally, 34 samples tested negative for NS1, IgG, and IgM across all years (2020: n = 5; 2021: n = 26; 2022: n = 3), suggesting possible admissions during the early phase of the disease. IgM and IgG positivity were used to distinguish between primary and secondary Dengue infections (Tables 2 and 3). Out of 404 confirmed cases, 377 could be classified as either primary or secondary Dengue. The primary and secondary infections differed significantly between severity groups (*P* = .008) (Table 3).

DENV Serotype Distribution and DENV Coinfections

Out of 426 samples, 404 (95%) tested positive for DENV RNA by real-time RT-PCR, with no detection of Zika or chikungunya viral RNA. A total of 22 samples (5%) tested negative for all 3 viruses. Among the 404 Dengue-positive samples, 375

Table 1. Characteristics of the Studied Patients on Admission

	2020 (n = 120)	2021 (n = 165)	2022 (n = 141)	*P* Value
Median age [range], y	44 [16, 94]	42 [12, 80]	52 [13, 86]	.223
Sex (male/female), No.	73/50	92/73	67/74	.64
Days of fever, median [range]	4 [1, 8]	3 [1, 7]	5 [1, 8]	**<.001**
Days of fever, mean (SD)	4.53 (2.36)	3.54 (1.52)	5.23 (1.31)	**<.001**
Clinical classification, No. (%)	…	…	…	**<.001**
Dengue without warning signs (DF)	97 (81)	131 (79)	47 (33)	
Dengue with warning signs (DWS)	23 (19)	32 (19)	83 (59)	
Severe Dengue (SD)	0 (0)	2 (2)	11 (8)	
Clinical presentations, No. (%)	…	…	…	
Headache	NA	145 (88)	130 (92)	.256
Retro ocular pain	NA	73 (44)	117 (83)	**<.001**
Myalgia	NA	101 (61)	130 (92)	**<.001**
Arthralgia	NA	81 (49)	128 (91)	**<.001**
Rash	NA	34 (21)	67 (48)	**<.001**
Abdominal pain	NA	16 (10)	14 (10)	1
Vomit	NA	31 (19)	25 (18)	1
Lethargy	NA	2 (1)	3 (2)	.665
Hepatomegaly	NA	0 (0)	3 (2)	.098
Shock	NA	2 (1)	4 (3)	.421
Respiratory distress	NA	1 (1)	5 (4)	.099
Edema	NA	1 (1)	32 (23)	**<.001**
Bleeding manifestation	18/120 (15)	48/165 (29)	92/141 (65)	
Subcutaneous	16 (13)	40 (24)	79 (56)	**<.001**
Mucosal	5 (4)	25 (15)	47 (33)	**<.001**
Severe	0 (0)	1 (1)	3 (2)	.342
Laboratory tests, median [range]	…	…	…	
Leucocytes, /µL	3.89 [0.82, 20.1]	4.04 [0.93, 16.9]	3.79 [1.44, 10.5]	.8896
Lymphocyte, %	NA	22.8 [2.5, 72.8]	27.2 [5.8, 58.1]	**.015**
Platelets, $\times 10^3$/µL	82.0 [6.0, 331]	123.0 [4.0, 384]	27.0 [4.0, 304]	**.003**
AST, U/L	56.5 [20.0, 678]	55.3 [15.1, 1790]	103 [18.5, 11100]	**<.001**
ALT, U/L	46.0 [13.0, 607]	38.4 [8.0, 928]	57.7 [8.2, 2190]	**<.001**
Serological tests, No. (%)	…	…	…	
NS1–positivity	95 (79)	92 (56)	76 (54)	**<.001**
IgM–positivity	6 (13)	24 (15)	33 (23)	.212
IgG–positivity	15 (13)	30 (18)	40 (28)	.302

P values were calculated by chi-square test for categorical variables and by analysis of variance, Kruskal-Wallis, or Wilcoxon test for continuous variables. Variables were summarized as percentage (%) or median with [range] or mean with (SD).

Significant *P*-values were emphasised in bold.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DENV, Dengue virus; IgG, immunoglobulin G; IgM, immunoglobulin M; NS1, nonstructural protein.

Table 2. Distribution of Dengue Serotypes by Years

	2020 (n = 120), No. (%)	2021 (n = 165), No. (%)	2022 (n = 141), No. (%)	Total (n = 426), No. (%)	*P* Value
DENV-1	21 (18)	48 (29)	12 (9)	81 (19)	<.001
DENV-2	58 (48)	63 (38)	82 (58)	203 (48)	
DENV-4	13 (11)	1 (1)	6 (4)	20 (5)	
DENV-1 & -2	0 (0)	35 (21)	21 (15)	56 (13)	
DENV-1 & -3	3 (3)	0 (0)	0 (0)	3 (1)	
DENV-2 & -4	0 (0)	5 (3)	7 (5)	12 (3)	
Unidentified	10 (8)	8 (5)	11 (8)	29 (7)	
DENV-negative	15 (13)	5 (3)	2 (1)	22 (5)	
Primary/secondary Dengue	81/23	65/72	68/68	377	<.001

P values were calculated by chi-square test.

Abbreviations: DENV, Dengue virus, serotypes 1, 2, 3, and 4 (coinfections are shown as DENV-1 & -2, DENV-1 & -3, DENV-2 & -4).

Table 3. Distribution of Dengue Serotypes by Clinical Classification

	DF (n = 275), No. (%)	DWS (n = 138), No. (%)	Severe (n = 13), No. (%)	Total (n = 426), No. (%)	*P* Value
DENV-1	54 (20)	22 (16)	5 (39)	81 (19)	.047
DENV-2	117 (43)	82 (59)	4 (31)	203 (48)	
DENV-4	15 (5)	4 (3)	1 (8)	20 (5)	
DENV-1 & -2	45 (16)	11 (8)	0 (0)	56 (13)	
DENV-1 & -3	2 (1)	1 (1)	0 (0)	3 (1)	
DENV-2 & -4	7 (3)	5 (4)	0 (0)	12 (3)	
Unidentified	17 (6)	10 (7)	2 (15)	29 (7)	
DENV-negative	18 (7)	3 (2)	1 (8)	22 (5)	
Primary/secondary Dengue	118/115	89/43	7/5	377	.008

P values were calculated by chi-square test.

Abbreviations: DENV, Dengue virus, serotypes 1, 2, 3, and 4 (coinfections are shown as DENV-1 & -2, DENV-1 & -3, DENV-2 & -4); DF, Dengue without warning signs; DWS, Dengue with warning signs; SD, severe Dengue.

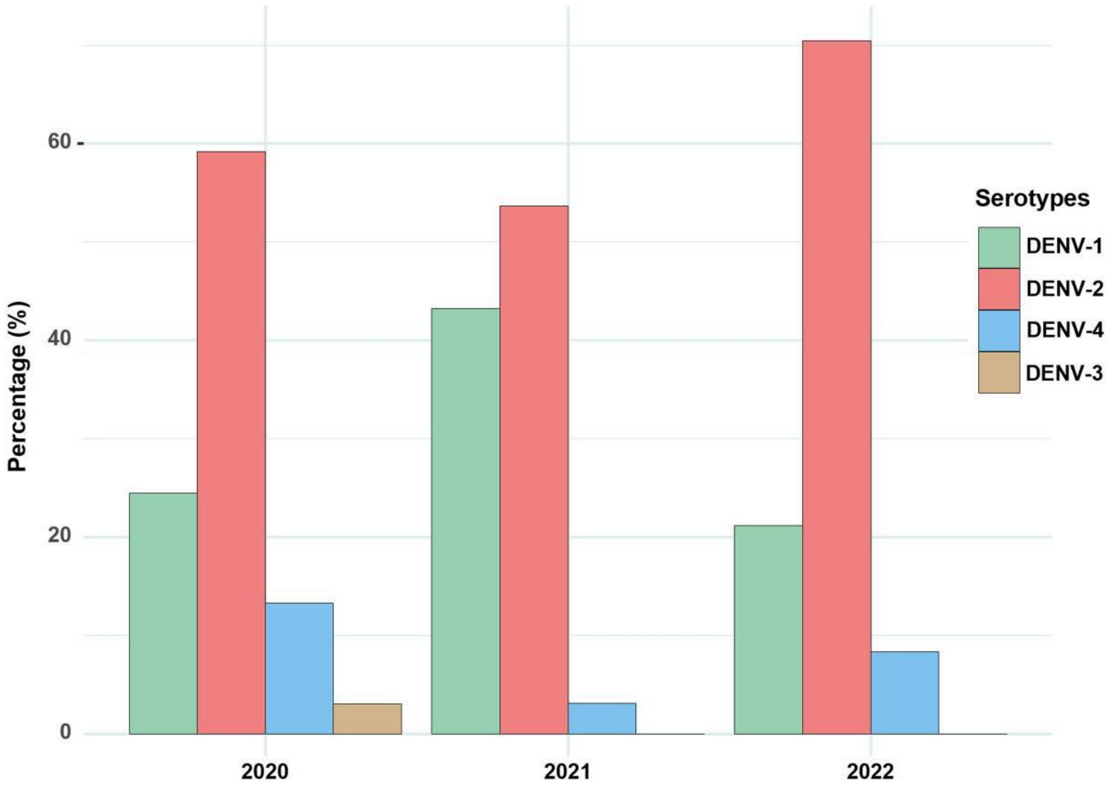

Figure 1. Distribution of Dengue serotypes in Northern Vietnam, 2020–2022.

were identified with specific DENV serotypes, leaving 29 without defined serotypes but still positive for DENV RNA (Figure 1 and Table 2). In 2020, all 4 DENV serotypes were present, while only DENV-1, DENV-2, and DENV-4 were detected in 2021 and 2022.

DENV-2 was the most frequently observed serotype across the 3 years (48%; 203/426), followed by DENV-1 (19%; 81/426) and DENV-4 (5%; 20/426) (Table 2). Coinfections were also identified, including DENV-1 and -2, DENV-1 and -3, and DENV-2 and -4. The most common coinfection was DENV-1 and - 2 (13%; 56/426), observed only in 2021 and 2022 (Table 2). When coinfections were accounted for in the single serotype count, DENV-2 remained the most prevalent (61%), followed by DENV-1 (31%) and DENV-4 (7%). DENV-3 was only detected in 2020, with a prevalence of <1%.

The distribution of clinical severity among the infected DENV serotypes showed borderline significance (*P* = .047) (Table 3). DENV-2 was associated with the highest number of Dengue cased with warning signs (DWS; 59%; 82/138), while DENV-1 was mostly associated with severe Dengue (SD; 39%; 5/13) (Table 3). No significant clinical differences were observed between single and multiple DENV serotype infections.

Phylogenetic Analysis of DENV Genotypes

Only a representative subset of samples (n = 102) from each DENV serotype (DENV-1, n = 29/81; DENV-2, n = 65/203; and DENV-4, n = 8/20) was selected for CprM region amplification (~511 bp). To determine the distribution of DENV genotypes, we aligned partial CprM gene sequences from our study with sequences from various geographical locations available in the NCBI database and performed a phylogenetic analysis. Figure 2, Supplementary Figure 2, and Supplementary Figure 3 illustrate the phylogenetic trees for DENV-1, DENV-2, and DENV-4, respectively. The phylogenetic analysis revealed that DENV-1 sequences (n = 29) clustered within genotype I (Supplementary Figure 2). Similarly, the DENV-4 sequences from our study were classified as genotype I (Supplementary Figure 3). For DENV-2, 18% (12/66) of the sequences were identified as genotype Asian I, while 82% (54/66) were classified as genotype Cosmopolitan (Figure 2).

The DENV-1 sequences from our study are closely related to those circulating in Vietnam in 2017 and in China in 2016. Similarly, the DENV-4 sequences exhibit a high degree of similarity to strains found in Southern Vietnam in 2018. The DENV-2 sequences of the Asian I clade show strong homology with DENV-2 strains circulating in Cambodia in 2019, while the Cosmopolitan genotype DENV-2 sequences are closely related to strains from China (2019) and Cambodia (2020). As Vietnam shares borders with China and Cambodia, it is likely that DENV spread between these countries.

DISCUSSION

Enhanced surveillance remains essential in clinical management and mitigating the impact of arbovirus infections, particularly in rapidly changing epidemic settings. This study aimed to elucidate the epidemiological patterns and genotype dynamics of Dengue and other arboviruses in Northern Vietnam from 2020 to 2022. This period was marked by significant fluctuations in the incidence and severity of Dengue cases (during COVID), as well as shifts in the circulation of different DENV serotypes and genotypes.

Of all 3 arboviruses analyzed, Dengue virus was detected in 95% of the samples tested. Neither Zika nor chikungunya virus RNA was detected in our study. A total of 5% (n = 22/426) of the Dengue/Zika/chikungunya RNA-negative samples probably indicate infections with other etiologies. The study findings on serotype distribution reveal that DENV-2 was the most prevalent serotype over the 3 years, with a notable presence of DENV-1 and DENV-4. The detection of DENV-3 exclusively in 2020 suggests that its circulation might have been transient or limited to specific regions or periods. The documented coinfections, especially between DENV-1 and DENV-2, shed light on the intricate interactions among various serotypes. Such coinfections can enhance virulence through genetic recombination, potentially leading to the emergence of more pathogenic strains that increase the severity of Dengue fever [20]. While our study demonstrated a consistent pattern of circulating DENV serotypes in Northern Vietnam during the 2020–2022 period, there has been a notable shift in the prevalence of DENV serotypes in Hanoi in recent years. Studies from 2017 and 2018 identified DENV-1 as the dominant serotype, but starting in 2019, DENV-2 emerged as the prevalent one [21–23]. For instance, subsequent infections with a different DENV serotype can lead to a phenomenon called antibody-dependent enhancement (ADE), where nonspecific antibodies may worsen the clinical course [24]. Given that DENV-1 was predominant in the population during 2017 and 2018 [25], the emergence of other DENV serotypes could have contributed to more widespread outbreaks and severe infections, as observed in the 2019 and 2020 outbreaks [26].

The results indicated variability in clinical severity among infections caused by different DENV serotypes. DENV-2 was associated with the highest number of DWS cases, while DENV-1 was linked to the most SD cases. DENV-2 has also been reported as the serotype most associated with severe Dengue in other studies [7, 27]. The clinical outcome of a DENV infection is influenced by various host and viral factors, making the virulence of the infecting serotype just one factor in determining disease severity. Our study found no significant differences in clinical severity between infections caused by a single DENV serotype vs multiple serotypes, nor between different genotypes within the same serotype.

Vietnam reported ~300 000 Dengue cases in 2019, ~133 000 cases in 2020, ~71 000 cases in 2021, and ~367 729 cases in 2022 [28]. The substantial outbreak in the northern region in 2022 may be attributable to the prevalence of the DENV-2 Cosmopolitan genotype, which aligns with the increased number of reported cases. This genotype is also noted for being widespread and associated with a higher degree of severity [7]. The lower amount of Dengue in 2021 might be due to reduced investigation efforts during the COVID-19 pandemic, which led to restrictions and a notable drop in reported cases compared with previous years. Our study indicates that patients admitted in 2022 experienced greater severity and more pronounced laboratory abnormalities than those in 2021 and 2020. However, as the progression to severe Dengue is influenced by both host and viral factors and considering that 2022 patients were older and presented at a more

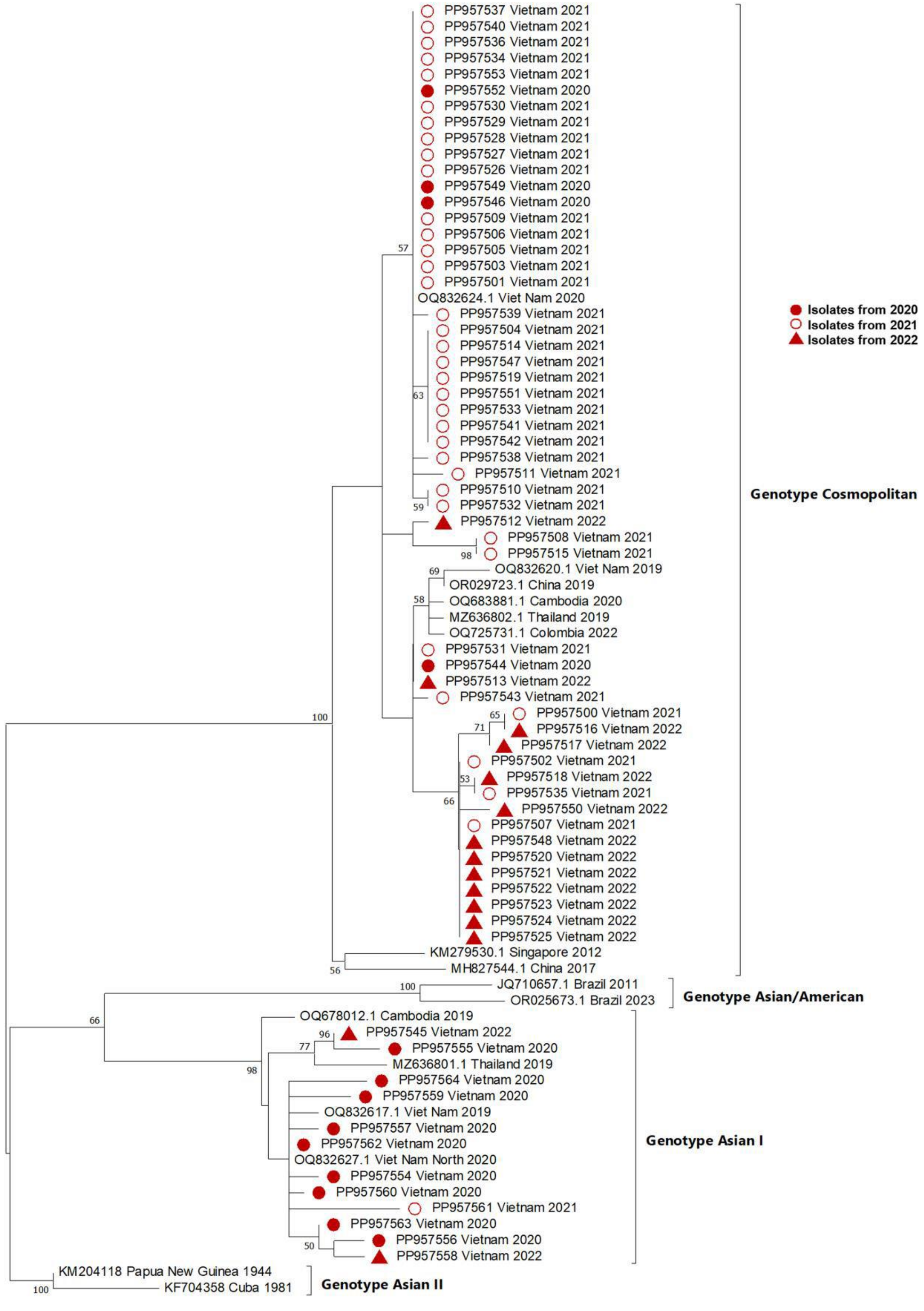

Figure 2. Phylogenetic analysis DENV-2 genotypes. Abbreviation: DENV-2, Dengue virus 2.

advanced stage of illness, comparing the virulence of the 2022 DENV-2 variant directly with other years remains challenging.

Different DENV genotypes are associated with varying degrees of immunogenicity and infection severity [7, 29]. This study also analyzed the CprM region, which has already proven to be a valuable comparator for the DENV envelope gene and/or the entire DENV genome [30, 31]. Our findings indicated a consistent prevalence of DENV-1 genotype 1 and DENV-4 genotype 1 over the 3-year study period. The phylogenetic analysis revealed that DENV-1 sequences clustered within genotype I, showing high similarity to DENV-1 sequences from Vietnam in 2017. Similarly, the DENV-4 sequences from our study were classified as genotype I, closely related to DENV-4 sequences from Southern Vietnam in 2018. Notably, 75% of the DENV-2 Asian I sequences were from 2020, indicating a potential shift from genotype Asian I to Cosmopolitan in the 2021–2022 outbreaks.

In 2022, Ho Chi Minh City, located in Southern Vietnam and home to ∼9.5 million residents, experienced a 3-fold increase in Dengue cases compared with 2020 and a 5-fold increase compared with 2021. A similar trend was observed in the southern region, where DENV-2 was the most prevalent serotype, followed by DENV-1 and DENV-4 [16]. Supporting our findings, Tran et al. reported the reemergence of the DENV-2 Cosmopolitan genotype in the southern region in 2022 [16]. Consequently, the DENV-2 Cosmopolitan genotype likely played a significant role in the sudden intensification of the outbreak in Vietnam that year. The surge in Dengue cases in 2022 underscores the growing public health challenge posed by Dengue in Northern Vietnam. This surge was accompanied by a shift toward more severe manifestations, including a higher incidence of Dengue with warning signs and severe Dengue.

A notable limitation of this study is that it is based on samples from a single hospital in Hanoi. This sampling approach may not fully capture the geographic and demographic diversity of Dengue cases in Northern Vietnam. In addition, the study focuses on hospitalized patients with hemorrhagic fever symptoms, implying that cases with milder manifestations may be underrepresented, which could affect the generalizability of the results. Longitudinal studies with larger sample sizes and more geographically diverse data could provide further insights into the factors driving the observed changes in virus co-circulation and disease severity. While population-wide seroprevalence studies for Dengue, Zika, and chikungunya can provide valuable information on the current or past prevalence of these viruses, this study did not include serological testing for ZIKV and CHIKV due to the high risk of serological cross-reactivity with Dengue in this endemic setting [14, 15].

In conclusion, this study provides valuable insights into the epidemiological trends, serotype distribution, and genotype dynamics of Dengue in Northern Vietnam. These findings enhance our understanding of arboviral diseases and support public health measures in the region.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Author contributions. T.P.V. designed and supervised the study and contributed to the study materials and assays. L.H.S. and T.P.V. were involved in the conceptualization of the study and contributed to the study materials. D.D.A. and L.T.L.K. performed the experimental procedures. D.D.A. performed the statistical analysis and validation of the results. N.T.T., N.X.H., N.L.T., L.H.S., and T.N.M. recruited the patients and contributed to the investigation materials for sampling procedures. D.A.A. and T.P.V. wrote the first draft. D.D.A., T.P.V., and P.G.K. reviewed the first draft. All authors read and approved the manuscript.

Availability of data and materials. All data generated or analyzed during this study are included in this article. A total of 102 successfully sequenced DENV genotype–specific sequences generated from this study were submitted to GenBank and were assigned the accession numbers PP957471–PP957572 (n = 102).

Ethical approval and consent to participate. All study participants provided signed informed consent before enrollment. The Institutional Review Board of the 108 Military Hospital and the University of Tübingen approved the study, titled "Host and Viral Factors Influencing Dengue Severity and Susceptibility" (Ethics Approval No. 274/2022B02). The study complies with the Nagoya Protocol, and authorization for the use of genetic resources in Germany was obtained from the Vietnamese Ministry of Natural Resources and Environment (Reference No. 2995/QĐ-BTNMT). All procedures followed GCP/GCLP guidelines.

Consent for publication. All authors read and consented to publication of the article.

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Dominance of DENV-1 and Flavivirus Serological Cross-Reactivity During the 2016 Dengue Outbreak in Vietnam

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Dominance of DENV-1 and Flavivirus Serological Cross-Reactivity During the 2016 Dengue Outbreak in Vietnam

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ABSTRACT

Flaviviruses such as dengue virus (DENV), Zika virus (ZIKV), and Japanese encephalitis virus (JEV) pose a major health burden in Vietnam, where overlapping clinical features and serological cross-reactivity complicate accurate diagnosis and outbreak control. This study aimed to investigate circulating DENV serotypes and assess serological cross-reactivity with other flaviviruses during the 2016 dengue outbreak in central Vietnam. Aretrospective study was conducted on 146 hospitalized dengue patients during the 2016 outbreak in Binh Dinh province. Laboratory diagnosis included NS1antigen testing, ELISA (IgM/IgG), and real-time RT-PCR for DENV serotyping. IgM and IgG cross-reactivity with five flaviviruses, including DENV, ZIKV, JEV, West Nile virus (WNV), and tick-borne encephalitis virus (TBEV), and onealphavirus, chikungunya virus (CHIKV), was evaluated using ELISA. DENV-1 positive samples were further analysed by sequencing the capsid-premembrane (CprM) gene. DENV-1 was the predominant serotype (86%), with all sequenced strains clustering within genotype I. Secondary infections were more frequent (64%) than primary infections (36%) and were associated with a significantly higher median age ($p = 0.003$) and elevated hs-CRP levels ($p = 0.029$). Strong IgG cross-reactivity was observed among flaviviruses, particularly DENV, JEV, WNV, and TBEV ($r > 0.85$), while ZIKV and CHIKV showed low seropositivity. Incontrast, IgM responses demonstrated greater virus specificity. Ten PCR-negative cases showed broad serological reactivity, suggesting possible misdiagnosis or late-stage infection. Our findings reveal that DENV-1 genotype I was the predominant serotype during the 2016 outbreak in central Vietnam. Extensive IgG cross-reactivity among flaviviruses hinders serological diagnosis, highlighting the need for integrated molecular surveillance to ensure accurate outbreak response.

Do Duc Anh and Do Huy Loc contributed equally to this study. Thirumalaisamy P. Velavan and Nguyen Linh Toan are shared senior authors.

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1 | Introduction

Flaviviruses, primarily transmitted by mosquitoes and ticks, present a significant public health challenge, particularly in tropical regions [1]. These viruses are responsible for a wide spectrum of clinical manifestations, ranging from mild febrile illness to severe, life-threatening complications such as hemorrhagic fever, encephalitis, and shock syndromes [2, 3]. The rapid spread of flaviviruses, combined with overlapping symptoms and limited diagnostic capacity in many endemic regions, further complicates timely detection, clinical management, and outbreak control [4].

In Vietnam, dengue virus (DENV) is endemic and responsible for recurrent outbreaks of viral hemorrhagic fever. The risk of transmission increases during and after the rainy season, when populations of *Aedes* mosquitoes, the primary vectors of DENV, surge [5]. Between 2000 and 2020, Vietnam recorded an annual average of approximately 95 000 dengue cases, with a peak of 294 707 cases in 2019 [6]. In 2022, dengue cases surged dramatically, showing a fivefold increase compared to 2021, highlighting the increased burden on local healthcare systems during outbreak periods [7]. The burden and intensity of dengue outbreaks are determined by a complex interplay of virological, ecological, immunological, and socio-environmental factors, including the co-circulation of all four dengue virus serotypes (DENV-1−DENV-4) in Vietnam [8]. Notably, the emergence or reintroduction of new DENV serotypes or genotypes has been associated with outbreak intensity in endemic regions [9].

Variations in immunogenicity among the serotypes have also been associated with differences in disease severity [10]. For instance, DENV-1 and DENV-2 have been associated with more severe clinical outcomes in our previous study [7, 11]. Moreover, genetic diversity within each serotype may contribute to variability in disease severity, transmissibility, and immune response among infected individuals [12, 13]. Subtypes within DENV serotypes have also been reported to exhibit increased infectivity and virulence [14].

In addition, several other flaviviruses, including Zika virus (ZIKV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and West Nile virus (WNV), have been documented in human and animal reservoirs in Vietnam [15–18]. These flaviviruses can circulate silently in the population and occasionally present with unspecific clinical symptoms that overlap with dengue, complicating clinical diagnosis [16]. A further layer of complexity in clinical management arises from serological cross-reactivity, which can lead to misdiagnosis in serological assays, particularly among closely related flaviviruses. This group of pathogens can also modulate immune responses through mechanisms such as antibody-dependent enhancement (ADE) [19]. Prior studies have shown that non-neutralizing, cross-reactive antibodies may facilitate viral entry into Fc gamma receptor-bearing cells, thereby enhancing viral replication and promoting inflammation [19, 20].

Given this complex epidemiological and immunological landscape, the present study aimed to reassess the circulation of DENV in hospitalized patients during a major dengue outbreak in 2016 at the Central Hospital of Binh Dinh province, central Vietnam [21]. In addition, we sought to investigate the potential serological cross-reactivity of DENV with other relevant flaviviruses, including JEV, WNV, TBEV, and ZIKV as well as the alphavirus chikungunya (CHIKV).

2 | Materials and Methods

2.1 | Ethical Approval Statement

All participants provided signed informed consent for the anonymized use of their blood samples in research, including testing for flaviviruses. For subjects under 18 years of age, consent was obtained from their parents or legal guardians. The study was approved by the Institutional Review Board of the Military Medical University in Hanoi, Vietnam (Approval no. 103MCH/RES/DENV-GER_V-D1-2016), the Binh Dinh Medical College in Gia Lai, Vietnam (Approval no. 466-QĐ/CĐYT), and the University of Tübingen, Germany (Approval no. 274/2022B02), for the project entitled “Host and Viral Factors Influencing Dengue Severity and Susceptibility.” Authorization for the use of Vietnamese genetic resources in Germany was granted in accordance with the Nagoya Protocol obtained from the Vietnamese Ministry of Natural Resources and Environment (Reference No. 2995/QĐ-BTNMT). All procedures followed GCP/GCLP guidelines and were in accordance with the ethical standards of the Helsinki Declaration.

2.2 | Study Population

Samples were collected during the 2016 dengue outbreak in Binh Dinh Central Hospital, spanning March to June in 2016. This retrospective study included 146 patients diagnosed with dengue who were admitted to the provincial Central Hospital. Dengue diagnoses followed the World Health Organisation diagnostic′s criteria [5], as adopted by the Vietnamese Ministry of Health. The inclusion criteria were patients presenting with fever within 7 days of onset, accompanied by at least two clinical signs or symptoms suggestive of dengue (e.g., nausea/vomiting, rash, body aches and pains, tourniquet test positive) and positive for at least one of the indirect diagnostic methods (serological rapid test NS1/IgG/IgM), as recommended and detailed in the WHO guideline 2009 [5]. Patients with bacterial or other viral infections, chronic diseases, or hematological disorders were excluded. A total of 10 mL of whole blood was collected from each participant at the time of admission, comprising samples for routine laboratory diagnostics and for research purposes. Plasma was separated and stored at −70°C until use. Clinical severity data for dengue cases were not available in this study cohort.

2.3 | Patients Laboratory Assessment

The dengue nonstructural protein 1 (NS1) antigen and anti-DENV immunoglobulin M (IgM) and G (IgG) antibodies were determined upon hospital admission to support clinical diagnosis. The following laboratory tests were conducted at the admitting hospital at the time of admission: white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), hematocrit (HCT), platelet count (PLT), urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (CRP.hs). Primary and secondary dengue infections were differentiated based on the IgM/IgG optical density (OD) ratio using ELISA, with patient plasma diluted at 1:101. According to WHO guidelines and supported by prior studies [5, 22], an OD ratio greater than 1.2 indicates primary infection, while a ratio below 1.2 suggests secondary infection.

2.4 | Dengue Serotype Detection by Real-Time RT-PCR

DENV serotypes in confirmed dengue cases were identified using the RealStar Dengue Type RT-PCR kit 1.0 (Altona Diagnostics GmbH, Hamburg, Germany) on a LightCycler480-II (Roche, Mannheim, Germany), following the manufacturer's instructions. All assays were performed in duplicate. Patients with identified DENV serotypes by RT-PCR were classified as confirmed dengue cases, whereas those without serotype identification but positive by NS1 and/or IgM/IgG tests were classified as probable dengue cases.

2.5 | Serological Assays

To investigate cross-reactivity with other viral hemorrhagic fever-related pathogens, plasma IgG and IgM antibodies against five flavivirus pathogens (DENV, JEV, ZIKV, WNV, and TBEV) and one alphavirus (CHIKV) were detected using commercial ELISA kits (Euroimmun, Lübeck, Germany). The specific kits utilized were anti-dengue virus type 1–4 ELISA (IgG/IgM), anti-JEV ELISA (IgG/IgM), anti-ZIKV ELISA (IgG/IgM), anti-WNV ELISA (IgG/IgM), anti-TBEV ELISA 2.0 (IgG/IgM), and anti-CHIKV ELISA (IgG/IgM).

All assays were performed in duplicate following the manufacturer's instructions. Briefly, plasma samples were diluted at a ratio of 1:101 and incubated at 37°C for 60 min. Samples were then incubated sequentially with the conjugate solution for 30 min and the substrate solution for 15 min at room temperature, with three washes performed between each step. The reaction was terminated using a stop solution, and absorbance was measured at wavelengths of 450 and 620 nm with a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany). Results were interpreted according to the manufacturer-defined cut-off indices provided with each kit.

2.6 | Amplification and Sequencing of the Dengue Capsid-Premembrane (CprM)

A representative subset of samples ($n = 30$) from DENV-1-positive patients was selected for amplification of the CprM region. cDNA was synthesized from viral RNA using the LunaScript RT-SuperMix, following the manufacturer's protocol. The primers for CprM region amplification were those described by Lanciotti et al. [23]. The PCR product from the first round of amplification (PCR-outer, lengths ~511 bp) were used for phylogenetic analysis. In brief: PCR reactions were performed in 20 μL reaction volume with 3 μL of synthesized cDNA (approx. 5 ng cDNA), 1x buffer (Qiagen GmbH, Hilden, Germany), 0.5 μM of each primer, 200 μM of dNTPs, and 1 U (unit) of Taq DNA polymerase (Qiagen GmbH, Hilden, Germany). The thermal cycling parameters consist of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation (30 s at 94°C), annealing (60 s at 55°C), extension (60 s at 72°C), followed by a final extension at 72°C for 10 min. PCR amplicons were stained with SYBR green and visualized on a 1.2% gel electrophoresis.

PCR products were purified using Exo-SAP-IT (Applied Biosystems, Beverly, MA, USA) and used for the sequencing reaction using the BigDye Terminator v.1.1 Cycle Sequencing Kit on an ABI 3130XL DNA sequencer (Applied Biosystems, Beverly, MA, USA). Sequencing reactions were done for both strands using forward and reverse primers. The sequences were assembled and checked for nucleotide ambiguities manually using Seqman version 6.1 (DNASTAR, Lasergene, USA). The consensus sequences were verified using National Center for Biotechnology Information (NCBI) BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7 | DENV Phylogenetic Analysis

The sequences were aligned using ClustalW in MEGA version 11 [24], with respective reference sequences for DENV-1: NC001477. The phylogenetic analysis was performed using MEGA. The phylogenetic tree was reconstructed using the maximum likelihood method based on the Kimura 2-parameter model with 1000 bootstrap iterations. Reference sequences from various geographical regions were obtained from NCBI genotyping tool, with accession numbers provided for each serotype. The sequences generated from this study were submitted to GenBank and were assigned the accession numbers PQ562260–PQ562289 ($n = 30$).

2.8 | Statistical Analysis

The data were analyzed and visualized using the software R version 4.3.2 (http://www.r-project.org). The patient data were presented as median values (with range) for quantitative variables and as absolute numbers (with percentages) for categorical variables. The normality of the distribution of quantitative variables was tested using the Shapiro–Wilk test. Categorical data were compared using the chi-square test, while continuous variables were compared using the Student's t-test or Wilcoxon test. Pearson correlation coefficients (as the data were parametric) were computed to evaluate relationships between antibody responses to different viruses. A p-value of < 0.05 is considered statistically significant.

3 | Results

3.1 | Demographic and Laboratory Data of Study Participants

All patients were of Kinh ethnicity and residents of Binh Dinh (now Gia Lai) province. Overall, patient ages ranged from 1 to

50 years, with 52% of the study population being female. There was no significant difference in sex distribution between patients with primary and secondary DENV infections. The median age of patients with primary infection (10.5 [1–40]) was significantly lower than that of those with secondary infection (20 [2–50]) ($p = 0.003$) (Table 1).

Laboratory parameters in the study population deviated from normal physiological ranges (Table 1). In our study population, the median WBC counts were low, consistent with viral infection, PLT counts were markedly reduced (median of $75 \times 10^3/\mu L$), and elevated levels of AST (median of 97 U/L) and ALT (median of 59 U/L) evidenced the liver damage in dengue (Table 1). Additionally, CRP.hs levels were elevated in majority of patients (> 1 mg/L), indicating systemic inflammation or acute infection. When comparing primary and secondary infections, CRP.hs levels were significantly higher in secondary dengue cases (median 9.15 [0.2–295] vs. 3.55 [0.4–192]; $p = 0.029$), suggesting a more pronounced inflammatory response in these patients (Table 1).

3.2 | DENV Serotyping and Phylogenetic Analysis

A total of 146 samples were subjected to DENV serotype differentiation. All four DENV serotypes were detected, with DENV-1 being the most prevalent, identified in 86% of cases ($n = 125/146$) (Table 2). DENV-4 was found in seven patients (5%), while DENV-2 and DENV-3 were each detected in only one case (1%). One patient (1%) presented a co-infection with

TABLE 1 | Patient characteristics on admission stratified by primary and secondary dengue infections.

	Primary infection ($n = 52$)	Secondary infection ($n = 94$)	p value
Age (years)	10.5 (1–40)	20 (2–50)	**0.003**
Sex (count female, %)	28 (54%)	48 (51%)	0.863
NS1 (count positive, %)	26 (50%)	49 (52%)	0.863
WBC ($\times 10^9$/L)	3.3 (1.3–11)	3.55 (1.3–12.4)	0.836
RBC ($\times 10^{12}$/L)	4.71 (3.24–5.88)	4.95 (2.51–8.5)	0.163
Hb (g/L)	138.5 (105–164)	138 (63–185)	0.798
HCT (%)	41.45 (31.5–51.9)	41.65 (11.3–53.2)	0.699
PLT ($\times 10^9$/L)	75.5 (6–225)	77 (8–186)	0.598
Urea (mmol/L)	3.58 (1.05–13.6)	3.8 (0.34–9.97)	0.608
Creatinine (μmol/L)	79 (27–109)	78 (36–123)	0.644
AST (U/L)	98 (16.6–1127.7)	97.8 (13.9–8112)	0.902
ALT (U/L)	60.55 (4.5–529.3)	57.2 (5–2835)	0.787
CRP.hs (mg/L)	3.55 (0.4–192)	9.15 (0.2–295)	**0.029**
Serotype (DENV-1) (positive, %)	47 (90%)	78 (83%)	0.391
IgG positivity			
DENV (count %)	36 (69%)	88 (94%)	**< 0.001**
JEV (count %)	44 (85%)	93 (99%)	**0.001**
TBEV (count %)	37 (71%)	90 (96%)	**< 0.001**
WNV (count %)	36 (69%)	89 (95%)	**< 0.001**
ZIKV (count %)	10 (19%)	32 (34%)	0.085
CHIKV (count %)	0 (0%)	5 (5%)	0.161
IgM positivity			
DENV (count %)	38 (73%)	37 (39%)	**< 0.001**
JEV (count %)	29 (56%)	28 (30%)	**0.003**
TBEV (count %)	9 (17%)	5 (5%)	**0.036**
WNV (count %)	13 (25%)	3 (3%)	**< 0.001**
ZIKV (count %)	0 (0%)	0 (0%)	**0.001**
CHIKV (count %)	0 (0%)	3 (3%)	0.553

Note: Variables are summarized as median (range) for continuous data and absolute counts (percentages) for categorical data. p-values were determined from the comparison between primary ($n = 52$) and secondary ($n = 94$) dengue infections, using the chi-square test for categorical variables and the student's t-test/Wilcoxon rank-sum test for continuous variables. p-value in bold: statistically significant.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHIKV, Chikungunya virus; CRP.hs, high-sensitivity C-reactive protein; DENV, dengue virus; Hb, hemoglobin; HCT, hematocrit; IgG, immunoglobulin G; IgM, immunoglobulin M; JEV, Japanese encephalitis virus; NS1, nonstructuralnonstructural protein 1; PLT, platelet count; RBC, red blood cell count; TBEV, Tick-borne encephalitis virus; WBC, white blood cell count; WNV, West Nile virus; ZIKV, Zika virus.

TABLE 2 | Distribution of dengue virus serotypes.

DENV-1 *n* (%)	DENV-2 *n* (%)	DENV-3 *n* (%)	DENV-4 *n* (%)	DENV-3 and DENV-4 *n* (%)	Unidentified *n* (%)	Total (*n*)
125 (86)	2 (1%)	1 (1%)	7 (5%)	1 (1%)	10 (7%)	146

Abbreviations: DENV-1,2,3,4, dengue virus serotype 1,2,3,4; DENV-3 and DENV-4, coinfection of dengue virus serotype 3 and dengue virus serotype 4.

DENV-3 and DENV-4. In 10 patients (7%), the DENV serotype could not be determined (Supporting Information S1: Table S2).

To assess the genetic characteristics of the predominant DENV-1 strain, partial sequences of the capsid-premembrane (CprM) gene from 30 DENV-1-positive samples were aligned with reference sequences from various geographic regions available in the NCBI database. Phylogenetic analysis revealed that all DENV-1 strains clustered within genotype I. These sequences showed high similarity to strains previously circulating in Vietnam (2003), Cambodia (2014–2015), China (2016), and New Caledonia (2014) (Figure 1). This suggests a continued circulation and regional persistence of DENV-1 subtype I in Vietnam, as similar strains were also reported in the country in 2003, 2017, and again between 2020 and 2022 (Figure 1).

3.3 | Serological Profiles in Study Participants

Serological profiles differed significantly between primary and secondary dengue infections. Patients with secondary infections showed markedly higher IgG seropositivity across multiple flaviviruses, including DENV (94% vs. 69%, $p < 0.001$), JEV (99% vs. 85%, $p = 0.001$), TBEV (96% vs. 71%, $p < 0.001$), and WNV (95% vs. 69%, $p < 0.001$) (Table 1). In contrast, IgM positivity was significantly higher in primary infections for DENV (73% vs. 39%, $p < 0.001$), JEV (56% vs. 30%, $p = 0.003$), TBEV (17% vs. 5%, $p = 0.036$), and WNV (25% vs. 3%, $p < 0.001$), suggesting probable recent infections (Table 1). No IgM positivity for ZIKV was observed in either group. Anti-CHIKV IgG/IgM positivity also remained low in the study population (Table 1).

Among the 10 samples with unidentified DENV serotypes, five showed anti-DENV IgM positivity, suggesting recent dengue infections (Supporting Information S1: Table S2). One case (sample ID: BD072) was more consistent with JEV infection, as indicated by the presence of anti-JEV IgM and the absence of IgM against other tested viruses. Another case (sample ID: BD087) showed probable infection with TBEV, based on the detection of anti-TBEV IgM alone (Supporting Information S1: Table S2). The remaining samples had broad IgM positivity across multiple flaviviruses, making the etiology unclear.

3.4 | Correlation of Antibody Responses

Strong correlations were observed among IgG responses to multiple flaviviruses, indicating substantial serological cross-reactivity within the study population. IgG against TBEV showed the highest correlation with WNV ($r = 0.94$) and DENV ($r = 0.86$), followed closely by its correlation with JEV ($r = 0.58$) (Figure 2 and Supporting Information S1: Table S1). Similarly, IgG responses to WNV were strongly correlated with DENV ($r = 0.92$) and moderately with JEV ($r = 0.54$), underscoring strong IgG cross-reactivity among four viruses: DENV, WNV, TBEV, and JEV (Figure 2 and Supporting Information S1: Table S1). In contrast, IgG responses to ZIKV and CHIKV showed weaker correlations with the other tested viruses ($r < 0.2$).

Among IgM responses, no samples tested positive for anti-ZIKV IgM. However, moderate correlations were noted, especially between JEV and DENV ($r = 0.50$) and between JEV and TBEV ($r = 0.36$), suggesting cross-reactive IgM responses among flaviviruses (Figure 2 and Supporting Information S1: Table S1). Notably, anti-WNV IgM was correlated with anti-TBEV IgM ($r = 0.48$) and anti-JEV IgM ($r = 0.35$), consistent with the patterns seen in IgG responses, where cross-reactivity was evident among DENV, WNV, TBEV, and JEV (Figure 2 and Supporting Information S1: Table S1). In contrast, anti-CHIKV IgM showed minimal or no correlation with other viruses, suggesting distinct or nonoverlapping immune responses.

4 | Discussion

Our retrospective analysis of the 2016 dengue outbreak in central Vietnam confirms the co-circulation of all four DENV serotypes during the study period, with DENV-1 genotype I identified as the predominant strain. Additionally, patients with active dengue infection exhibited strong IgG and IgM cross-reactivity with TBEV, JEV, and WNV, underscoring the challenges associated with serology-based diagnostic methods.

The large 2016 dengue outbreak in Binh Dinh Province (now Gia Lai Province since July 2025), which occurred in the same year as Vietnam's first confirmed ZIKV cases, presents a notable opportunity to investigate arboviral co-circulation and diagnostic challenges in the region [21, 25]. Most hospitalized patients in our study had secondary dengue infections, with their significantly higher median age supporting a higher likelihood of prior dengue exposure. Secondary infections have been associated with more severe clinical outcomes, characterized by heightened inflammatory responses that contribute to increased vascular permeability, plasma leakage, and multi-organ involvement [19]. In our cohort, secondary infections were associated with elevated levels of high-sensitivity CRP, a nonspecific but sensitive biomarker of systemic inflammation. Elevated CRP may reflect the intensity of immune activation in dengue infection, which can trigger a cytokine storm characterized by an exaggerated release of pro-inflammatory cytokines, and is often associated with poor prognosis [26]. Although patients were enrolled within 7 days of fever onset, the exact day of illness onset was not uniformly recorded. This limits the resolution with which IgM/IgG ratio dynamics can be interpreted and precludes accurate assessment of disease

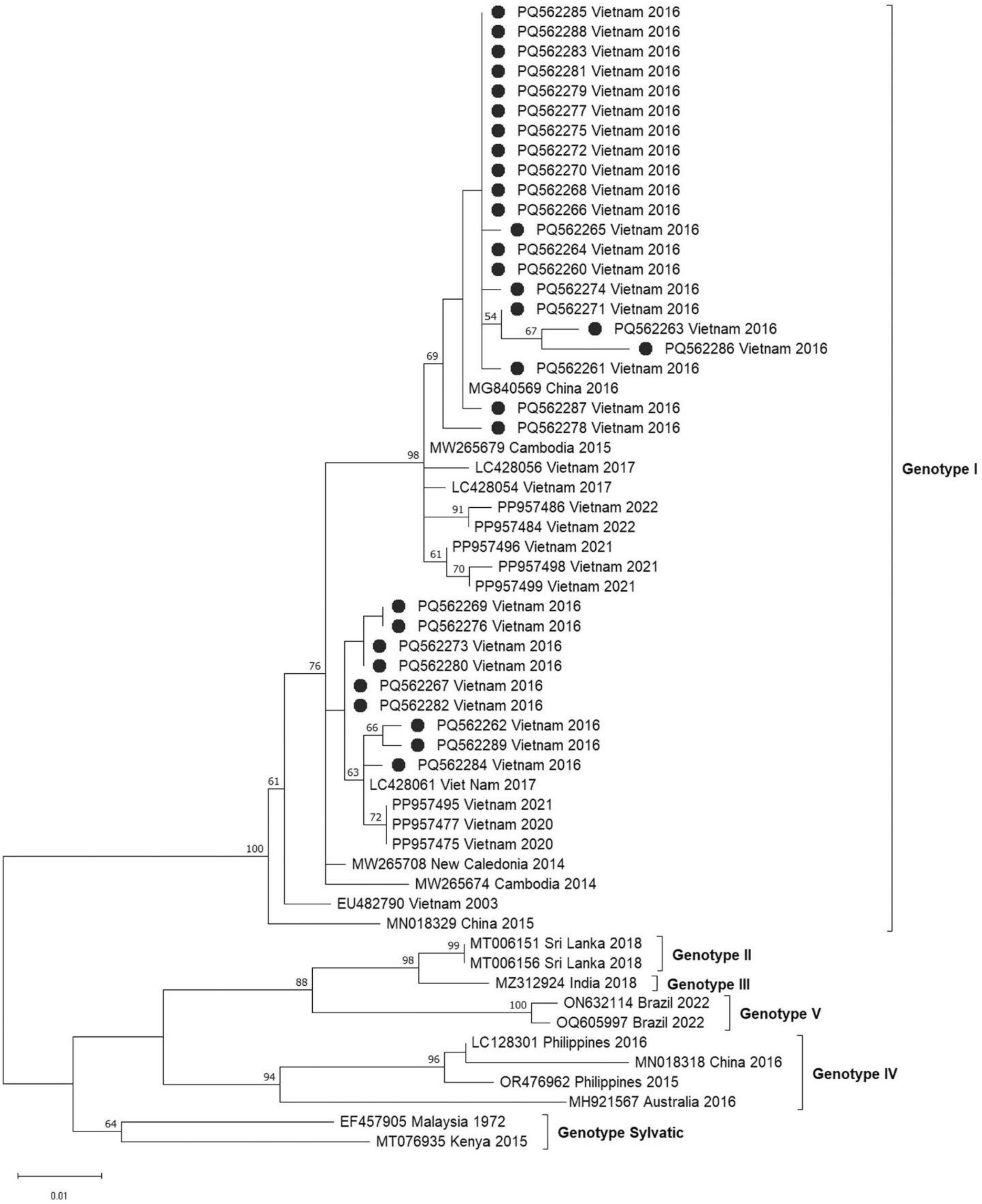

FIGURE 1 | Phylogenetic analysis of dengue virus serotype 1. The phylogenetic tree was reconstructed using the maximum likelihood method based on the Kimura 2-parameter model with 1000 bootstrap iterations. The scale bar indicates a sequence divergence of 0.1. The sequence obtained from this study is highlighted with black circle.

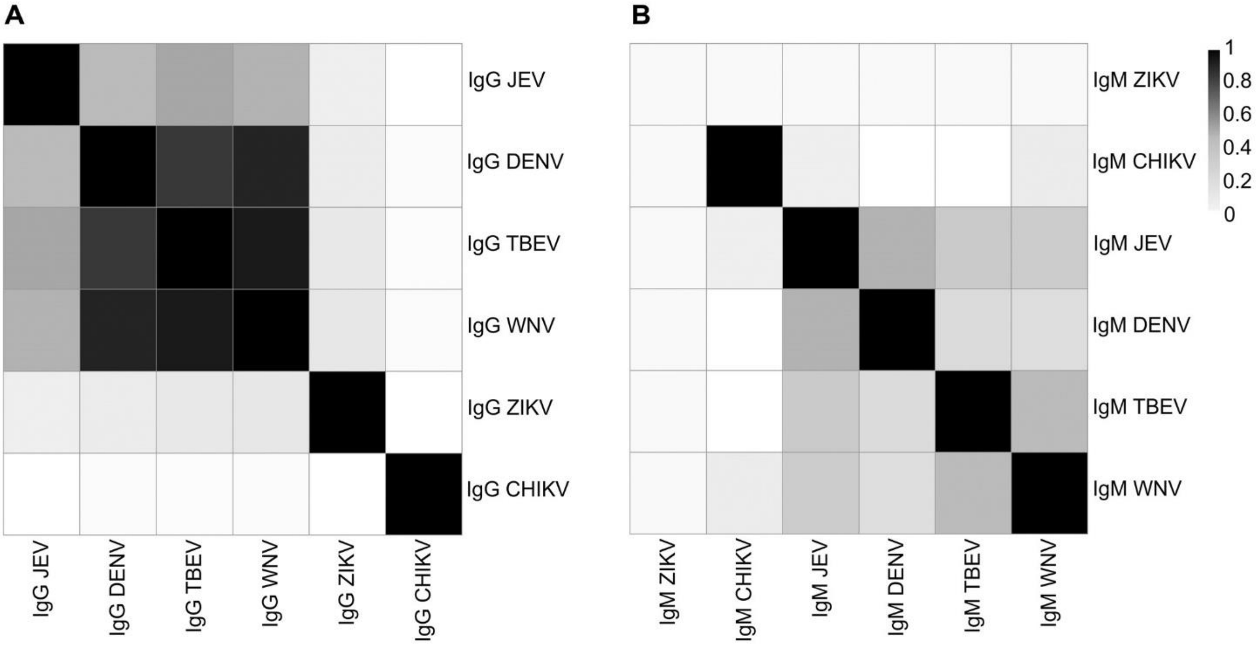

FIGURE 2 | Correlation of IgG (A) and IgM (B) antibody responses. CHIKV, Chikungunya virus; DENV, dengue virus; IgG, immunoglobulin G; IgM, immunoglobulin M; JEV, Japanese encephalitis virus; TBEV, Tick-borne encephalitis virus; WNV, West Nile virus; ZIKV, Zika virus.

severity or correlation with specific clinical manifestations in this cohort. Furthermore, the small sample size and group imbalance constrain the generalizability of our findings, particularly in comparisons involving PLT, HCT, and liver enzymes, which are key indicators in dengue pathophysiology.

DENV-1 accounted for 86% of all infections in our study. This serotype has been frequently associated with symptomatic dengue and has been implicated in more severe clinical outcomes in both primary and secondary infections [27, 28]. In Vietnam, DENV-1 and DENV-2 have been the predominant circulating serotypes over the past decade, with a shift from DENV-1 to DENV-2 observed during the 2018–2019 period across the country [29, 30]. While DENV-2, DENV-3, and DENV-4 were also detected in our study, their prevalence was considerably lower. Although DENV-1 was the predominant serotype identified in this cohort, the detection of DENV-2, DENV-3, and DENV-4, at low levels suggests continued circulation of less prevalent serotypes, which may act as hidden reservoirs for future outbreaks. Previous studies have demonstrated that such serotypes can rapidly displace the dominant serotype, particularly when shifts in population-level immunity create ecological niches for resurgence [9, 29]. These transitions may be further influenced by ADE, which can exacerbate both viral transmission and disease severity upon secondary infection.

Notably, we identified one case of co-infection with DENV-3 and DENV-4, highlighting the genetic diversity and simultaneous circulation of multiple serotypes in the region. While co-infections with multiple DENV serotypes were not commonly detected, even isolated occurrences may have significant evolutionary implications, including the potential for genetic recombination or reassortment, which could lead to the emergence of novel viral variants with altered pathogenicity, transmissibility, or immune escape capacity [31]. These findings reinforce the need for high-resolution genomic surveillance, particularly in hyperendemic regions, where overlapping epidemics and fluctuating serotype dominance increase the complexity of outbreak prediction and control.

To better understand DENV-1 circulation, we performed phylogenetic analysis using a fragment of the capsid-premembrane gene. All DENV-1 sequences belonged to genotype I, a lineage introduced into Vietnam from Thailand in the 1980s–1990s and later from Cambodia in the 2000s [29]. Our sequences clustered with Vietnamese isolates from 2003, 2017, and 2020–2022, indicating sustained local circulation. Although data from southern Vietnam during 2016–2017 are limited, DENV-1 genotype I was also the dominant strain in a major northern Vietnam outbreak in 2017 and persisted through 2022 [7, 30]. The dominance of a particular DENV strain is difficult to predict, as it can be influenced by various factors, including climatic and ecological conditions, human mobility, population immunity, and vector transmission dynamics [32]. Since shifts in circulating DENV strains can significantly influence outbreak intensity, these complexities underscore the importance of continuous molecular surveillance to strengthen outbreak preparedness and enable timely public health responses. Due to sample limitations, we were unable to perform sequencing for the DENV-2, DENV-3, and DENV-4 cases identified in this study. This limitation restricts our ability to comprehensively characterize the genotypic diversity and evolutionary relationships of all circulating DENV serotypes during the 2016 outbreak.

Vietnam is endemic for multiple flaviviruses, many of which co-circulate and share overlapping clinical features, making accurate diagnosis particularly challenging during dengue outbreaks [2, 16]. Our study revealed that IgG and IgM antibodies to JEV, WNV, and TBEV were frequently detected in dengue-confirmed patients with identified DENV serotypes, suggesting that these anti-JEV/WNV/TBEV serological positives likely reflect cross-reactivity or limited specificity of the assays rather than true co-infections or prior exposures to JEV, WNV, or TBEV. The rarity and sporadic reporting of human cases involving these viruses in Vietnam further supports this interpretation [15–18]. Nonetheless, in the case of JEV, widespread vaccination since 1997 may have contributed to the high IgG seroprevalence observed in our study population, particularly given the median age of overall patients of 19 years [17]. These uncertainties in distinguishing between cross-reactive IgG responses and true past exposures highlight the need for confirmatory testing, such as virus-specific neutralization assays, to better clarify the sero-epidemiological landscape of flaviviruses in the region. The lack of neutralization assays, such as the plaque reduction neutralization test (PRNT), is a limitation of this study, as it restricts our ability to differentiate true past flavivirus exposure from cross-reactive antibody responses. Future studies should incorporate PRNT or NS1-based, serotype-specific ELISAs to enhance the accuracy of seroprevalence estimates and to clarify infection histories, especially in regions with known co-circulation of multiple flaviviruses.

In contrast, IgM responses demonstrated higher virus specificity, as reflected by lower correlations in seropositivity between different flaviviruses compared to IgG responses. This likely originates from the early-phase nature of IgM production by naïve B cells, which have not yet undergone somatic hypermutation or affinity maturation, resulting in reduced cross-reactivity [33, 34]. Particularly, our observation of absent anti-ZIKV IgM and significantly lower anti-ZIKV IgG seropositivity in all dengue cases supports the hypothesis that ZIKV elicits less cross-reactive responses with DENV than other flaviviruses, consistent with previous reports [35]. These findings highlight the diagnostic utility of IgM in identifying recent infections and improving specificity in resource-limited settings. It is important to consider that co-infections or prior exposures to different flaviviruses may contribute to broader IgM positivity, potentially affecting diagnostic interpretation. However, this possibility was beyond the scope of this study and warrants further investigation.

Recent studies have highlighted that serological cross-reactivity among flaviviruses can not only confound diagnosis but also affect clinical outcomes [16, 20]. In individuals with prior flavivirus exposure or vaccination, non-neutralizing antibodies may contribute to enhanced immune responses during secondary DENV infection, potentially increasing disease severity and hospitalization risk. Emerging evidence further suggests that immune priming from unrelated viral infections, such as SARS-CoV-2 may modulate host immune responses during dengue infection, although the exact mechanisms remain incompletely understood [36, 37]. These findings underscore the importance of integrated immunological surveillance in regions where flaviviruses and other viral pathogens co-circulate.

In our study, we were unable to determine the DENV serotype in 10 patients, and several factors may account for this. One possibility is misdiagnosis, as some patients may have been infected with other flaviviruses such as JEV or TBEV (Supporting Information S1: Table S2), which can elicit cross-reactive antibodies and result in false-positive dengue serology [38, 39]. Another explanation is that these patients may have presented during the later stages of dengue infection, when viral RNA levels typically fall below detectable limits, leading to false-negative PCR results (Supporting Information S1: Table S2). Real-time RT-PCR is highly effective for detecting acute DENV infections during the early phase of illness and is considered the gold standard for dengue diagnosis, with optimal sensitivity within the first 2–7 days of symptom onset [40, 41]. In contrast, IgM ELISA played an important role by detecting recent infections that may have been missed by PCR due to declining viremia at the time of sample collection, as IgM antibodies typically begin to rise after Day 4–5 of illness and persist during the convalescent phase [10].

An additional limitation of our study is the extended storage duration of blood samples, which were collected during the 2016 outbreak and stored for several years before being subjected to research analyses. Even under optimal storage conditions, long-term preservation may lead to partial degradation of viral RNA or reduced antibody stability, which could affect the sensitivity and reliability of both molecular and serological assays. This factor may partly account for the non-confirmed DENV cases and the complex serological profiles observed in a subset of patients. Additional diagnostic approaches, such as PCR targeting highly conserved genomic regions or viral genome sequencing, may be beneficial for confirming the etiology of these cases and enhancing overall diagnostic accuracy [42]. These findings suggest that strengthening surveillance systems, particularly through the ongoing identification of circulating flaviviruses, will be essential for improving outbreak prediction, guiding public health responses, and mitigating the impact of future hemorrhagic fever epidemics.

5 | Conclusion

This study highlights the predominance of DENV-1 genotype I and the high proportion of secondary infections during the 2016 dengue outbreak in central Vietnam, with elevated high-sensitive CRP levels indicating a heightened inflammatory response in secondary infections. Extensive IgG cross-reactivity among flaviviruses, particularly DENV, JEV, WNV, and TBEV, complicated serological diagnosis, while IgM responses remained more virus-specific. These findings underscore the need for integrated molecular and serological surveillance strategies to improve diagnostic accuracy and support effective outbreak management in flavivirus-endemic regions.

Author Contributions

T.P.V., N.L.T. designed, supervised the study, and contributed to the study materials and assays. L.H.S. and T.P.V. were involved in the conceptualization and contributed to the study materials. D.D.A., D.H.L. performed the experimental procedures, statistical analysis, and validation of the results. D.T.H.D., N.T.H., N.H.L., and N.T.H.N. recruited the patients and contributed to the investigation materials for sampling procedures. D.A.A. and D.H.L. wrote the first draft. D.D.A., D.H.L, T.P.V., and P.G.K. reviewed the first draft. All authors have read and approved the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/, reference number PQ562260-PQ562289. All data are available in the main text or the supplementary materials.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supplementary Table1: Correlation Matrix of IgG and IgM antibody responses. **Supplementary Table2:** Serological Profile of Samples without Identified DENV Serotype.

Publication No.3

Diagnostic challenges of arboviral infections and dengue virus serotype distribution in febrile patients in East Java, Indonesia

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Diagnostic challenges of arboviral infections and dengue virus serotype distribution in febrile patients in East Java, Indonesia

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ABSTRACT

Objectives: Arboviruses pose significant public health threats worldwide, with Southeast Asia being a hotspot for these infections. This study aimed to reassess the incidence of dengue, Zika, and chikungunya viruses in patients clinically diagnosed with dengue in East Java, Indonesia in 2023.

Methods: The study included 108 patients admitted to hospitals in Jember, with blood samples collected on admission. Multiplex reverse transcription-polymerase chain reaction was used to detect viral RNA for dengue, Zika, and chikungunya, whereas dengue serotypes were identified using real-time polymerase chain reaction.

Results: A total of 67 of 108 (62%) patients tested positive for dengue virus (DENV), one patient tested positive for chikungunya, and no cases of Zika were detected. Differences in laboratory parameters between patients who were DENV RNA–negative and confirmed dengue cases suggest possible misdiagnosis of dengue. Serotyping of DENV-positive samples revealed DENV serotype 3 as the predominant serotype in Jember, accounting for 34% of cases (n = 23 of 67), followed by DENV serotype 1 and DENV serotype 2 at 19% each (n = 13 of 67) and 6% for DENV-4 (n = 4 of 67), whereas 21% (n = 14 of 67) remained untyped.

Conclusions: This study highlights the nature of the dengue outbreak in Jember in 2023, where all four DENV serotypes were in circulation, and underlines the need for serological or nucleic acid-based methods to improve arbovirus diagnosis in the region.

Introduction

Vector-borne diseases account for over 17% of all infectious diseases worldwide, representing a significant public health threat [1]. In the Americas and Southeast Asia, the co-circulation of dengue, chikungunya, and Zika has emerged as an escalating epidemiological risk, marked by increasing case numbers, complications, and disease severity. These arboviral infections, transmitted primarily by *Aedes aegypti* and *Aedes albopictus*, can range from mild febrile illnesses to severe neurological complications. The overlapping transmission cycles of these viruses are especially common in Southeast Asia, where the warm, humid climate and high population density create optimal conditions for Aedes mosquitoes. This environment leads to frequent outbreaks, often peaking during the rainy season, which can place significant strain on already overburdened health care systems [2].

Dengue is hyperendemic in Southeast Asia, with cases increasing rapidly in the last decade [3]. Transmitted by the bites of *Aedes* mosquitoes, dengue is caused by one of four genetically distinct dengue virus (DENV) serotypes (DENV1-4). Severe forms of the disease, such as dengue hemorrhagic fever (DHF), were first documented in Indonesia during outbreaks in Jakarta and Surabaya in 1968 [4] and in Central Java in 1976, with the latter linked to DENV-3 [5]. In Indonesia,

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recurring dengue outbreaks, along with fluctuations in co-circulating DENV serotypes, continue to pose a major public health challenge and contribute to significant mortality [6,7]. Notably, DENV-4 emerged as the predominant serotype during the 2019-2020 outbreak in Jember, East Java [8].

Reports of Zika virus (ZIKV) circulation in Southeast Asia remain limited [9]. The first documented case of Zika in Indonesia dates to 1981 in Central Java [10], where patients were asymptomatic, complicating the diagnosis of febrile illnesses [11]. Sporadic reports of travelers returning from Indonesia with Zika infections further suggest underdiagnosis in the region [12,13]. The clinical overlap of Zika with other prevalent febrile illnesses, such as dengue and chikungunya, further complicates accurate diagnosis in Indonesia.

Chikungunya virus (CHIKV), of the *Alphavirus* family, is endemic to Africa and Asia. Transmitted by *Aedes* mosquitoes, it generally causes a mild, self-limiting illness. Indonesia experienced significant chikungunya outbreaks between 2009 and 2010, with 137,655 reported cases nationwide [14]. A considerable proportion of chikungunya, Zika, and dengue infections may present asymptomatically or with mild symptoms. When symptoms are present, they are often non-specific among the three etiologies: headache, myalgia, arthralgia, rash, and retro-orbital pain, making clinical differentiation difficult. Algorithms comparing clinical manifestations of these diseases have been proposed, but their sensitivity and specificity remain unvalidated [15].

Given this context, the current study re-evaluated febrile patients who presented with at least two clinical symptoms of viral hemorrhagic fever and were diagnosed as dengue cases by attending physicians. Using molecular diagnostics, the study reinvestigated the presence of dengue, Zika, and chikungunya and characterized the circulating pathogens by serotype.

Materials and methods

Study design and sample collection

A total of 108 febrile patients diagnosed with dengue, admitted to four hospitals in Jember, East Java Province, Indonesia, between January and July 2023, were included in this study. At the local hospitals, patients with dengue were diagnosed solely on the basis of clinical signs and symptoms. Inclusion criteria were febrile patients with a body temperature of ≥38°C for less than 7 days, accompanied by at least two clinical symptoms of dengue, such as headache, myalgia, retro-orbital pain, arthralgia/bone pain, or rash, characterized based on the World Health Organization South-East Asia Regional Office 2011 guidelines [16] (https://iris.who.int/handle/10665/204894). Blood samples were collected, and plasma was separated and stored at −20°C for further analysis. Conventional hematologic tests were carried out on admission.

Ethical approval and consent to participate

Written informed consent was obtained from all hospitalized patients and/or their relatives, and from parents if subjects were under 18 years old. This study was approved by the research ethics committee at the University of Jember, Indonesia, with the reference number 1854/UN25.8/KEPK/DL/2023.

RNA extraction and detection of dengue, Zika, and chikungunya viruses

Viral RNA was extracted from 140 µl of plasma using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of the extracted RNA was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was then tested for the presence of DENV, ZIKV, and CHIKV RNA using the Fast Track Diagnostics (FTD) Zika/Dengue/Chikungunya Multiplex reverse transcription-polymerase chain reaction (RT-PCR) kit (Siemens Healthineers Company, Luxembourg). Briefly, the polymerase chain reaction (PCR) cycle conditions for FTD screening were as follows: reverse transcription at 50°C for 15 minutes, initial denaturation at 94°C for 1 minute, followed by 45 cycles of 8 seconds at 94°C and 1 minute at 60°C. Two master mixes were prepared: Master Mix 1 for ZIKV and Master Mix 2 for DENV and CHIKV. The reaction mix contained 1 µL of enzyme, 12.5 µl of buffer, and 1.5 µl of primer and probe. The mix was pipetted into a 96-well PCR plate, with 9 µl of sample and 1 µl of internal control. The plate was briefly mixed, and real-time PCR was performed using the LightCycler 480 Instrument II (Roche, Basel, Switzerland).

DENV serotyping

DENV serotypes were identified using RealStar Dengue Type RT-PCR kit 1.0 (Altona Diagnostics, Hamburg, Germany) according to the manufacturer's instructions. Briefly, the PCR cycle conditions for DENV serotyping were as follows: reverse transcription at 55°C for 20 minutes, initial denaturation at 95°C for 2 minutes, followed by 45 cycles of 15 seconds at 95°C and 45 seconds at 55°C and 15 seconds at 72°C. Two master mixes were prepared: Master Mix 1 for DENV-1/4 and Master Mix 2 for DENV-2/3. The reaction mix contained 5 µl of Master A, 15 µl of Master B, and 1 µl of internal control 1. A total of 20 µl of the reaction mix was pipetted into a 96-well PCR plate, with 10 µl of sample. Reverse transcription and amplification cycles were conducted using the LightCycler 480 Instrument II (Roche, Basel, Switzerland).

Statistical analysis

Categorical variables were reported as absolute counts and were compared using chi-square tests. Quantitative variables were presented as medians with ranges, and comparisons were made using Student's *t*-test or Mann–Whitney U tests as appropriate. The normality of distribution of the quantitative variables was tested using the Shapiro–Wilk test. All statistical analyses were conducted using R software version 4.3.2 (http://www.r-project.org). $P < 0.05$ was considered statistically significant for statistical comparisons in the study.

Results

Detection of dengue, Zika, and chikungunya viruses

A total of 67 of 108 (62%) samples tested positive for DENV RNA, and one sample (1%) tested positive for CHIKV RNA using Multiplex RT-PCR. All tested samples were negative for ZIKV RNA. A total of 40 samples (n = 40; 37%) tested negative for DENV/ZIKV/CHIKV RNA (patients without DENV/ZIKV/CHIKV).

Patient characteristics

Available data of the enrolled patients are summarized in Table 1. A total of 50 patients were male (46%) and 58 were female (54%), indicating an almost equal representation of both sexes in the study population. There was no significant difference in sex distribution between patients with confirmed infectious etiology (DENV) and patients without DENV/ZIKV/CHIKV. The clinical data and the age of the patients were not available for the analysis.

Hematologic profile

The conventional blood laboratory tests from 70 of 108 patients were available for analysis. The results showed that the red blood cell count was within the normal range for the majority of patients (median [range] red blood cell = 4.67 [3.01-6.24]). However, the white blood cell (WBC) count was slightly decreased in overall, with the median

Table 1
Laboratory parameters of patients.

	Available patients (n = 70)	Non-DENV/ZIKV/CHIKV (n = 26)	DENV (n = 43)	CHIKV (n = 1)	*P*-value*
Sex (Male/Female)	40/30	14/12	25/18	Male	0.921
RBC ($\times 10^6/\mu l$)	4.67 [3.01, 6.24]	4.53 [3.01, 5.70]	4.78 [3.52, 6.24]	4.67	0.079
HGB (g/dl)	3.4 [8.7, 18.4]	13.0 [8.7, 15.8]	13.7 [9.20, 18.4]	13.1	0.065
HCT (%)	39.3 [25.6, 53.6]	37.8 [25.6, 46.5]	40.8 [26.5, 53.6]	37.8	**0.039**
WBC ($\times 10^6/\mu l$)	4.75 [1.2, 21.4]	5.70 [1.20, 21.4]	4.70 [1.30, 15.1]	5.2	0.437
PLT ($\times 10^3/\mu l$)	105.0 [13.0, 244.0]	119 [41.0, 244]	96.0 [13.0, 187]	119	0.160
LYM (%)	33.5 [3.0, 71.0]	29.0 [3.0, 56.0]	35.5 [8.0, 71.0]	32	0.388
MONO (%)	10.0 [2.0, 33.0]	11.0 [2.0, 29.0]	9.50 [6.0, 33.0]	7	0.960
EO (%)	0.0 [0.0, 14.0]	0 [0, 14.0]	0 [0, 9.0]	0	0.629
BASO (%)	1.0 [0.0, 4.0]	1.0 [0, 3.0]	1.0 [0, 4.0]	2	0.176
NEUT (%)	54.5 [10.0, 94.0]	57.0 [23.0, 94.0]	52.0 [10.0, 84.0]	59	0.512

BASO, basophil percentage; CHIKV, Chikungunya virus; DENV, Dengue virus; EO, eosinophil percentage; HCT, hematocrit; HGB, hemoglobin; LYM, lymphocyte percentage; MONO, monocyte percentage; NEUT, neutrophil percentage; RBC, red blood cell count; PLT, platelet count; WBC, white blood cell count; ZIKV, Zika virus.
Available data from 70 patients; variables are presented in median with range [min, max].
[a] *P*-value from the statistical comparison between the non-DENV/ZIKV/CHIKV group and the DENV group using the chi-square test, Student's *t*-test, or the Mann-Whitney U-test, as appropriate.

WBC count close to the lower limit of the physiological range (median [range] WBC = 4.75 [1.2-21.4]), indicating possible viral infections.

In addition, some patients presented elevated levels of monocytes and eosinophils, suggesting chronic infection and/or inflammation (Table 1). Six patients (n = 6 of 70) had anemia, defined as hemoglobin levels below 12 g/dl, whereas a significant number (n = 62 of 70) had thrombocytopenia, with a platelet (PLT) count below 150,000 PLT/μl. The low median platelet count suggests the presence of DHF, with possible development of severe thrombocytopenia (Table 1).

Notably, comparing patients with confirmed DENV with patients without DENV/ZIKV/CHIKV, we found that hematocrit (HCT), an important marker of DHF, was significantly higher in patients with confirmed DENV (median = 40.8 [26.5-53.6]) than in the non-DENV/ZIKV/CHIKV group (median = 37.8 [25.6-46.5]) (P = 0.039). A higher median monocyte count was also observed in the non-DENV/ZIKV/CHIKV group than patients with confirmed DENV, although this difference was not statistically significant.

The patient with confirmed CHIKV infection had laboratory values within the normal range, except for a low platelet count (PLT = 119 $\times 10^3/\mu l$) indicating mild thrombocytopenia.

Distribution of DENV serotypes

DENV-positive samples (n = 67 of 108) were subjected to serotype differentiation using the RealStar Dengue Type RT-PCR kit. The results indicated that n = 13 of 67 (19%) samples were identified as DENV-1, n = 13 of 67 (19%) samples as DENV-2, n = 23 of 67 (34%) samples as DENV-3, and n = 4 of 67 (6%) samples as DENV-4. In addition, n = 14 of 67 (21%) samples could not be assigned to any of the four DENV serotypes using RT-PCR methods (Table 2). No significant differences in the hematologic profile of patients were observed between the four DENV serotypes.

Table 2
Distribution of dengue, Zika, and chikungunya viruses among the patients.

Arbovirus detected	Febrile patients n =108 (%)
Dengue (DENV)	67 (62%)
DENV-1	13 (19%)
DENV-2	13 (19%)
DENV-3	23 (34%)
DENV-4	04 (06%)
Unidentified dengue serotypes	14 (21%)
Chikungunya	1 (1%)
Zika	0 (0)
Negative for dengue, Zika, and chikungunya	40 (37%)

DENV-1, 2, 3, 4: dengue virus serotype 1, 2, 3, and 4.

Discussion

The results of this study provide insights into the ongoing co-circulation of dengue and chikungunya in Jember, Indonesia and highlight the complex landscape of arboviral infections in this hyperendemic region. Dengue remains a significant public health concern, with 62% of suspected cases testing positive and DENV-3 emerging as the predominant serotype during the 2023 outbreak. This is in line with previous studies that have shown fluctuations in serotype predominance in Indonesia over the years [6,7]. Although DENV-4 was predominant during the 2019-2020 outbreak in the region [8], our current study shows a shift toward DENV-3, emphasizing the dynamic nature of dengue transmission in Indonesia.

The absence of ZIKV detection is consistent with limited reports of Zika circulation in Southeast Asia [9]. However, the asymptomatic or mild presentation of Zika infections raises concerns about potential underdiagnosis, particularly, in regions such as Indonesia where clinical resources may be limited, and Zika symptoms overlap with other arboviral infections. The unspecific clinical symptoms of dengue, Zika, and chikungunya complicates accurate clinical diagnosis, further emphasizing the need for molecular diagnostics in such settings. This study identified a case of chikungunya infection, indicating the ongoing circulation of the virus. Previous research has also highlighted that chikungunya continues to be an underdiagnosed cause of acute febrile illness in Indonesia [17].

The hematologic findings in this study align with known markers of dengue infections. Most notably, the significantly higher HCT levels observed in patients with confirmed DENV compared with non-DENV/ZIKV/CHIKV cases support the utility of HCT as a diagnostic marker for DHF. In addition, the widespread occurrence of thrombocytopenia in DENV patients is consistent with typical clinical presentations of dengue and is a well-established marker for identifying potential severe cases. The high prevalence of thrombocytopenia also suggests that some patients may have progressed to severe dengue, warranting close clinical monitoring. However, the clinical data of the patients were not available for the analysis. To confirm DENV infections and prevent misdiagnosis, laboratory diagnostics are essential [18], as demonstrated by the significantly lower HCT levels in patients without detectable DENV/ZIKV/CHIKV RNA than in patients with confirmed DENV. Rapid diagnostic tests (for non-structural protein 1, immunoglobulin G, and immunoglobulin M) can reduce false positives despite cross-reactivity within the Flaviviridae family [19] and aid in classifying primary and secondary dengue infections [20], which can support disease management. Furthermore, nucleic acid testing for DENV detection and serotype identification offer high specificity and sensitivity, making these methods valuable, particularly, during the early phase of infection [21].

Globalization has been linked to the rapid spread of diseases, whereas climate change has contributed to the geographic expansion of arthropod-borne vectors [22]. Reports have highlighted ZIKV, yellow fever virus, Japanese encephalitis virus, West Nile virus, tick-borne encephalitis virus, and DENV as predominant arboviral infections. Indonesia, a hyperendemic country for dengue, has seen a rising number of cases over the past decade [23], with 143,266 cases reported in 2022 across 467 cities and regions, resulting in an incidence rate of 42.25 per 100,000 population. Although research on DENV is well-documented in Surabaya, one of Indonesia's largest cities, there is a lack of studies in other areas, including Jember Regency, the third largest city in East Java. Reports show that Jember experienced the highest number of cases in 2019 (988 cases), with a significant decline in 2021 (447 cases), followed by an increase to 781 cases in 2022 [8]. The highest cases in Jember were reported in Kaliwates, where our study samples were collected.

The factors influencing dengue severity include host and viral characteristics, particularly, the infecting DENV serotype [24]. In Indonesia, DENV-3 caused dengue epidemics since its identification in 1970 until it was displaced by DENV-1 and DENV-2 in the early 2000s [23]. Although all DENV serotypes are endemic in Indonesia, DENV-3 has been reported to cause severe infections, including encephalitis [25]. In addition, the predominance of DENV serotypes varies across the Southeast Asia from 2021 to 2023, with Vietnam reporting DENV-2 [26], Singapore reporting DENV-3 [27], and Malaysia reporting DENV-4 [28]. This diversity raises concerns because shifts in predominant serotypes could impact the intensity of future outbreaks due to the phenomenon of antibody-dependent enhancement [29].

One of the key limitations of this study is the inability to assign 21% of DENV-positive samples to any of the four serotypes, which may be attributed to low viral loads in these samples. Further investigation using next-generation sequencing or alternative molecular techniques may be necessary to better understand the genetic diversity of circulating DENV serotypes. Furthermore, our study focused only on symptomatic patients presenting at local hospitals, which limits the generalizability of the findings. Therefore, further studies on dengue seroprevalence and vector surveillance could provide a broader and more comprehensive epidemiological overview of dengue and other arboviruses.

Conclusion

In conclusion, this study emphasizes the need for ongoing surveillance and molecular diagnostics to accurately detect and differentiate between arboviral infections in hyperendemic regions such as Indonesia. The shift in DENV serotype predominance, together with the potential for co-infections and misdiagnosis, underscores the need to improve diagnostic capabilities. This improvement is not only important to optimize patient outcomes but also to effectively support public health.

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Ethical approval

Written informed consent was obtained from all hospitalized patients and/or their relatives and from parents if subjects were under 18 years old. This study was approved by the research ethics committee at the University of Jember, Indonesia, with the reference number 1854/UN25.8/KEPK/DL/2023.

Author contributions

TPV and KS conceptualized and designed the study. TPV contributed to the study materials and assays. RR recruited the patients. LMS, DDA, NI, and TTTH performed the experimental procedures. DDA and HVT were involved in the statistical analysis and validation of the results. TNM, TPV, RO, TTTH, and LHS supervised experimental procedures. TPV, HVT, and DDA wrote the first draft. All authors have read and approved the manuscript.

Declarations of competing interest

The authors have no competing interests to declare.

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2.2 Chapter 2: Host and Immune Determinants of Dengue Severity

Publication No.4

Characterization of dengue patients in Vietnam: Clinical, virological, and IL-10 profiles during 2021- 2022 outbreaks

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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Characterization of dengue patients in Vietnam: Clinical, virological, and IL-10 profiles during 2021– 2022 outbreaks

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Abstract

Background

The pathogenesis of dengue is attributed to a complex interaction between the dengue virus (DENV) and the host immune system. The aim of this study is to investigate the clinical, virological, and Interleukin-10 (IL-10) profiles of dengue patients in Vietnam from two consecutive outbreaks in 2021 and 2022.

Methods

A total of n=306 dengue patients were examined, who were clinically stratified according to dengue without warning signs (DF; n=178), dengue with warning signs (DWS; n=115) and severe dengue (SD; n=13). Patients were screened for dengue, Zika and chikungunya viruses. DENV were subjected to serotype specific real-time RT-PCR. Interleukin-10 (IL-10) levels were measured by ELISA, and *IL-10* promoter variants (-1082G/A; -819C/T; -592C/A) were genotyped by direct Sanger sequencing to determine a possible association with susceptibility to dengue and disease severity.

Results

No chikungunya or Zika viruses were detected. Patients were infected by one of the three different DENV serotypes (DENV-1, -2, -4). Plasma IL-10 levels were significantly elevated in patients (DF vs. DWS, p=0.004; DF vs. SD, p=0.001; DWS vs. SD, p=0.015). While the *IL-10* allele -819C contributed to an increased risk of dengue (OR = 1.5, 95% CI = 1.1-2.0, p=0.04), genotype -1082GA showed a protective role against the disease (OR = 0.45, 95% CI = 0.27-0.72, p=0.009), and allele -1082G showed a protective role against DWS (OR = 0.44, 95% CI = 0.22-0.81, p=0.049). Also, the *IL-10* GTA (-1082G/-819T/-592A) haplotype was observed to confer protection (OR = 0.31, 95% CI = 0.14-0.67, p< 0.003).

Data availability statement: The authors confirm that the data supporting the findings of this study are available within the article.

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Competing interests: The authors have declared that no competing interests exist.

Conclusion

While DENV-1 and DENV-2 were the predominant serotypes in circulation, plasma IL-10 levels and *IL-10* promoter variants were also significantly associated with dengue and its severity.

Author summary

Dengue places a significant burden especially in low- and middle-income countries, including Vietnam. The pathogenesis of dengue is attributed to a complex interaction between the dengue virus and the host immune system. We explicitly investigated on the occurrence of arboviruses (CHIKV, DENV and ZIKA) during two consecutive outbreaks in 2021 and 2022 from Northern Vietnam and compared the results with those of previous outbreaks. From the study cohort of cases of dengue without warning signs, dengue with warning signs and severe dengue, we investigated whether levels of the human cytokine IL-10 and *IL-10* promoter variants were associated with susceptibility and with their clinical course. The results show that DENV-1, DENV-2 and DENV-4 were the circulating serotypes in 2021-2022. Serotypes DENV-1 and DENV-2 were more frequently observed in dengue cases with warning signs and in severe dengue, while no cases of chikungunya or Zika viruses were detected in the studied period. Plasma IL-10 levels and *IL-10* promoter variants were significantly modulated in patients with varying degrees of severity.

Introduction

Dengue, caused by dengue virus (DENV), is a mosquito-borne viral disease that is a major public health problem in Southeast Asia. While the Americas and Asia carry >70% of the global dengue burden, the number of dengue cases has increased more than twofold in the last decade, from 2010 to 2019 [1]. Dengue is caused by one of the four genetically distinct serotypes (DENV-1,2,3 and -4) and the pathogenesis is influenced by a complex interplay between DENV serotypes, host factors and differential host immune responses.

While infections with DENV-2 have a higher tendency to develop severe dengue than other serotypes [2,3], host factors such as innate and adaptive immunity, antibody-dependent enhancement (ADE), cross-reactive memory T cells are also important determinants of dengue severity [4,5]. DENV-2 and DENV-3 were observed to elicit a stronger cytokine response than other serotypes [6], these differences in immunogenicity and pathogenesis are associated with distinct DENV serotypes.

Endothelial dysfunction leading to vascular leakage is the hallmark of severe dengue [7]. It is known that altered cytokine levels are associated with increased endothelial cell damage and plasma leakage [8]. Previous studies have reported that DENV infection can induce the production of interleukin-10 (IL-10) by monocytes, which dampens anti-DENV immune responses and virus control [9,10]. While elevated IL-10 levels have been shown to be associated with severe dengue [11] and to be a predictive marker for secondary DENV infection [12], the *IL-10* promoter variants rs1800872 (-592C/A) and rs1800871 (-819C/T), located on chromosome 1q31-32, have been shown to dysregulate IL-10 serum levels [13].

Population-specific genetic variations in *IL-10* are known to be associated with susceptibility and varying clinical courses of dengue. Therefore, the study of IL-10 levels and its genetic variants in the context of dengue is important to decipher the complex interplay between the host immune response and the DENV, and similarly provide insights about *IL-10* variants in the Vietnamese population. In this context, firstly, dengue patients with varying degrees of severity from two consecutive seasonal outbreaks (2021 and 2022) were screened for dengue, Zika and chikungunya viruses in this study cohort and DENV serotypes were studied. Secondly, IL-10 plasma levels were quantified, and *IL-10* variants were genotyped to determine a possible association with susceptibility to dengue and disease severity.

Materials and methods

Ethics statement

Written and signed informed consent was obtained from all study participants prior to enrolment. The study was approved by the Institutional Review Board of the 108 Military Hospital and by the University of Tübingen for the project 'Host and viral factors influencing dengue severity and susceptibility' (Ethics Approval No. 274/2022B02). The study has adopted and implemented the Nagoya Protocol and received approval for the utilization of genetic resources in Germany from the Vietnamese Ministry of Natural Resources and Environment (Reference: No.2995/QĐ-BTNMT).

Study premise and study population

The study employed a convenience sampling method, including patients who were admitted to the central hospital in Hanoi, Vietnam, during two consecutive seasonal dengue outbreaks between October 2021 and December 2022. A total of 306 civilian patients with symptoms of haemorrhagic fever admitted to the 108 Military Central Hospital in Hanoi, Vietnam, participated in the study. Patients with bacterial or other viral infections, chronic diseases or haematological disorders were excluded. The infection was diagnosed based on the diagnostic criteria for dengue according to the World Health Organization (https://apps.who.int/iris/handle/10665/44188) approved by the Vietnamese Ministry of Health [14] and the positivity of NS1 antigen or/and anti-DENV immunoglobulin M and G (anti-DENV IgM and IgG). Serological tests for dengue infection were carried out on admission. Blood samples from all dengue patients were collected at admission and plasma samples were separated and stored at -70°C. In addition, 200 µL of whole blood was collected in QIAcard FTA Indicating Mini (Qiagen GmbH, Hilden, Germany) and stored at room temperature. Similarly, blood samples from 300 healthy blood donors who tested negative for HBsAg, anti-HCV and anti-HIV were collected from the transfusion department.

Patients clinical parameters

Patients were categorized into three groups based on the 2009 WHO guidelines: Dengue without warning signs (DF), dengue with warning signs (DWS) and severe dengue (SD). Measurements of aspartate aminotransferase (AST), alanine aminotransferase (ALT), white blood cell (WBC) count, red blood cell (RBC) count, haematocrit (HCT), platelet count (PLT) and dengue diagnosis were conducted in real time at 108 Military Central Hospital in Hanoi.

Screening for dengue, Zika and chikungunya viruses

Total RNA was extracted from 140µL patient plasma using QIAmp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. To exclude other arboviruses and confirm dengue infection, all samples (n=306) were screened for dengue/Zika/chikungunya by multiplex real-time PCR using the Fast Track Diagnostics Kit (Siemens

Healthcare GmbH, Erlangen, Germany) on a LightCycler480-II (Roche, Mannheim, Germany) according to the manufacturer's instructions. All samples were tested in duplicate to ensure accuracy.

Dengue virus serotyping

All dengue positive patient DENV RNA samples (n=299/306) were serotyped using the RealStar Dengue Type real-time PCR kit 1.0 (Altona Diagnostics GmbH, Hamburg, Germany) following manufacturer's instructions, on a LightCycler480-II (Roche, Mannheim, Germany). All samples were tested in duplicate to ensure accuracy.

Quantification of interleukin-10

IL-10 levels were quantified from all DENV RNA-positive patient samples (n=299/306) using the Bio-Plex IL-10 Pro Human Cytokine Screening Panel (Bio-Rad Laboratories GmbH, Feldkirch, Germany) according to the manufacturers protocol in a Bio-Plex 200 system and the values were then quantified using Bio-Plex Manager 6.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

Genotyping of interleukin-10 variants

Genomic DNA was extracted from dried blood spots (n=299) from patients and blood pellets (n=300) from healthy controls using the commercially available QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The quality and quantity of extracted DNA were checked using the NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). The *IL-10* promoter region containing the polymorphisms rs1800896 (-1082A/G), rs1800871 (-819C/T), and rs1800872 (-592C/A) were amplified by PCR with specific primer pairs IL-10F 5′-GAA GAA GTC CTG ATG TCA CTGC-3′ (forward) and IL-10R 5′-TAG GTC TCT GGC CTT AGT TTC-3′ (reverse) [15].

In brief: PCR reactions were performed in 15µL reaction volume with 5 ng of genomic DNA, 1x HotStarTaq Master mix (Qiagen GmbH, Hilden, Germany), and 0.5 µM of each primer. The thermal cycling parameters were an initial denaturation at 95 °C for 15 minutes, followed by 35 cycles of denaturation (30 seconds at 94 °C), annealing (60 seconds at 62 °C), extension (60 seconds at 72 °C), followed by a final extension at 72 °C for 10 minutes. PCR amplicons were stained with SYBR green and run on a 1.2% gel electrophoresis gel. A ~760 bp product was visualized with a UV transilluminator. PCR products were purified using Exo-SAP-IT PCR (Applied Biosystems, Beverly, MA, USA) and purified amplicons were used as a sequencing template using the BigDye Terminator v.1.1 Cycle Sequencing Kit on an ABI 3130XL DNA sequencer (Applied Biosystems, Beverly, MA, USA). All *IL-10* sequences (~760 bp) were aligned to an *IL-10* reference gene (NG_012088.1) using Bio-edit 7.2 software (https://bioedit.software.informer.com/7.2/) and genotypes were labelled as either homozygous or heterozygous, which was visually confirmed using the respective electropherograms.

Statistical analysis

Data was analysed and visualized using the R software version 4.3.2 (http://www.r-project.org). A p-value < 0.05 was considered statistically significant. Clinical and demographic data were presented either as median or mean values with range for quantitative variables and absolute numbers with percent for categorical variables. The normality of distribution in the quantitative variables was tested using the Shapiro–Wilk test. Categorical data were compared using Chi-square or Fisher's exact tests, while continuous variables were compared using t-test or Kruskal-Wallis test as appropriate. Dunn's test was applied as post-hoc pairwise tests with

Bonferroni adjustment. The Benjamini-Hochberg Procedure was applied for multiple testing due to the increased risk of type I error with false discovery rate equal to 0.05.

Allele, genotype, or haplotype frequencies were determined by simple gene counting and deviations from Hardy-Weinberg equilibrium were tested. The association between IL-10 genetic variants and dengue was assessed using logistic regression, adjusting for age and sex, under three inheritance models: dominant (comparing homozygotes for the major allele to heterozygotes and homozygotes for the minor allele), recessive (comparing homozygotes for the major allele and heterozygotes to homozygotes for the minor allele), and over-dominant (comparing heterozygotes to homozygotes for both the major and minor alleles). The *IL-10* haplotypes were estimated using the R package "SNPassoc" [16] version 2.1.0 and "haplo.stats" [17] version 1.9.3. The linkage disequilibrium (LD) analysis was performed with the program Haploview v.4.1 (https://www.broadinstitute.org/haploview/haploview).

Results

Baseline characteristics of study subjects

The patients and healthy controls were from the Hanoi metropolitan area and were of Kinh ethnicity. Patients were stratified according to the severity of dengue: dengue without warning signs (DF) (n=178), dengue with warning signs (DWS) (n=115) and severe dengue (SD) (n=13). The demographic and clinical characteristics of the patients are summarized in Table 1. No significant differences in age and sex were observed (Table 1). Significant differences were observed in the days of fever before admission, blood parameters, liver enzymes and bleeding manifestations (Table 1). While NS1 positivity was not significant between the groups, IgM and IgG showed significant differences in the distribution between the analyzed groups (Table 1).

Dengue, Zika and chikungunya detection and dengue virus serotyping

Neither Zika nor chikungunya viral RNA was detected in any of the 306 tested cases. The multiplex PCR assay confirmed that 299/306 cases were positive for DENV RNA. Seven cases (7/306) were not recognized as DENV RNA-positive by the real-time PCR. Of the 299 DENV-positive cases, the DENV serotypes were determined in 280 samples (Fig 1a and Table 1). Three DENV serotypes (DENV-1,-2 and -4) were detected except for the DENV-3 serotype. The prevalence and dominance trend of DENV serotypes remained consistent during the two outbreaks (2021 and 2022), with DENV-2 being the most frequently detected, followed by DENV-1 and DENV-4. Co-infections with DENV-1 and DENV-2 (n=56, 20%) were predominant, followed by DENV-2 and DENV-4 co-infections (n=12, 4%). All three serotypes contributed to the severity of the infection. DENV-1, followed by DENV-2 and DENV-4 contributed to the severe cases. (Table 1).

Plasma IL-10 levels

Significant differences in IL-10 levels were observed: DF (median = 9.9 pg/mL, range [1.1 – 3060]), DWS (median = 18.0 pg/mL, range [1.4 – 183]) and SD (median = 46.3 pg/mL, range [8.3 – 128]). The distribution of IL-10 levels differed significantly between the study groups (DF vs. DWS, $p = 0.004$; DF vs. SD, $p = 0.001$; DWS vs. SD, $p = 0.015$) (Fig 2).

IL-10 variants with DENV infection, severity, and serotypes

All *IL-10* variants analyzed in patients and controls were in Hardy-Weinberg equilibrium. The association of the investigated variants was analyzed using different genetic models, including

Table 1. Patient characteristics on admission during seasonal dengue outbreaks.

Characteristics	Dengue without warning signs (DF) (n=178)	Dengue with warning signs (DWS) (n=115)	Severe dengue (SD) (n=13)	p-value
Median age (Range)	45 [12-86]	47 [15-82]	50.5 [19-80]	0.298**
Sex (Male/Female)	92/80	59/56	5/7	0.71*
Days of Fever Mean (±SD)	3.65 (1.56)	5.24 (1.33)	5.25 (0.866)	< 0.001**
Days of Fever Median [Range]	4.00 [1.00, 8.00]	5.00 [1.00, 8.00]	5.00 [4.00, 7.00]	< 0.001**
Leucocytes/μL Median [Range]	4.01 [0.930, 16.9]	3.71 [1.33, 11.6]	5.00 [1.45, 10.5]	0.799**
Lymphocyte (%) Median [Range]	22.8 [2.50, 72.8]	28.3 [6.10, 56.3]	21.0 [6.80, 53.0]	0.015**
Platelets ×10^3/μL Median [Range]	115 [9.00, 384]	20.0 [4.00, 228]	29.0 [4.00, 125]	< 0.001**
AST Median U/L [Range]	54.0 [15.1, 1210]	115 [16.0, 1040]	257 [31.0, 11100]	< 0.001**
ALT Median U/L [Range]	38.7 [8.00, 855]	66.9 [8.20, 636]	113 [25.6, 2190]	< 0.001**
Bleeding manifestation n (%)	34 (20%)	97 (84%)	9 (75%)	< 0.001*
Serotypes				
DENV-1 n (%)	37 (22%)	18 (16%)	5 (42%)	NA
DENV-2 n (%)	70 (41%)	71 (62%)	4 (33%)	NA
DENV-4 n (%)	3 (2%)	3 (3%)	1 (8%)	NA
DENV-1/2 n (%)	45 (26%)	11 (10%)	0 (0%)	NA
DENV-2/4 n (%)	7 (4%)	5 (4%)	0 (0%)	NA
Unidentified	10 (6%)	7 (6%)	2 (17%)	NA
Serological tests				
NS1 – positivity (%)	127 (74%)	82 (71%)	9 (75%)	0.881*
IgM – positivity (%)	62 (36%)	74 (64%)	5 (42%)	< 0.001*
IgG – positivity (%)	56 (33%)	70 (61%)	7 (58%)	< 0.001*

**P-values were calculated by Chi-square test;*

***P-values were calculated by Kruskal-Wallis test. Variables were summarized in Percentage, Mean (standard deviation) or Median [range]. AST: Aspartate aminotransferase; ALT: Alanine Aminotransferase; DENV: Dengue virus; NS1: non-structural protein; IgG: immunoglobulin G; IgM: immunoglobulin M.*

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allelic, dominant, recessive, and over dominant. The variant alleles -1082A, -819T, and -592A were predominant in the Vietnamese population (Table 2). Linkage disequilibrium (LD) analysis revealed a strong LD (D' > 0.90) between -819C/T and -592C/A. The frequency of allele -819C was significantly higher in dengue patients than in healthy controls (OR = 1.5, 95%CI = 1.1-2.0, p-value = 0.04) (Table 2). The frequency of genotype -1082GA was higher in healthy control than in patients (OR = 0.45, 95%CI = 0.27-0.72, p-value = 0.009), suggesting a protective effect (Table 2). The dominant model demonstrated a significant association between genotype -1082 GG+GA and a reduced risk of dengue (OR = 0.5, 95% CI = 0.31-0.8, p-value = 0.024). Conversely, the over-dominant model indicated a positive association between genotype -1082 GG+AA and dengue (OR = 2.28, 95% CI = 1.41-3.71, p-value = 0.009).

Association of *IL-10* variants with varying severity and DENV serotypes

Significant associations were only observed among DWS patients and controls (Table 3): the frequency of the -1082G allele was found higher in controls compared to DWS patients (OR = 0.44, 95% CI = 0.22-0.81, p-value = 0.049), suggesting that the IL-10 SNP rs1800896 -1082A/G is associated with protection from DWS. Additionally, the recessive model revealed that the genotype -1082GG was associated with protection from developing DWS (OR = 0.4, 95% CI = 0.19-0.77, p-value = 0.049), while the genotype -1082GA was associated with increased risk of DWS under the over-dominant model. No significant difference in IL-10 plasma levels between the *IL-10* variants was observed in our study.

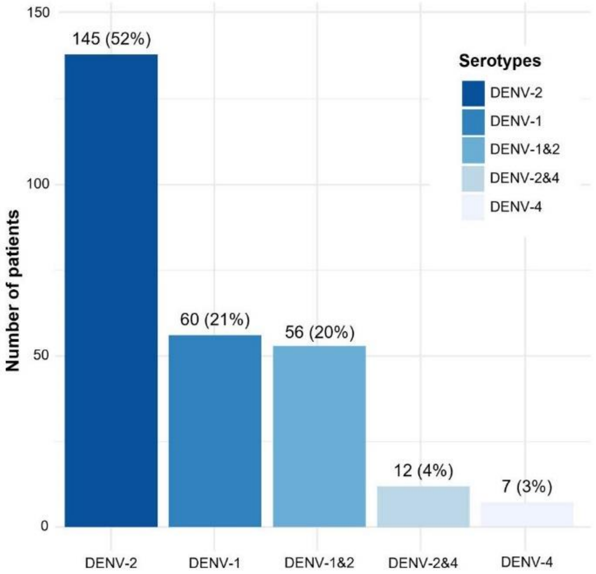

Fig 1. Distribution of DENV serotypes among patients. Data are presented as number of observations and percentages.

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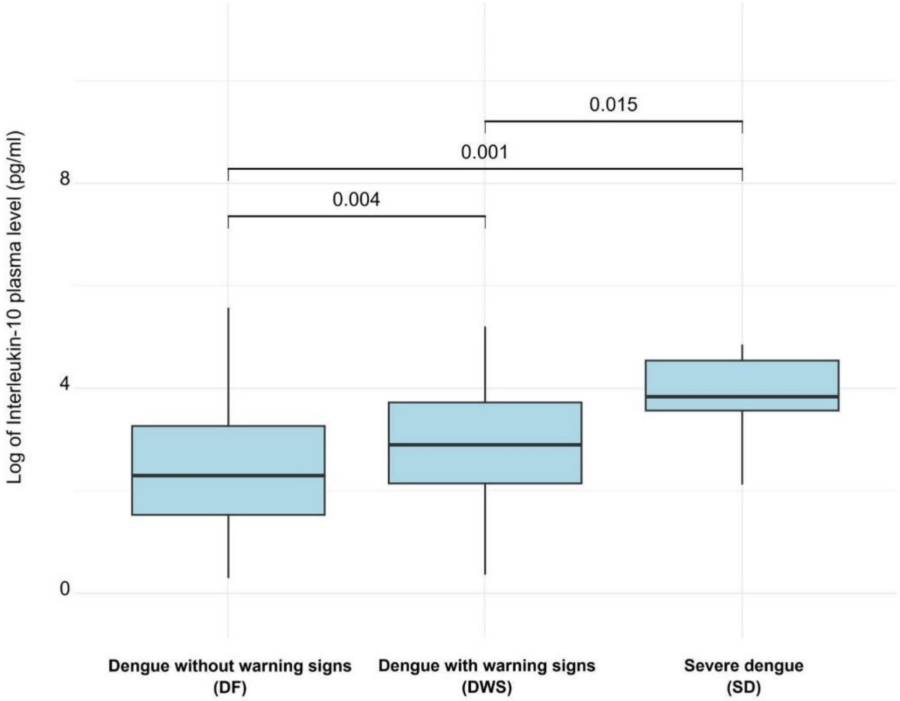

Fig 2. IL-10 plasma levels in dengue patients with different clinical severities.

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Table 2. Genotype and allele distribution of *IL-10* polymorphisms in dengue patients and healthy controls. Bold values indicate statistical significance. Odds ratio and p-value were calculated by using binary logistic regression models adjusted for age and sex. All p-values were adjusted by the Benjamini-Hochberg procedure.

SNP	Variable	Genotypes	DENV n = 287	Control n = 282	OR (95% CI)	p-value
rs1800872 -592C/A	Genotypes	AA	159 (55.4)	170 (60.3)	Ref	
		CA	102 (35.5)	97 (34.4)	1.19 (0.81-1.76)	0.398
		CC	26 (9.1)	15 (5.3)	1.96 (0.95-4.17)	0.130
	Allele	A	420 (73.2)	437 (77.5)	Ref	
		C	154 (26.8)	127 (22.5)	1.34 (1.04-1.8)	0.096
	Dominant	AA	159 (55.4)	170 (60.3)	Ref	
		CC + CA	128 (44.6)	112 (39.7)	1.32 (0.91-1.91)	0.195
	Recessive	CA + AA	261 (90.9)	267 (94.7)	Ref	
		CC	26 (9.1)	15 (5.3)	1.83 (0.9-3.83)	0.164
	Over-dominant	CC + AA	185 (64.5)	185 (65.6)	Ref	
		CA	102 (35.5)	97 (34.4)	0.88 (0.6-1.29)	0.527
rs1800871 -819C/T	Genotypes	TT	160 (55.7)	178 (63.1)	Ref	
		CT	101 (35.2)	91 (32.3)	1.31 (0.89-1.94)	0.218
		CC	26 (9.1)	13 (4.6)	2.28 (1.08-5)	0.085
	Allele	T	421 (73.3)	447 (79.3)	Ref	
		C	153 (26.7)	117 (20.7)	1.5 (1.11-2.04)	**0.04**
	Dominant	TT	160 (55.7)	178 (63.1)	Ref	
		CC + CT	127 (44.3)	104 (36.9)	1.5 (1.03-2.18)	0.085
	Recessive	CT + TT	261 (90.9)	269 (95.4)	Ref	
		CC	26 (9.1)	13 (4.6)	2.21 (1.06-4.8)	0.085
	Over-dominant	CC + TT	186 (64.8)	191 (67.7)	Ref	
		CT	101 (35.2)	91 (32.3)	0.81 (0.55-1.18)	0.303
rs1800896 -1082G/A*	Genotypes	AA	238 (84.4)	169 (72.2)	Ref	
		GA	39 (13.8)	64 (27.4)	0.45 (0.27-0.72)	**0.009**
		GG	5 (1.8)	1 (0.4)	4.77 (0.63-98.94)	0.218
	Allele	A	515 (91.3)	402 (85.9)	Ref	
		G	49 (8.7)	66 (14.1)	0.61 (0.4-0.94)	0.085
	Dominant	AA	238 (84.4)	169 (72.2)	Ref	
		GG + GA	44 (15.6)	65 (27.8)	0.5 (0.31-0.8)	**0.024**
	Recessive	GA + AA	277 (98.2)	233 (99.6)	Ref	
		GG	5 (1.8)	1 (0.4)	6.24 (0.84-128.23)	0.173
	Over-dominant	GA	39 (13.8)	64 (27.4)	Ref	
		GG + AA	243 (86.2)	170 (72.6)	2.28 (1.41-3.71)	**0.009**

*Analysis was performed with 282 patients and 234 control

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Haplotype association

We reconstructed the *IL-10* haplotypes based on three studied SNPs. The frequencies of these observed haplotypes were significantly different between compared groups (Table 5). Data showed that ATA (-1082G/-819T/-592A) is the most frequently observed haplotype in both patient and controls groups (70% of overall haplotypes). The GTA haplotype (-1082A/-819T/-592A) is associated with protection from dengue (OR = 0.31, 95% CI = 0.14 - 0.67, p = 0.003), while the ACC haplotype was found more frequently in dengue patients, and is associated with a higher risk of dengue with borderline significance (OR = 1.36, 95% CI = 0.99 – 1.89, p

Table 3. Genotype and allele distribution of *IL-10* polymorphisms in dengue patients with different severity and healthy controls. Data are shown in number and percentage. Bold values indicate statistical significance. Odds ratio and p-value were calculated by using binary logistic regression models adjusted for age and sex. All p-values were adjusted by the Benjamini-Hochberg procedure.

SNP	Variable	Geno-types	DF n = 162	Control n = 282	OR (95% CI)	p-value	DWS n = 113	OR 2 (95% CI)	p-value	SD n = 12	OR 3 (95% CI)	p-value
rs1800872 -592C/A	**Genotypes**	AA	91 (56.2)	170 (60.3)	Ref		61 (54.0)	Ref		7 (58.3)		
		CA	57 (35.2)	97 (34.4)	1.19 (0.76-1.87)	0.464	40 (35.4)	1.07 (0.62-1.83)	0.842	5 (41.7)	0.94 (0.19-3.81)	0.941
		CC	14 (8.6)	15 (5.3)	1.88 (0.81-4.34)	0.227	12 (10.6)	2.06 (0.77-5.3)	0.298	0 (0.0)	–	–
	Allele	A	239 (73.8)	437 (77.5)	Ref		162 (71.7)	Ref		19 (79.2)	Ref	
		C	85 (26.2)	127 (22.5)	1.31 (0.93-1.85)	0.214	64 (28.3)	1.26 (0.84-1.9)	0.430	5 (20.8)	0.67 (0.16-2.11)	0.941
	Dominant	AA	91 (56.2)	170 (60.3)	Ref		61 (54.0)	Ref		7 (58.3)	Ref	
		CC + CA	71 (43.8)	112 (39.7)	1.75 (0.76-3.97)	0.270	52 (46.0)	1.99 (0.76-4.99)	0.298	5 (41.7)	0.7 (0.14-2.81)	0.941
	Recessive	CA + AA	148 (91.4)	267 (94.7)	Ref		101 (89.4)	Ref		12 (100.0)		
		CC	14 (8.6)	15 (5.3)	1.3 (0.85-2)	0.307	12 (10.6)	1.18 (0.71-1.96)	0.679	0 (0.0)	–	–
	Over-dominant	CC + AA	105 (64.8)	185 (65.6)	Ref		73 (64.6)	Ref		7 (58.3)	Ref	
		CA	57 (35.2)	97 (34.4)	0.88 (0.57-1.37)	0.577	40 (35.4)	1.03 (0.61-1.76)	0.908	5 (41.7)	1.18 (0.29-5.92)	0.941
rs1800871 -819C/T	**Genotypes**	TT	92 (56.8)	178 (63.1)			60 (53.1)			8 (66.7)	Ref	
		CT	56 (34.6)	91 (32.3)	1.25 (0.8-1.97)	0.392	41 (36.3)	1.23 (0.72-2.1)	0.622	4 (33.3)	0.89 (0.18-3.62)	0.941
		CC	14 (8.6)	13 (4.6)	2.17 (0.92-5.15)	0.193	12 (10.6)	2.47 (0.9-6.61)	0.188	0 (0.0)	–	–
	Allele	T	240 (74.1)	447 (79.3)			161 (71.2)			20 (83.3)		
		C	84 (25.9)	117 (20.7)	1.45 (1.02-2.06)	0.193	65 (28.8)	1.49 (1.08-2.25)	0.173	4 (16.7)	0.77 (0.18-2.5)	0.941
	Dominant	TT	92 (56.8)	178 (63.1)			60 (53.1)			8 (66.7)		
		CC + CT	70 (43.2)	104 (36.9)	2.16 (0.92-5.09)	0.193	53 (46.9)	2.58 (0.94-6.89)	0.177	4 (33.3)	0.82 (0.16-3.35)	0.941
	Recessive	CT + TT	148 (91.4)	269 (95.4)			101 (89.4)			12 (100.0)		
		CC	14 (8.6)	13 (4.6)	1.43 (0.93-2.2)	0.202	12 (10.6)	1.43 (0.86-2.39)	0.299	0 (0.0)	–	–
	Over-dominant	CC + TT	106 (65.4)	191 (67.7)			72 (63.7)			8 (66.7)		
		CT	56 (34.6)	91 (32.3)	0.84 (0.54-1.31)	0.464	41 (36.3)	0.88 (0.52-1.5)	0.702	4 (33.3)	1.06 (0.26-5.32)	0.941
			DF n = 158	**Control n = 234**			**DWS n = 112**			**SD n = 12**		
rs1800896 -1082G/A	**Genotypes**	AA	131 (82.9)	64 (27.4)			95 (84.8)			12 (100.0)		
		GA	23 (14.6)	1 (0.4)	0.53 (0.29-0.91)	0.193	16 (14.3)	0.39 (0.19-0.76)	**0.049**	0 (0.0)	–	–
		GG	4 (2.5)	169 (72.2)	8.24 (1.07-171.34)	0.193	1 (0.9)	0.18 (0-9.2)	0.606	0 (0.0)	–	–
	Allele	A	285 (90.2)	129 (27.6)			206 (92.0)			24 (100.0)		
		G	31 (9.8)	339 (72.4)	0.78 (0.48-1.26)	0.392	18 (8.0)	0.44 (0.22-0.81)	**0.049**	0 (0.0)	–	–
	Dominant	AA	131 (82.9)	64 (27.4)			95 (84.8)			12 (100.0)		
		GG + GA	27 (17.1)	170 (72.6)	10.58 (1.39-218.45)	0.193	17 (15.2)	0.31 (0-19.03)	0.702	0 (0.0)	–	–
	Recessive	GA + AA	154 (97.5)	65 (27.8)			111 (99.1)			12 (100.0)		
		GG	4 (2.5)	169 (72.2)	0.63 (0.36-1.06)	0.196	1 (0.9)	0.4 (0.19-0.77)	**0.049**	0 (0.0)	–	–
	Over-dominant	GG + AA	135 (85.4)	233 (99.6)			96 (85.7)			12 (100.0)		
		GA	23 (14.6)	1 (0.4)	1.96 (1.13-3.49)	0.193	16 (14.3)	2.48 (1.28-5.09)	**0.049**	0 (0.0)	–	–

Genotype and allele distribution of *IL-10* polymorphisms in dengue infected patients with different DENV serotypes were investigated (Table 4). No significant difference was found between compared groups.

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Table 4. Genotype and allele distribution of *IL-10* polymorphisms in dengue patients with different DENV serotypes and healthy controls. Data are shown in number and percentage. Bold values indicate statistical significance. Odds ratio and p-value were calculated by using binary logistic regression model and adjusted for age and sex. P-values were adjusted by the Benjamini-Hochberg procedure.

SNP	Variable	Genotypes	DENV-1 n = 105	Control n = 282	OR (95% CI)	p-value	DENV-2 n = 199	OR 2 (95% CI)	p-value	DENV-4 n = 19	OR 3 (95% CI)	p-value
rs1800872 -592C/A	**Genotypes**	AA	61 (58.1)	170 (60.3)	Ref		111 (55.8)	Ref		9 (47.4)	Ref	
		CA	33 (31.4)	97 (34.4)	1.04 (0.61-1.75)	0.967	70 (35.2)	1.18 (0.76-1.81)	0.458	9 (47.4)	1.31 (0.44-3.74)	0.781
		CC	11 (10.5)	15 (5.3)	1.78 (0.7-4.37)	0.360	18 (9.0)	2.05 (0.92-4.62)	0.160	1 (5.2)	1.54 (0.08-9.64)	0.781
	Allele	A	155 (73.8)	437 (77.5)	Ref		292 (73.4)	Ref		27 (71.1)	Ref	
		C	55 (26.2)	127 (22.5)	1.22 (0.82-1.8)	0.456	106 (26.6)	1.34 (0.96-1.87)	0.160	11 (28.9)	1.2 (0.51-2.62)	0.781
	Dominant	AA	61 (58.1)	170 (60.3)	Ref		111 (55.8)	Ref		9 (47.4)	Ref	
		CC + CA	44 (41.9)	112 (39.7)	1.74 (0.7-4.19)	0.360	88 (44.2)	1.92 (0.88-4.24)	0.177	10 (52.6)	1.35 (0.07-7.93)	0.781
	Recessive	CA + AA	94 (89.5)	267 (94.7)	Ref		181 (91.0)	Ref		18 (94.7)	Ref	
		CC	11 (10.5)	15 (5.3)	1.15 (0.7-1.87)	0.753	18 (9.0)	1.31 (0.87-1.98)	0.247	1 (5.3)	1.25 (0.44-3.5)	0.781
	Over-dominant	CC + AA	72 (68.6)	185 (65.6)	Ref		129 (64.8)	Ref		10 (52.6)	Ref	
		CA	33 (31.4)	97 (34.4)	1.03 (0.62-1.74)	0.967	70 (35.2)	0.9 (0.59-1.38)	0.636	9 (47.4)	0.84 (0.3-2.52)	0.781
rs1800871 -819C/T	**Genotypes**	TT	62 (59.0)	178 (63.1)			111 (55.8)			9 (47.4)		
		CT	31 (29.5)	91 (32.3)	1.08 (0.63-1.83)	0.931	69 (34.7)	1.29 (0.83-1.99)	0.308	9 (47.4)	1.35 (0.45-3.88)	0.781
		CC	12 (11.4)	13 (4.6)	2.41 (0.95-6.04)	0.201	19 (9.5)	2.42 (1.08-5.57)	0.119	1 (5.2)	1.83 (0.09-11.72)	0.781
	Allele	T	155 (73.8)	447 (79.3)			291 (73.1)			27 (71.1)		
		C	55 (26.2)	117 (20.7)	1.43 (0.95-2.12)	0.211	107 (26.9)	1.53 (1.09-2.14)	0.090	11 (28.9)	1.38 (0.58-3.03)	0.781
	Dominant	TT	62 (59.0)	178 (63.1)			111 (55.8)			9 (47.4)		
		CC + CT	43 (41.0)	104 (36.9)	2.54 (1.01-6.3)	0.201	88 (44.2)	2.41 (1.08-5.5)	0.119	10 (52.6)	1.93 (0.1-11.96)	0.781
	Recessive	CT + TT	93 (88.6)	269 (95.4)			180 (90.5)			18 (94.7)		
		CC	12 (11.4)	13 (4.6)	1.31 (0.79-2.14)	0.434	19 (9.5)	1.5 (0.99-2.27)	0.139	1 (5.3)	1.45 (0.51-4.07)	0.781
	Over-dominant	CC + TT	74 (70.5)	191 (67.7)			130 (65.3)			10 (52.6)		
		CT	31 (29.5)	91 (32.3)	1 (0.59-1.69)	0.989	69 (34.7)	0.83 (0.54-1.27)	0.440	9 (47.4)	0.77 (0.27-2.29)	0.781
			DENV-1 n = 101	**Control n = 234**			**DENV-2 n = 195**			**DENV-4 n = 19**		
rs1800896 -1082G/A	**Genotypes**	AA	86 (85.1)	169 (72.2)			162 (83.1)			16 (84.2)		
		GA	13 (12.9)	64 (27.4)	0.46 (0.22-0.89)	0.201	29 (14.9)	0.52 (0.3-0.87)	0.090	3 (15.8)	0.59 (0.13-1.97)	0.781
		GG	2 (2.0)	1 (0.4)	7.88 (0.65-186.15)	0.254	4 (2.1)	5.41 (0.57-122.13)	0.246	0 (0.0)	–	–
	Allele	A	185 (91.6)	402 (85.9)			353 (80.5)			35 (92.1)		
		G	17 (8.4)	66 (14.1)	0.68 (0.37-1.2)	0.360	37 (9.5)	0.7 (0.43-1.11)	0.195	3 (7.9)	0.63 (0.15-1.91)	0.781
	Dominant	AA	86 (85.1)	169 (72.2)			162 (83.1)			16 (84.2)		
		GG + GA	15 (14.9)	65 (27.8)	10.59 (0.9-247.04)	0.201	33 (16.9)	7.27 (0.79-161.08)	0.177	3 (15.8)	0.61 (0.13-2.02)	0.781
	Recessive	GA + AA	99 (98.0)	233 (99.6)			191 (97.9)			19 (100.0)		
		GG	2 (2.0)	1 (0.4)	0.54 (0.27-1.02)	0.201	4 (2.1)	0.58 (0.35-0.97)	0.120	0 (0.0)	–	–
	Over-dominant	GG + AA	88 (87.1)	170 (72.6)			166 (85.1)			16 (84.2)		
		GA	13 (12.9)	64 (27.4)	2.21 (1.14-4.54)	0.201	29 (14.9)	1.96 (1.16-3.37)	0.090	3 (15.8)	1.61 (0.48-7.32)	0.781

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Table 5. Association of *IL-10* haplotypes and dengue. The alleles in the haplotype were ranked in the SNP order of rs1800896, rs1800871 and rs1800872. Associations were tested using logistic regression adjusted for age and sex.

Haplotypes	Number of observation (%)		OR (95% CI)	p-value
	DENV (n=574)	Control (n=564)		
ATA	402 (70.0%)	394 (69.8%)	Ref	
GTA	12 (2.0%)	38 (6.7%)	0.31 (0.14 - 0.67)	0.003
ACC	108 (19.0%)	78 (14.0%)	1.36 (0.99 - 1.89)	0.061
GCC	34 (6.0%)	34 (6.4%)	1.08 (0.64 - 1.82)	0.783
Minor	18 (3.0%)	20 (3.1%)	0.66 (0.35 - 1.22)	0.182

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= 0.061) No significant difference in IL-10 serum levels between varying haplotypes was found (Fig 3).

Discussion

The study investigated host and viral genetic factors for dengue susceptibility and severity. We report the incidence of dengue in two consecutive outbreaks in 2021-2022 and showed that DENV-1 and DENV-2 were the predominant serotypes in circulation and distributed among severe cases. Plasma levels of the host cytokines IL-10 were significantly elevated in dengue patients of varying severity. The promoter variants of *IL-10*, which have been described to regulate IL-10 levels [13], were also differentially distributed in dengue patients and in healthy controls.

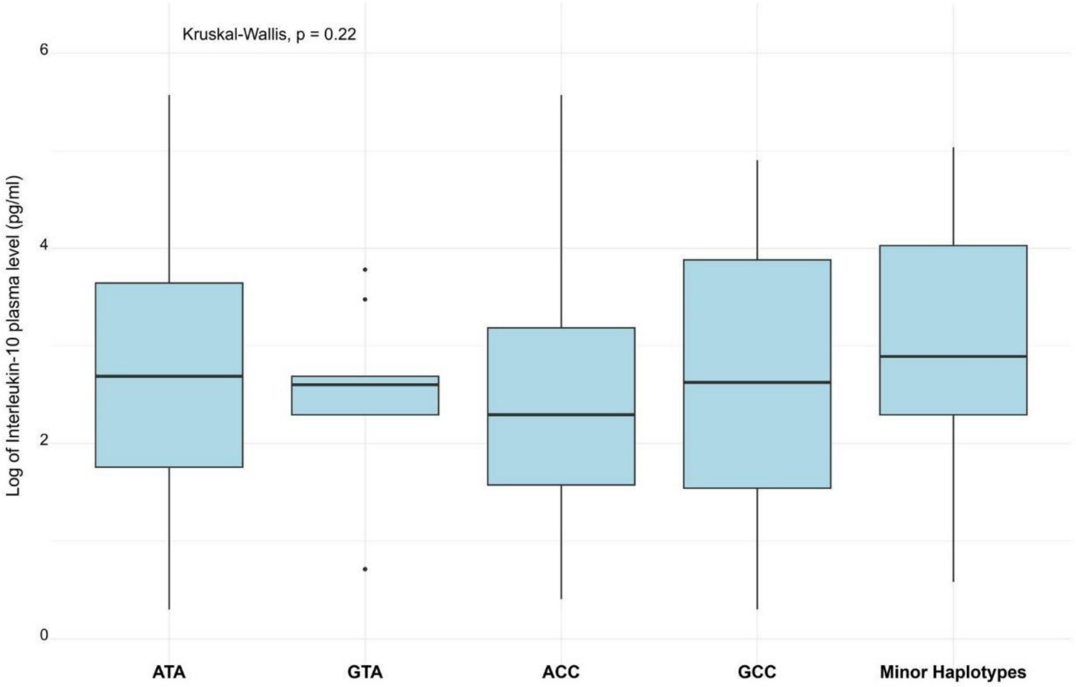

Fig 3. IL-10 plasma levels in different IL-10 haplotypes.

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The main DENV serotypes circulating during the 2021-2022 outbreak were DENV-2 and DENV-1, followed by DENV-4. In northern Vietnam, the distribution of DENV-1,-2 serotypes have undergone changes in recent years. DENV-1 was previously observed as the predominant serotype during the years 2017-2019 [18]. However, during the outbreaks in 2019-2020, there was a shift to DENV-2 dominance [19], a trend that persisted throughout the 2021-2022 period, as observed in our study. The DENV-1,-2,-4 serotypes were persisting in the northern population compared to other regions, where all four serotypes co-existed [20]. It is well described that DENV-2 is associated with severe disease progression [2], and more than 61% of DENV-2 infections were observed in patients with DWS and severe dengue in our study. This finding is consistent with data from previous outbreaks that posed an increased burden on public health [21]. While DENV-3 serotype were not observed in our study, this appears to be more regional in Vietnam, as reported in previous outbreaks [20].

The host cytokine plasma IL-10, which is well described as an anti-inflammatory marker in dengue [22], was significantly associated to dengue severity. While patients without warning signs had lower IL-10 plasma levels, higher levels were indicative of disease deterioration, as observed in severe cases. Elevated IL-10 levels in dengue pathogenesis have been shown to reflect a reduced type I interferon response and thus a delayed viral clearance during acute episodes [22]. During DENV infection, there is a reduction in IFN-γ production by T cells, and an early increase in IL-10 production, leading to less IFN- γ and more viral persistence, as shown from other viral infections [9,23].

Exacerbated inflammatory response and altered vascular function are hallmarks of dengue [24]. IL-10 acts as an immunoregulatory cytokine in both the Th1 and Th2 responses to maintain immune homeostasis and to prevent excessive inflammation and tissue damage. While an optimal IL-10 response might mitigate immune-mediated pathology, an excessive IL-10 milieu could hinder effective viral clearance and potentially aggravate the clinical course of the disease.

Upon monocyte infection with DENV, the expression of *IL-10* gradually rises over time and more in the case of ADE [22]. Secondary infections trigger an earlier and higher release of IL-6 and IL-10 compared to primary infections, indicating that pre-existing immune memory accelerates cytokine production in subsequent infections [25]. Several transcription factors are involved in the modulation of *IL-10* transcription, and approximately 75% of the variation in IL-10 secretion capacity derives from genetic factors [26,27]. Further, a study has demonstrated a strong genetic influence on cytokine production, including IL-10, upon ex vivo stimulation [28]. This suggests that genetic factors may influence IL-10 levels, thereby affecting the severity and susceptibility to dengue. However, no significant difference in IL-10 plasma levels was observed between the genetic variants in our study. It is also important to note that dengue pathogenesis is complex and involves multiple factors, ranging from the infected viral serotype, sequence of infections (primary/secondary) to the dynamics of other immune modulators upon infection. Therefore, anticipating the level of IL-10 in dengue patients is complex and dependent on multiple factors.

Nonetheless, an earlier study found that ACC haplotype (-1082A/-819C/-592C) is associated with downregulated IL-10 level in dengue patients [29], which consistent with our preliminary observation, but the results from our study did not reach the statistical significance level (Fig 3). The study findings show a marked association between *IL-10* promoter rs1800896 (-1082G/A) and rs1800871 (-819C/T) with DENV infection. Allelic frequencies observed in our study align with data published on the Ensembl database (https://www.ensembl.org/index.html) for the Kinh ethnicity (KHV): rs1800896 (A>G), rs1800871 (T>C), and rs1800872 (A>C).

Our observations suggest that the allele -819C correlates with a significantly increased risk of DENV infection compared to -819T. On the other hand, the genotype -1082GA and allele -1082G were found to be protective factors against developing dengue and DWS. Haplotype analysis similarly revealed that the GTA haplotype (-1082G/-819T/-592A) conferred protection from dengue in the Vietnamese population. Aligned with our findings, the GTA haplotype was reported to be associated with protection from other infectious diseases such as schistosomiasis and malaria in the Nigerian population [15] and in the Brazilian population [30]. Considering that the predominant IL-10 genetic variant in the Vietnamese population is ATA (-1082A/-819T/-592A), with the GTA haplotype being less frequent, the high susceptibility and burden of dengue observed in the Vietnamese population may be partially due to genetic characteristics of the Vietnamese population.

In comparison to a recent study from Mexico, where the allelic distribution is different from Vietnam (Mexico: rs1800896 (A<G), rs1800871 (T<C), and rs1800872 (A<C); https://www.ensembl.org/index.html), we found that the distribution of the SNPs rs1800872 (-592C/A) and rs1800871 (-819C/T) among DENV infected patients are different. A study found that -819T and -592A increase the risk of DENV infection in the Mexican population [27], while our study revealed that these two alleles appear to have a protective role against DENV infection in the Vietnamese population. However, given that the allelic distributions of the three studied SNPs are opposite in these two populations, the association of *IL-10* genetic variants with dengue might be influenced, thus affecting the generalization of the study findings.

Although our study is the first to investigate the association between *IL-10* SNPs and DENV infection in the Vietnamese population, a relatively large sample size would be helpful for further studies to justify our findings. While a significant association between IL-10 levels of varying severity and the association of promoter *IL-10* variants was observed in our study, other cytokines and chemokines may also regulate the course of infection. Further studies are warranted, such as the explicit investigation of a large panel of cytokines and chemokines to understand how they are co-regulated during dengue infection, and if so, how they are modulated during the clinical course of DENV infection.

In summary, this study underscores the importance of understanding genetic predispositions and immune response mechanisms in dengue severity, particularly in endemic regions like Vietnam. The identification of IL-10 plasma levels and genetic variants, specifically the protective role of the -1082G allele, provides valuable insights into host-pathogen interactions and susceptibility to severe dengue. Such findings can guide the development of targeted public health strategies, including early risk stratification, personalized treatment approaches, and the design of immunogenetic-based interventions. Furthermore, the consistent dominance of DENV-2 and the associated higher severity highlights the need for serotype-specific monitoring and vaccination campaigns to mitigate the growing burden of dengue in Southeast Asia. This integrative approach, combining clinical, genetic, and serotype data, is pivotal in enhancing preparedness and response efforts in dengue-endemic regions.

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RESEARCH ARTICLE OPEN ACCESS

Elevated Soluble HLA-G Levels Associate With Dengue Severity in Vietnamese Patients

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Keywords: clinical severity | dengue | sHLA-G | Vietnam

ABSTRACT

The pathogenesis of dengue remains complex and incompletely understood. One proposed mechanism involves the virus evading host immune responses through the upregulation and/or secretion of immune-inhibitory molecules. This study investigates the association between plasma levels of soluble human leukocyte antigen G (sHLA-G), a known immunoregulatory molecule, and dengue severity in hospitalized patients. A total of 238 dengue patients and 118 healthy controls were enrolled. Dengue infection was confirmed by real-time RT-PCR, and patients were clinically categorized as having dengue fever without warning signs (DF), dengue with warning signs (DWS), or severe dengue (SD), according to WHO guidelines. Laboratory parameters were assessed upon hospital admission, and plasma sHLA-G levels were measured using ELISA. sHLA-G levels were significantly elevated in dengue patients compared to healthy controls (median [range]: 42.7 [7.10–1300] U/mL vs. 11.1 [4.7–620] U/mL; $p < 0.001$). After adjusting for age, sex and disease severity, a significant association was observed between sHLA-G levels and days of illness ($\beta = 0.1$, $p = 0.03$). Patients requiring close medical monitoring (DWS/SD) showed higher sHLA-G levels (51.0 [7.17–525] U/mL) than those having dengue fever without warning signs (38.0 [7.10–1300] U/mL); $p = 0.011$. While ALT and AST were positively correlated with sHLA-G levels in all patients, total lymphocyte counts were inversely correlated with sHLA-G in severe cases ($r = -0.78$, $p = 0.002$). Elevated sHLA-G levels are associated with dengue severity and may serve as a useful marker for identifying high-risk cases and for guiding clinical monitoring.

Clinical trial registration: Not applicable.

1 | Introduction

The dengue virus (DENV) is responsible for massive outbreaks of viral haemorrhagic fever worldwide, particularly in tropical and subtropical regions [1]. Vietnam experiences a high annual incidence of dengue with a significant impact on the health and economic system [2]. Clinically, the disease can range from nonspecific acute febrile illness to haemorrhagic manifestations

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with altered haemostasis, possibly leading to shock or multi-organ failure [3]. The pathogenesis of dengue is complex and has not yet been elucidated in detail, as it depends on both viral and host factors, with the host immune system playing a decisive role.

After infection, dendritic cells (DCs) presenting the DENV antigen recruit monocytes and macrophages to the site of infection. Instead of effectively eliminating the virus, these recruited immune cells - important components of the innate immune response - become primary targets for DENV [4]. This process facilitates viral replication and dissemination to other host immune cells in the lymph nodes, bone marrow, spleen and liver. The molecular and immunological mechanisms by which DENV subverts the host's immune response to viral antigens, remains unclear.

One possible explanation is that DENV preferentially infects DCs, monocytes and macrophages thereby impairing the development of an efficient early antiviral response. During viral replication, DENV secretes nonstructural proteins (NS), specifically NS1, NS2B and NS4, which disrupt the signalling pathway of type I interferon (IFN), a critical component of the innate immune defence [5]. This disruption delays the host's antiviral response and allows the virus to persist and propagate. In addition, secondary DENV infections can lead to antibody-dependent enhancement, in which non-neutralising antibodies facilitate viral replication and thus increase the disease burden for the host [5].

Furthermore, the production of immune checkpoints (ICP) during DENV infection can impair the host's immune response [6, 7]. These molecules trigger T cells dysfunction and inhibit immune cells activity and are associated with impaired immune defence in various diseases, including cancer and infectious diseases [7, 8]. One of the potential immune inhibitors is the HLA-G ligand (Human Leukocyte Antigens G), which is a ligand for receptors in B cells, T cells, monocytes, DCs and subsets of natural killer cells (NKs) [9]. HLA-G molecules are involved in the inhibition of NK cells activity, the maturation of CD4 + T lymphocytes and DCs, the apoptosis of CD8+ cytotoxic T cells and the development of regulatory T cells (Tregs) [9].

The expression and regulation of HLA-G is highly dynamic: four membrane-bound forms (HLA-G1 to G4) and three soluble, secreted forms (sHLA-G5 to G7) generated by alternative splicing of the primary transcript [10]. The HLA-G1 transmembrane isoform can produce a soluble form (sHLA-G) by proteolytic shedding, which retains all the functions of the membrane counterpart, potentially expanding immunoregulatory activities on a systematic scale. Studies have reported an upregulation of HLA-G antigens upon DENV infections suggesting a role of the ICPs in the pathogenesis of dengue [11]. Furthermore, it has also been observed that the level of sHLA-G is modulated at different stages in arboviral infections [6].

While the role of HLA-G in organ transplantation, pregnancy and cancer is well documented, its involvement in viral infections has not yet been sufficiently studied. This study examines sHLA-G levels in dengue patients to assess their association with disease severity.

2 | Methods

2.1 | Ethical Approval Statement

All study participants provided signed informed consent before enrolment. The Institutional Review Board of the 108 Military Hospital and the University of Tübingen approved the study, titled “Host and Viral Factors Influencing Dengue Severity and Susceptibility” (Ethics Approval No. 274/2022B02). The study complies with the Nagoya Protocol and authorization for the use of genetic resources in Germany was obtained from the Vietnamese Ministry of Natural Resources and Environment (Reference No. 2995/QĐ-BTNMT). All procedures followed GCP/GCLP guidelines.

2.2 | Study Population

Samples were collected during two consecutive seasonal outbreaks in northern Vietnam, spanning September to November in 2021 and 2022. The study population consisted of 238 civilian patients suspected of having dengue who agreed to be enrolled in the study and were admitted to the 108 Military Central Hospital in Hanoi. The dengue diagnoses followed the World Health Organisation diagnostic's criteria [3] (https://apps.who.int/iris/handle/10665/44188), as adopted by the Vietnamese Ministry of Health. The inclusion criteria are patients presenting with fever and at least two clinical signs or symptoms suggestive of dengue (e.g. nausea/vomiting, rash, body aches and pains, positive tourniquet test) and/or a positive result from at least one indirect diagnostic method (serological rapid test), as recommended in the WHO 2009 guidelines [3]. Patients with bacterial or other viral infections, chronic diseases, or haematological disorders were excluded. A total of 118 Vietnamese healthy blood donors were recruited and considered as healthy controls in the study. Blood samples were collected at admission and plasma was separated and stored at −70°C until analysis.

2.3 | PCR Confirmation of Dengue

Total viral RNA was isolated from 140 µL of patient plasma utilizing the QIAmp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. All samples ($n = 238$) underwent multiplex real-time PCR analysis for dengue, Zika, and chikungunya viral RNA using the Fast-Track Diagnostics Kit (Siemens Healthcare GmbH, Erlangen, Germany) on a LightCycler480-II (Roche, Mannheim, Germany), following the manufacturer's guidelines. Testing was performed using internal controls and provided standards from the manufacturer. Confirmed dengue cases were identified by the presence of dengue viral RNA through real-time RT-PCR and absence of Zika and chikungunya viral RNA.

2.4 | Dengue Severity Classification and Laboratory Assessment

In Vietnam, admitted patients were clinically classified into three severity levels according to WHO guidelines [3]: dengue without warning signs (DF, $n = 103$), dengue with warning signs (DWS, $n = 122$) and severe dengue (SD, $n = 13$). In this classification, warning signs include the presence of abdominal pain, persistent vomiting, mucosal bleeding, clinical fluid accumulation, lethargy/restlessness, liver enlargement and a rapid decrease in platelet count with rising haematocrit. Severe dengue is defined by severe plasma leakage leading to shock or respiratory distress, severe bleeding or severe organ impairment such as hepatitis, myocarditis or encephalopathy [3]. Patients who required close monitoring and hospitalization included both those with DWS and SD, as recommended by the WHO [3]. Accordingly, these groups were combined for the analysis of sHLA-G levels to highlight clinically relevant differences and ensure statistical power. Clinical presentations were documented upon admission. Laboratory parameters assessed during admission include a complete hemogram: Erythrocyte (RBC), Haemoglobin (Hb), Haematocrit (HCT), Platelet (PLT), Leucocyte (WBC), Neutrophile (NEU), Lymphocyte (LYM), Monocyte (MONO), Eosinophile (EOS), Basophile (BASO), Large Unstained Cells (LUC), and liver enzymes: Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) levels.

2.5 | Measurement of sHLA-G Plasma Levels

Plasma levels of sHLA-G (sHLA-G1 and sHLA-G5) were quantified in 238 patients and 118 healthy controls using the sHLA-G enzyme-linked immunosorbent assay (ELISA) kit (BioVendor–Laboratorní medicína a.s., Brno, Czech Republic), a sandwich enzyme immunoassay for the quantitative measurement of sHLA-G, according to the manufacturer's instructions, whose detection limit is 0.38 ng/mL. Absorbance was measured using an ELISA reader (Infinite 200 PRO-TECAN, Maennedorf, Switzerland) at 450 nm with the reference wavelength set to 630 nm. sHLA-G concentrations were determined from a five-point standard curve prepared from serial dilutions of calibrator (7.81, 15.63, 31.25, 62.5, and 125 U/mL) purchased by the kit as standard reagent. Results were expressed as Units/millilitre (U/mL).

2.6 | Statistical Analysis

Data were analysed and visualized using the R software version 4.3.2 (http://www.r-project.org). Clinical and demographic data were presented as median values (with range) for quantitative variables and absolute numbers with percent for categorical variables. The normality of distribution in the quantitative variables was tested using the Shapiro-Wilk test. Categorical data were compared using Chi-square test, while continuous variables were compared using Kruskal-Wallis or Wilcoxon test as appropriate. Dunn test was applied as post-hoc test. Spearman correlation with Bonferroni adjustment was applied to assess the correlation between levels of sHLA-G and other blood parameters. A $p < 0.05$ was considered statistically significant.

Multiple linear regression was performed to estimate the relationship between sHLA-G levels and days of illness, adjusted for age, sex and clinical severity. In our study, days of illness are defined as the number of days from the onset of fever until admission. The sHLA-G values were log-transformed before inclusion in the regression analysis to reduce skewness and heteroscedasticity.

3 | Results

3.1 | Demographic and Clinical Characteristics of Dengue Patients

All patients and healthy controls belonged to the Kinh ethnic group and were residents of Hanoi metropolitan area living in various communes. The patient group comprised 127 males and 111 females, with a median age of 47 years (range: [14–87] years). The healthy control group consisted of 66 male and 52 female with the median age of 45 years (range: [25–56] years). Detailed demographic and clinical data of the recruited patients are presented in Table 1. There were no significant differences in age and sex between patient severity groups, or between patients and controls (Table 1).

All patients were confirmed dengue-positive by RT-PCR. Patients with DWS and SD were hospitalized later in the disease course (median 5 days) compared to those with DF (median 3 days). Patients with DWS and SD more frequently presented with rash and bleeding manifestations compared to those with DF (Table 1).

3.2 | Laboratory Parameters of Dengue Patients

Patients' laboratory parameters are summarized in Table 1. There was no difference in the total leukocyte counts between the three severity grades. DF patients had lower lymphocyte counts (median [range]: 0.69 [0.17–3.56] $\times 10^6/\mu$L) compared to DWS (median [range]: 0.99 [0.22–4.09] $\times 10^6/\mu$L) ($p = 0.001$) (Table 1 and Supporting Information S1: Table S1). In contrast, neutrophil and platelet counts were significantly higher in DF (NEU median [range]: 2.56 [0.47–13.1] $\times 10^6/\mu$L; PLT median [range]: 138 [14.0–384] $\times 10^3/\mu$L) compared to DWS (NEU median [range]: 1.77 [0.45–6.21] $\times 10^6/\mu$L; PLT median [range]: 21.0 [4.00–228] $\times 10^3/\mu$L) (Table 1 and Supporting Information S1: Table S1). In addition, liver enzymes including AST and ALT were higher in DWS (AST median [range]: 111 [16.0–1040] U/L; ALT median [range]: 66.2 [8.20–636] U/L) compared to DF (AST median [range]: 46.3 [17.3–350] U/L; ALT median [range]: 35.3 [8.00–503] U/L) (Table 1 and Supporting Information S1: Table S1).

3.3 | sHLA-G Plasma Levels in Healthy Controls and in Dengue Patients

The levels of sHLA-G were significantly higher in dengue patients (median [range]: 42.7 [7.10–1300] U/mL) compared to healthy controls (median [range]: 11.1 [4.7–620] U/mL) ($p < 0.001$) (Figure 1a). In addition, sHLA-G levels differed significantly across severity groups: between DWS (median

TABLE 1 | Characteristics of dengue patients.

	Dengue without warning signs ($n = 103$)	Dengue with warning signs ($n = 122$)	Severe dengue ($n = 13$)	p value
Demographic data				
Age (years)	46 [14–87]	49 [17–83]	49 [21–82]	0.627
Gender (male/female)	56/47	65/57	6/7	0.855
Clinical manifestation				
Day of disease (days)	3 [1–5]	5 [1–8]	5 [4–7]	< 0.001*
Headache	97 (94.2%)	106 (86.9%)	12 (92.3%)	0.192
Retro-ocular pain	54 (52.4%)	84 (68.9%)	12 (92.3%)	0.003*
Myalgia	75 (72.8%)	92 (75.4%)	10 (76.9%)	0.637
Arthralgia	62 (60.2%)	85 (69.7%)	11 (84.6%)	0.116
Rash	13 (12.6%)	70 (57.4%)	6 (46.2%)	< 0.001*
Abdominal pain	0 (0%)	20 (16.4%)	5 (38.5%)	< 0.001*
Vomit	15 (14.6%)	31 (25.4%)	6 (46.2%)	0.008*
Lethargy	0 (0%)	1 (0.8%)	4 (30.8%)	< 0.001*
Edema	0 (0%)	26 (21.3%)	6 (46.2%)	< 0.001*
Hepatomegaly	0 (0%)	3 (2.5%)	0 (0%)	0.248
Shock	0 (0%)	0 (0%)	5 (38.5%)	< 0.001*
Respiratory distress	0 (0%)	0 (0%)	6 (46.2%)	< 0.001*
Bleeding manifestations	15 (14.9%)	102 (83.6%)	10 (76.9%)	< 0.001*
Subcutaneous	15 (14.9%)	82 (67.2%)	8 (61.5%)	< 0.001*
Mucosal	0 (0%)	64 (52.5%)	6 (46.2%)	< 0.001*
Severe	0 (0%)	0 (0%)	4 (30.8%)	< 0.001*
Laboratory tests				
Erythrocyte $\times 10^6/\mu L$	4.83 [3.66–6.26]	5.14 [3.97–7.62]	5.08 [2.76–5.89]	< 0.001*
Haemoglobin g/L	146 [110–187]	153 [113–190]	150 [70.0–172]	0.003*
Haematocrit	43 [31.8–53.4]	44.7 [34.8–60.5]	43.7 [21.4–51.6]	0.001*
Platelet $\times 10^3/\mu L$	138 [14.0–384]	21.0 [4.00–228]	29.0 [4.00–125]	< 0.001*
Leucocyte $\times 10^6/\mu L$	4.08 [1.27–16.9]	3.71 [0.96–11.6]	4.70 [1.45–10.5]	0.762
Neutrophile $\times 10^6/\mu L$	2.56 [0.47–13.1]	1.77 [0.45–6.21]	1.97 [0.81–7.70]	< 0.001*
Lymphocyte $\times 10^6/\mu L$	0.69 [0.17–3.56]	0.99 [0.22–4.09]	1.03 [0.31–2.81]	0.003*
Monocyte $\times 10^6/\mu L$	0.39 [0.06–1.35]	0.31 [0.05–2.53]	0.39 [0.10–0.74]	0.189
Eosinophile $\times 10^6/\mu L$	0.01 [0–0.21]	0.02 [0–0.45]	0.01 [0–0.07]	0.036*
Basophile $\times 10^6/\mu L$	0.03 [0–1.18]	0.07 [0–1.76]	0.09 [0.01–0.26]	< 0.001*
LUC $\times 10^6/\mu L$	0.10 [0.02–3.49]	0.36 [0.02–5.82]	0.40 [0.05–2.45]	< 0.001*
Neutrophile %	65.8 [18.7–93.4]	49.7 [17.3–82.3]	56.5 [26.1–76.1]	< 0.001*
Lymphocyte %	19.2 [2.5–72.8]	27.9 [6.1–56.3]	21.1 [6.8–53.0]	< 0.001*
Monocyte %	9.10 [1.6–20.9]	7.80 [2.9–36.6]	8.50 [3.6–11.8]	0.098
Eosinophile %	0.40 [0–4.00]	0.65 [0–11.6]	0.20 [0.10–2.30]	0.002*
Basophile %	0.60 [0–11.3]	2.20 [0–15.1]	1.40 [0.60–4.70]	< 0.001*
LUC %	2.70 [0.4–35.0]	10.0 [1.30–55.6]	6.55 [3.30–26.1]	< 0.001*
AST U/L	46.3 [17.3–350]	111 [16.0–1040]	186 [31–11100]	< 0.001*
ALT U/L	35.3 [8.00–503]	66.2 [8.20–636]	90 [25.6–2190]	< 0.001*

Note: Variables were summarized in percentage (%) or median with [range]. p values were calculated by Chi-square and Kruskal-Wallis test.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; LUC, large unstained cells.
*Statistically significant.

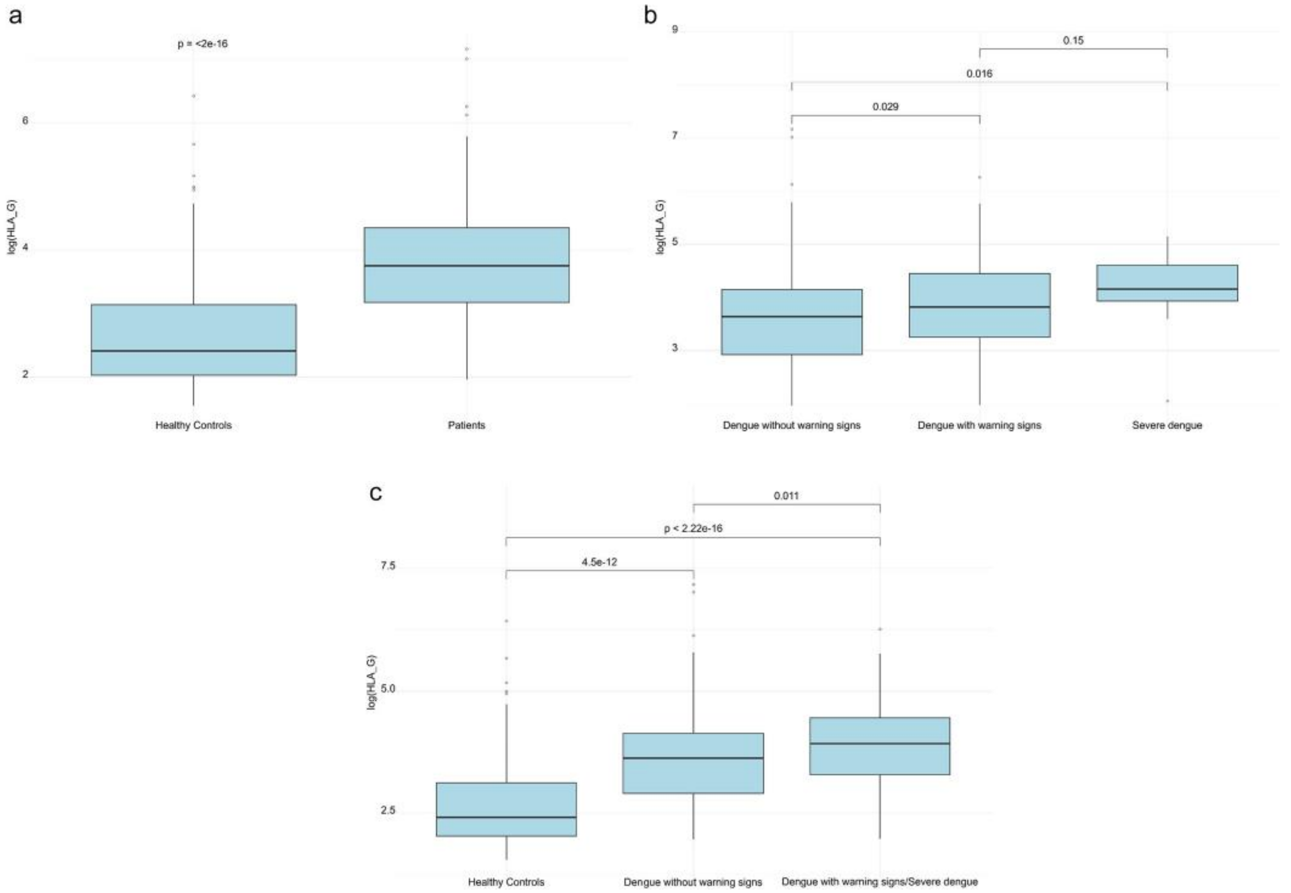

FIGURE 1 | sHLA-G plasma levels in dengue patients and its clinical relevance. (a) sHLA-G plasma levels in dengue patients and healthy controls. (b) sHLA-G plasma levels in dengue patients of varying severity. (c) sHLA-G plasma levels in dengue patients by medical monitoring requirements. sHLA-G plasma levels were determined using a commercially available ELISA as described in Methods. The data are provided as box plots as log-transformed sHLA-G (U/mL).

[range]: 45.6 [7.17–525] U/mL) and DF (median [range]: 38.0 [7.10–1300] U/mL) ($p = 0.029$); and between SD (median [range]: 63.9 [7.73–172] U/mL) and DF ($p = 0.016$) (Figure 1b and Table 2). Notably, significantly higher sHLA-G levels were observed in patients requiring close medical monitoring (DWS/SD) (median [range]: 51.0 [7.17–525] U/mL) compared to those with dengue fever (DF) (median [range]: 38.0 [7.10–1300] U/mL), ($p = 0.011$). Further stratification into DWS and SD subgroups revealed no significant difference in sHLA-G levels. Multiple linear regression adjusting for age, sex, severity was performed to estimate the association between sHLA-G levels (log-transformed) and days of illness. The results showed a positive association between two variables ($\beta = 0.1$, $p = 0.033$) (Supporting Information S1: Table S2 and Figure 2).

3.4 | Plasma Levels of sHLA-G Correlated With Laboratory Parameters in Dengue

Thirteen laboratory parameters: WBC, NEU, LYM, MONO, EOS, BASO, LUC, RBC, Hb, HCT, PLT, AST and ALT were included in the analysis (Table 3). While LUC and liver enzymes positively correlated with sHLA-G levels in all dengue patients (LUC: $r = 0.21$, $p = 0.004$; AST: $r = 0.21$, $p = 0.001$; ALT: $r = 0.14$, $p = 0.038$), PLT and NEU showed inverse correlations (PLT: $r = -0.21$, $p = 0.001$; NEU: $r = -0.16$, $p = 0.013$) (Table 3). In addition, LYM and LUC inversely correlated with sHLA-G levels in severe cases (LYM: $r = -0.78$, $p = 0.002$; LUC: $r = -0.81$, $p = 0.005$) (Table 3).

4 | Discussion

The severity of dengue primarily depends on immunopathogenic mechanisms, where an impaired immune response not only contributes to the progression of the disease, but also hinders the elimination of DENV and disease complications [5]. HLA-G is known for its tolerogenic role suppressing the activity of effector cells, in particular NK cells and CD8+ cytotoxic T lymphocytes [12] and thus might be potentially involved in the pathogenesis of dengue. Based on this assumption, our study aimed to investigate the association between sHLA-G and dengue in Vietnamese patients. The results suggested that sHLA-G levels were associated with dengue and might serve as a marker for disease severity.

In our study, plasma levels of sHLA-G were significantly higher in dengue-confirmed patients, compared to healthy controls.

TABLE 2 | Levels of sHLA-G in dengue patients of varying clinical severities.

	Dengue without warning signs ($n = 103$)	Dengue with warning signs ($n = 122$)	Severe dengue ($n = 13$)	p value
Mean (SD)	77.8 (174)	68.1 (68.2)	76.2 (42.4)	0.013*
Median [Min, Max]	38.0 [7.10–1300]	45.6 [7.17–525]	63.9 [7.73–172]	
	Dengue without warning signs ($n = 103$)	**Dengue with warning signs and severe dengue ($n = 135$)**	**Healthy controls ($n = 118$)**	
Mean (SD)	77.8 (174)	68.9 (66.1)	29.1 (67.1)	< 0.001*
Median [Min, Max]	38.0 [7.10–1300]	51.0 [7.17–525]	11.1 [4.70–620]	

Note: p values were calculated by Kruskal-Wallis test.
*Statistically significant.

The expression of HLA-G is restricted to certain tissues under physiological conditions, but is upregulated during pregnancy and in pathological conditions such as tumours, viral infections and inflammatory diseases [13]. Soluble HLA-G proteins are formed by two mechanisms: alternative splicing and proteolytic release mediated by metalloproteinases. Among two mechanisms, it is likely that proteolytic release is a backup to alternative splicing to control specific immunomodulatory functions [14]. The production of sHLA-G is regulated by various factors, including genetic, hormonal, pathological and immunological signals. For example, Park and co-authors reported that proteolytic release is particularly pronounced in pathological conditions, in which mutations occur at the splice sites of the HLA-G gene [14].

Previous studies have suggested that the expression of sHLA-G during dengue pathogenesis can be modulated by various mechanisms. In particular, interleukin-10, interferon-gamma and matrix metalloproteinases (MMP-2 and MMP-9), all of which are elevated in severe dengue, have been shown to enhance the production of sHLA-G [15–17]. Notably, MMP-2 and MMP-9 levels are significantly elevated in severe dengue and have been associated with impaired immune responses and increased disease complications in affected patients [18]. These findings suggest that sHLA-G may contribute to immune suppression during dengue through multiple mechanisms, potentially leading to reduced activity of NK cells, $CD8^+$ T cells, and regulatory T cells.

In contrast, Renata and co-authors observed decreased sHLA-G levels during the acute phase arbovirus infection in Brazilian patients compared to the recovery phase that reflects the stable state of the patient's immune system [6]. Nevertheless, patients in this study were confirmed to have Zika and chikungunya with neurological complications. These differences in viral aetiology and clinical presentation may account for the discrepancy in sHLA-G profiles compared to our dengue-infected cohort. However, our findings align with other studies reporting elevated sHLA-G levels in patients infected with human cytomegalovirus, herpes, hepatitis and SARS-CoV-2 viruses compared to healthy controls [19]. In addition, polymorphisms within the HLA-G gene can influence plasma concentrations, potentially varying across different populations [20, 21].

Time of symptom onset is recognized as a key determinant of clinical severity in dengue [22]. In our study, multiple linear regression analysis revealed a positive association between the number of illness days and plasma sHLA-G levels after adjusting for age, sex and disease severity. These findings suggest that sHLA-G levels increase during the course of dengue infection, underscoring the dynamic nature of this biomarker. This also highlights the importance of considering illness duration when evaluating sHLA-G as a potential indicator of disease progression. Despite our findings demonstrate a clear association between sHLA-G levels and clinical severity indicators, the data should be interpreted as correlative. Further longitudinal studies are needed to determine the temporal dynamics of sHLA-G elevation and its potential mechanistic role in dengue pathogenesis.

In addition, our study revealed correlations between sHLA-G levels and key parameters for dengue, such as NEU, PLT, AST

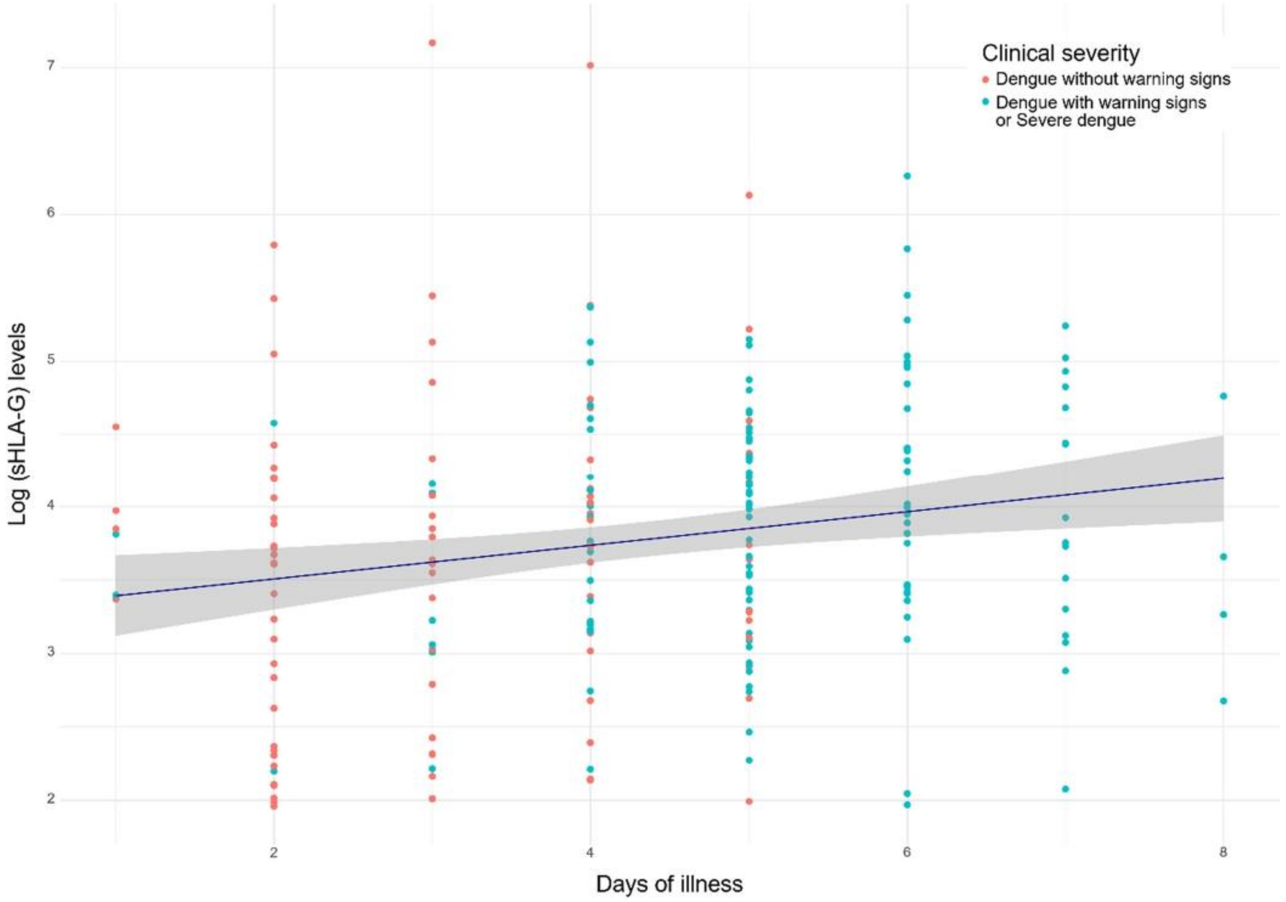

FIGURE 2 | sHLA-G levels and day of illness multiple linear regression was performed adjusting disease severity to estimate the association between log-transformed sHLA-G levels and days of illness.

TABLE 3 | Correlation of sHLA-G levels with laboratory parameters.

	WBC	NEU	LYM	MONO	EOS	BASO	LUC	RBC	Hb	HCT	PLT	AST	ALT
All patients													
R	−0.04	**−0.16**	0.11	−0.05	0.03	0.13	**0.21**	0	0.04	0.09	**−0.21**	**0.21**	**0.14**
p value	0.557	0.013*	0.097	0.488	0.7	0.051	0.004*	0.975	0.518	0.184	0.001*	0.001*	0.038*
DF													
R	**−0.2**	**−0.21**	0.03	−0.13	**−0.23**	−0.03	0.09	−0.08	−0.09	−0.01	−0.1	0.06	0.01
p value	0.041*	0.032*	0.731	0.199	0.019*	0.763	0.444	0.418	0.341	0.917	0.301	0.547	0.883
DWS													
R	0.14	−0.04	0.18	0.11	**0.26**	**0.2**	0.2	0	0.12	0.12	−0.14	0.13	0.05
p value	0.132	0.645	0.06	0.259	0.006*	0.032*	0.049	0.992	0.225	0.195	0.112	0.166	0.575
SD													
R	−0.44	−0.23	**−0.78**	−0.28	−0.01	−0.39	**−0.81**	0	0.03	−0.02	−0.2	0.07	0.19
p value	0.135	0.448	0.002*	0.352	0.976	0.188	0.005*	1	0.922	0.943	0.502	0.817	0.529

Note: Significant correlations are highlighted in bold.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BASO, basophile; DF, dengue without warning signs; DWS, dengue with warning signs; EOS, eosinophile; Hb, haemoglobin; HCT, haematocrit; LUC, large unstained cells; LYM, lymphocyte; MONO, monocyte; NEU, neutrophile; PLT, platelet; R, Spearman correlation coefficient; RBC, erythrocyte; SD, severe dengue; WBC, Leucocyte.
*Statistically significant.

and ALT [3, 22]. These findings highlight the potential added value of integrating sHLA-G with routine clinical markers to support triage and enhance understanding of the disease course. Lymphocytes are critical for antiviral immunity and their depletion, a hallmark of severe dengue and other viral infections, is associated with impaired immune response and increased infection consequences [23]. In particular, a strong inverse correlation between sHLA-G levels and total lymphocyte counts was observed in severe cases. These data suggested that elevated sHLA-G may contribute to lymphocyte depletion by exerting immune suppressive effects, such as inducing apoptosis or inhibiting lymphocyte proliferation, thereby exacerbating immune dysfunction and contributing to disease severity [24].

Also, large unstained cell (LUC) count was inversely correlated with sHLA-G level in severe patients. LUC includes large, activated lymphocytes and other atypical cells, such as virocytes, blasts cells and hematopoietic stem cells. LUC count is considered a potential indicator of a patient's immune response status during viral infections [25, 26]. A reduced LUC count may indicate impaired immune activation and is thus associated with increased infection severity. Correlations between LUC and CD8+ as well as CD4 + T cells were also noted in HIV patients [27] and LUC was suggested to be a predictive biomarker for hematologic toxicities in cancer and hematologic malignancies [28]. The observed negative correlation between LUCs and sHLA-G levels in patients with severe clinical manifestations may reflect a reduction in activated lymphocytes in response to elevated sHLA-G suggesting a potential role for sHLA-G in suppressing immune defense. Nonetheless, a significant positive correlation between LUC count and sHLA-G levels was found when all patients with varying degrees of severity were analysed together suggesting that LUC counts may respond differently to changes in sHLA-G levels across different stages and severities.

Notably, strong and statistically significant correlations between sHLA-G levels and laboratory parameters, including lymphocyte and large unstained cell counts, were observed exclusively in severe dengue cases. However, these findings must be interpreted with caution due to the small number of patients in the severe dengue group ($n = 13$). This represents a key limitation of our study, as it limits the statistical power and generalizability of the subgroup analyses. Despite this constraint, our results provide preliminary evidence that molecular immune checkpoints, such as sHLA-G, may contribute to the complex immunopathogenesis of severe dengue and warrant further investigation in larger cohorts. Furthermore, the possibility to assess the level of sHLA-G only at one time point in each patient limited the understanding of sHLA-G dynamics.

5 | Conclusion

Immune inhibitors potentially have an important role in the pathogenesis of dengue. In conclusion, our findings suggest that sHLA-G levels are associated with dengue severity, highlighting its potential as a marker for future clinical monitoring and early identification of severe cases.

Author Contributions

Barbara Seliger and Thirumalaisamy P. Velavan designed, supervised the study and contributed to the study materials and assays. Le Huu Song and Thirumalaisamy P. Velavan were involved in the conceptualization of the study. Do Duc Anh and Anja Mueller performed the experimental procedures, the statistical analysis and validation of the results. Nguyen Trong The and Le Huu Song recruited the patients and contributed to the investigation materials for sampling procedures. Do Duc Anh wrote the first draft. Do Duc Anh, Thirumalaisamy P. Velavan and Barbara Seliger reviewed the first draft. All authors have read and approved the manuscript.

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Ethics Statement

All study participants provided signed informed consent before enrolment. The Institutional Review Board of the 108 Military Hospital and the University of Tübingen approved the study, titled "Host and Viral Factors Influencing Dengue Severity and Susceptibility" (Ethics Approval No. 274/2022B02). The study complies with the Nagoya Protocol, and authorization for the use of genetic resources in Germany was obtained from the Vietnamese Ministry of Natural Resources and Environment (Reference No. 2995/QĐ-BTNMT). All procedures followed GCP/GCLP guidelines.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supplementary 1: *p* value from post hoc Dunn test. **Supplementary 2:** Multiple linear regression for sHLA-G level and day of illness.

Publication No.6

Risk Stratification of Dengue Cases Requiring Hospitalisation

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RESEARCH ARTICLE OPEN ACCESS

Risk Stratification of Dengue Cases Requiring Hospitalization

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ABSTRACT

Dengue pathogenesis involves immune-driven inflammation that contributes to severe disease progression. This study assessed a machine learning model to identify a minimal, yet highly predictive biomarker set, aiming to support clinical decision-making and patient triage. A total of 48 inflammatory mediators were quantified from plasma samples collected at admission from confirmed dengue patients, classified as either dengue without warning signs (DF) or dengue with warning signs/severe dengue (DWS/SD). A random forest approach was applied to identify the most predictive biomarkers associated with disease severity requiring hospitalization, based on admission-time variables. Among the 48 immune mediators, 43 were differentially expressed in dengue patients versus healthy controls, and 26 showed significant differences between DF and DWS/SD cases. Lymphocyte counts negatively correlated with IL-1RA, while liver enzymes showed positive correlations with HGF and SCGF-beta; platelet counts also negatively correlated with these markers. Key severity-associated markers included HGF, TNF-beta, MIP-1-beta, and SCGF-beta. A model incorporating these markers and fever duration achieved nearly 80% accuracy in distinguishing DWS/SD from DF cases, independent of clinical examination. The findings suggest that targeted cytokine profiling may guide early hospitalization decisions and ease healthcare burdens in dengue-endemic regions.

Abbreviations: Beta-NGF, beta nerve growth factor; CTACK, cutaneous T-cell attracting chemokine; Eotaxin, eotaxin (CCL11); FGF-basic, fibroblast growth factor basic; GM-CSF, granulocyte macrophage colony stimulating factor; GRO-alpha, growth regulated oncogene alpha; G-CSF, granulocyte colony stimulating factor; HGF, hepatocyte growth factor; IFN-alpha, interferon alpha; IFN-gamma, interferon gamma; IL-10, interleukin 10; IL-12p40, interleukin 12 subunit p40; IL-12p70, interleukin 12 subunit p70; IL-13, interleukin 13; IL-15, interleukin 15; IL-16, interleukin 16; IL-17, interleukin 17; IL-18, interleukin 18; IL-1RA, interleukin 1 receptor antagonist; IL-1-alpha, interleukin 1 alpha; IL-1-beta, interleukin 1 beta; IL-2, interleukin 2; IL-2R-alpha, interleukin 2 receptor alpha; IL-3, interleukin 3; IL-4, interleukin 4; IL-5, interleukin 5; IL-6, interleukin 6; IL-7, interleukin 7; IL-8, interleukin 8; IL-9, interleukin 9; IP10, interferon gamma-induced protein 10; LIF, leukemia Inhibitory Factor; MCP1/MCAF, monocyte chemoattractant protein1/monocyte chemotactic and activating factor; MCP3, monocyte chemoattractant protein 3; MIF, macrophage migration inhibitory factor; MIG, monokine induced by gamma interferon; MIP1-alpha, macrophage inflammatory protein 1 alpha; MIP1-beta, macrophage inflammatory protein 1 beta; M-CSF, macrophage colony stimulating factor; PDGF-BB, platelet derived growth factor BB; RANTES, regulated on activation, normal t cell expressed and secreted; SCF, stem cell factor; SCGF-beta, stem cell growth factor beta; SDF-1-alpha, stromal cell derived factor 1 alpha; TNF-alpha, tumor necrosis factor alpha; TNF-beta, tumor necrosis factor beta; TRAIL, tumor necrosis factor related apoptosis inducing ligand; VEGF, vascular endothelial growth factor.

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1 | Introduction

Dengue is the most prevalent mosquito-borne human disease and is caused by the dengue virus (DENV). Clinical manifestations range from asymptomatic to severe and sometimes fatal disease. Severe cases often require hospitalization where timely monitoring and intervention can reduce the overall mortality rate to less than 1% [1, 2]. The World Health Organization (WHO) classifies symptomatic dengue into three clinical categories: dengue without warning signs (DF; dengue fever), dengue with warning signs (DWS), and severe dengue (SD) [3]. Warning signs, such as mucosal bleeding, persistent vomiting, hepatomegaly, low platelet counts, and abruptly elevated liver enzymes, indicate the need to monitor patients closely, while milder cases can be treated at home [3]. Furthermore, cases with warning signs can eventually progress to SD leading to life-threatening complications, such as hypovolaemic shock, severe bleeding, and multiorgan failure [4].

The symptoms of dengue are often difficult to differentiate from other viral fevers and concomitant illnesses, even during critical phases of infection [5]. This presents a significant challenge for clinicians attempting to provide an accurate diagnosis and prognosis of the disease, particularly in countries with a high prevalence of dengue. An inaccurate diagnosis may result in unnecessary hospitalization and subsequently an increased burden on the healthcare system [6]. On the other hand, the accurate identification of severe cases is critical to prevent fatalities. Clinical and conventional laboratory markers, including white blood cell (WBC) counts, platelet counts (PLT), and liver enzyme levels can help to distinguish severe cases of dengue from DF [7, 8]. However, clinical manifestations may be delayed and can either follow or be triggered by changes in humoral inflammatory profiles [9]. Therefore, inflammatory mediators such as cytokines and chemokines may serve as early direct indicators of the systemic inflammatory status and have potential for prognostication of tissue damage before clinical manifestations of disease severity [10].

Cytokines are fundamental to the pathogenesis and clinical manifestations of dengue. They are involved in both protection and pathogenesis by playing a crucial role in the control of DENV replication and the regulation of inflammatory processes. Dysregulated cytokine production causing excessive release of pro-inflammatory cytokines may contribute to increased vascular permeability and increase disease severity and the risk of complications [11, 12]. Overproduction of Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-alpha), interleukin (IL)-1, IL-6, and IL-8, and the down-regulation of anti-inflammatory cytokines such as IL-4, IL-10, IL-13, and tumor necrosis factor beta (TGF-beta), can lead to systemic inflammation and vascular damage, worsening the dengue prognosis [12, 13].

This study aimed to develop a robust method for differentiating the severity of dengue infections, thereby facilitating rapid triage decisions based on a comprehensive assessment of humoral inflammatory markers. To achieve this, a broad spectrum of humoral immune mediators, including 48 cytokines, chemokines, and growth factors, was analyzed alongside clinical and laboratory parameters of dengue patients in Vietnam. A supervised machine learning approach was used to identify the most predictive biomarkers for determining disease severity requiring hospitalization, based on admission-time variables. The analyses identified a small subset of cytokines capable of reliably distinguishing dengue severity, offering potential utility in early clinical decision-making and patient triage.

2 | Materials and Methods

2.1 | Ethical Approval Statement

Signed informed consent was obtained from all study participants before enrollment. The study was approved by the Institutional Review Board of the 108 Military Hospital and the University of Tübingen for the project entitled "Host and viral factors influencing dengue severity and susceptibility" (ethics approval no. 274/2022B02). The study adhered to the Nagoya Protocol and received approval from the Vietnamese Ministry of Natural Resources and Environment (Reference: No.2995/QĐ-BTNMT). All procedures followed GCP/GCLP guidelines.

2.2 | Study Population

Samples were collected during two consecutive seasonal dengue outbreaks between September to December in 2021 and 2022. The study included civilian patients ($n = 306$) with symptoms of viral hemorrhagic fever, who were admitted to the 108 Military Central Hospital in Hanoi, Vietnam. Dengue was diagnosed according to the diagnostic criteria of the World Health Organisation (https://apps.who.int/iris/handle/10665/44188), which were adopted by the Vietnamese Ministry of Health. The inclusion criteria were patients presenting fever with at least two of the clinical sign/symptoms suggesting dengue (e.g., nausea/vomiting, headache, retro-ocular pain, rash, body aches, Tourniquet test positive) and/or positive for at least one of the indirect diagnostic methods (serological rapid tests), as detailed in the WHO guideline 2009 [3]. Exclusion criteria included patients with bacterial or other viral infections, chronic diseases, or hematological disorders. Blood samples were collected from all dengue patients on admission. Similarly, blood samples were also collected from healthy blood donors ($n = 118$) who had tested negative for HBsAg, anti-HCV and anti-HIV from the hospital transfusion department. Plasma was separated from blood and stored at −70°C until use.

2.3 | Dengue Serological Tests

Samples were subjected to nonstructural protein 1 (NS1) DENV antigen testing and anti-DENV immunoglobulin M and G (IgM and IgG) antibody tests using the Bioline Dengue Duo kit (Abbott, Santa Clara, USA; formerly Alere Inc, Waltham, USA), following the manufacturer's instructions. Among dengue patients, those testing IgG positive within 8 days after the onset of fever were categorized as secondary infections, while cases testing positive only for NS1 or IgM were classified as primary infections. Tertiary and quaternary infections were indistinguishable from secondary infections in this study.

2.4 | Dengue RNA Positivity and Exclusion of Zika/Chikungunya RNA

Total viral RNA was isolated from 140 µL of patient plasma utilizing the QIAmp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's guidelines. To exclude possible infections with other arboviruses circulating in Vietnam and to confirm dengue infection, all samples ($n = 306$) were subjected to multiplex real-time PCR analysis for dengue/Zika/chikungunya viral RNA using the Fast Track Diagnostics Kit (Siemens Healthcare GmbH, Erlangen, Germany) on a LightCycler480-II (Roche, Mannheim, Germany), following the manufacturer's protocol. Confirmed dengue cases ($n = 299$) were identified as those with detectable DENV RNA by real-time RT-PCR and tested negative for Zika/chikungunya RNA.

2.5 | Clinical Severity and Laboratory Parameters

Patients were categorized clinically into three severity levels based on WHO guidelines [3]: dengue without warning signs (DF), DWS, and SD. The clinical presentation was recorded on admission, and the patient's sex (male or female) was noted as specified at birth. Laboratory parameters (see Table 1), including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, leukocytes (WBC) count, lymphocytes (LYM) count, neutrophils (NEU) count, Eosinophils (EOS) count, Basophils (BASO) count, erythrocytes (RBC) count, monocytes (MONO) count, platelet (PLT) count, hemoglobin (Hb), and hematocrit (HCT), were determined at admission to hospital.

2.6 | Human Cytokines Screening Using Bio-Plex Panel

Plasma samples from confirmed dengue cases by RT-PCR ($n = 299/306$) and healthy controls ($n = 118$) were analyzed for 48 inflammatory markers, including cytokines ($n = 35$), chemokines ($n = 9$), and growth factors ($n = 4$). The assay was carried out using the magnetic bead-based Bio-Plex Pro Human Cytokine Screening Panel 48-plex (Bio-Rad Laboratories GmbH, Feldkirch, Germany) following the manufacturer's protocol on a Bio-Plex 200 system.

The panel involves a biologically relevant array of adaptive immunity cytokines, pro-inflammatory cytokines, and anti-inflammatory cytokines, as: FGF-basic, Eotaxin, G-CSF, GM-CSF, IFN-gamma, IL-1-beta, IL-1RA, IL-1-alpha, IL-2R-alpha, IL-3, IL-12p40, IL-16, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, GRO-alpha, HGF, IFN-alpha, LIF, MCP3, IL-10, IL-12p70, IL-13, IL-15, IL-17, IP10, MCP1/MCAF, MIG, Beta-NGF, SCF, SCGF-beta, SDF-1 alpha, MIP1-alpha, MIP1-beta, PDGF-BB, RANTES, TNF-alpha, VEGF, CTACK, MIF, TRAIL, IL-18, M-CSF, and TNF-beta (detailed in the abbreviations list).

Each assay utilized a 15 µL volume of plasma sample, with the assay controls, standards, and patient/healthy control samples distributed into each well of the assay 96-well plate. All assay steps were conducted as per the manufacturer's instructions. The resulting data were processed using Bio-Plex Manager 6.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 | Statistical Analysis

The data were analyzed and visualized using R software version 4.3.2 (http://www.r-project.org). A p-value < 0.05 was considered statistically significant for statistical comparisons in the study. Demographic, clinical, and laboratory data of the patients were summarized, with quantitative variables presented as median values with ranges, and categorical variables as absolute numbers with percentages. The titers of quantified biomarkers were expressed as median values with ranges (pg/mL). The distribution of quantitative variables was evaluated for normality using the Shapiro–Wilk and D'Agostino–Pearson tests, with additional visual assessment based on skewness and kurtosis. Categorical variables were compared using chi-square tests, while continuous variables were assessed using the Wilcoxon rank-sum test. Nonparametric Spearman correlation with Holm corrections for multiple testing was utilized to assess correlations between different markers and laboratory parameters.

2.8 | Predictive Modelling

A random forest machine learning approach [14], using the "*randomForest*" package in R [15], was taken to identify the most important variables for differentiating dengue cases requiring close medical observation (DWS/SD) from milder cases (DF) [3]. For this, the study considered two different sets of predictors. The first was based solely on the plasma levels inflammatory mediators together with the duration of fever before hospital admission (days of fever). The second was based on more traditional dengue severity-related parameters that included age, sex, days of fever, type of infection (primary/secondary dengue), clinical features, and laboratory parameters (as listed in Table 1). Warning signs, such as mucosa/severe bleeding manifestations, abdominal pain, lethargy, hepatomegaly, shock, and respiratory distress were excluded due to DWS/SD being partially defined based on the presence of these symptoms. Seven inflammatory biomarkers (GRO-alpha, beta-NGF, IL-15, IL-3, IL-5, VEGF and IL-6) with a missing rate of >30% were excluded from the analysis (Table 2); other missing values were imputed using the "*missForest*" package in R [16]. Feature selection was carried out using the "*VSURF*" R package" [17]. Model metrics, including accuracy, specificity, sensitivity, and F1-score, were generalized following 10-fold cross-validation. The receiver operating characteristic curve was constructed and the area under the curve (AUROC) was calculated using the "*pROC*" package in R [18].

3 | Results

3.1 | Confirmation of Dengue Infection and Classification of Primary and Secondary Dengue

Zika and chikungunya viral RNA were not detected in any of the 306 tested samples. The multiplex real-time RT-PCR assay confirmed the presence of DENV RNA in 299 out of 306 cases; the other seven cases were excluded from the study. IgM/IgG positivity was used to differentiate primary dengue infections (133/299) from secondary infections (140/299). Among DENV RT-PCR confirmed cases included in the study, 26 cases

TABLE 1 | Patient characteristics on admission.

	Dengue without warning signs (DF) ($n = 172$)	Dengue with warning signs (DWS) and severe dengue (SD) ($n = 127$)	p value
Demographic data			
Median age (years)	47 (14–87)	49 (17–83)	0.233
Sex (% male)	54	50	0.64
Clinical presentations			
Days of fever (days)	4 (1–8)	5 (1–8)	< 0.001
Headache, n (%)	157 (91.3%)	112 (88.2%)	0.437
Retro ocular pain, n (%)	95 (55.2%)	92 (72.4%)	0.003
Myalgia, n (%)	129 (75.0%)	97 (76.4%)	0.781
Arthralgia, n (%)	112 (65.1%)	92 (72.4%)	0.198
Rash, n (%)	28 (16.3%)	73 (57.5%)	< 0.001
Vomit, n (%)	23 (13.4%)	33 (26.0%)	0.01
Abdominal pain, n (%)	0 (0%)	22 (17.3%)	NA
Lethargy, n (%)	0 (0%)	5 (3.9%)	NA
Hepatomegaly, n (%)	0 (0%)	3 (2.4%)	NA
Shock, n (%)	0 (0%)	5 (3.9%)	NA
Respiratory distress, n (%)	0 (0%)	6 (4.7%)	NA
Edema, n (%)	0 (0%)	31 (24.4%)	< 0.001
Bleeding manifestation			
Subcutaneous, n (%)	33 (19.2%)	86 (67.7%)	< 0.001
Mucosal, n (%)	0 (0%)	65 (51.2%)	< 0.001
Severe, n (%)	0 (0%)	4 (3.1%)	0.031
Laboratory tests			
Leukocytes $\times 10^3/\mu L$	4.01 (0.93–16.9)	3.71 (1.33–11.6)	0.947
Lymphocyte $\times 10^3/\mu L$	0.78 (0.17–4.57)	0.99 (0.22–4.09)	0.003
Neutrophils $\times 10^3/\mu L$	2.18 (0.45–203)	1.80 (0.74–7.70)	0.004
Eosinophils $\times 10^3/\mu L$	0.01 (0.01–0.24)	0.02 (0.01–0.45)	0.132
Basophils $\times 10^3/\mu L$	0.03 (0.01–1.18)	0.07 (0.02–1.76)	< 0.001
Monocytes $\times 10^3/\mu L$	0.36 (0.05–1.35)	0.31 (0.08–2.53)	0.140
Platelets $\times 10^3/\mu L$	115 (9.00–384)	20.0 (4.00–228)	< 0.001
Erythrocytes $\times 10^6/\mu L$	4.90 (3.66–7.62)	5.10 (2.76–6.81)	0.001
Hemoglobin g/dL	14.6 (11.0–18.7)	15.1 (10.0–19.0)	0.003
Hematocrit (%)	43.1 (31.8–54.7)	44.5 (21.4–60.5)	0.002
AST (U/L)	54.0 (15.1–1210)	119 (16.0–11 100)	< 0.001
ALT (U/L)	38.7 (8.00–855)	66.9 (8.20–2190)	< 0.001
Serological tests			
NS1—positivity (%)	127 (73.8%)	91 (71.7%)	0.695
IgM—positivity (%)	62 (36.0%)	79 (62.2%)	< 0.001
IgG—positivity (%)	56 (32.6%)	77 (60.6%)	< 0.001

Note: Variables were summarized in absolute count with percentage or median with (range). Categorical variables were compared using chi-square test, and quantitative variables were compared using Wilcoxon test. p-value < 0.05 is considered significant.
Abbreviations: AST, aspartate aminotransferase; ALT, alanine Aminotransferase; DENV, dengue virus; IgG, immunoglobulin G; IgM, immunoglobulin M; NS1, nonstructural protein.

TABLE 2 | Plasma levels of inflammatory mediators in dengue patients.

	Dengue without warning signs (DF) ($n = 172$)	Dengue with warning signs (DWS) and severe dengue (SD) ($n = 127$)	p value
Pro-inflammatory			
IL-17	8.7 (1.2–49)	11 (1.6–36)	0.001
MIG	1084 (61–51 615)	2140 (63–60 703)	0.010
MIP1-alpha	3.9 (0.28–36)	5.9 (0.81–64)	0.010
SCGF-beta	119 349 (5887–1 806 133)	384 276 (9043–1 093 594)	< 0.001
GRO-alpha*	766 (92–2500)	399 (54–2242)	< 0.001
IFN-gamma	40 (0.87–346)	32 (0.61–269)	0.005
MCP1/MCAF	81 (4.2–1505)	57 (6.1–977)	< 0.001
Beta-NGF*	4.1 (0.06–86)	3.7 (0.03–67)	0.390
IL-15*	374 (23–774)	101 (14–284)	0.009
IL-18	70 (2.1–947)	92 (6–557)	0.091
IP10	10 234 (80–615 856)	13 824 (333–388 336)	0.641
M-CSF	49 (3.9–298)	60 (2.5–343)	0.415
MCP3	4 (0.12–194)	4.1 (0.38–193)	0.802
TNF-alpha	58 (6.4–361)	60 (11–454)	0.539
TRAIL	79 (2.5–558)	117 (1.2–444)	0.365
Eotaxin	91 (7.7–960)	131 (13–480)	0.045
IL-3*	0.4 (0.04–9)	0.68 (0.02–51)	0.058
MIF	444 (19–5570)	494 (27–15 730)	0.88
PDGF-BB	355 (1–6773)	326 (3.6–4513)	0.458
MIP1-beta	140 (7.6–783)	68 (6.4–283)	< 0.001
RANTES	1129 (63–59 856)	484 (29–14 771)	< 0.001
TNF-beta	146 (11–404)	64 (2.4–357)	< 0.001
GM-CSF	2.4 (0.03–12)	2.2 (0.03–11)	0.4
IL-5*	53 (4.5–278)	23 (7–112)	0.008
SDF-1-alpha	1920 (218–14 677)	1585 (268–16 000)	0.295
Anti-inflammatory			
CTACK	950 (27–9892)	1682 (68–6116)	0.003
IL-10	9.9 (0.07–461)	19 (0.03–183)	< 0.001
IL-1RA	1231 (66–20 578)	756 (82–26 298)	< 0.001
IL-4	2.3 (0.25–13)	2.2 (0.33–5.7)	0.608
IL-9	185 (20–562)	89 (5.8–523)	< 0.001
IL-7	9.6 (0.18–108)	8.3 (0.18–107)	0.12
Pro/anti-inflammatory			
HGF	600 (64–3059)	1294 (86–12 358)	< 0.001
IL-1-alpha	27 (0.12–205)	53 (2.2–342)	< 0.001
IL-1-beta	6.6 (0.31–34)	7.8 (1.4–20)	0.032
IL-12p40	77 (0.85–456)	95 (6.9–524)	0.015
IL-13	5 (0.12–61)	7 (0.15–40)	0.002
LIF	44 (3.8–327)	82 (3.8–315)	0.003
IL-2R-alpha	77 (4.5–689)	97 (6.5–472)	0.063
IFN-alpha	13 (0.08–104)	11 (0.63–293)	0.169

(Continues)

TABLE 2 | (Continued)

	Dengue without warning signs (DF) ($n = 172$)	Dengue with warning signs (DWS) and severe dengue (SD) ($n = 127$)	p value
IL-6*	4.9 (0.03–360)	4.7 (0.09–502)	0.774
IL-12p70	2.8 (0.07–39)	3.8 (0.07–40)	0.239
IL-16	83 (5.4–1272)	104 (9.9–2236)	0.104
IL-2	6.4 (0.04–50)	5.1 (0.1–23)	0.26
IL-8	16 (0.9–181)	24 (2.9–790)	0.002
Growth factors			
FGF-basic	36 (0.46–234)	66 (1.8–291)	0.002
G-CSF	131 (8.8–937)	136 (12–8649)	0.333
SCF	83 (8.3–287)	94 (11–885)	0.203
VEGF*	299 (10–1010)	93 (21–876)	0.006

Note: Variables were summarized in median with range (pg/mL). The names of 48 inflammatory mediators are detailed in the abbreviation list. *Variables with > 30% values missing from measurement. Levels of variables were compared using Wilcoxon test. p-value < 0.05 is considered significant.

($n = 26/299$) with negative dengue serological tests (negative for NS1, IgG, and IgM assays) were retained in the analysis due to their positive RT-PCR results, although they could not be classified as either primary or secondary infections.

3.2 | Baseline Characteristics of the Study Participants

Both patients and healthy controls resided in Hanoi metropolitan areas and were of Kinh ethnicity. No significant differences were observed in age or sex distribution between controls and patients, nor between the different dengue severity groups (median age, DF vs. DWS/SD: 47 vs. 49 years, $p = 0.233$; proportion male, DF vs. DWS/SD: 54% vs. 50%, $p = 0.64$) (Table 1). Patients were categorized into two groups based on the need for hospitalization: those with dengue without warning signs (DF, $n = 172$), and those with DWS or SD (DWS/SD, $n = 127$; including $n = 114$ with DWS and $n = 13$ with SD).

The clinical data of the patients are summarized in Table 1. Significant variations were observed in the duration of fever before admission (days of fever), dengue-related clinical manifestations, blood parameters, and liver enzymes between the DF and DWS/SD groups (Table 1). Retro-ocular pain ($p = 0.003$), rash ($p < 0.001$), vomiting ($p = 0.01$), and bleeding manifestations ($p < 0.001$) were more frequently reported in DWS/SD compared to DF patients (Table 1). DWS/SD patients exhibited significantly lower PLTs ($p < 0.001$) and higher levels of liver enzymes AST ($p < 0.001$), and ALT ($p < 0.001$) compared to DF patients (Table 1).

3.3 | Level of Inflammatory Mediators Differ Between Dengue Patients and Healthy Controls

Plasma concentrations of $n = 43/48$ markers were found to be significantly different between dengue patients and healthy controls, except for $n = 5/48$ markers including TRAIL ($p = 0.082$), TNF-beta ($p = 0.991$), GM-CSF ($p = 0.405$), IL-5 ($p = 0.078$), and IL-7 ($p = 0.051$) (Supporting Information S1: Table S1). In addition, significant differences between DF patients and DWS/SD patients were observed in $n = 26/48$ markers (Table 2), including Pro-inflammatory markers ($n = 13$): IL-17, MIG, MIP1-alpha, SCGF-beta, GRO-alpha, IFN-gamma, MCP1/MCAF, IL-15, Eotaxin, MIP1-beta, RANTES, TNF-beta, IL-5; anti-inflammatory markers ($n = 4$): CTACK, IL-10, IL-1RA, IL-9; Pro/anti-inflammatory markers ($n = 7$): HGF, IL-1-alpha, IL-1-beta, IL-12p40, IL-13, LIF, IL-8; and two growth factors ($n = 2$): FGF-basic, VEGF (Table 2).

3.4 | Correlations of Inflammatory Mediators and Laboratory Parameters in Dengue

Spearman correlations were employed to determine the relationship between the plasma levels of 48 inflammatory mediators and conventional laboratory parameters in dengue patients, as summarized in Figure 1 and Supporting Information S1: Table S2. Notably, a negative correlation was observed between lymphocyte counts and IL-1RA ($\rho = -0.5$, $p < 0.001$), while positive correlations were noted between liver enzymes and HGF (AST: $\rho = 0.5$, $p < 0.001$; ALT: $\rho = 0.5$, $p < 0.001$) and SCGF-beta (AST: $\rho = 0.5$, $p < 0.001$; ALT: $\rho = 0.4$, $p < 0.001$) (Figure 1). PLTs were also observed to negatively correlated with HGF ($\rho = -0.4$, $p < 0.001$) and SCGF-beta ($\rho = -0.5$, $p < 0.001$), suggesting the potential associations of HGF and SCGF-beta with dengue (Figure 1 and Supporting Information S1: Table S2).

In addition, other blood cell counts (WBC, NEU, and MONO) displayed inverse correlations with majority of markers, except for IL-5, IL-6, IL-7, IL-15, TNF-beta, and VEGF (Figure 1). Correlations among 48 inflammatory mediators were also summarized in Figure 2 and Supporting Information S1: Table S3. While strong correlations were found between majority of mediators, lower correlations with other cytokines were observed in IL-5, IL-6, and IL-7 (Figure 2). These results

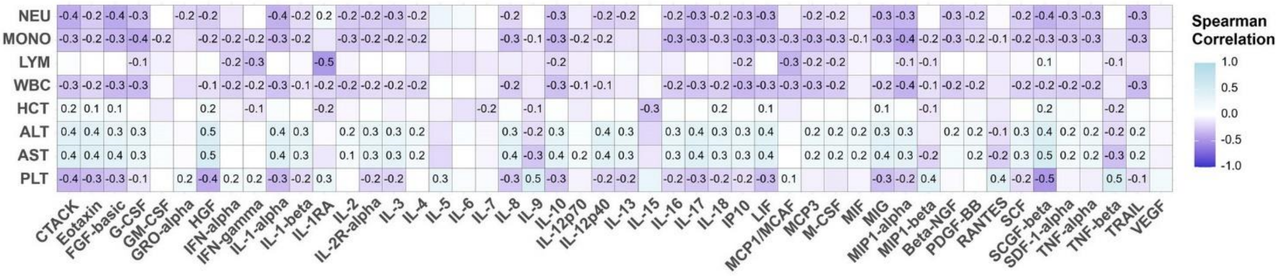

FIGURE 1 | Correlations between laboratory parameters and inflammatory biomarkers. The names of inflammatory mediators are detailed in the abbreviation list. The correlation coefficient was calculated using Spearman's method with Holm corrections. p-value < 0.05 is considered significant. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCT, haematocrit; LYM, lymphocytes count; MONO, monocytes count; NEU, neutrophils count; PLT, platelet count; WBC, leucocytes count.

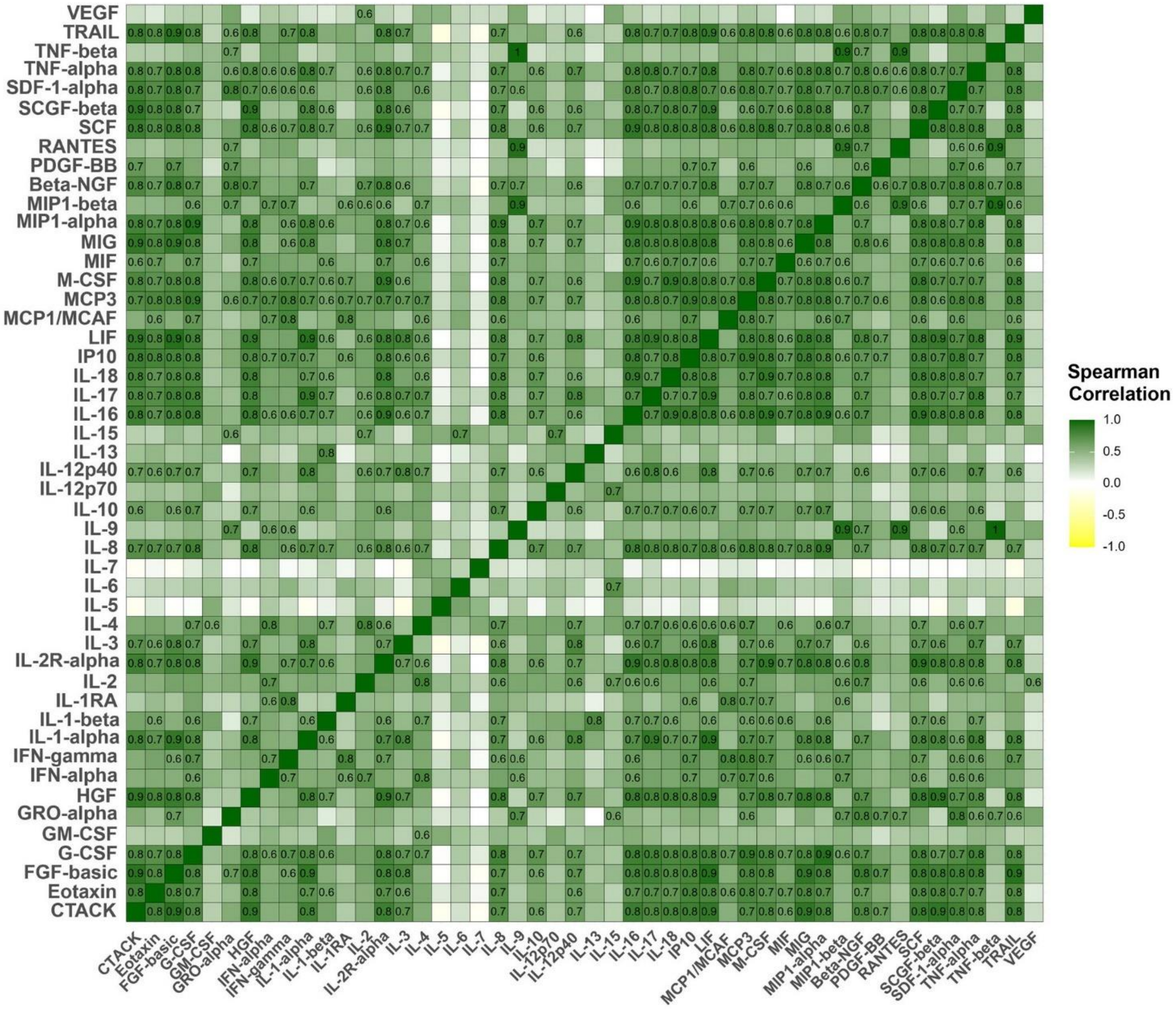

FIGURE 2 | Correlations among inflammatory biomarkers. The names of inflammatory mediators are detailed in the abbreviation list. The correlation coefficient was calculated using Spearman's method with Holm corrections. p-value < 0.05 is considered significant.

indicate correlations among all variables analyzed in the study, including inflammatory biomarkers and conventional dengue parameters, suggesting the presence of multicollinearity.

3.5 | Cytokine-Based Classification of Dengue Severity

The ability of inflammatory mediators to differentiate patients by dengue severity, and thus their need for hospitalization, was subsequently investigated. Following a feature selection procedure (see Section 2), four cytokines (TNF-beta, HGF, MIP1-beta, and SCGF-beta), alongside the days of fever, were identified as robust predictors for disease severity. A model based on these features (Model 1) showed a predictive accuracy in differentiating DWS/SD from DF of 0.78 and an AUROC of 0.86 (Figure 3 and Table 3), with model sensitivity and specificity of 0.81 and 0.77, respectively. Importantly, model performance based on these features alone was comparable to a model based on traditional dengue markers including clinical and laboratory parameters (Model 2) (accuracy = 0.77, AUROC = 0.87, sensitivity = 0.78, specificity = 0.78) (Figure 4 and Table 3), implying that these cytokines could be used as robust biomarkers of dengue severity for rapid patient triaging. “A reduced model derived from Model 2, incorporating the strongest predictors, including days of fever, TNF-beta, HGF, MIP1-beta, and SCGF-beta achieved comparable, and slightly improved, performance relative to the full Model 2 (accuracy = 0.78, AUROC = 0.88, sensitivity = 0.79, specificity = 0.76) (Supporting Information S1: Figure S1).”

4 | Discussion

In dengue, patient triaging remains challenging due to the nonspecific signs and symptoms of the disease. Conventional classification of dengue cases relies on astute clinical interpretation and laboratory findings [5]. This approach requires experienced physicians and may not be optimal during dengue outbreaks. Therefore, this study aimed to determine potential biomarkers that could assist physicians in making timely admission decisions for dengue patients based on inflammatory biomarkers, independently of clinical symptoms.

The study revealed significant differences in plasma levels of inflammatory mediators between dengue patients and healthy controls, as well as between DF and DWS/SD patients. A total of 43 out of 48 markers were differentially regulated between dengue patients and healthy controls, highlighting the altered humoral inflammatory profile associated with dengue infection. Markers that did not show significant differences between patients and controls suggest that these distinct cytokines may not play a prominent role in dengue-associated immune dysregulation, or that their levels remain stable across different stages of the disease.

Significant differences were found in 26 of 48 markers between DF and DWS/SD dengue patients. Among the pro-inflammatory markers, levels of IFN-γ, MIP-1β, RANTES, and TNF-β were significantly higher in DF patients compared to those with DWS/SD, whereas SCGF-β levels were significantly elevated in the DWS/SD group (Table 2). These markers play

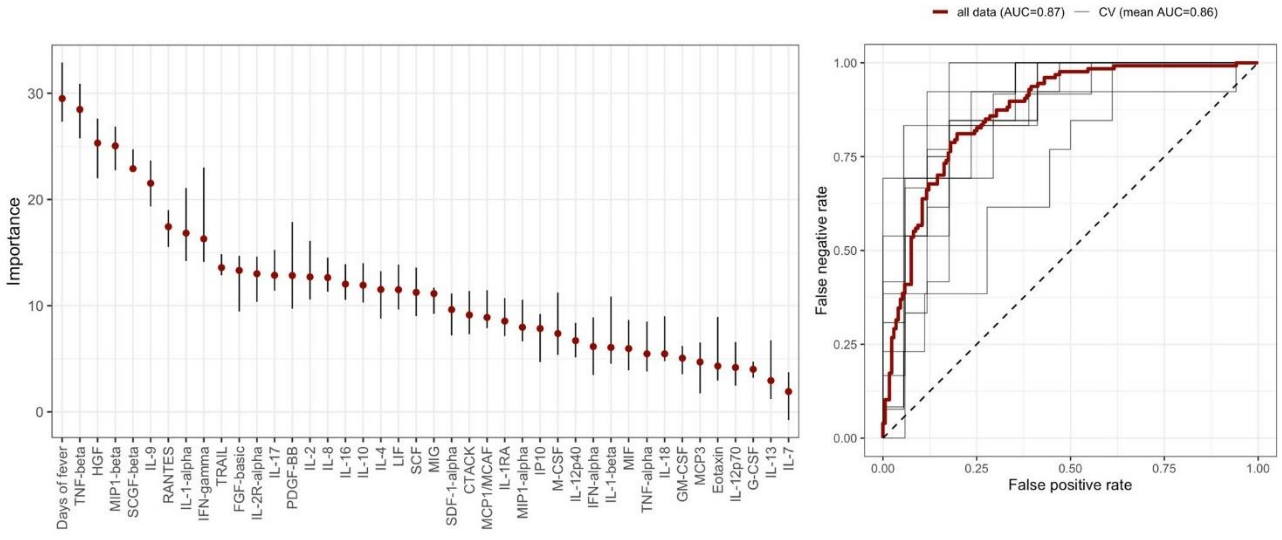

FIGURE 3 | Feature importance plot and AUROC curve of Model 1. The names of inflammatory mediators are detailed in the abbreviation list. AUC, area under the curve; CV, cross-validation.

TABLE 3 | Parametric of Random Forest models after 10-fold cross-validation.

	Accuracy	AUROC	Sensitivity	Specificity	F1-score
Model 1	0.78	0.86	0.81	0.77	0.81
Model 2	0.77	0.87	0.78	0.78	0.79

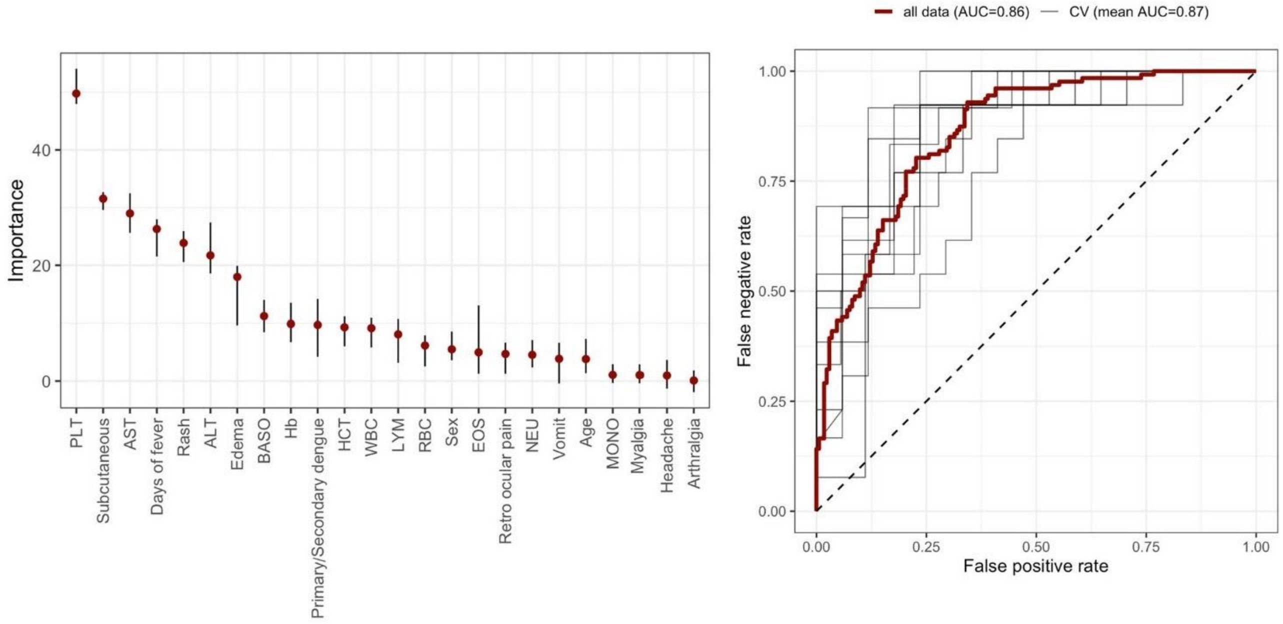

FIGURE 4 | Feature importance plot and AUROC curve of Model 2. ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BASO, basophils count; CV, cross-validation; EOS, eosinophils count; Hb, haemoglobin; HCT, haematocrit; LYM, lymphocytes count; MONO, monocytes count; NEU, neutrophils count; PLT, platelet count; RBC, erythrocytes count; WBC, leucocytes count.

key roles in immune cell activation, chemotaxis, and the inflammatory cascade, processes typically upregulated during the acute phase of dengue infection [12, 19]. Strong associations with severity were also found in anti-inflammatory markers, including IL-10, IL-1RA and IL-9, suggesting possible disruptions of the balance between pro-inflammatory and anti-inflammatory responses in cases that required hospitalization. Additionally, inflammatory markers with both pro-inflammatory and anti-inflammatory effects, such as HGF, IL-1-alpha, and IL-1-beta, were significantly elevated in DWS/SD patients. This may reflect the complex interplay between inflammation and immune resolution during disease progression [11]. Our results indicate that FGF-basic levels are elevated in DWS/SD patients, while VEGF levels are reduced, highlighting a potential imbalance that may impair effective tissue repair and contribute to increased vascular permeability during infection. Elevated FGF-basic levels in DWS/SD patients may reflect a compensatory response to greater endothelial damage or tissue injury in more severe disease, triggering enhanced fibroblast activation and repair mechanisms [20]. Notably, these observations contrast with findings by Furuta et al., who reported reductions in both FGF-basic and VEGF levels in DHF patients compared to DF patients, suggesting that differences in timing, disease kinetics, or host response dynamics may influence growth factor profiles [21]. Further studies with longitudinal data are needed to elucidate the temporal dynamics and regulatory roles of these growth factors in dengue pathogenesis.

With several inflammatory mediators as potential markers for distinguishing DWS/SD from DF cases, a machine learning approach was applied. After a robust feature selection process, the final model comprises five variables: days of fever, TNF-beta, HGF, MIP-1-beta, and SCGF-beta, which can differentiate DWS/SD from DF cases with nearly 80% accuracy. For years, conventional laboratory parameters such as PLT, HCT, and liver enzymes have been used as indicators of disease severity, alongside clinical manifestations [8, 22]. A comprehensive clinical examination provides substantial data, whereas a less thorough approach may compromise the specificity and sensitivity of outcome prediction [22, 23]. Therefore, the model's independence from clinical examination in this study is a key advantage, making it particularly useful when clinical presentation is unclear and patient numbers are high during an outbreak [5].

The time from symptom onset plays a crucial role in determining the severity of dengue. This variable is often measured from the onset of initial symptoms, most commonly fever, which is both easily recognized and widely reported by patients. A recent study has also shown that the time since dengue symptom onset is one of the most important predictors for the length of hospital stays, independent of the assigned severity score [7]. This variable is closely linked to viraemia levels, which significantly impact disease progression [24]. In addition, the disease time-point is strongly associated with dynamic humoral changes, including fluctuations in inflammatory mediators and immune responses that influence dengue severity [19]. The study analyses thus reconfirm the importance of considering the number of days of fever alongside other dengue-related variables.

During its lifecycle, DENV infects hepatocytes and Kupffer cells, resulting in liver damage and further exacerbation of liver dysfunction [25]. HGF, a protein produced in response to liver injury, is elevated in SD patients and correlates with liver enzyme levels. Consistent with previous studies [26], HGF was also identified as a potential predictor of DWS/SD. Meta-analyses have similarly shown that the initial acute inflammatory response with hepatic involvement is a key determinant of disease progression in dengue [27], suggesting the promising value of HGF for dengue severity prediction.

In contrast to HGF, TNF-beta levels were significantly higher in patients with DF (milder cases) compared to those with DWS/SD, indicating an association between elevated TNF-beta and less severe clinical presentations. Since TNF-beta is known to inhibit viral replication and synergize with other interferons [28], reduced TNF-beta levels may favor DENV activity and increase host damage. The results also demonstrated that TNF-beta plasma levels were significantly higher in healthy controls than in dengue patients, suggesting a potential protective role for this cytokine against dengue infection and progression to severe disease. In addition to the possible protective role of TNF-beta, MIP1-beta showed a strong association with DF patients. Consistent with the finding from this study, Bozza et al. identified MIP1-beta as a predictive factor with a protective effect for SD [29].

During dengue progression, there is a notable decrease in leukocyte count [29], which is counteracted by increased haematopoiesis following the upregulation of bone marrow-stimulating factors, such as SCGF-beta [30]. Previous reports have indicated an increased secretion of SCGF-beta following infection with respiratory syncytial virus [31] and in cases of liver cancer [32]. This study revealed significant correlations between SCGF-beta levels and monocyte, leukocyte, and PLTs, as well as AST and ALT levels, suggesting an upregulation of hematopoietic activity to compensate for blood and other tissue damage.

Changes in laboratory parameters reflect the systemic impact of dengue, with thrombocytopenia and transaminitis as key indicators of disease severity. It is thus rational to find strong correlations between most inflammatory mediators and dengue-related laboratory parameters, which also aligns with previous studies that have shown how these factors collectively contribute to immune responses and disease pathogenesis [11, 27]. Furthermore, the findings from this study proposed that inflammatory markers could serve as early indicators of disease progression, potentially before these could be detected through laboratory-assessed abnormalities, such as thrombocytopenia or elevated liver enzymes [9]. Moreover, and as clearly demonstrated here, a small number of measured cytokines are sufficient to identity patients in need of close medical observation as reliable as traditional assessment based on time-consuming laboratory findings.

The complexity of dengue pathogenesis arises from the interaction of various factors, including viral and host elements, with the host immune system playing a pivotal role. This study demonstrates that among other biomarkers, TNF-beta, HGF, MIP-1 beta, and SCGF-beta are strongly associated with dengue, and together with duration of symptoms before hospital admission, these markers could help identify cases requiring hospitalization on admission. Nonetheless, a comprehensive clinical examination and case-by-case decision-making remain vital in medical practice. It is also important to note that the classification of DF and DWS/SD in this study was based on patients' clinical evaluations, and the possibility of misclassification by attending physicians cannot be excluded. Therefore, a longitudinal study with severity assessments at different time points may provide further insights into the role of various humoral mediators in dengue. One additional limitation is that this was a single center study conducted in Vietnam. Therefore, the generalizability of these findings to other geographic regions and dengue serotypes requires further investigation. Nonetheless, this study was able to identify distinct humoral inflammatory profiles corresponding to different levels of dengue severity and proposed potential biomarkers as indicators for patient admission. While the large panel of inflammatory markers examined in this study provided comprehensive insights into dengue immunopathogenesis, it was intentionally applied to cast the net wide and identify a small set of robust predictors of severity. Routine measurement of all analytes is not proposed; instead, future efforts should focus on validating a minimal, cost-effective biomarker subset with comparable predictive power for clinical application. As dengue outbreaks progress rapidly and strain healthcare systems, this approach is promising to reduce the burden on medical facilities in endemic areas.

5 | Conclusion

Key inflammatory biomarkers and the duration of symptoms before admission can together inform hospitalization decisions in dengue patients. Targeted cytokine profiling integrated with machine learning offers a practical approach to improve triage and reduce healthcare burden in dengue-endemic areas. Further longitudinal studies are needed to validate these findings.

Author Contributions

T.P.V. and L.H.S. conceptualized and designed the study. L.H.S., P.G.K., and T.P.V. contributed to the study materials and assays. N.T.T recruited the patients. D.D.A. performed the experimental procedures. D.D.A., M.R., and S.K. were involved in the statistical analysis and validation of the results. T.P.V., D.D.A., and M.R. wrote the first draft. All authors have read and approved the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supporting information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supplementary Table S1. Plasma levels of inflammatory mediators in dengue patients and healthy controls. **Supplementary Table S2.** Correlation matrix of plasma levels of inflammatory mediators and laboratory parameters in dengue patients. **Supplementary Table S3.** Correlation matrix of plasma levels of inflammatory mediators in dengue patients. **Supplementary Figure S1.** Feature importance plot and AUROC curve of Model with the most robust predictors.

3. DISCUSSION

This dissertation provides novel insights into the complex interplay between viral characteristics, host factors, and immunological mechanisms that influence the clinical course and severity of dengue virus (DENV) infection. Focusing on Vietnam, an endemic country with recurrent outbreaks and high serotype diversity, the findings highlight that both viral genetic variations, particularly the emergence of the DENV-2 Cosmopolitan genotype, and host immune responses significantly shape disease dynamics. Furthermore, the studies underscore ongoing diagnostic challenges due to serological cross-reactivity and the co-circulation of other arboviruses such as ZIKV, CHIKV, and various flaviviruses. In addition, our studies identified key biomarkers associated dengue severity. Leveraging these biomarkers, we developed a machine learning algorithm capable of classifying dengue severity independently of clinical assessment. These findings emphasize the urgent need for improved surveillance and diagnostic strategies. The following discussion critically examines the key virological and immunological findings in the context of existing literature and explores their implications for clinical management and public health preparedness.

3.1 Epidemiology and Evolutionary Dynamics of Dengue and Co-Circulating Arboviruses

3.1.1 Co-circulation of Arboviruses and Diagnostic Implications

Vietnam, as a tropical country, experiences a high diversity of circulating arboviruses (Nguyen-Tien et al., 2019; Quyen et al., 2017). Among these pathogens, Zika and chikungunya viruses are of particular concern due to their clinical similarity to dengue, which can complicate diagnosis and surveillance efforts. Despite their generally low mortality rates, these viruses can lead to markedly different long-term health consequences, from birth defects caused by Zika to persistent joint pain from chikungunya (Martins et al., 2020).

Outbreaks of Zika and chikungunya have occurred over the past decade in regions such as the Americas, Africa, and the Indian subcontinent (World Health Organization, 2016, 2022). Their overlapping clinical presentations with dengue, characterized by fever, rash, arthralgia, and headache, combined with serological cross-reactivity, pose a significant diagnostic challenge (Andrew et al., 2022; Chan et al., 2022). This diagnostic ambiguity raises concerns that undetected co-circulation of these arboviruses contribute to the increased frequency and severity of dengue outbreaks in Vietnam (Quyen et al., 2017).

In our three-year surveillance conducted in northern Vietnam (2020-2022), no Zika or chikungunya cases were detected via molecular screening. Similarly, our retrospective cohort during the 2016 dengue outbreak in Binh Dinh province (central Vietnam) found no confirmed cases of these two viruses, suggesting a lower burden of these infections compared to dengue. However, a Zika outbreak was reported in southern Vietnam between 2016 and 2017 (Phan et al., 2019), and chikungunya cases were identified in several provinces bordering Cambodia (Pham Thi et al., 2017). Taken together, Zika and chikungunya may be present in Vietnam, but their transmission appears limited and has not resulted in a pronounced impact or a large number of symptomatic cases.

In contrast, a parallel study conducted in Jember, East Java, Indonesia, illustrated a common challenge in endemic regions: the misdiagnosis of chikungunya as dengue (Anh et al., 2025). The chikungunya infected patient presented with dengue-like clinical manifestations and laboratory parameters and was diagnosed and treated as a dengue case at the local medical centre (Anh et al., 2025). In Indonesia, a dengue endemic country, the diagnosis of dengue relied solely on clinical manifestations, whereas in Vietnam and other countries, serological tests including NS1, IgG, and IgM were used to reduce the risk of misdiagnosis (Anh et al., 2025). This result emphasises the need to improve dengue diagnostics in endemic regions. Despite the potential cross-reactivity, the use of serological tests remains essential and can improve the accuracy of dengue diagnosis.

In addition, the Binh Dinh cohort included patients who tested negative for dengue by PCR but exhibited IgG/IgM seropositivity to DENV and other flaviviruses, including JEV, WNV, and TBEV, suggesting prior exposure to these or related pathogens. These antibodies responses to JEV/WNV/TBEV were also frequently detected in PCR-confirmed dengue cases, suggesting that the observed seropositivity likely reflects serological cross-reactivity or limited assay specificity, rather than true co-infections or past exposures. Some patients may have presented during the late phase of dengue infection, when viral RNA levels fall below the detection threshold, leading to false-negative DENV-PCR results (Chong et al., 2020; Chung et al., 2015). While real-time RT-PCR is considered the gold standard for diagnosing acute dengue infections, with highest sensitivity during the first 2-7 days of illness (83, 84). Conversely, IgM ELISA is valuable in identifying recent infections that may be missed by PCR, as IgM antibodies typically rise after days 4-5 and persist into the convalescent phase (85).

Furthermore, a small subset of cases in our studies exhibited IgM positivity exclusively to JEV or TBEV, which may indicate recent infections with these viruses. Notably, the characteristics of these patients did not differ significantly from other individuals in the cohort. Additional diagnostic approaches, such as PCR targeting highly conserved genomic regions or viral genome sequencing, may be beneficial for confirming the aetiology of these cases and enhancing overall diagnostic accuracy (Scaramozzino et al., 2001). Overall, integrated molecular-serological surveillance strategies are essential for accurate diagnosis and effective outbreak management in flavivirus-endemic regions.

3.1.2 Spatiotemporal Patterns and Genotypic Shifts in DENV Circulation

The presence of all four dengue virus serotypes has been recognized in Vietnam and other endemic countries in the region (World Health Organization, 2023). While dengue outbreaks occur annually, the predominance of DENV serotypes varies across both temporal and spatial scales.

Our study revealed that DENV-2 was the most prevalent serotype in northern Vietnam during the 2020-2022 period, followed by DENV-1 and DENV-4. Notably, the exclusive detection of DENV-3 in 2020 suggests that the circulation of this serotype may have been transient or restricted to specific regions or timeframes. A similar epidemiological dengue profile was observed in southern Vietnam during the same period, where DENV-2 was the dominant serotype, followed by DENV-1 and DENV-4 (Tran et al., 2023). In contrast, our analysis of the 2016 dengue outbreak in Binh Dinh, central Vietnam, identified DENV-1 as the predominant serotype, followed by DENV-4, DENV-2, and DENV-3. These variations in serotype dominance highlight the distinct temporal patterns of DENV circulation observed throughout Vietnam. Notably, while DENV-3 remained limited in both Vietnamese cohorts, it was the predominant serotype in Jember, Indonesia, during the 2023 outbreak, highlighting regional differences in DENV serotype circulation (Anh et al., 2025).

Factors influencing DENV serotypes distribution include viral evolution, host immunity, human mobility, vector ecology and environmental factors (Allicock et al., 2020).

Genetic mutations contribute to the emergence of new virus strains with increased transmissibility or the potential to escape the vector and host immune system (Holmes & Twiddy, 2003). Additionally, population-level immunity following large outbreaks can drive serotype replacement, as individuals develop long-term immunity to one serotype while probably remaining susceptible to others (Endy et al., 2002). While people moving between regions can spread new viruses, mosquito numbers and weather conditions also affect how DENV serotypes propagate (Messina et al., 2014).

Over the past decade, a notable shift in DENV serotype distribution has been observed in northern Vietnam. While DENV-1 was the predominant serotype in 2017 and 2018, a clear transition toward DENV-2 dominance emerged from 2019 onward (Alied et al., 2023; Guo et al., 2022; Mourad et al., 2022). This shift coincided with the intensified 2019 dengue outbreak in Hanoi, located in north-central Vietnam, which recorded the highest number of cases to date. Similar serotype transitions have been linked to increased outbreak severity in previous studies (Phadungsombat et al., 2023; Verma et al., 2023). In addition to shifts in serotype distribution, our study also revealed shifts in genotypic diversity within individual serotypes. While DENV-1 genotype I and DENV-4 genotype I remained consistently detected throughout the study period, a notable shift from DENV-2 genotype Asian I to the Cosmopolitan genotype was observed during the 2021-2022 outbreak. These findings underscore the importance of continuous molecular surveillance to inform timely and effective public health interventions.

3.1.3 Emergence of DENV-2 Cosmopolitan Genotype and Disease Severity

As discussed previously, circulating DENV variants potentially contribute to the intensity and severity of dengue outbreaks. The major outbreak in northern Vietnam in 2022, marked by a sharp increase in both overall incidence and the number of cases with warning signs or severe manifestations, appears closely linked to the emergence and dominance of the DENV-2 Cosmopolitan genotype (Anh et al., 2025).

This genotype is known for its widespread distribution and association with increased disease severity (Suppiah et al., 2018). Laboratory studies have demonstrated that the DENV-2 Cosmopolitan genotype exhibits higher infection titres in both mammalian and mosquito cell lines compared to other DENV variants, which may enhance its transmission potential and pathogenicity (Samune et al., 2024). A similar phenomenon was reported in Thailand, where the emergence of this genotype was linked to increased disease severity and altered transmission dynamics during dengue outbreaks (Phadungsombat et al., 2018). In 2018-2019, DENV-2 genotype I was the predominant strain circulating in Vietnam, particularly in northern regions. However, since 2020, the emergence and increasing prevalence of the DENV-2 Cosmopolitan genotype has been observed (Phadungsombat et al., 2023). Our studies showed that this genotype completely predominated over the DENV-2 Asian I genotype in 2021, a trend that continued in 2022, when one of the largest dengue outbreak in the region was observed (Anh et al., 2025).

Different DENV serotypes were reported to be associated with different degrees of clinical severity. The cohort results from northern Vietnam indicated that DENV-2 was associated with the highest number of dengue cases presenting with warning signs, whereas DENV-1 was linked to the greatest number of severe dengue cases (Anh et al., 2025). These results are consistent with other studies where DENV-2 and DENV-1 was reported as the serotypes most strongly associated with unfavourable clinical outcomes (Suppiah et al., 2018; Vicente et al., 2016). DENV-1 and DENV-2 also demonstrated higher replication rates in vitro compared to the other serotypes, suggesting their higher virulence (Quintero-Gil et al., 2018; Rathore et al., 2021).

However, no notable differences in clinical severity were observed between infections involving a single DENV serotype and those involving multiple serotypes, or among different genotypes within the same serotype. It is important to note that clinical outcomes in dengue are influenced by a complex interplay of host, viral, and environmental factors, making comparisons of virulence between DENV variants challenging. The small sample size and limited genetic diversity of DENV variants in our cohort may constrain the generalizability of these findings. Future studies incorporating larger, geographically diverse datasets are needed to further clarify the relationship between DENV genetic variation and disease severity.

3.2 Host and Immune Determinants of Dengue Severity

3.2.1 Host Genetic Susceptibility and Role of IL-10 Promoter Polymorphisms

Host genetic polymorphisms have been reported to influence susceptibility to dengue (Xavier-Carvalho et al., 2017). This thesis focused on evaluating single nucleotide polymorphisms of *IL-10* promoter region in Vietnamese population and their association with dengue. Our findings revealed significant associations between *IL-10* genetic variants, rs1800896 (-1082G/A) and rs1800871 (-819C/T), and dengue susceptibility. Particularly, the -819C allele was associated with an increased risk of dengue, whereas the -1082G allele seemed to act as a protective factor. Moreover, individuals with the -1082GA genotype or carrying the -1082G allele showed a reduced risk of developing dengue with warning signs.

Our analysis revealed an association between IL-10 plasma levels and dengue severity, with the highest levels observed in severe cases, followed by patients with warning signs, and then those without warning signs. Consistent with our findings, other studies have reported that elevated IL-10 levels are associated with dengue-like viral infections, including Zika and chikungunya, further supporting its role in the pathogenesis of viral haemorrhagic fevers (Krishnan et al., 2021; Tappe et al., 2016). Nonetheless, no significant associations were observed between *IL-10* genetic variants and IL-10 plasma levels in our cohort.

However, previous in vitro studies have shown that the -1082G and -819T alleles significantly downregulate IL-10 production, and the -592A allele has been associated with reduced IL-10 promoter activity and lower expression levels (Xiu et al., 2016). Based on these findings, it is possible that the GTA haplotype (-1082G/-819T/-592A) contributes to reduced IL-10 production compared to other haplotypes.

The GTA haplotype (-1082G/-819T/-592A) was found to be protective against dengue in our study. This haplotype may modulate IL-10 secretion during early dengue infection, potentially enhancing viral clearance in the initial phase and reducing the risk of subsequent complications. Notably, the GTA haplotype has also been associated with protection against other parasitic diseases, such as schistosomiasis and malaria in Nigerian and Brazilian populations, suggesting a broader role in modulating immune responses to infection (Adedoja et al., 2018; Paula Carolina Valença Silva, 2014). Since the GTA haplotype of the *IL-10* promoter region is relatively rare in the Vietnamese population, it is plausible that this genetic variation contributes to increased susceptibility to dengue and the disease burden. However, IL-10 secretion and the clinical severity of dengue are influenced by multiple factors, making the role of this anti-inflammatory cytokine in dengue pathogenesis complex and not yet fully understood.

Although our study was the first one that investigated *IL-10* SNPs in relation to dengue within the Vietnamese population, further in vivo and in vitro studies are needed to provide direct evidence on how these genetic variants influence cytokine production and disease severity.

3.2.2 Soluble HLA-G as an Immunomodulator in Dengue Pathogenesis

Our study revealed that plasma levels of sHLA-G were significantly elevated in dengue patients compared to healthy controls and varied according to disease severity. The role of sHLA-G in viral infections is multifaceted, given its well-established tolerogenic properties, particularly its ability to suppress the activity of effector immune cells, including NK cells and T lymphocytes (Contini et al., 2020). Elevated sHLA-G levels have been reported in various viral infections, such as human cytomegalovirus, herpesvirus, hepatitis viruses, and SARS-CoV-2 (Jasinski-Bergner et al., 2022).

Cytokines like IL-10 and IFN-gamma, which are associated with dengue pathogenesis, are known to induce sHLA-G expression, explaining the higher sHLA-G titres observed in patients with more severe clinical manifestations (Malavige et al., 2013; Morandi et al., 2007; Moreau et al., 1999). After adjusting for potential confounders, we observed that sHLA-G levels increased over the course of dengue, suggesting a host-driven compensatory response to limit immune-mediated tissue damage. This pattern aligns with the known immunosuppressive function of HLA-G and suggests a potential role for this molecule in disease monitoring (Contini et al., 2020).

A low level of sHLA-G in the early phase of infection facilitates more efficient viral clearance, while its upregulation in the later phases could help control excessive immune activation, thereby mitigating immunopathology (Adikari et al., 2014; Chandele et al., 2016). This dual role is similar to that of IL-10 and other anti-inflammatory cytokines, which are known to stimulate HLA-G expression (Moreau et al., 1999).

Significant correlations between sHLA-G levels and key dengue laboratory markers, including lymphocyte count, platelet count, and liver enzyme levels were found in our study. Notably, a strong inverse correlation was noted between sHLA-G and total lymphocyte counts in severe dengue. Lymphopenia is a hallmark of severe dengue and may result from lymphocyte apoptosis, peripheral tissue redistribution, and functional exhaustion of CD8+ T cells (Guo et al., 2021). Other studies have reported that sHLA-G can inhibit $CD8^+$ T cell proliferation by binding to inhibitory receptors such as ILT2 and ILT4, thereby dampening cytotoxic activity and potentially impairing viral clearance, thus allowing DENV to persist and replicate more effectively within the host (Shiroishi et al., 2003).

Nonetheless, the observed correlation between sHLA-G levels and lymphopenia should be interpreted with caution, as the relatively small number of severe dengue cases in the study limits both statistical power and the generalizability of the findings. Despite this limitation, our results provide preliminary evidence that molecular immune checkpoints such as sHLA-G potentially influence the immunopathogenesis of dengue and suggest further investigation in larger cohorts. Overall, the dynamic regulation of sHLA-G over time, along with its association with key clinical and laboratory markers, highlights its potential as a valuable marker for disease monitoring and progression in dengue infection.

3.2.3 Inflammatory Mediators and Immune Dysregulation in Dengue Severity

To better understand the inflammatory response in dengue, we measured the concentrations of 48 key inflammatory mediators in patients with different levels of clinical severity. The analysis revealed significant differences in inflammatory mediator levels between dengue patients and healthy controls, as well as across disease severities, underscoring the central role of immune activation and cytokine dysregulation in the pathogenesis of dengue.

Patients were stratified by hospitalization requirement, with those showing warning signs or severe symptoms (DWS/SD) receiving close medical monitoring (World Health Organization, 2009). Significant differences in 26 of the 48 inflammatory biomarkers were observed between mild dengue fever (DF) and DWS/SD cases. Among pro-inflammatory markers, notable differences between the two groups were found in SCGF-beta, IFN-gamma, MIP-1-beta, RANTES, and TNF-beta. These cytokines are involved in immune cell activation, chemotaxis, and the inflammatory cascade, processes that are typically upregulated in severe dengue (Bhatt et al., 2024; Patro et al., 2019). Similar cytokine profiles have been reported in other acute viral infections, such as influenza and COVID-19, where elevated levels of IFN-gamma, TNF-alpha, and MIP-1-beta contribute to the cytokine storm phenomenon and are associated with lung injury (Channappanavar et al., 2016; Huang et al., 2020).

Variations were also observed in anti-inflammatory markers, including IL-10, IL-1RA, and IL-9. In line with our findings, previous studies have demonstrated that IL-10 and IL-1RA are significantly upregulated in severe viral illnesses and are associated with disease severity (Zhao et al., 2020). These cytokines have also been suggested as potential biomarkers for differentiating active versus latent tuberculosis, highlighting their role in ongoing inflammatory processes (Suzukawa et al., 2016). Other inflammatory mediators that have both pro- and anti-inflammatory roles, such as HGF, IL-1-alpha, and IL-1-beta, were also significantly elevated in DWS/SD patients compared to those with DF. These observations underscore the complex interplay between inflammation and immune regulation during disease progression, where the effects of individual cytokines may shift over time (Khanam et al., 2022). Additionally, two growth factors, FGF-basic and VEGF, showed significant differences between groups, suggesting a role in tissue repair and vascular permeability, both key elements in dengue pathophysiology.

Correlation analyses revealed strong relationship between the inflammatory mediators and key dengue-related laboratory parameters, such as platelet count, lymphocyte count, and liver enzymes. Our findings are consistent with previous studies showing that cytokines and chemokines contribute to both immune response and disease progression (Khanam et al., 2022; Moallemi et al., 2023). These inflammatory mediators may serve as early indicators of disease severity, as changes in their levels can occur prior to clinical manifestation and conventional paraclinical markers, such as thrombocytopenia or transaminitis (Shimabukuro-Vornhagen et al., 2018).

3.3 Biomarker-Based Machine Learning Model and Prediction of Dengue Severity

Given the potential of several inflammatory mediators to distinguish between DWS/SD and DF, we applied a machine learning approach for classification. Specifically, we employed a Random Forest algorithm, which is well-suited for managing non-linear relationships, high-dimensional data, missing values, and multicollinearity, while also offering insights into feature importance (Lindner et al., 2022).

Following a robust feature selection process, our final model included five key variables: time since symptom onset (days of fever), and plasma levels of TNF-beta, HGF, MIP-1-beta, and SCGF-beta. This model achieved nearly 80% classification accuracy, being comparable to a model that incorporated all clinical and laboratory parameters. These findings suggest the potential utility of a biomarker-driven approach, particularly in guiding admission decisions when clinical presentations are unclear or when healthcare systems are overburdened during outbreaks.

Traditionally, dengue severity has been assessed using laboratory markers such as platelet count, haematocrit, and liver enzymes, combined with clinical manifestations (Lee et al., 2024; Tsheten et al., 2021). However, this approach relies on comprehensive clinical evaluations (Marois et al., 2021), which can be time-consuming and may lack specificity or sensitivity if assessments are limited (Lee et al., 2024). In contrast, our model functions independently of detailed clinical assessments, making it highly applicable in outbreak settings where rapid and reliable triage is essential (Wong et al., 2020). Moreover, clinical manifestations may be delayed and can either follow or be triggered by changes in humoral inflammatory profiles (Shimabukuro-Vornhagen et al., 2018). Therefore, inflammatory mediators such as cytokines and chemokines may serve as early direct indicators of the systemic inflammatory status and have potential for prognostication of tissue damage before clinical manifestations of disease severity (Liu et al., 2021).

Among the selected biomarkers, HGF, a protein released in response to liver injury, was significantly elevated in DWS/SD patients. This is consistent with DENV's known tropism for hepatocytes and Kupffer cells, which contributes to liver dysfunction (Samanta & Sharma, 2015). Our findings align with prior studies showing HGF levels correlate with liver enzyme elevations and are associated with severe dengue outcomes (Fiestas Solórzano et al., 2022; Nakamura & Mizuno, 2010; Voraphani et al., 2010).

Conversely, TNF-beta was associated with milder dengue cases. Known for its role in inhibiting viral replication and synergizing with interferons (Wong & Goeddel, 1986), higher levels of TNF-beta may offer protective effects. In our study, controls exhibited significantly higher TNF-beta levels compared to dengue patients, supporting its possible role in limiting infection severity. Similarly, MIP-1-beta was more strongly associated with DF than DWS/SD. This is consistent with findings by Bozza et al., who identified MIP-1-beta as a predictive marker with protective effects against severe dengue (Bozza et al., 2008).

SCGF-beta, another key biomarker in our model, is involved in haematopoiesis. Dengue infection is characterized by leukopenia, which may trigger a compensatory response via upregulation of bone marrow-stimulating factors like SCGF-beta (Tsai et al., 2012; Wong et al., 2012). Our results revealed significant correlations between SCGF-beta and monocyte, leukocyte, and platelet counts, as well as AST and ALT levels, suggesting its involvement in restoring haematological balance and mitigating tissue damage. These findings are supported by earlier studies linking SCGF-beta to other viral infections and hematopoietic responses (Foronjy et al., 2014; Kimura et al., 2024).

Accurate and early triage of dengue patients remains a major challenge due to the non-specific nature of initial clinical presentations. Misclassification can lead to unnecessary hospitalizations, adding pressure to healthcare systems (Hadinegoro, 2012). Classical dengue classification relies heavily on clinical judgment and lab results (Wong et al., 2020), which may not be feasible during large outbreaks or in resource-limited settings. Our study demonstrates that a limited panel of cytokines, combined with symptom duration, can effectively identify patients requiring close medical monitoring, with accuracy comparable to traditional clinical assessments. However, while promising, this classification model should be viewed as complementary to standard medical practice, not a replacement. Clinical judgment remains essential, and further validation in larger, diverse patient cohorts is necessary to assess generalizability and utility.

3.4 Conclusion

In conclusion, this thesis offers a comprehensive and multidisciplinary examination of the viral, host genetic, and immunological determinants that influence dengue virus (DENV) infection dynamics and disease severity, with a particular focus on Vietnam and other endemic regions in Southeast Asia. Through integrated virological surveillance, molecular characterization, and immune-profiling, my work identifies critical shifts in circulating DENV serotypes and genotypes, most notably the emergence and dominance of the DENV-2 Cosmopolitan genotype as key drivers of recent outbreaks and more severe clinical presentations. On the host side, the identification of IL-10 promoter polymorphisms and elevated soluble HLA-G levels as markers of susceptibility and disease severity highlights the importance of host immunogenetic background in shaping clinical outcomes. Moreover, the application of machine learning to cytokine profiles has led to the development of a promising biomarker-based model for early risk stratification and triage, with potential utility in outbreak settings where health systems are under pressure. Collectively, these findings advance our understanding of dengue pathogenesis and provide a foundation for improving early diagnosis, clinical management, and surveillance strategies in resource-limited settings. The thesis underscores the need for continuous molecular and immunological monitoring to inform timely public health responses and ultimately reduce the burden of dengue in endemic regions.

4. SUMMARY

This doctoral thesis explores the complex interplay between viral, host genetic, and immunological factors that influence susceptibility to dengue virus (DENV) infection and progression to severe disease, with a particular focus on Vietnam. Dengue is the most widespread mosquito-borne viral disease, transmitted primarily by *Aedes* mosquitoes, and co-circulates with other arboviruses such as Zika (ZIKV), chikungunya (CHIKV), and flaviviruses like Japanese encephalitis virus (JEV) and West Nile virus (WNV). The increasing incidence of dengue is attributed to climate change, rapid urbanization, and expanding vector ranges. DENV exists as four distinct serotypes (DENV-1 to DENV-4), each with multiple genotypes, contributing to varied disease severity and presenting diagnostic and clinical challenges.

The thesis is structured around six interlinked studies: three focusing on viral factors and three on host factors. On the viral side, a prospective study conducted in northern Vietnam (2020-2022) analysed 426 suspected dengue cases to characterize circulating DENV serotypes and genotypes and their relationship with disease severity, while also screening for ZIKV and CHIKV. A retrospective study during the 2016 outbreak in central Vietnam evaluated 146 hospitalized patients for DENV serotype distribution and flavivirus serological cross-reactivity. A third study in East Java, Indonesia (2023), investigated 108 febrile patients and highlighted the diagnostic challenges between dengue and chikungunya due to overlapping clinical presentations and limited diagnostics. These studies revealed dynamic shifts in serotype dominance across regions and time, with DENV-2, particularly the Cosmopolitan genotype, emerging as a key contributor to more severe outbreaks in recent years. Serological cross-reactivity among flaviviruses was also found to complicate accurate diagnosis.

On the host side, the thesis examined immunological and genetic markers associated with dengue severity. One study involving 306 Vietnamese patients from the 2021-2022 outbreaks analysed clinical severity alongside interleukin-10 (IL-10) levels and its genetic variants, identifying IL-10 as a potential marker of disease progression. Another study assessed plasma levels of soluble human leukocyte antigen G (sHLA-G), an immunoregulatory molecule, in 238 dengue patients and 118 healthy controls and found elevated levels correlated with increasing severity and immune dysregulation. A third

study applied machine learning to a comprehensive panel of 48 inflammatory mediators measured in the 306-patient cohort. Using a Random Forest model, five key features: days since fever onset and levels of TNF-beta, HGF, MIP-1-beta, and SCGF-beta were sufficient to classify patients by disease severity with nearly 80% accuracy. This approach demonstrated the potential of biomarker-based tools for early triage, particularly valuable during outbreaks when clinical resources are overwhelmed.

Genetic polymorphisms in the IL-10 promoter region were found to influence susceptibility and severity. The GTA haplotype (-1082G/-819T/-592A) was associated with protection against dengue, potentially by modulating IL-10 expression and limiting immune-mediated damage. Similarly, elevated sHLA-G levels were associated with suppression of effector immune functions, possibly contributing to immune evasion by DENV. The humoral immune profiling revealed that both pro-inflammatory and anti-inflammatory cytokines played roles in shaping disease outcomes. Notably, the biomarker-based model outperformed traditional clinical assessments in predicting severe cases, suggesting its value for outbreak response and patient stratification.

In conclusion, this thesis provides a comprehensive investigation into the factors driving dengue pathogenesis and severity. It highlights the role of viral genetic diversity, particularly the emergence of the DENV-2 Cosmopolitan genotype, in influencing outbreak dynamics and clinical outcomes. At the host level, immunogenetic markers such as IL-10 polymorphisms and sHLA-G levels offer insight into individual susceptibility and immune responses. By integrating molecular epidemiology, immunological profiling, and computational modelling, the thesis proposes practical approaches for early diagnosis, risk stratification, and targeted clinical management in dengue-endemic regions. These findings have broad implications for improving public health surveillance and guiding clinical practice in Southeast Asia and other high-transmission settings.

5. ZUSAMMENFASSUNG

Diese Doktorarbeit untersucht das komplexe Zusammenspiel zwischen viralen, genetischen und immunologischen Faktoren, die die Anfälligkeit für eine Dengue-Virus-(DENV)-Infektion und den Krankheitsverlauf bis hin zu schweren Verläufen beeinflussen, mit besonderem Fokus auf Vietnam. Dengue ist die weltweit am weitesten verbreitete, durch Mücken übertragene Virusinfektion, primär durch *Aedes*-Mücken, und tritt oft gemeinsam mit anderen Arboviren wie Zika (ZIKV), Chikungunya (CHIKV) und Flaviviren wie dem Japanischen Enzephalitis Virus (JEV) oder dem West-Nil-Virus (WNV) auf. Die zunehmende Inzidenz von Dengue wird unter anderem durch den Klimawandel, die rasante Urbanisierung und die Ausbreitung der Vektoren begünstigt. DENV existiert in vier unterschiedlichen Serotypen (DENV-1 bis DENV-4), die jeweils mehrere Genotypen umfassen und zu unterschiedlichen Krankheitsverläufen führen, was Diagnostik und Klinik erheblich erschwert.

Die Dissertation basiert auf sechs miteinander verknüpften Studien, drei konzentrieren sich auf virale Faktoren und drei auf Wirtsfaktoren. Auf der viralen Seite analysierte eine prospektive Studie im Norden Vietnams (2020-2022) 426 Verdachtsfälle, um zirkulierende DENV-Serotypen und Genotypen in Bezug auf Krankheitsverläufe zu charakterisieren und gleichzeitig ZIKV und CHIKV zu detektieren. Eine retrospektive Studie untersuchte 146 hospitalisierte Patienten während eines Ausbruchs 2016 in Zentralvietnam auf die Verteilung von Serotypen sowie Kreuzreaktionen mit anderen Flaviviren. Eine dritte Studie in Ost-Java, Indonesien (2023), analysierte 108 fieberhafte Patienten und zeigte diagnostische Herausforderungen auf, insbesondere im Hinblick auf die Unterscheidung zwischen Dengue und Chikungunya aufgrund ähnlicher Symptome und eingeschränkter Diagnostik. Diese Studien belegten eine dynamische Verschiebung der dominierenden Serotypen über Zeit und Regionen hinweg, wobei sich insbesondere DENV-2 (Cosmopolitan-Genotyp) als treibender Faktor schwerer Ausbrüche in den letzten Jahren herauskristallisierte. Serologische Kreuzreaktionen zwischen Flaviviren erschwerten zusätzlich die exakte Diagnostik.

Auf der Wirtsseite untersuchte die Arbeit immunologische und genetische Marker, die mit der Schwere der Dengue-Erkrankung assoziiert sind. Eine Studie mit 306 vietnamesischen Patienten aus den Ausbrüchen der Jahre 2021-2022 analysierte die klinische Schwere zusammen mit den Interleukin-10 (IL-10) -Spiegeln und dessen genetischen Varianten und identifizierte IL-10 als potenziellen Marker für den Krankheitsverlauf. Eine weitere Studie analysierte die Plasmaspiegel des löslichen humanen Leukozyten Antigens G (sHLA-G) bei 238 Patienten und 118 gesunden Kontrollen. Höhere sHLA-G-Werte korrelierten mit zunehmender Krankheitsintensität und Immunregulationsstörungen. In einer dritten Studie wurde ein Machine-Learning-Ansatz (Random Forest) auf ein Panel von 48 inflammatorischen Mediatoren angewendet, die aus dem Plasma derselben Patientenkohorte quantifiziert wurden. Fünf Parameter – Fieberdauer sowie die Spiegel von TNF-beta, HGF, MIP-1-beta und SCGF-beta – reichten aus, um den Schweregrad mit einer Genauigkeit von rund 80 % zu klassifizieren. Dieses Modell unterstreicht das Potenzial biomarkerbasierter Werkzeuge zur Frühtriage, insbesondere bei überlasteten Gesundheitssystemen während Ausbrüchen.

Genetische Polymorphismen in der IL-10-Promotorregion wurden mit Anfälligkeit und Schweregrad der Erkrankung in Verbindung gebracht. Besonders das GTA-Haplotyp (-1082G/-819T/-592A) war mit einem Schutz vor schwerem Verlauf assoziiert möglicherweise über eine reduzierte IL-10-Expression und Begrenzung immunvermittelter Schäden. Erhöhte sHLA-G-Spiegel wiederum gingen mit einer Suppression von Effektorzellen einher, was eine mögliche Rolle bei der Immunflucht von DENV nahelegt. Die Analyse der humoralen Immunantwort zeigte, dass sowohl proinflammatorische als auch antiinflammatorische Zytokine eine entscheidende Rolle für den Krankheitsverlauf spielen. Auffällig war, dass das biomarkerbasierte Vorhersagemodell herkömmliche klinische Einschätzungen in der Prädiktion schwerer Fälle übertraf, ein vielversprechender Ansatz zur Risikostratifizierung.

Zusammenfassend liefert diese Dissertation einen umfassenden Beitrag zum Verständnis der Faktoren, die Pathogenese, Schweregrad und Dynamik von Dengue-Ausbrüchen beeinflussen. Sie hebt die Bedeutung der genetischen Diversität des Virus – insbesondere des DENV-2 Cosmopolitan-Genotyps für Ausbruchsmuster und klinische Verläufe hervor. Auf Wirtsseite bieten immunogenetische Marker wie IL-10-Polymorphismen und sHLA-G-Spiegel wertvolle Einsichten in individuelle Anfälligkeiten und Immunantworten. Durch die Integration von molekularer Epidemiologie, Immunprofiling und rechnergestütztem Modellieren schlägt diese Arbeit praxisnahe Ansätze für

Frühdiagnostik, Risikostratifizierung und gezielte Versorgung in endemischen Gebieten vor mit weitreichender Bedeutung für die öffentliche Gesundheit in Südostasien und anderen Regionen mit hoher Übertragungsrate.

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7. DECLARE OF CONTRIBUTIONS

We hereby declare that the doctoral dissertation entitled " **Viral and Host Determinants of Dengue Susceptibility and Severity in Endemic Southeast Asia**", submitted to the members of the PhD Board at the Faculty of Medicine, University of Tübingen, represents original work conducted by Do Duc Anh and co-authors at the Institute of Tropical Medicine, University of Tübingen, under the supervision of Prof. Dr. Thirumalaisamy P. Velavan. Six publications form the backbone of this doctoral dissertation, all authored primarily by Do Duc Anh:

Publication 1: Open forum infectious diseases. 2024 Dec 24; 12(1):ofae753.
Publication 2: Journal of Medical Virology. 2025 Aug 21; 97(8):e70569.
Publication 3: IJID regions. 2024 Dec 4; 14:100512.
Publication 4: PLOS Neglected Tropical Diseases. 2025 Mar 28; 19(3):e0012954.
Publication 5: Journal of Medical Virology. 2025 Sep 5; 97(9):e70594.
Publication 6: Journal of Medical Virology. 2025 Aug; 97(8):e70511.

Do Duc Anh has made substantial contributions to all six manuscripts, including study design, sampling procedures, experimental implementation, data analysis, and manuscript preparation. A detailed statement of the individual contributions of all co-authors for each publication, are as follows:

Contributions of PhD candidates and other co-authors

Publication 1:

Anh DD, The NT, My TN, Linh LTK, Hoan NX, Kremsner PG, Toan NL, Song LH, Velavan TP. Epidemiology and Genotype Dynamics of Dengue in Hospitalized Patients in Northern Vietnam Between 2020 and 2022. Open Forum Infectious Diseases. 2024 Dec 24; 12(1):ofae753. PMID: 39834749. **IF: 3.8**.

Do Duc Anh: Methodology, Investigation, Data curation, Formal analysis, Validation, Software and Visualization, Investigation, Writing - Original draft, Writing - Review and Editing. **Nguyen Trong The**: Investigation, Resources. **Truong Nhat My**: Investigation, Resources. **Le Thi Kieu Linh**: Methodology, Investigation. **Nghiem Xuan Hoan**: Investigation, Resources. **Peter G. Kremser**: Writing - Review and Editing. **Nguyen Linh Toan**: Investigation, Resources. **Le Huu Song**: Conceptualization, Resources, Investigation. **Thirumalaisamy P. Velavan**: Conceptualization, Supervision, Resources, Writing - Original draft, Writing - Review and Editing, Project administration, Funding acquisition.

Publication 2:

Anh DD, Loc DH, Tong HV, Dieu DTH, Hang NT, Lanh NH, Nhung NTH, Kremsner PG, Song LH, Toan NL, Velavan TP. Dominance of DENV-1 and Flavivirus Serological Cross-Reactivity During the 2016 Dengue Outbreak in Vietnam. Journal of Medical Virology. 2025 Aug 21; 97(8):e70569. **IF: 4.6**.

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Publication 3:

Anh DD, Sani LM, Riyanti R, Istinaroh N, My TN, Van Tong H, Oktarianti R, Huyen TTT, Song LH, Senjarini K, Velavan TP. Diagnostic challenges of arboviral infections and dengue virus serotype distribution in febrile patients in East Java, Indonesia. IJID Region. 2024 Dec 4; 14:100512. PMID: 39850333. **IF 1.5**.

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Rike Oktarianti: Supervision. **Tran Thi Thanh Huyen**: Investigation, Supervision. **Le Huu Song**: Supervision. **Kartika Senjarini**: Conceptualization, Methodology. **Thirumalaisamy P. Velavan**: Conceptualization, Methodology, Resources, Supervision, Writing - Original draft, Writing - Review & editing, Project administration, Funding acquisition.

Publication 4:

Anh DD, Vugrek L, The NT, Hafza N, My TN, Linh LTK, Loc HD, Jonas SC, Toan NL, Kremsner PG, Song LH, Velavan TP. Characterization of dengue patients in Vietnam: Clinical, virological, and IL-10 profiles during 2021- 2022 outbreaks. PLoS Negl Trop Dis. 2025 Mar 28; 19(3):e0012954. PMID: 40153381. **IF 3.4**.

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Publication 5:

Anh DD, The NT, Song LH, Anja M, Velavan TP, Barbara S. Elevated Soluble HLA-G Levels Associate with Dengue Severity in Vietnamese Patients. Journal of Medical Virology. 2025 Sep 5; 97(9):e70594. **IF: 4.6**.

Do Duc Anh: Methodology, Investigation, Data Curation, Formal analysis, Validation, Software and Visualization, Writing - Original draft, Writing - Review & editing. **Nguyen**

Trong The: Investigation, Resources. **Le Huu Song**: Conceptualization, Methodology, Resources. **Anja Mueller**: Methodology, Investigation, Formal analysis, Validation. **Thirumalaisamy P. Velavan**: Conceptualization, Supervision, Resources, Writing - Original draft, Writing - Review & editing. **Barbara Seliger**: Conceptualization, Supervision, Resources, Writing - Original draft, Writing - Review & editing.

Publication 6:

Anh DD, Recker M, The NT, Krishna S, Kremsner PG, Song LH, Velavan TP. Risk Stratification of Dengue Cases Requiring Hospitalization. Journal of Medical Virology. 2025 Aug; 97(8):e70511. **IF 4.6**.

Do Duc Anh: Methodology, Investigation, Formal analysis, Data Curation, Validation, Software and Visualization, Writing - Original draft, Writing - Review & editing. **Mario Recker**: Formal analysis, Validation, Writing - Original draft, Writing - Review & editing. **Nguyen Trong The**: Investigation, Resources. **Sanjeev Krishna**: Formal analysis, Validation. **Peter G. Kremsner**: Conceptualization, Methodology. **Le Huu Song**: Conceptualization, Methodology, Resources. **Thirumalaisamy P. Velavan**: Conceptualization, Methodology, Writing - Original draft, Writing - Review & editing Project administration, Funding acquisition.

I also declare that I wrote the general introduction and discussion in this thesis.

Sincerely,
Tübingen, 15 September 2025

PhD Candidate	Primary Supervisor
Do Duc Anh, MD	**Prof. Dr. Thirumalaisamy P. Velavan**

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