Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

Department of Biomedical Sciences

Assessing the antibody responses against dengue virus in the context of emerging arboviruses

From basic immunology to new diagnostics

Dissertation for the degree of doctor in biomedical sciences
at the University of Antwerp to be defended by

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Front Cover: Mapping of the antibody reactivity against a peptide library covering the entire dengue polyprotein. Each bar corresponds to a peptide of 15 amino acid length.
Back Cover: Picture of the Itaya River taken from Malecon of Tarapaca. Iquitos, Loreto, Peru.

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Het beoordelen van antilichaam responsen tegen het dengue virus in het kader van de opkomende arbovirussen – *Van basis immunologie naar nieuwe toepassingen voor diagnostiek*

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Summary

Dengue virus (DENV) is the etiological agent of dengue fever. It is the most prevalent arthropod-borne viral disease in the world and therefore a significant public health concern. DENV has a wide-ranging geographical distribution throughout the tropics and subtropics putting nearly half of the world population at risk of infection.

The diagnosis of dengue poses a double challenge at the clinical and laboratory level. At the clinical level, dengue presents with non-specific symptoms that can be confused with the clinical manifestations of other febrile diseases that geographically overlap with dengue. Therefore, diagnosis cannot be made on clinical and epidemiological data alone and thus requires sensitive and specific laboratory techniques.

Molecular techniques are highly sensitive and specific; however, they are difficult to perform in low-resource settings and have a short diagnostic window. Antibody-based assays represent a good opportunity due to their simplicity and affordability. However, they deal with low specificity as a consequence of the high genetic and antigenic similarity of DENV with other members of the *Flaviviridae* family. This renders cross-reactive antibodies that recognize epitopes located in highly conserved flavivirus regions. When antigens with these flavivirus-cross-reactive epitopes are used as antigens in serological tests, instead of capturing DENV-specific antibodies only, false positive results can appear due to the detection of the cross-reactive antibodies.

Consequently, the selection of the appropriate biomaterials to capture specific anti-dengue antibodies is crucial for accurate diagnosis. Hence, there is a tendency to replace whole viral lysates with virus-specific antigenic regions at a higher amino acid resolution. For this purpose, the main goal of this PhD thesis was to delineate the dynamics of the antibody responses against DENV at epitope level using a high throughput flavivirus peptide microarray to identify those regions able to capture DENV-specific Abs only, with the ultimate goal to identify/discover novel biomarkers for serological diagnostic purposes.

The overall strategy was to use a high-throughput peptide microarray library to map peptides that are highly recognized by Abs from DENV infected individuals. The next step was to select the DENV-specific antigens for further testing in a multiplexed bead-

based immunoassay, ultimately leaving only a few promising peptides that can be used to develop a serological test with low complexity that can be translated to low-resource settings.

In this thesis, a successful characterization of the temporal evolution of the IgG and IgM response was done by using a custom-designed high-density peptide microarray containing linear epitopes spanning the entire proteome and diversity of DENV1-4, zika virus and yellow fever virus. For this, sera from a cohort of dengue infected individuals from Peru (representative of secondary infections) of which four follow-up samples were collected in a six-month period, and overseas travelers (as representatives of primary infections) of which two follow-up samples were collected.

Linear epitopes represented by short overlapping peptides retained their immunogenicity. Thus, allowing the delineation of the dynamics in the humoral response against DENV and simultaneously presented an efficient platform for the discovery of virus-specific peptides for diagnostic purposes.

Additionally, important detailed information regarding immunodominant epitopes was obtained through the identification of novel linear epitopes located in structural proteins as well as in non-structural proteins.

The diagnostic potential of 20 synthetic peptides to detect dengue virus infection was further assessed by setting-up a bead-based multiplex peptide immunoassay using the Luminex technology to determine antibody binding levels to each of these peptides in a large sear panel of carefully selected human serum samples.

It was found that although single peptides offered satisfactory diagnostic performance to be used as potential biomaterials in the development of better serological tests for DENV diagnosis, the combination of multiple peptides using a machine learning algorithm improved the sensitivity and specificity compared to the use of single peptides.

In summary, this work added valuable information by mapping the immunodominant linear epitopes across the DENV polyprotein. Besides, the feasibility of the use of synthetic peptides as substitutes of full antigens for dengue serology was confirmed in a bead-based multiple peptide immunoassay. This offers a promising future for the development of highly accurate next-generation dengue serological tests.

Nederlandse samenvatting

Het denguevirus (DENV) is de etiologische veroorzaker van knokkelkoorts. Het is de meest voorkomende door geleedpotigen overgedragen virale ziekte ter wereld en veroorzaakt daarom een aanzienlijk probleem voor de volksgezondheid. DENV heeft een brede geografische spreiding over de tropen en subtropen, daardoor loopt ongeveer de helft van de wereldbevolking het risico op infectie.

De diagnose van dengue vormt een dubbele uitdaging op klinisch en laboratoriumniveau. Op klinisch niveau presenteert dengue zich met niet-specifieke symptomen die kunnen worden verward met de klinische manifestaties van andere koortsachtige ziekten die geografisch overlappen met dengue. Daarom kan de diagnose niet alleen worden gesteld op basis van deze klinische en epidemiologische gegevens, maar vereist gevoelige en specifieke laboratoriumtechnieken.

De huidige moleculaire technieken zijn zeer gevoelig en specifiek, maar moeilijk uitvoerbaar in een omgeving waar de toegang tot middelen beperkt is. Daarnaast vertonen deze testen een kort diagnostisch venster. De op antilichamen gebaseerde testen vormen meer opportuniteit vanwege hun eenvoud en betaalbaarheid. Ze hebben echter te maken met een lage specificiteit door de hoge genetische en antigene gelijkenis van DENV met andere leden van de *Flaviviridae* familie. Hierdoor worden kruisreactieve antilichamen ontwikkeld die epitopen herkennen afkomstig van een sterk geconserveerde flavivirusgebieden. Wanneer antigenen met deze flavivirus-kruisreactieve epitopen worden gebruikt als antigenen in serologische testen, in plaats van alleen de DENV-specifieke antilichamen op te vangen, vals-positieve resultaten optreden ten gevolge van de aanwezigheid van deze kruisreactieve antilichamen.

De selectie van de juiste biomaterialen om specifieke anti-dengue-antilichamen te vangen is cruciaal voor het stellen van een nauwkeurige diagnose. Daarom is er de neiging om hele virale lysaten te vervangen door virus-specifieke antigene gebieden met een hogere aminozuur resolutie.

Het doel van deze doctoraatsthesis is daarom om de dynamiek van de antilichaamresponsen tegen DENV op epitoop niveau af te bakenen met behulp van een flavivirus-peptide-microarray om die regio's te identificeren die alleen DENV-specifieke

antilichamen op kunnen vangen, met als uiteindelijk doel het identificeren/ontdekken van nieuwe biomarkers voor serologische diagnostische doeleinden.

De algemene strategie ging van het gebruik van een high-throughput peptide-microarray-bibliotheek om peptiden in kaart te brengen die worden herkend door Abs van met DENV geïnfecteerde individuen. De DENV-specifieke antigenen vervolgens te selecteren om ze verder te testen in een multiplex immunoassay, om uiteindelijk een paar veelbelovende peptiden over te houden die op hun beurt gebruikt kunnen worden gebruikt voor de ontwikkeling van een serologische test met een lage complexiteit die kan worden vertaald naar gebieden met een beperkte toegang tot middelen.

In deze thesis werd er een succesvolle karakterisering van de temporele evolutie van de IgG- en IgM-respons gedaan door gebruik te maken van een speciaal ontworpen hoge densiteit peptide-microarray met lineaire epitopen die het gehele proteoom en de diversiteit van DENV1-4, het zika virus en het gele koorts virus. Hiervoor werden sera van een cohort van met dengue geïnfecteerde individuen uit Peru (vertegenwoordiger van secundaire infecties) gebruikt waarvan vier follow-upmonsters werden verzameld in een periode van zes maanden, en overzeese reizigers (als vertegenwoordigers van primaire infecties), waarvan er twee opvolg monsters werden verzameld.

Lineaire epitopen die zijn weergegeven door korte overlappende peptiden behielden hun immunogeniciteit. Zo kon de dynamiek in de humorale respons tegen DENV worden afgebakend en werd er tegelijkertijd een efficiënt platform gepresenteerd voor het ontdekken van virus specifieke peptiden voor diagnostische doeleinden.

Bovendien werd belangrijke en gedetailleerde informatie over immuun-dominante epitopen verkregen door de identificatie van nieuwe lineaire epitopen die zich zowel in de structurele eiwitten als de niet-structurele eiwitten bevinden.

Het diagnostische potentieel van 20 synthetische peptiden om dengue virusinfectie te detecteren, werd verder beoordeeld door het opzetten van een multiplex peptide immunoassay met behulp van de Luminex-technologie om de antilichaambindingsniveaus voor elk van deze peptiden te bepalen in een groot panel van zorgvuldig geselecteerde menselijke serummonsters.

Er werd vastgesteld dat hoewel enkele peptiden bevredigende diagnostische prestaties boden om te worden gebruikt als potentiële biomaterialen bij de ontwikkeling van betere

serologische tests voor DENV-diagnose, de combinatie van meerdere peptiden met behulp van een machine learning-algoritme de gevoeligheid en specificiteit verbeterde in vergelijking met het gebruik van individuele peptiden.

Samenvattend heeft dit werk waardevolle informatie toegevoegd door de immuun-dominante lineaire epitopen over het DENV-polyproteïne in kaart te brengen. Bovendien werd de haalbaarheid van het gebruik van de synthetische peptiden als vervangers van volledige antigenen voor gebruik in de dengue-serologie bevestigd met de multiplex immunoassay. Dit biedt een veelbelovende toekomst voor de ontwikkeling van zeer nauwkeurige volgende generatie dengue serologische testen.

Abbreviations

DGD	Belgian government
ITM	Institute of Tropical Medicine Antwerp
MoH	Ministry of Health
WHO	World Health Organization
PAHO	Pan American Health Organization (PAHO)
ENSO	Humboldt and El Niño Southern Oscillation (ENSO)
ECLAC	Economic and Social Commission for Latin America and the Caribbean (ECLAC, 2021)
ENSO	Humboldt and El Niño Southern Oscillation
PAHO	Pan American Human Organization
HSGY	Santa Gema Hospital, Yurimaguas
LMICs	Low- or middle-income countries
RLSs	Resource limited settings
NIH	National Institute of Health
TDR	Special Programme for Research and Training in Tropical Diseases
DF	Dengue fever
DHF/DSS	Dengue hemorrhagic fever/Dengue shock syndrome
DENV	Dengue virus
DENV-1	Dengue virus type 1
DENV-2	Dengue virus type 2
DENV-3	Dengue virus type 3
DENV-4	Dengue virus type 4
ZIKV	Zika virus
CHIKV	Chikungunya virus
YFV	Yellow fever virus
TBEV	Tick borne encephalitis virus
JEV	Japanese encephalitis virus
WNV	West Nile virus
SLEV	Saint Louis encephalitis
ROCV	Rocio virus
ILHV	Ilheus virus
ONNV	O'nyong-nyong virus
MAYV	Mayaro virus
RRV	Ross River virus
HIV	Human Immunodeficiency virus
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic acid
+ssRNA	Positive-sense single-stranded RNA
ORF	Open reading frame
ELISA	Enzyme linked Immunosorbent Assay
PRNT	plaque-reduction neutralization test
MPIA	Multiplex peptide immunoassay
IFA	Immunofluorescence assay

IIFT	Indirect immunofluorescent Test
NAATs	Nucleic acid nucleic acid amplification techniques
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
POCTs	Point of care tests
RDTs	Rapid diagnostic tests
LFAs	lateral flow assays
OR	Odd's ratio
95% CI	95% Confidence interval
BSA	Bovine serum albumin
OVA	Ovalbumin
APC	antigen-presenting cell
LCs	Langerhans cells
MCs	mast cells
DCs	dendritic cells
PRRs	Pattern recognition preceptors
TNF	Tumor necrosis factor
IFN	interferon
LN	lymph node
Th1	T helper 1
FcgRs	fragment crystallizable gamma receptors
TCR	T cell receptor
NO	Nitric Oxide
ADE	Antibody-dependent enhancement
Abs	Antibodies
Ags	Antigens
TS-Abs	Type-specific antibodies
CR-ABS	Cross-reactive antibodies
ATR	Antibody target regions
RF	Random forest
ROC	Receiver operating characteristic
AUC	Area under the curve
MDA	Mean decrease accuracy
MDG	Mean decrease gini

Chapter 1: Dengue

1.1 Dengue

1.1.1 Distribution

Dengue virus (DENV) is a vector-borne virus that belongs to the *Flaviviridae* family, genus *Flavivirus*. The genus *Flavivirus* currently represents a threat to global health due to the (re)emergence of many of its members expanding from the (sub)tropics to more temperate regions worldwide (1). Flaviviruses are enveloped viral particles that have a positive-sense single-stranded RNA genome (+ssRNA) (2). DENV is currently recognized as the most widespread arbovirus and has a high burden on global public health with approximately 400 million infections each year (3). With the pandemic emergence of Zika virus (ZIKV) in 2015 (4), the recent re-emergence of yellow fever virus (YFV) in the Americas and parts of Africa (5), the rapid spread of West Nile virus (WNV) in North America and more recently in parts of Europe, the high distribution of Japanese encephalitis virus (JEV) in Asia, and the potential risk of emergence of other flaviviruses, places this group of viruses high on the list of continuing global public health challenges (1) (Figure 1).

The antigenic relationships between flaviviruses were established early in 1989, grouping them into serocomplexes, that were defined based on the ability of polyclonal antisera of one flavivirus to neutralize others (6). This resulted in 8 serocomplexes plus 17 independent viruses that were not antigenically similar enough to be included in a serocomplex (6). This antigenic relatedness among flaviviruses is currently also supported by genomic similarities based on phylogenetic analysis and on their arthropod vector association (7) (Figure 2). DENV constitutes a unique serocomplex comprised by the four DENV serotypes (DENV1-4), meaning that the antibodies induced after exposure against one serotype are able to cross-neutralize the other serotypes to a certain degree (7).

Dengue virus is transmitted to humans through the bite of infected female mosquitoes of the *Aedes Stegomyia sub*genus. *Aedes aegypti* is considered the primary vector for DENV, and it is distributed worldwide, but found in high densities across tropical countries (8,9). while *Aedes albopictus* is a secondary vector that has succeeded in colonizing temperate zones in the USA and Europe (9,10). However, other mosquitoes

of the *A. scutellaris* complex (i.e. *Ae. polynesiensis*, *Ae. katheriensis, Ae. scutellaris* and *Ae. hensilli*) have also been implicated as vectors of DENV (Ritchie Scott 2014).

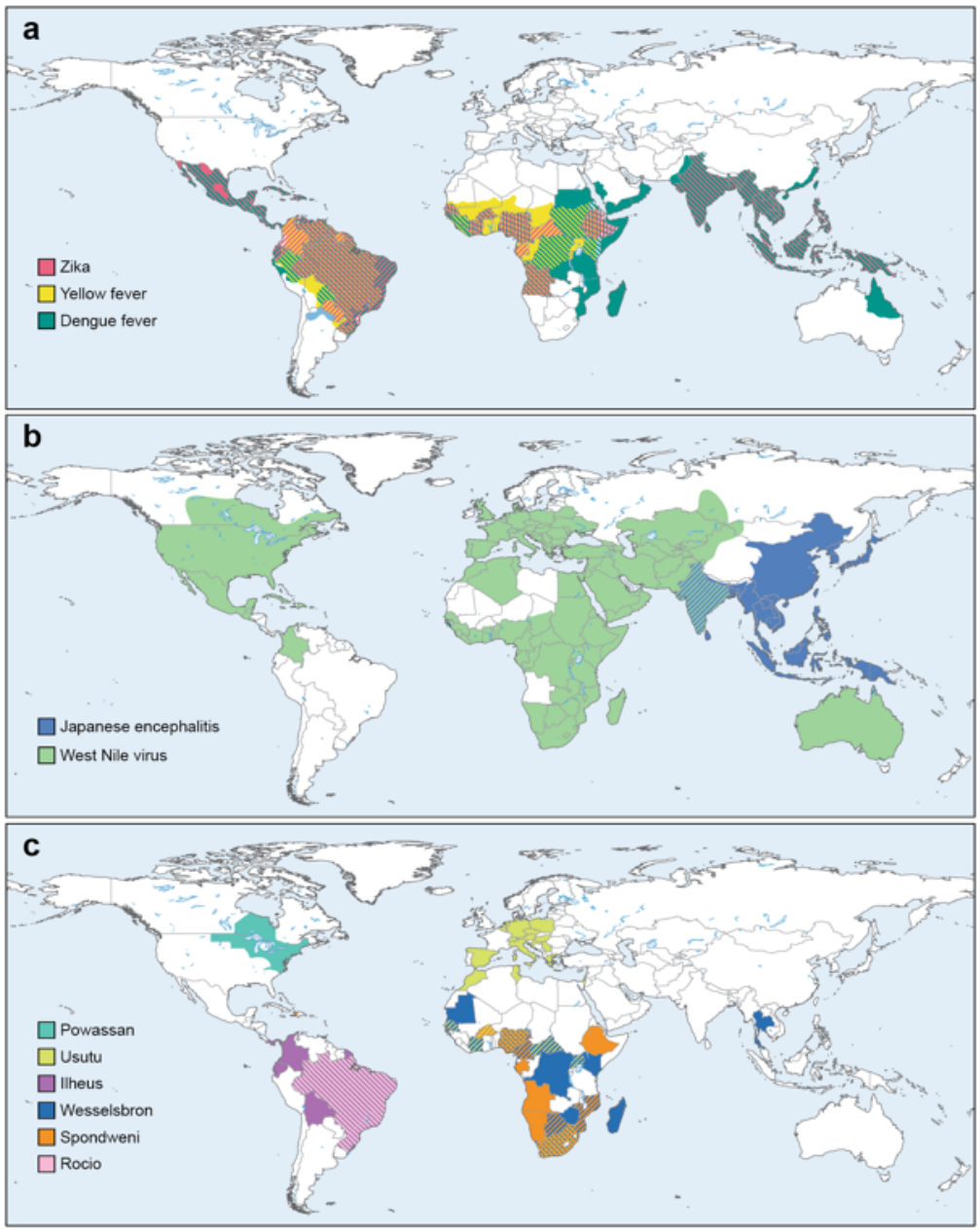


Figure 1. Global distribution of flaviviruses. a. Global distribution of ZIKV, YFV and DENV. b. Global distribution JEV and WNV. c. Geographic locations of flaviviruses with the potential for emergence in human populations. Taken from Pierson and Diamond. 2020 (1)

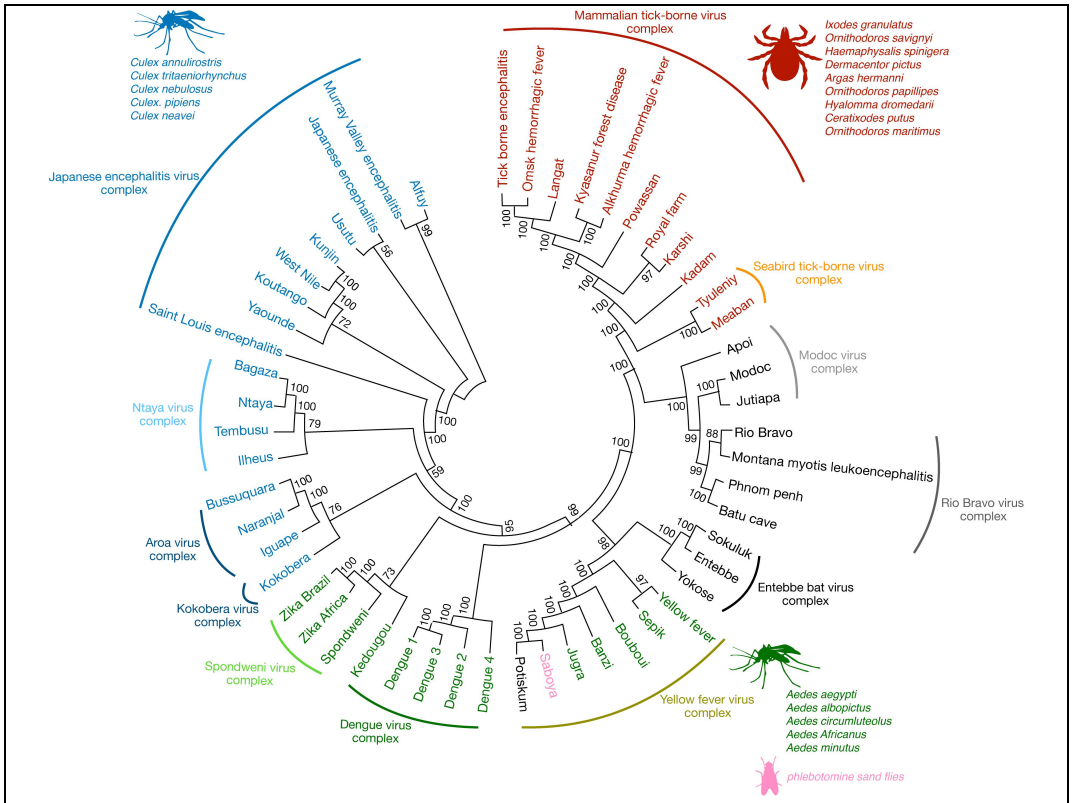
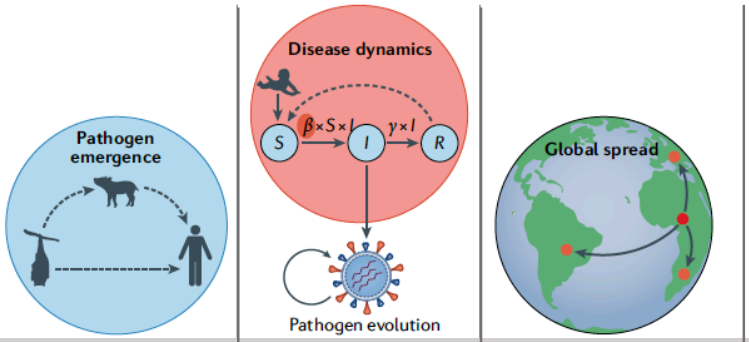


Figure 2. The antigenic relationships among flaviviruses. The phylogenetic analysis of the full-length polyprotein amino acid sequences demonstrates that flaviviruses cluster based on antigenic similarity and also according to their known transmission vectors. Each arched line corresponds to a different serocomplex, of which those flavivirus members belonging to the JEV, Spondweni, DENV, YFV, and mammalian TBV serocomplexes, are important human pathogens. The figure also shows the most common vectors of each serocomplex, such as the mosquito species *Culex* (blue) and *Aedes* (green) and various species of ticks (red). Flaviviruses with no known vector are shown in black. Exceptionally, the Saboya virus (pink) that belongs to the YFV complex which members are mainly transmitted by *Aedes*, has been successfully isolated from the phlebotomine sand flies. Figure taken from Rathore and St. John (7)

The burden of dengue has dramatically increased in the past two decades (11). This is mainly due to the spread and adaptation of the mosquito vector to new ecological niches as a consequence of massive unplanned urbanization, failure of vector control programs, changing land use patterns, increased international trade and travel and climate change (2,8) (Figure 3). Returning infected travelers have especially contributed to the global expansion of dengue infections with rising reports of autochthonous cases and outbreaks in regions previously considered as dengue-free (12,13). As a result, DENV is currently

distributed across 130 countries meaning that roughly half of the world's population lives in areas suitable for dengue transmission, with the vast majority in Asia, followed by Africa and the Americas. Asian countries hold about 70% of the total dengue burden, followed by the Americas and the western Pacific (2,3).



Climatic change	Drives range shifts for reservoir species	Affects transmission and susceptibility	Affects the geographical range of vectors
Technological change			
Transportation	Improved global surveillance		Air transit and high-speed rail affect pace and range of spread
Health care		Vaccination affects dynamics	Improved care reduces burden
Demographic change			
Population groth and land use	Increased contact with reservoir species	Population numbers affect evolution, birth rates affect dynamics	Larger population travelling
Urbanization	Depends on species	Density affects contact rate	Urban population more connected
Ageing	Imunosenescence affects spillover risk	Againg population increases transmission	Possible larger burden

Figure 3. Effects of climatic, technological and demographic change on disease emergence, dynamics and spread. The increase of dengue burden in the past decades can be explained by recent anthropogenic climatic, demographic and technological changes (rows) impacting on its re- emergence, its dynamics pattern at local- scale and its global spread (columns). An example susceptible (S), infected (I), recovered (R) model is shown, where β represents the transmission rate and γ is the recovery rate. Figure taken from Baker et al., 2021 (14).

1.1.2 The virus

DENV is a small spherical viral particle, 40 - 50 nm in diameter, that surrounds a +ssRNA genome of approximately 11,000 nucleotides (11 kilobases). A single uninterrupted open reading frame (ORF) is translated into a polyprotein precursor that is cleaved by cellular and virus-encoded proteases to produce ten individual functional proteins. Three of the

proteins, Capsid (C), pre-Membrane (prM) and envelope (E), are structural, while the other seven proteins are non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Figure 4a) (1,2).

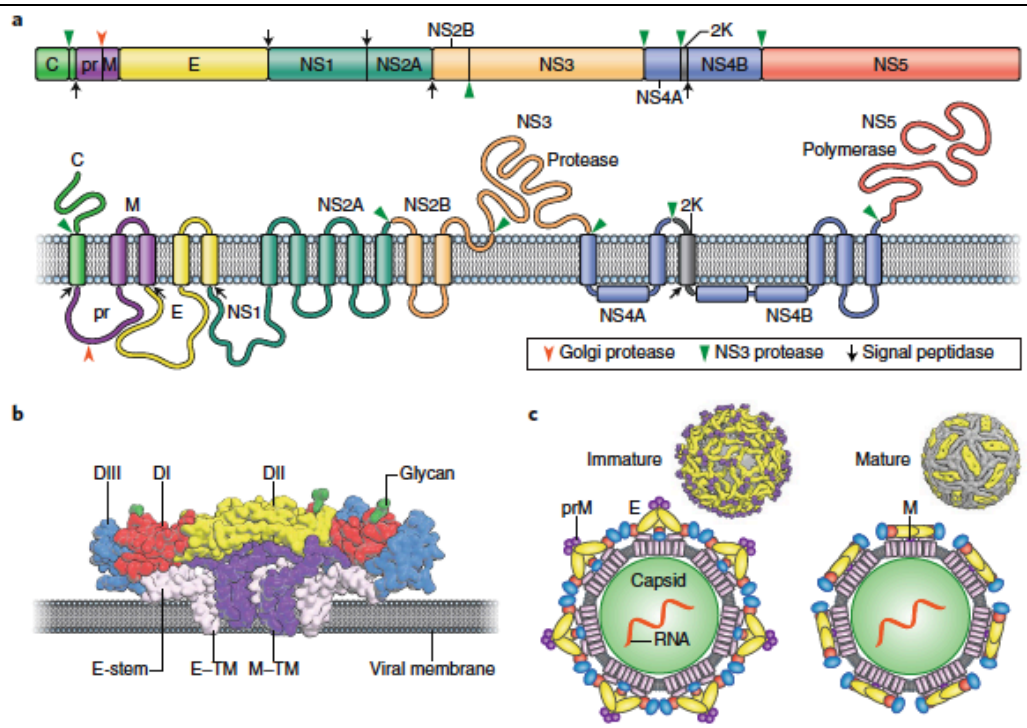


Figure 4. Organization and structure of DENV. a, Schematic diagram of the DENV genome encoding a single open reading frame that is translated at the ER into a polyprotein, which is processed via several proteolytic cleavages by viral and host cell proteases, resulting in ten functional proteins including three structural proteins, C, prM and E, and seven non-structural proteins. b, Structure of the M-E heterodimer. E proteins are composed of three-domain structures anchored to the viral membrane by a stem and two antiparallel transmembrane (TM) domains. Domain I (DI), domain II (DII) and domain III (DIII) are indicated in red, yellow and blue respectively. The M protein, also attached to the viral membrane by two transmembrane domains, is shown in purple. c, Representations of the spike-like and herringbone arrangement of E proteins on immature (left) and mature (right) forms of the virion. Taken from Pierson and Diamond. 2020 (1)

The DENV genomic RNA is surrounded by an icosahedral nucleocapsid which is at the same time covered by a host cell-derived lipid envelope in which two viral proteins, i.e. the E and the prM/M proteins, are embedded by their C-terminal hydrophobic anchors and forming heterodimeric complexes (14,15). The E protein is a viral hemagglutinin

that is essential for the attachment and membrane fusion during the viral entry into cells and is also the major antigenic determinant of DENV. E protein consists of three structural domains (DI, DII, and DIII) that lie parallel to the viral membrane as dimeric or trimeric units, with each monomeric unit anchored to the lipid bilayer by a helical stem region (1) (Figure 4b).

The DENV particle is assembled intracellularly in an immature form consisting of a lattice-like structure formed by 180 copies each of the prM and the E proteins (15). In its immature form, the DENV particles have a rough surface characterized by 60 spikes. Each spike consisting of a prM-E trimer complex (16) that has an icosahedral symmetry which prevents the premature fusion of the virion with the host membrane during the egress (1) (Figure 4c)

1.1.3 Virus entry and replication

The entry of the virus in mammalian cells begins with a myriad of different non-specific interactions mainly mediated by the E protein. These interactions facilitate the concentration of the virus at the cell surface for attachment to one or more receptors. Little is known about the identification of specific DENV receptors, but different studies suggest that DENV does not use a unique receptor but recognizes and binds different molecules on the cell surface (17). These interactions include: (i) binding of asparagine-linked sugars present on the E protein with multiple C-type lectins including dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), (ii) binding of charged molecules of the E protein to glycosaminoglycans on cell surfaces, and (iii) interactions between the viral lipid envelope and proteins of the T-cell immunoglobulin domain and mucin domain (TIM) and Tyro3, Axl and Mertk (TAM) family of phosphatidylserine receptors (1) (Figure 5).

Once DENV is attached to cells, the particle is internalized mainly through clathrin-mediated endocytosis (1) and in a minor degree through non-classical clathrin-independent endocytic pathways, depending on the cell host and the virus serotype or strain (17). The endosome maturation involves acidification, the low pH triggers membrane fusion through irreversible conformational changes in the E protein. The E-DII exposes the fusion loop that penetrates the host endosomal membrane, this causes a

rearrangement of the E-DIII providing the energy for the merger of the viral envelope and the endosomal membrane (18) (Figure 5).

The mechanism involving the C protein uncoating of the viral RNA is not fully understood. The released viral genome is transported through the cytoskeletal machinery towards the rough endoplasmic reticulum (ER). Here the +ssRNA serves as template for both the translation for the further synthesis of viral proteins (required for RNA replication) and the synthesis of the complementary negative strand. Later, this negative strand is used as a template for the synthesis of newly +ssRNA by the viral polymerase NS5 (19).

Virus assembly takes place in the ER. For the encapsidation of the +ssRNA, the C-protein accumulated on lipid droplets, is cleaved by NS2B-NS3 (serine-protease) which now can be recruited to bind to the viral RNA, forming the nucleocapsid (NC) (20). Alongside, the newly formed prM and E proteins are also recruited as heterodimers facing the ER lumen. Then, both proteins form trimers to assist the budding of the NC into the ER which travels through the secretory pathway forming the immature particles (18) (Figure 5).

Immature virions, in which the prM caps the fusion loop of the E protein in order to avoid premature fusion with the host membrane, follow the secretory pathway arriving at the Golgi. While passing through the low pH present in the trans-Golgi compartments, the E protein experiences N-linked glycosylation, ubiquitylation and conformational changes, where the trimeric prM-E heterodimers that characterize DENV immature particles, evolves into homodimers (18). This change exposes the cleavage site of the prM, which is cleaved by the cellular protease furin, leaving the membrane (M) protein and allowing the formation of infectious mature virions. However, due to the low pH, the pr peptide remains bound to the E protein, preventing the premature fusion of the viral envelope with the cell membrane. Once the virus is released to the outside environment, the neutral pH causes the dissociation of the pr molecules from the virus particle. The dissociation of the pr peptide does not occur simultaneously or with the same efficiency, leading to the release of heterogeneous mixtures of immature, mature and partially mature virions. The conformation of the E proteins in mature infectious virions exhibit a dynamic motion

as consequence of the conformation flexibility of its domains, a phenomenon referred to as virus breathing (18) (Figure 5).

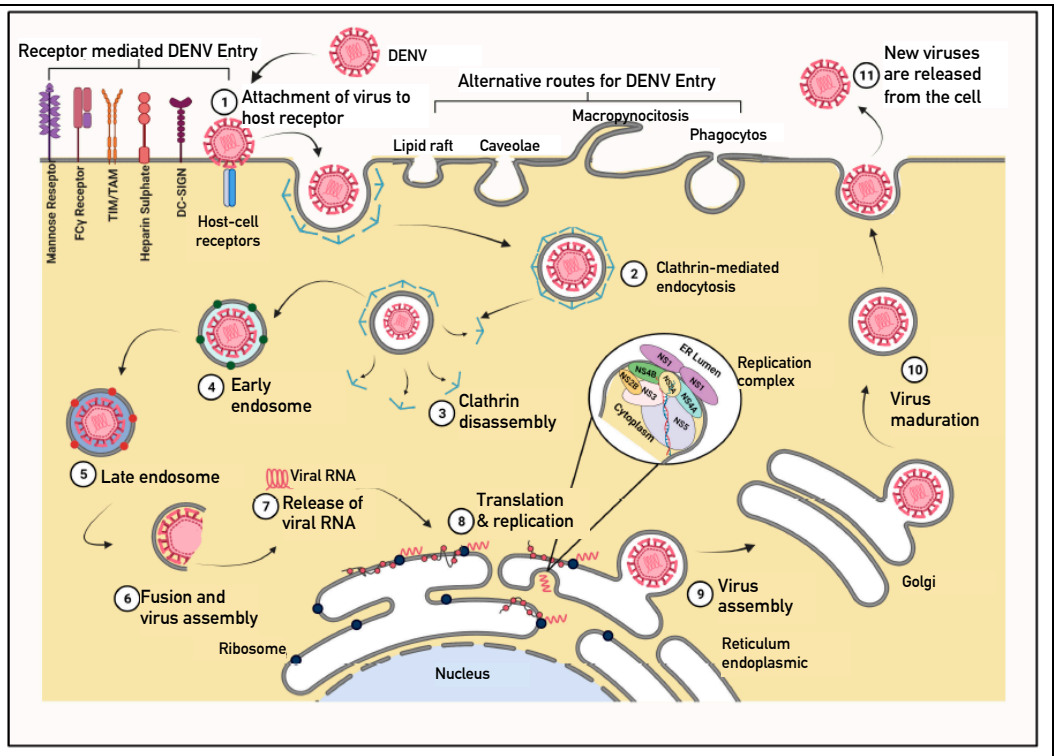


Figure 5. Replication cycle of Dengue virus: The replication cycle begins with the binding of the virus to host cell receptors. 1. After binding, the virus enters by receptor-mediated endocytosis, mainly clathrin-mediated endocytosis. 2. The clathrin-coated cavity internalize the virus. 3. Later, the clathrin molecules are disassembled from the endosome. 4. Early endosomes are characterized by a pH 6.5 and the presence of the Rab5 protein (green solid spheres). 5. The endosome maturation process involves the exchange of Rab5 by Rab7 (red solid spheres) and at a lower pH, i.e., around 5.5. 6. This acidification induces conformational changes of the E protein provoking the fusion of the viral envelope and the host membrane. 7. This fusion leads to the release of the nucleocapsid into the cytoplasm. 8. The viral RNA serves as a template for translation and replication in the ER. The translated NS proteins remain bound to the ER membrane forming the Replication complex. 9. Viral particles are assembled in the ER as immature virions. 10. The maturation of the viral particle occurs in the Trans-Golgi Network and involves the cleavage of the pr peptide from the prM protein mediated by host-furin proteases. 11. Mature particles are finally exocytosed from the infected cell. Figure taken from Nanaware et al., 2021. (18)

1.1.4 DENV transmission cycle

Two types of DENV transmission cycle have been identified: (i) the urban endemic/epidemic cycle, maintained in an *Aedes*-human-*Aedes* cycle, takes place in urban/peri urban areas in the tropics and in more temperate regions, and (ii) the sylvatic cycle, reported in Africa and Asia, where non-human primates are asymptomatically infected and transmit the virus to the mosquito fauna present in these regions.

The endemic/epidemic cycle represents the most important cycle, responsible for the bulk of urban epidemics and burden on public health. This cycle is especially efficient due to the behavior and ecology of the main dengue vectors, *Ae. aegypti* and *Ae. albopictus,* which can easily disperse throughout areas with approx. 300 m radius to seek oviposition sites. *Ae. aegypti* is the main vector for dengue transmission because of its high vector competence, its skipping oviposition behavior (laying eggs from the same batch in multiple sites), its desiccation-resistant eggs, the requirement for multiple feeds per gonotrophic cycle, its preference for human blood and its adaptation to reside and breed in man-made habitats, hence its clear anthropophilic character. Although *Ae. albopictus* has demonstrated high competence for DENV transmission in different laboratory settings, it is not as efficient as *Ae. aegypti* in nature. Aspects in the ecology of *Ae. albopictus*, related to its preference for peri-urban, rural and forested areas, in comparison to the highly anthropophilic *Ae. aegypti*, diminish its capacity as a vector (10,21).

When a mosquito bites a viremic individual, the period for the mosquito to become infectious can vary depending on the viral load, the temperature, the intrinsic vectorial competence and the virus genotype. This period is also known as “extrinsic incubation period”, where the midgut cells are first infected, then the dissemination and replication in different tissues takes place and finally, the infection and replication in the salivary glands can take between 4 – 10 days at ambient temperatures between 25 ºC – 28 ºC degrees. Once DENV is in the salivary glands of the mosquito it can transmit the virus to other humans during blood-feeding. The female mosquito can remain infective for its life-time, being capable of transmitting the virus with every blood meal taken. The virus can also be passed through the eggs to the mosquito offspring (vertical transmission), thus increasing the pool of DENV-infected vectors. After the mosquito bites, the infected

human can manifest the first signs of the disease ("intrinsic incubation period") between day 3 to 14, with an average of 4 – 7 days (22) (Figure 6). The viremic phase, and consequently the period during which the individual can transfer the virus to the mosquito upon a blood meal, ranges between two days before symptom onset until two days after fever has resolved.

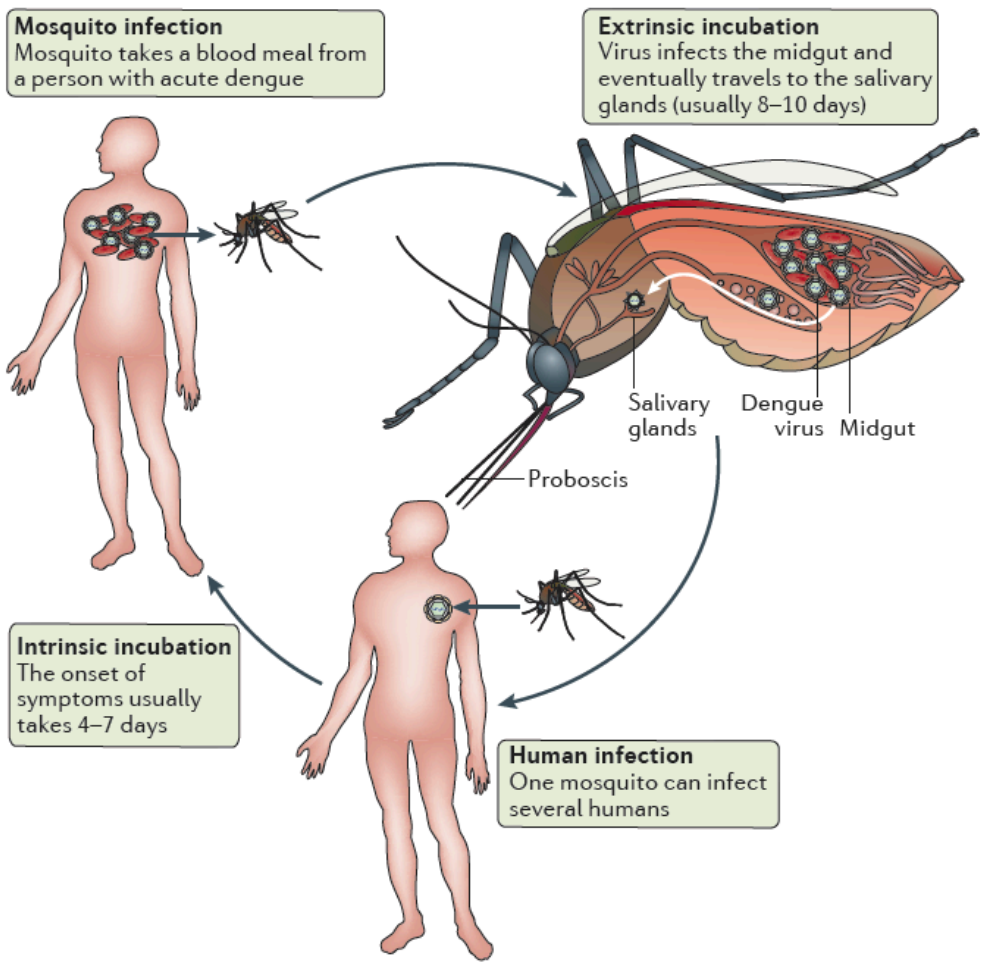


Figure 6. The urban dengue virus cycle in humans and mosquitoes. Taken from Guzman et al., 2016. (22)

1.1.5 Clinical manifestations

It is estimated that approximately 75% of the DENV infections are asymptomatic or with the presence of very subtle symptoms (3). When symptoms are present, they commonly manifest as a febrile disease known as dengue fever, accompanied by mild-to-moderate

debilitating symptoms that include skin erythema; headache; retro-orbital or orbital, muscle, joint, and bone pain; and rash (12,23).

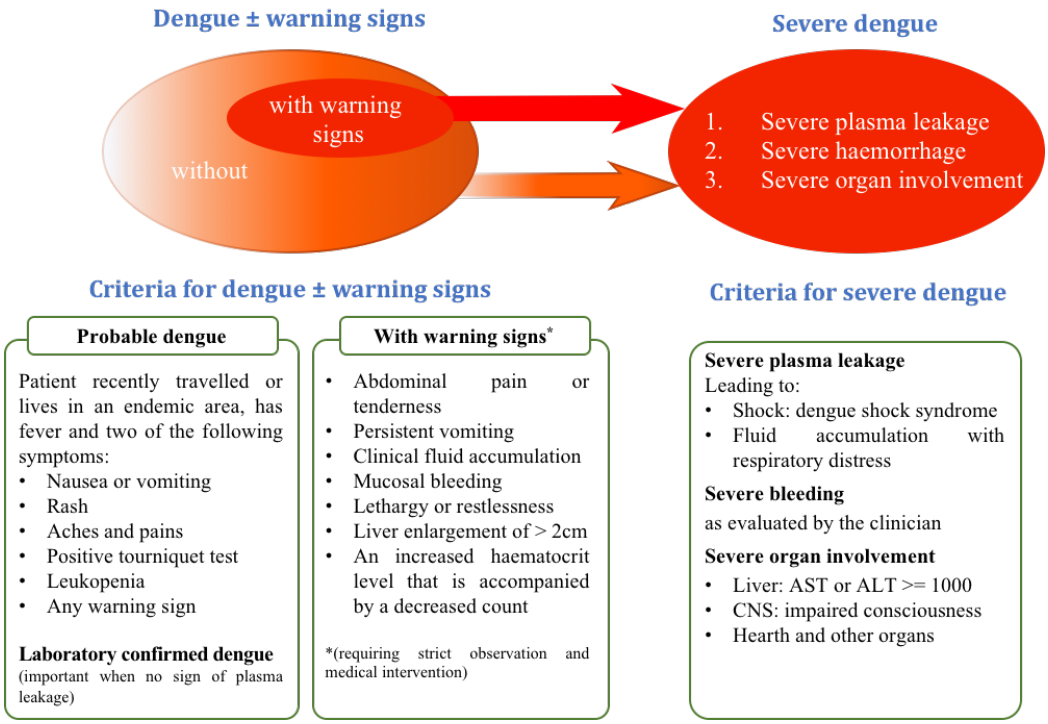


Figure 7. The 2009 WHO dengue clinical classification. ALT, alanine aminotransferase; AST, aspartate aminotransferase. Taken from WHO/TDR, Dengue Guidelines for Diagnosis, Treatment, Prevention and Control — New Edition, World Health Organization and Special Programme for Research and Training in Tropical Diseases, WHO Press, Figure 1.4, 2009. (12)

The current clinical classification system of the World Health Organization (WHO) categorizes the symptomatic patients based on the level of severity as having dengue with and without warning signs or severe dengue (Figure 7). This classification system was installed by the WHO in 2009 and replaced the previous classification systems that categorized dengue into dengue fever and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (12). This new classification scheme has proved to be more sensitive in capturing severe dengue cases thus helping clinicians in the early identification of symptoms that can evolve into DHF/DSS and facilitating the clinical management of cases in a timely manner (24). The simplicity of the new scheme in classifying the disease into dengue and severe dengue seems to also help in improving

the quality of epidemiological data collected globally, because surveillance data is not only better recorded but also more uniformized data sets across different clinical sites are obtained (24–26).

1.1.6 Dengue pathogenesis

The different clinical manifestations of dengue can be caused by any of the four dengue serotypes, and serotypes cannot be differentiated on clinical presentation. The infection with one serotype elicits long-term immunity to viruses of the same serotype upon re-exposure. This response is cross-protective against other serotypes but only for approx. 2-3 months; after this time, a secondary infection with a heterotypic serotype puts the patient in higher risk to develop severe disease (2,27).

The underlying mechanisms of development and progression of DENV infections to severe conditions are not well understood. The fact that severe symptoms such as vascular damage appear after the viremic phase suggest that this pathology is highly mediated by an exacerbated immune response (28). However, a synergistic interplay between viral and host factors has been postulated to explain this pathogenesis (Figure 8) (28), of which the best described are the antibody-dependent enhancement (ADE) and the NS1 protein.

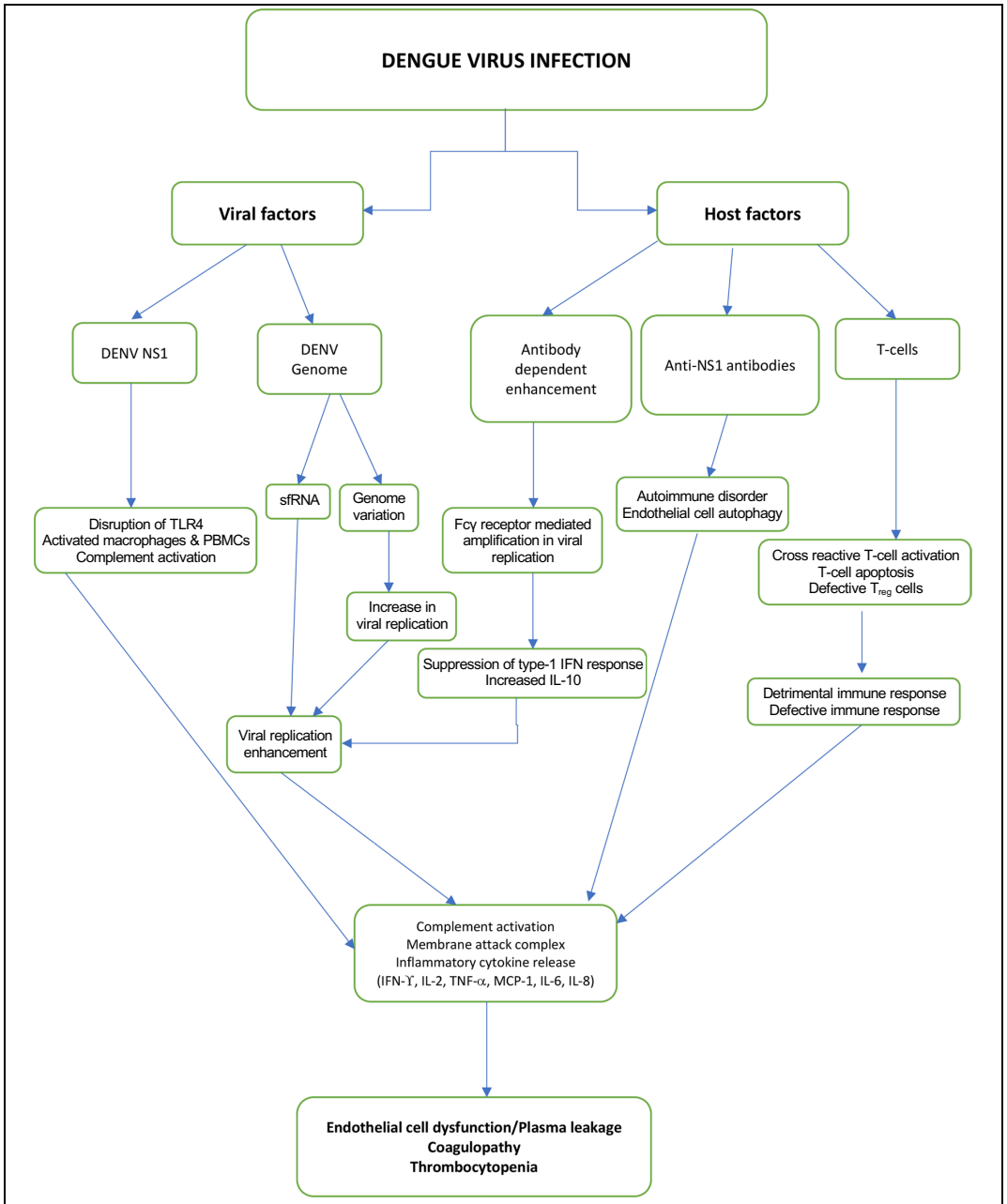


Figure 8. Complex interplay of viral and host factors in pathogenesis of dengue virus infection. sfRNA: subgenomic flaviviral RNAs**.** Taken from Bhatt et al., 2021 (28).

In primary infections, the acute phase of the disease is characterized by high viremia that coincides with high fever that is commonly self-limiting (Figure 9a). However, when the viremia decreases, a small proportion of patients can present life-threatening

complications including systemic vascular leakage syndrome, coagulation abnormalities that can involve bleeding, and organ involvement, typically hepatic or neurological (2,27).

During a secondary infection, the immunological memory changes the course of infection, where viremia peaks earlier but also resolves in a shorter time compared to a primary infection (Figure 9b). The high viremia levels observed during a secondary heterotypic DENV infection as well as primary dengue infections in infants of dengue-immune mothers enhances the probability to develop DHF/DSS (23).

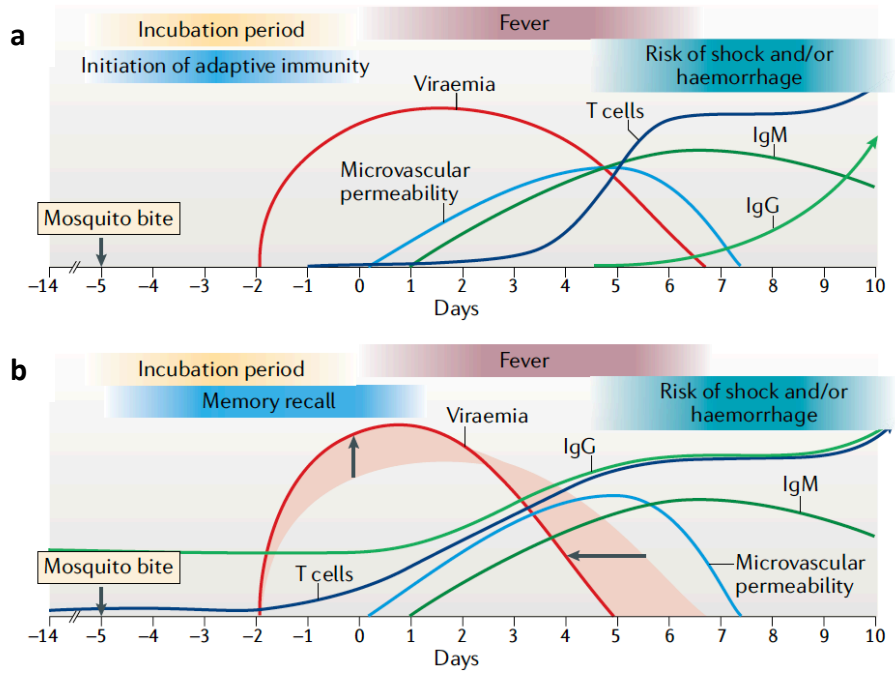


Figure 9. Time course of acute infection and immune responses during symptomatic dengue virus infection. (a) In a primary infection, after the bite of a mosquito infected with DENV, there is an incubation period of 4–7 days before fever develops. High viremia titers coincide with high fever. T cell response rises days before defervescence and is characterized for being cross-reactive. Once viremia has diminished, a fraction of patients could experience severe forms of the disease including hemorrhaging and/or shock that is thought to be immune- mediated. (b) A secondary DENV infection is characterized by a shorter viremia likely owing to pre-existing immunity, including antibodies that appear early in the acute phase. Also, an earlier and stronger T cell response, compared to primary infection, is promoted by memory T cells. This T cell response aids in the viral clearance against a homologous infection; however, it can potentially exacerbate immune pathology in a heterologous secondary exposure, increasing the risk to develop severe dengue. The critical phase in DENV infection is depicted with temperature dropping and the appearance of microvascular permeability. Taken from St.John and Rathore (27)

1.1.6.1 Antibody-dependent enhancement (ADE)

The best characterized factor associated with a higher risk of presenting severe dengue during a secondary infection with a different DENV serotype is ADE (29). It has been demonstrated that humans experiencing a secondary heterologous infection, a narrow range of pre-existing low antibody titers predicted increased disease severity, while high antibody titers were protective (30). However, to better understand the underlying mechanism of ADE, we first need to describe the immune response against dengue, therefore, this topic will be discussed in greater depth in Section 1.1.7.

1.1.6.2 NS1 induced pathogenesis

Non-structural protein-1 (NS1) is a highly conserved 48-kDa glycoprotein, produced as a monomer, but it dimerizes in the ER, forming part of the viral replication complex and remaining bound to the ER membrane where it has a role in the viral assembly through the interaction with host and viral proteins (2,23). NS1 can also oligomerize forming a hexamer which is secreted and circulates in the blood during acute illness. The correlation found between NS1 antigenemia and disease severity put this protein in the highlights and it is currently known that it plays a variety of roles in DENV pathogenesis (23,28) (Figure 10).

The best characterized mechanism of NS1-induced pathogenesis is through the endothelial disruption. It occurs at two levels: (i) NS1 interacts with the surface of microvascular endothelial cells in capillary beds provoking the degradation of the endothelial glycocalyx layer that results in barrier disfunction (Figure 10a), and (ii) through inducing autophagy of intercellular junctions leading to a transient disruption of the cell-to-cell contacts (23,28,31) (Figure 10b), both mechanisms resulting in vascular leak.

NS1 also plays a role in inducing secretion of inflammatory cytokines in innate immune cells. The activation of macrophages and human peripheral blood mononuclear cells (PBMCs) through Toll-like receptor 4 (TLR 4) induces the secretion of inflammatory cytokines such as IL-6, and the transcription of TNF-a, IL-1b and IL-8 genes (Figure 10c). Additionally, it has been suggested that NS1 could indirectly induce the secretion

of other soluble molecules through the interaction with other cells such as mast cells and platelets, however the exact mechanism remains to be elucidated (Figure 10d).

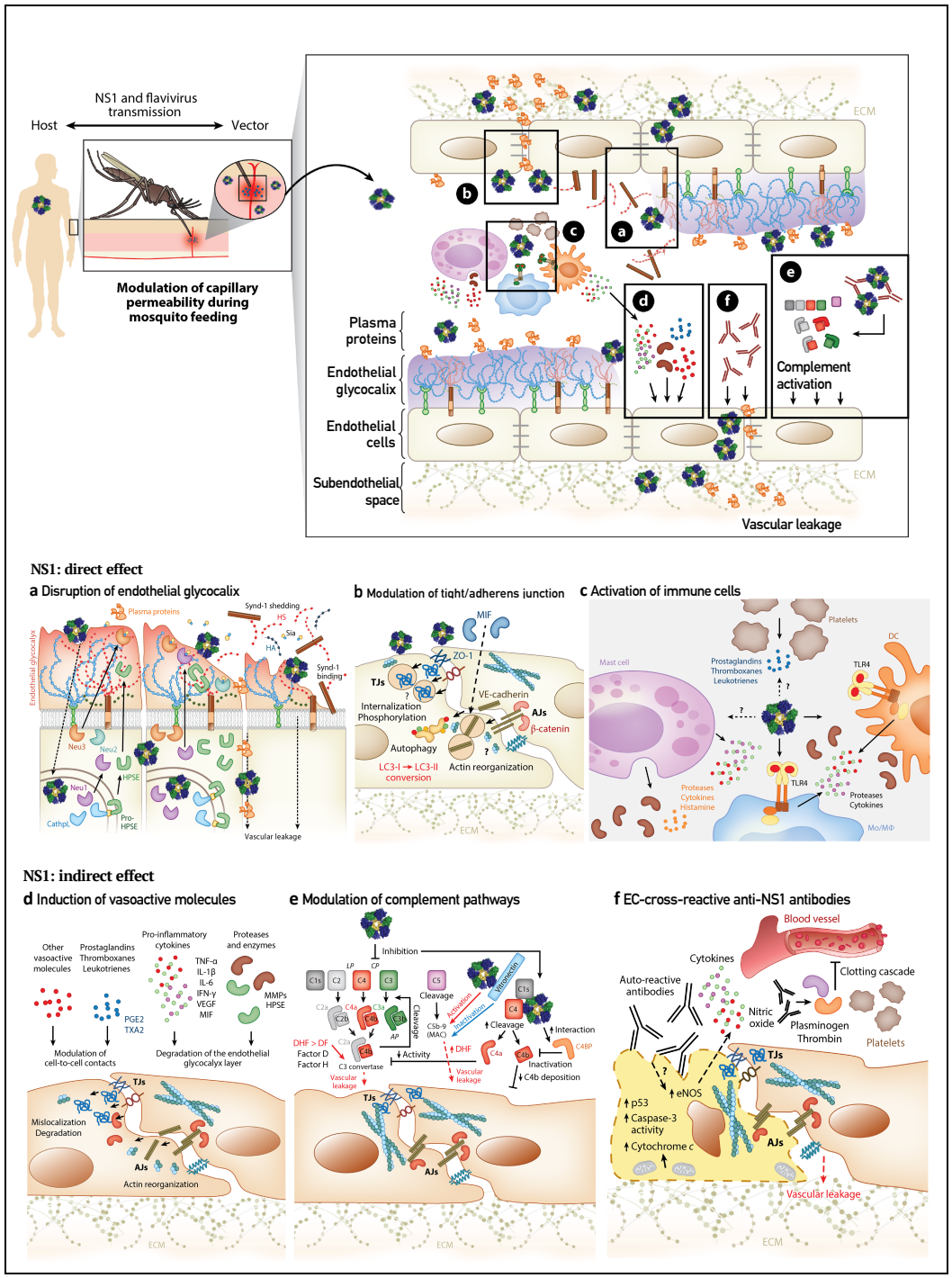


Figure 10. Mechanisms of DENV-NS1 pathogenesis leading to disease during DENV infection. During the bite of the mosquito to humans, the secreted form of NS1, present in the blood of viremic humans, is acquired together with the virions. NS1 would be necessary to overcome the midgut barrier in the mosquito for its efficient infection and replication of DENV (32). Secreted NS1 is also present in the saliva of an infected mosquito and it seems to modulate the capillary permeability in the dermis during the bite, helping in the DENV dissemination in humans. (a) NS1 interacts directly with endothelial cells, inducing the expression of sialidases (Neu1, Neu2, and Neu3) and activation of cathepsin L and heparanase; this leads to the shedding of key glycocalyx components (HS, Synd-1, Sia) resulting in barrier disfunction of the endothelial layer. (b) NS1 also causes the disruption of intercellular tight and adherens junctions proteins resulting in endothelial hyperpermeability. (c,d) NS1 can directly activate immune cells through the interaction with TLR4 to trigger a dysregulated production of proinflammatory cytokines that can contribute to vascular leak. NS1 may also stimulate secretion of other soluble molecules with vasoactive and proteolytic activities that can affect endothelial barrier integrity. (e) NS1 contributes to immune evasion via interaction with components of the complement pathway, leading to their activation (e.g., C3 convertase; Factor D, Factor H, C5b-9) or degradation (e.g., C4b, C5b-9). Hence, through the interaction with the complement, NS1 evades viral clearance and lysis of infected cells, leading to increased viremia and potentially contributing to endothelial injury. (f) Although there is no clear evidence of the pathogenic role of anti-NS1 antibodies in humans, there is evidence that these antibodies bind to platelets and components of the clotting cascade (e.g., plasminogen, thrombin) and may recognize autoreactive epitopes expressed on the surface of endothelial cells, triggering endothelial cell apoptosis. Abbreviations: AJ, adherens junctions; AP, alternative pathway; CathpL, cathepsin L; CP, classical pathway; DC, dendritic cell; DENV, dengue virus; DHF, dengue hemorrhagic fever; DF, dengue fever; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; HPSE, heparanase; HS, heparan sulfate; IFN, interferon; IL, interleukin; LC3, light chain 3; LP, lectin pathway; MAC, membrane attack complex; MIF, migration inhibitory factor; MMP, matrix metalloproteinases; Mo, monocyte; Mø, macrophage; Neu, neuraminidase; NS1, nonstructural protein 1; PGE, prostaglandin; Sia, sialic acid; Synd-1, syndecan-1; TJ, tight junction; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TXA, thromboxane; VE, vascular endothelial; VEGF, vascular endothelial growth factor; ZO, zonula occludens. Taken from Glasner et al., 2018 (23).

It has also been demonstrated that NS1 can bind to different complement components inducing either their activation (e.g., C3 convertase; Factor D, Factor H, C5b-9) or degradation (e.g., C4b, C5b-9). Higher levels of C5a are observed in DHF patients compared to patients with an ongoing mild/intermediate dengue fever, resulting in increased vascular leak and pathogenesis (18). These interactions of NS1 with the complement seem to protect DENV from two important viral defense mechanisms: complement-mediated neutralization and lysis of DENV-infected cells. Consequently, higher viral loads are observed, which induces injuries at the endothelium, finally resulting in vascular leakage (Figure 10e).

Lastly, it has also been reported that antibodies generated against NS1 may contribute to dengue pathogenesis. Although the exact mechanism remains to be defined, it appears

that the anti-NS1 antibodies react against self-molecules thus inducing the secretion of inflammatory cytokines, the apoptosis of endothelial cells through the Nitric Oxide (NO)-regulated pathway and the mimicry of some regions of NS1 with some self-proteins thus inducing autoimmunity (23,28)(Figure 10f).

1.1.7 Immune response against dengue

After the inoculation of DENV by the bite of an infected mosquito in the dermis, the cells that are targeted by the virus are immune cells from myeloid lineage, including monocytes, macrophages, Langerhans cells (LCs), mast cells (MCs) and dendritic cells (DCs). The innate immune response against DENV in these cells is triggered by the activation of pattern recognition receptors (PRRs), that at the same time start a cascade of inflammatory cytokines, primarily tumor necrosis factor (TNF) and interferon (IFN)-α. These molecules attract NK cells, NK T cells and CD8+T cells (cytotoxic T lymphocytes) that play a crucial role in controlling viral replication and pathogenesis at early stages mainly through the production of IFN, granzymes and perforin.

MCs are rapidly degranulated after encounter with DENV, which also induces the secretion of chemokines, cytokines and proteases. Besides, more macrophages and DCs are recruited to the site of infection, which once activated migrate to peripheral lymph nodes (LNs) in a CCR7-dependent and CCL21-dependent manner where the start of the adaptive immune response takes place through the presentation of the antigen to CD4+ and CD8+ T cells. (18,27) (Figure 11).

In order to achieve viremia, DENV needs to migrate to draining LNs, where it can infect more DCs and monocytes. In the LNs, T cells are activated after the presentation of DENV-antigens by DCs and polarized to a T helper 1 (Th1) cell phenotype (Figure 12). DENV-infected cells in the skin and the peripheral LNs secret type I and II interferons, that recruit cytotoxic cells including natural killer, natural killer T cells and CD8+ T cells. These cells are implicated in the direct killing of DENV infected cells and also control the infection through the production of pro-inflammatory cytokines such as IFNγ and TNF-α and other cytotoxic molecules including granzyme and perforin. Besides, once activated, CD4+ T cells also aid in viral clearance through their cytotoxic activities, enhancement of B and CD8+ cells, and by promoting memory responses.

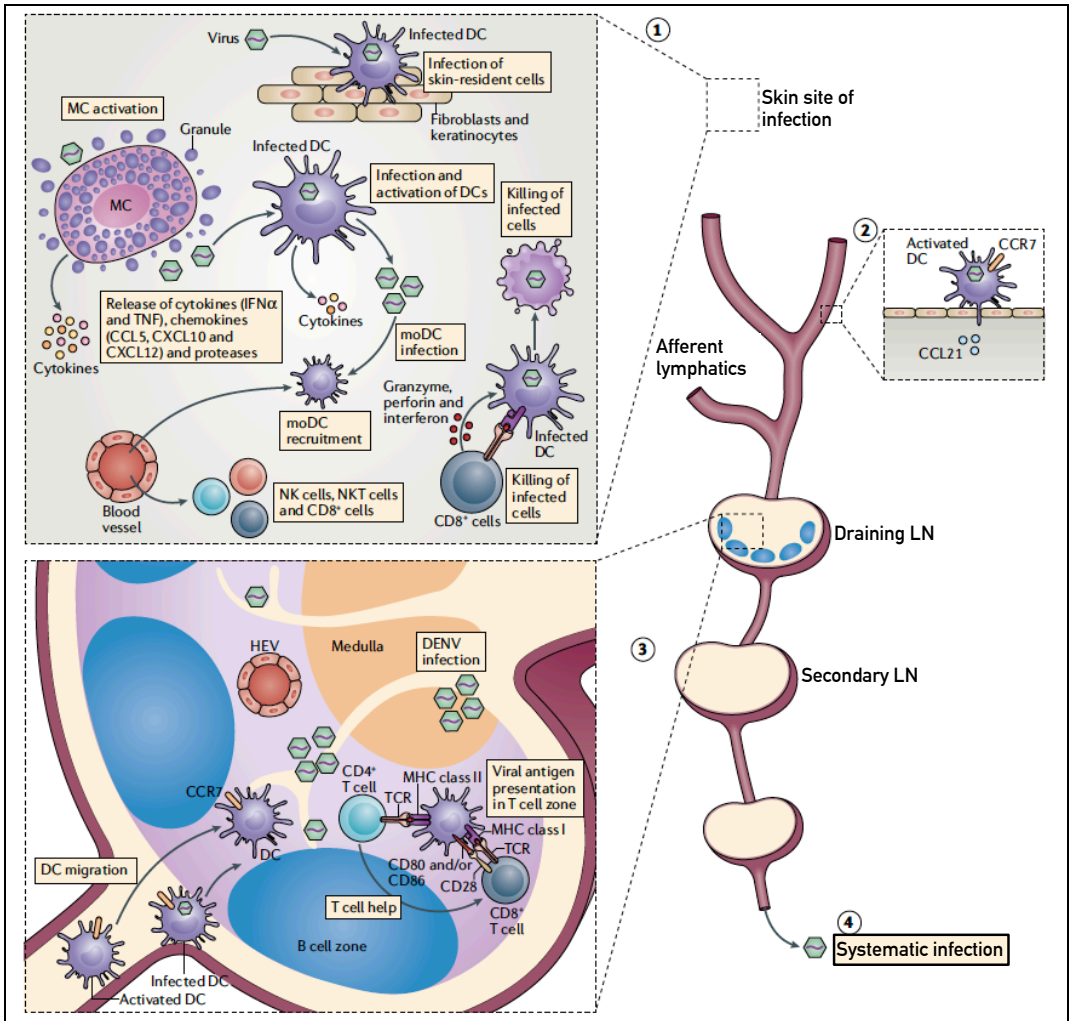


Figure 11. Initiation of anti- dengue virus immunity in the skin and draining lymph nodes. |*1*| Immune cells that patrol the epidermis in the search of pathogens, such as Langerhans cells (LCs), dendritic cells (DCs) and mast cells (MCs) constitute the first barrier to fight DENV after its inoculation through a mosquito bite. Once DCs are infected they release cytokines, chemokines and proteases that activate MCs and help to recruit more antigen-presenting cells to the site of infection |*2*| DENV infects and replicates in skin- resident dermal DCs, macrophages and recruited moDCs and also present DENV antigens after processing. After activation, DCs express CCR7 and migrate to the draining lymph node (LN) also helped by the CCL21 chemokine. |*3*| Once in the LN, activated DCs upregulate the expression of co- stimulatory molecules such as CD80 and CD86, and they present antigen to $CD4^+$ and $CD8^+$ T cells in T cell zones for the initiation of the adaptive immune response. DENV infects more cells in the LNs, serving as amplification centers for the virus to progress to a, |*4*| systemic infection. HEV, high endothelial venule; TCR, T cell receptor. Taken from St John and Rathore 2019 (27).

The type of immune response driven by T cells after recognizing antigen-derived peptides (T-cell epitopes) bound to HLA class I and II molecules will depend on the ability of the peptide to bind the HLA and its immunogenicity able to trigger either a strong protective T cell response that contribute to the control of the infections (27), or whether it will have a pathological effect characterized by a dysregulated production of inflammatory cytokines, known as "cytokine storm" that results in an increase vascular permeability (23). The detrimental effect of the T cell response is explained by the "original antigenic sin" by which cross-reactive T cells that are specific to the causative DENV serotype in a primary infection, become predominant in a heterologous secondary infection, thus inducing a T cells that have low affinity for their cognates epitopes and mounting a weak ineffective response (33).

Studies on the recognition of T cell epitopes have revealed that CD8+ T cell epitopes are primarily found in NS3, followed by Capsid, NS5, NS4A and NS4B proteins. By contrast, CD4+ T cells epitopes are predominantly located in proteins that are also targeted by antibodies, such as the Capsid followed by the E and NS1 proteins, and to a minor degree in NS3, NS2A/B and NS5 proteins (33).

The interaction between $CD4^+$ T helper cells and B cells in the germinal center is important for the generation of B-cells able to produce neutralizing (contributing in blocking DENV infectivity and viral clearance) as well as non-neutralizing antibodies (Figure 12). Most of the secreted antibodies are non-neutralizing and mainly directed against the structural proteins, while the E protein seems to concentrate the majority of neutralizing antibodies, and they are particularly directed against the EDIII region.

After primary infection, within days 5 – 10 following infection, the hallmark of the humoral response is the first induction of IgM antibodies (34), followed by an IgG response characterized for being highly serotype specific, although antibodies that cross-react with heterologous serotype or related flaviviruses are also produced. Nevertheless, the fraction of protective antibodies, those able to neutralize the virus, tend to cross-neutralize heterologous serotypes but these antibodies are short lived, they normally wane between 2 – 6 months after infection (35); following this period the protection is conferred only against the homologous serotype. Conversely, during a secondary

infection, the humoral response is marked by a reduced IgM but an enhanced IgG response that appears soon after the symptoms onset.

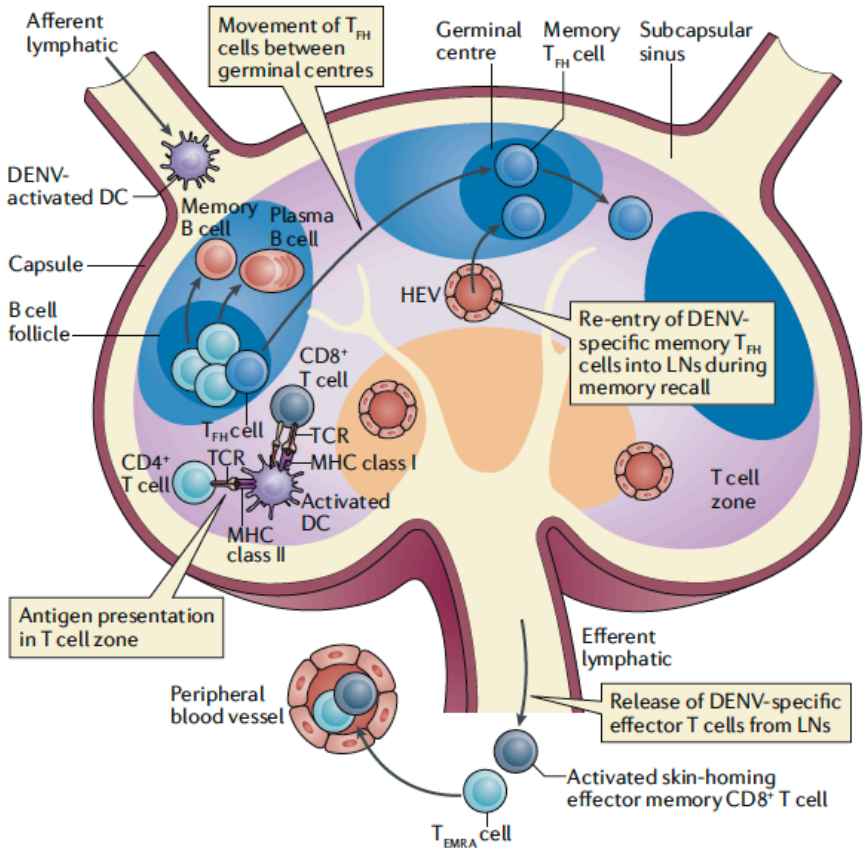


Figure 12. Adaptive T cell responses during dengue virus infection. Activated/infected dendritic cells (DCs) migrate to draining LN through afferent lymphatics and present the antigen to $CD4^+$ and $CD8^+$ T cells. Some activated $CD4^+$ T cells differentiate into T follicular helper (T_{FH}) cells. These cells, located in the germinal center, migrate to the periphery of the B cell follicle in the LN to promote the activation of B cell, development of DENV- specific memory B cells and plasma cells. T_{FH} cells can also traffic to new follicles and to re-enter germinal centers. During a secondary DENV infection, memory $CD8^+$ T cells acquire a skin- homing phenotype enabling them to exit the LN through efferent lymphatics and to patrol the skin for clearance of DENV. Similarly, some $CD4^+$ T cells can acquire a cytotoxic phenotype and are known as T effector memory RA (T_{EMRA}) cells, also contributing in the DENV clearance. During a secondary infection, clonal expansion of B cells can be triggered by memory T_{FH} cells, also enhancing the antibody production and affinity maturation. GPR56, G protein- coupled surface receptor 56; HEV, high endothelial venule; TCR, T cell receptor. Taken from St John and Rathore 2019 (27).

It is documented worldwide that during a secondary exposure with a serotype different from the first infection, the infected individual is at higher risk to develop DHF/DSS. As a result of the highly genetic and structural similar immunogens the immunological memory mounts an anamnestic humoral and T cell response instead of a specific immune response, as consequence of the "original antigenic sin". Thus, cross-reactive non-neutralizing antibodies are thought to increase disease severity through a mechanism known as the antibody dependent enhancement (ADE), that mediates (i) higher viral load (ii) mast cells activation and (iii) higher cytotoxicity mediated by natural killer (NK) cells (18,27).

DCs and monocytes are susceptible to ADE because they express the fragment crystallizable (Fc) gamma receptors (FcgRs) which bind to the sub-neutralizing antibodies. The higher viral load induced by ADE occurs in the presence of a narrow range of sub-neutralizing antibodies from a previous heterotypic infection which, rather than neutralizing, enhance the active transportation of DENV particles via opsonization into antigen-presenting cells.

Antibody-virus complexes are opsonized by the DCs leading to an increased viral replication in the cells. These infected cells contribute with more virions able to infect more cells, increasing the viral load which triggers immunopathological events that finally translates into more severe clinical manifestations of the disease (30) (Figure 13). Besides, further characterization of the ADE mechanism indicates that patients with severe dengue presented the Fc of their IgG associated with glycans. Moreover, it was discovered that the composition of Fc-associated glycan modulates the affinity of the IgG molecule for the Fc receptor FcγRIIIA (36). Recently, it was demonstrated that an increased abundance of afucosylated IgG1 glycoforms, represented a robust prognostic tool for dengue disease, since higher levels of afucosylated IgG were predictive of dengue disease severity (37).

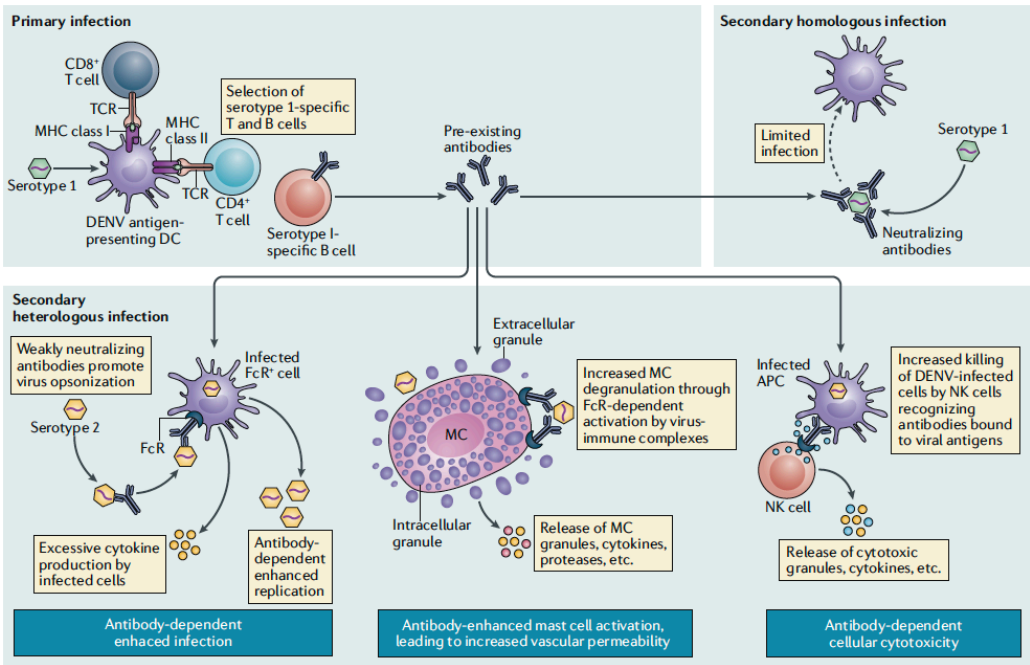


Figure 13. Antibody- dependent pathologies during dengue virus infections. In a secondary infection, pre-existing serotype- specific high- affinity antibodies produced during the primary infection, when present at optimal concentrations, can neutralize DENV and limit the infection (indicated by dashed arrow). However, during secondary heterologous infection, pre-existing cross- reactive, sub- neutralizing antibodies may lead to opsonization of virus particles and enhanced uptake, resulting in increased virus replication, a phenomenon termed antibody-dependent enhancement (ADE). The high viral load in infected cells leads to an exacerbated production of cytokines. Similarly, antibodies opsonizing DENV particles can be recognized by crystallizable fragment receptor (FcR) expressed by mast cells (MCs), activating the release of granules, cytokines and proteases, which lead to increased vascular permeability and leakage during DENV infection. Antibodies bind to DENV-infected cells can promote the lysis of these cells through antibody- dependent cellular cytotoxicity, mediated by cytotoxic natural killer (NK) cells. Activated NK cells also contribute to the cytokine storm, and the tissue damage through the release of cytotoxic granules and cytokines. APC, antigen-presenting cell; DC, dendritic cell; TCR, T cell receptor; DENV, dengue virus. Taken from St John and Rathore 2019 (27).

1.1.8 Dengue serotypes

DENV is subdivided into four serotypes, i.e. DENV-1, DENV-2, DENV-3 and DENV-4. The four serotypes share between 65 – 70% amino acid sequence identity that makes them genetically related but antigenically distinct (10). Genetically distinct groups within each serotype are referred to as genotypes (Table 1), for which the envelope protein differ

from each other by ≤10% at the amino acid level (38); genotypes in turn can also be divided into different lineages.

DENV endemic regions are characterized by the co-circulation of the four serotypes, which is especially associated with large cities. This hyperendemicity was accelerated after World War II due to important changes in human population growth, uncontrolled urbanization and massive human movement (2,22).

The dynamics in the transmission of the DENV serotypes in a given location frequently starts with an intense diversification followed by lineage extinction and replacement (39). These DENV strain substitutions in a specific population are also related with a fluctuation in the dynamics of the epidemic patterns, with alternated large epidemics (high disease severity rates) and small outbreaks (mild disease manifestations).

The forces driving these transmission dynamics are not very clear, but have been related with demographic factors such as host age - higher incidence of dengue in younger age groups- (22,40), host genetics and history of infections, along with the evolutionary genetic of DENV as result of natural selection, immune pressure and population bottlenecks – such as the impose when a decrease in the population of mosquitoes takes place (38,41).

However, the genetic differences between the DENV serotypes or between lineages within the same serotype does not alone explain the differences at the clinical and epidemiological level, because large DENV epidemics have been reported when a new serotype/genotype/lineage is introduced in a specific population. Therefore, the epidemic potential does not seem to be related with a specific DENV genotype or strain.

Recently, long-term studies have shown more evidence supporting the hypothesis that large epidemics tend to occur when the herd immunity against a particular infecting DENV strain wanes over time leading to the introduction/emergence of a related but distinct viral lineage. The observation that large epidemics are also characterized by higher rates of DHF/DSS, supports the hypothesis that host genetics and immunity in human populations with an increased circulation of different DENV serotypes/genotypes also increases the probability of heterotypic secondary infections and thus the risk to develop ADE (46).

Serotype	Genotype	Distribution	First detection in Peru
DENV-1	I	Southeast Asia, China and the Middle East (Saudi Arabia)	
	II	Asia-Pacific region (Thailand, Malaysia, Cambodia, Myanmar, Vietnam and Australia)	
	III	(sylvatic?): Malasya	
	IV	Pacific Rim, the Western Pacific islands and Australia	
	V	Americas, strains from West Africa and Asia	1990 / 1995 /2019
DENV-2	Asian I	Thailand, Malaysia, Cambodia, Myanmar, Vietnam and Australia	
	Asian II	China, Indonesia, The Philippines, Taiwan, Sri Lanka, India, Honduras and Mexico	
	Asian/American	Southeast Asia, Central and South America and the Caribbean	2001 / 2010
	Cosmopolitan	East and West Africa, the Middle East, the Indian subcontinent, Indian and Pacific Ocean Islands and Australia. Recently detected in Mexico, Peru	2019
	American	Central and South America, the Caribbean and older strains from Indian subcontinent and the Pacific Islands	
	Sylvatic		
DENV-3	I	Southeast Asia, the Philippines and the South Pacific islands	
	II	Southeast Asia	
	III	Asia, the Pacific, East Africa and Latin America	2001
	IV	Puerto Rico and Tahiti.	
	V	Philippines, Japan, China, Brazil	
DENV-4	I	The Philippines, Thailand, Vietnam, Myanmar, Malaysia, Sri Lanka, India, Japan, China, Brasil	
	IIa	Asia (Thailand)	
	IIb	Southeast Asia, China, islands of the Western Pacific Ocean, Australia, the Caribbean and the Americas	2001
	III	India, Thailand	
	IV	Sylvatic (Malaysia)	

Table 1. Dengue serotypes and genotypes and their distribution. Adapted from Chen and Vasilakis, 2011 (42–45).

In this context, the genetic similarity between the DENV serotypes induces short-term immunological cross-protection after primary infection which becomes more type-specific in the long-term; however, the heterogenous antigenic relationship among DENV strains would also play a critical role in the effectivity of the anamnestic immunity in secondary DENV infections. Despite the fact that DENV is under evolutionarily constraint due to its alternation between the vertebrate and invertebrate host (47), there are regions in the genome, mainly located in the structural proteins, that are under antigenic evolution due to their continue exposure to immune pressure (48).

Mutations that affect the antigenic phenotype of DENV strains – i.e. that induce changes in structural proteins – are of particular importance because they modify the antibody

binding (49–51). The analysis of these antigenic differences is critical for DENV surveillance, because the antibodies generated by prior heterotypic infection would not only be ineffective in secondary infections, but rather used by the virus to generate more disease through ADE.

Therefore, the analysis of the genetic evolution of DENV through phylogenetic analysis should be complemented by the monitoring of antigenic evolution using antibody neutralization assays. Antigenic cartography constitutes an elegant visual method to monitor genetic and antigenic evolution by positioning the virus and the antiserum in an antigenic map, such that the distance between them directly corresponds to the measured neutralizing titers. This method, first applied to influenza viruses (52), and later adapted to DENV by Katzelnick et al., demonstrated that DENV serotypes tend to group together based on genetic similarity, but these isolates can be as antigenically similar to a virus of the same serotype as to viruses from another serotype, and comparable distances were measured between and within serotypes (53)

A recent long-term study conducted in Thailand by Katzelnick et al. shows more clear evidence supporting the hypothesis that the clade/strain replacement patterns that coincides with epidemic dynamics is associated with the antigenic variation among the DENV serotypes. It was shown that the pressure imposed by homotypic immunity, would force the virus to antigenically evolve thus causing large outbreaks mainly explained by ADE. This antigenic diversity is reduced over time resulting in smaller antigenic differences between serotypes and again, in order to evade homotypic immunity, the DENV strains change antigenically to resemble other serotypes, thus weak cross-neutralization takes place leading to enhance disease severity (38).

1.1.9 Dengue in Peru

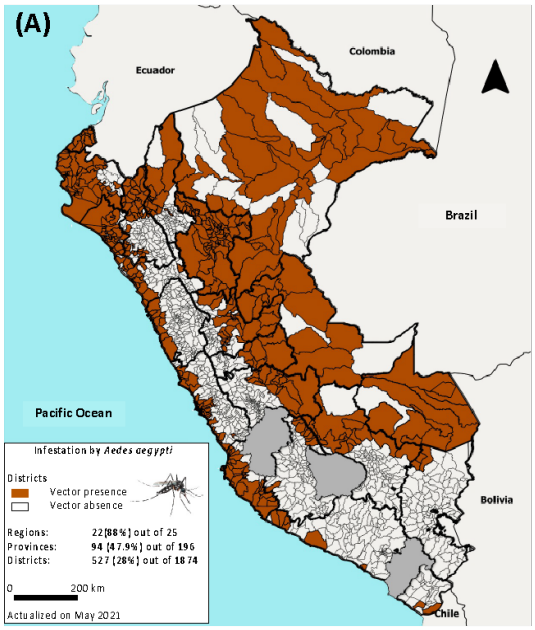
Peru is an upper middle-income South-American country with a population of 32.5 million and a surface area of approximately 1.2 million Km^2. Its location and territory hold the second largest part of the Amazon Rainforest after Brazil, which covers 60% of the national territory. The Peruvian geography is complex and diverse with dry lands facing the Pacific Ocean, Andean valleys in the north and southeast and Amazonian lands with a marked biodiversity in the east.

Despite its location in the tropics, the presence of mountain ranges, topography variations and two ocean currents (Humboldt and El Niño Southern Oscillation (ENSO)) give Peru a large diversity in climatology. Unfortunately, this diversity also propitiates the presence of various vector-transmitted diseases to which Peru is an endemic area, including malaria, dengue, leishmaniasis, Zika, chikungunya, Chagas, Bartonellosis among others. Peru is responsible for only 0.5% of global warming, its population is particularly vulnerable to the consequences of climate change since it strongly affects the dynamics of transmission of vector-borne diseases. Warmer temperatures and the variations in the amplitude and frequency of the ENSO, together with unplanned urbanization characterized by inadequate water supply and poor management of solid residues have favored the conditions for the expansion and multiplication of the vector *Ae. aegypti* from equatorial latitudes down to the south.

In addition to environmental factors, the distribution of these diseases in Peru is strongly influenced by socio-economic factors that, together with health inequities, places the poorest in the most vulnerable situation. In an analysis of the health situation in Peru in 2010, it was shown that about 41.2% of dengue cases were associated with the lack of drinking water (54). The poverty in Peru increased with the onset of the COVID-19 pandemic, from 16.5% in 2019 to 25.8% in 2020, while the extreme poverty duplicated (Economic and Social Commission for Latin America and the Caribbean (ECLAC, 2021)). Together with the discontinuation of several vector-disease control programs, this has manifested in a negative impact on the incidence of vector-borne diseases.

As in many parts of South America, dengue is one of the most important re-emerging diseases in Peru and confronts the country with a nation-wide public health problem. The first reports of dengue in Peru date from 1818 with a register of 50,000 cases (55). Despite the eradication of *Ae. aegypti* from Peru in 1958, the vector re-infested the country in 1984 and the first dengue cases post eradication occurred in 1990 in Iquitos, one of the most important cities in the Peruvian Amazon. After the reintroduction of the vector, it expanded to other regions in the Amazon basin, later to the northern cities and in 2000 it reached Lima city. Currently, *Ae. aegypti* is present in 22 out of 25 regions of the country which correlates with the districts reporting dengue cases (Figure 14). Based on criteria such us population at risk, dengue incidence rate, case history, entomological parameters

as aedic indices and dengue outbreak history, it was calculated that in Lima with a population of 10,896,863 inhabitants, about 14.5% (1,575,811) of people live in districts with a high risk of dengue outbreaks.



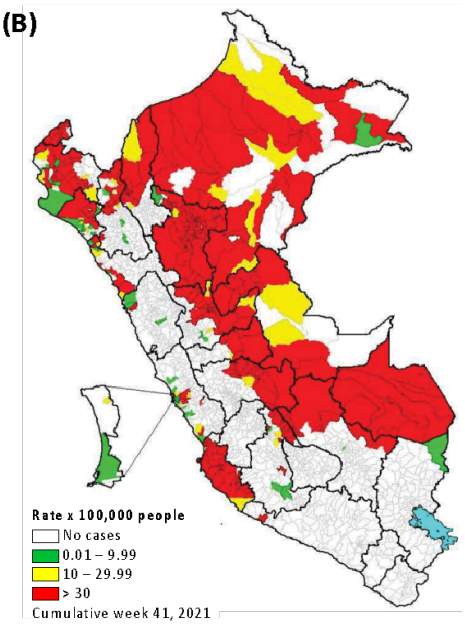


Figure 14. (A) Distribution of the dengue vector *Aedes aegypti* in Peru. Taken from http://www.digesa.minsa.gob.pe/DCOVI/infestacion.pdf. Accessed October, 2021. **(B) Cumulative incidence of dengue in Peru.** Epidemiologic week Nº 41. Taken from: Centro Nacional de Epidemiologia, Prevención y Control de Enfermedades – MINSA. Accessed October, 2021.
https://www.dge.gob.pe/epipublic/uploads/dengue/dengue_202141_26_091955.pdf

1.1.9.1 Dengue burden in Peru

According to the WHO, Peru is considered as one of the ten most highly endemic countries in the American Region with circulation of the different dengue virus (DENV) serotypes. Since the resurgence in 1990, outbreaks have been reported in different regions of the country, with a periodic frequency of re-appearance, followed by years of decreasing numbers of cases (Figure 15).

Large DENV epidemics have occurred in 2001, 2010, 2015, 2017 and 2020 that have been associated with (i) the introduction of the genotype III of DENV-3 in 2001 and the Asian/American genotype of DENV-2 in 2010, (ii) the ENSO climatic event in 2017 that

mainly affected the Northern coastal cities with extensive flooding, drinking water restrictions, sewer collapse and overcrowding creating the ideal conditions for the largest dengue epidemic in the country since its re-emergence in 1990, caused mainly by the DENV-2 Asian-American genotype; and (iii) the recent 2020 dengue epidemic associated with the introduction of Genotype V of DENV-1 in the Loreto Region the previous year and the Cosmopolitan genotype of DENV-2 in Madre de Dios Region in 2019 where case numbers were exacerbated as a consequence of interruptions of Vector Control Programs due to COVID-19 pandemic.

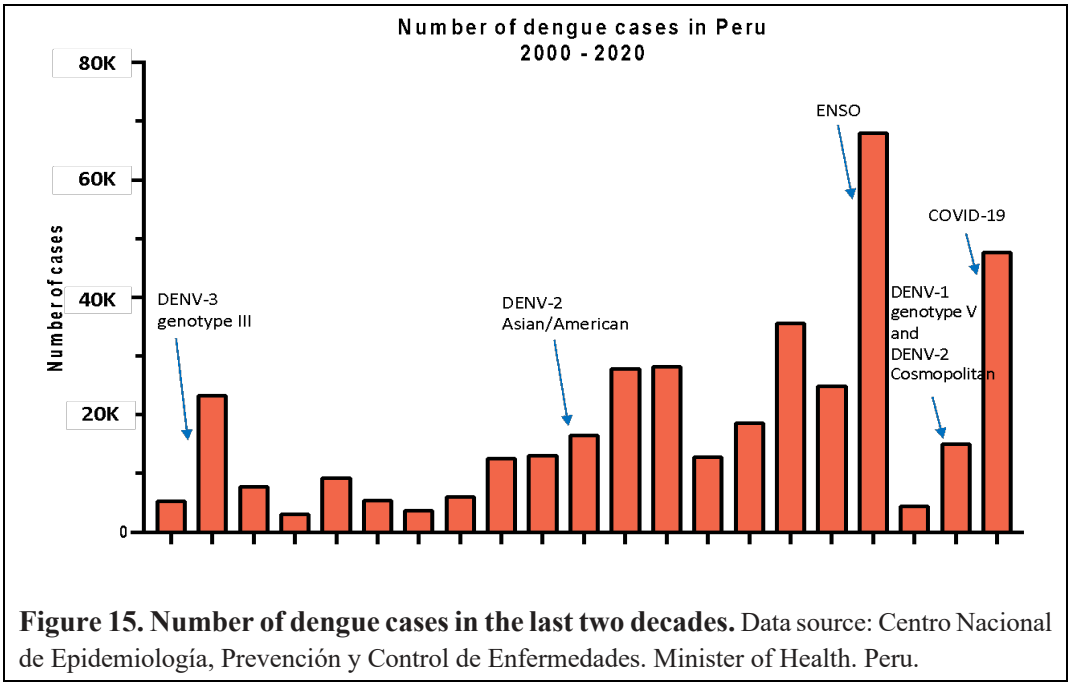


Figure 15. Number of dengue cases in the last two decades. Data source: Centro Nacional de Epidemiología, Prevención y Control de Enfermedades. Minister of Health. Peru.

In the past two decades, from 2000 to 2020, approximately 400,000 dengue cases were reported in the country, causing the death of more than 500 people. The dengue burden has scaled up through years, where the number of cases between 2011 and 2020 were approximately 2.5 times higher than during the previous decade.

The dengue burden is not evenly distributed across the Peruvian territory, with the most affected areas located in the Peruvian Amazon that holds more than 75% of the reported cases. Social determinants such as lack of water accessibility, higher poverty percentages and poor access to health services characterize these regions (Figure 16) (56).

Figure 16. Peruvian population with access to public water system 24 hours a day by department. Public water system, includes water by public system inside the house, outside the house but inside the building or by pipe for public use. Source: National Institute of Statistics and Informatics - Technical report "Peru: Forms of Access to Water and Basic Sanitation", elaborated with results of the National Survey of Budgetary Programs and National Household Survey 2019. Accessed November 2021. https://www.inei.gob.pe/media/MenuRecursivo/boletines/boletin-el-agua-y-saneamiento.pdf

The epidemiological and clinical characteristics of DENV together with the lack of efficient and reliable surveillance and notification systems make that a large proportion of infections remain undetected. In 2010, it was estimated that in the Americas the number of apparent dengue infections was around (13.3 million) seven times higher than the number reported by the WHO, while particularly for Peru it was estimated that the number of apparent infections in 2010 was 472,445 cases (95% CI, 316,552 – 677,480),

almost 25 times higher than the 19,005 cases reported by the WHO (3). The underestimation of the dengue burden has negative repercussions in the preventive and control strategies adopted by the policymakers, because unreliable assessment of resources and interventions take place.

The failure in calculating the true extent of symptomatic dengue cases occurs at two levels. First, the under-ascertainment of cases at the community level, related to the failure in capturing cases that do not seek healthcare due to problems such as limited access to primary care, costs, alternative health-care providers or milder dengue cases resolved at home. Second, the underreporting of cases at the healthcare-level, corresponding to symptomatic cases that seek healthcare but are incorrectly reported due to insufficient testing, poor deployment of diagnostic tools or misdiagnosis with symptomatically similar illnesses.

Bhatt et al., calculated that only around 30% of apparent dengue infections will seek treatment at official healthcare facilities. For these patients that do seek healthcare, it was estimated that for the Americas about 55.5% of cases are lost at the clinical level due to underreporting, while the contribution of Peru for the underreporting is one of the highest in the region, estimating that only about 13% of cases are finally reported to the PAHO (57) (Figure 17).

Besides the estimation of the true extent of dengue, the measure of the economic burden associated with dengue cases is also substantial for the adoption of control measurements by policymakers. It was calculated that the economic cost of dengue in Latin America and the Caribbean was about $1.73 billion annually. To our knowledge, studies about the economic burden of dengue in Peru are scarce, the only attempt to estimate the direct and indirect costs associated to dengue infection was in 2010, Shepard et al., calculated that cost of non-fatal cases admitted to the hospital was of US$723, while the costs of ambulatory cases were calculated in US$259. Unfortunately, the number of hospitalizations is not available for 2010, however considering a small 10% of hospitalizations, the annual cost of dengue illness in Peru for the 19,005 cases reported to WHO in 2010 was approximately US$5·8 million, while if the costs based on the estimated 472,445 cases is calculated, the economic burden of dengue in Peru would be

approximately US$144 million, which represented the 2.4% of the budget allocated to Public Health in Peru in 2010.

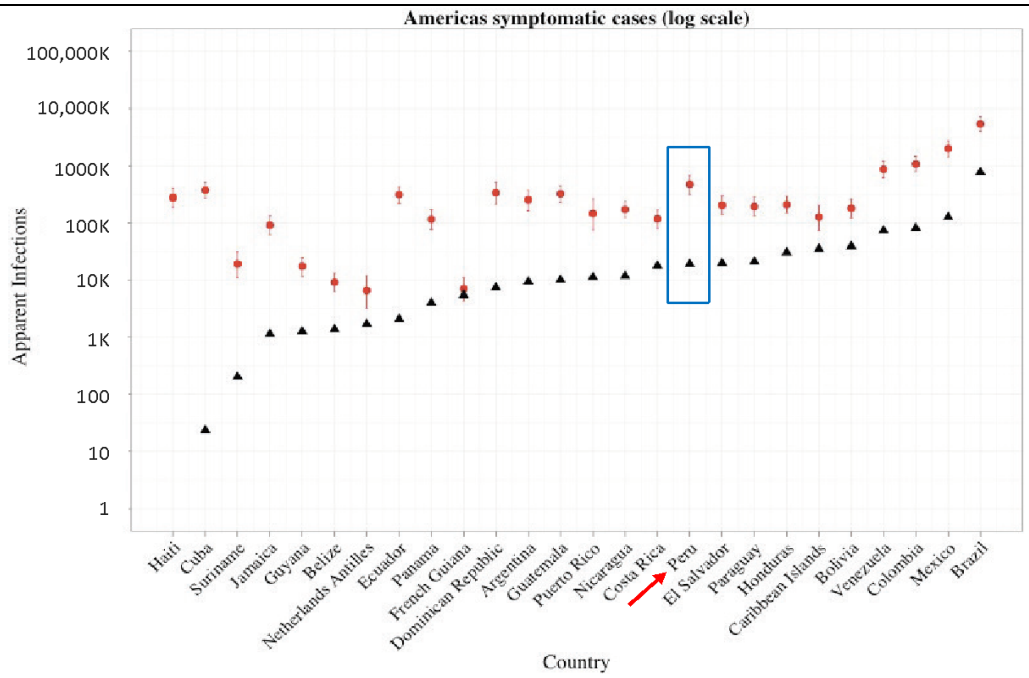


Figure 17. Number of apparent infections (red) and number of diagnosed dengue cases reported to the WHO (black) per country in the Americas. Taken from Bhatt et al., 2013.

1.1.9.2 Circulation of dengue serotypes in Peru

The four dengue serotypes have been reported in Peru, but a different serotype typically dominates each epidemic season (58). Besides, each serotype/genotype/lineage dengue shift in Peru has coincided with an increase in disease severity (48,58,59).

Therefore, the characterization of the genetic diversity of DENV is important not only to elucidate the evolutionary process of the virus, but also because viral factors have shown to have an impact on the dynamics of disease manifestation and outbreak emergence in specific populations (41,46).

As previously mentioned, there is more strong recent evidence that the complex interplay between cross-protection and ADE would represent the major forces leading to the antigenic evolution of DENV and would explain the phylogenetic distances observed between DENV serotypes.

In Peru the serotyping is performed by the NIH on a percentage of samples testing positive to dengue, this percentage was 31% in 2016, only 13.6% in 2017 and approx. 24% in 2018 and 2019. The 4-plex RT-PCR has replaced the plaque reduction neutralization test and the indirect immunofluorescence assay as the preferred method to serotype acute dengue samples. Between 2016 to 2019, DENV-2 was the main circulating serotype. Additionally, the distribution of the four dengue serotypes over the country in the last four years is shown in Figure 18A, and DENV-2 was found in all the regions reporting dengue cases. The number of samples serotyped does not necessarily correlate with the region with the highest dengue burden, which is probably explained by the capacity of the reference laboratories to perform the analysis (Figure 18B, 18C).

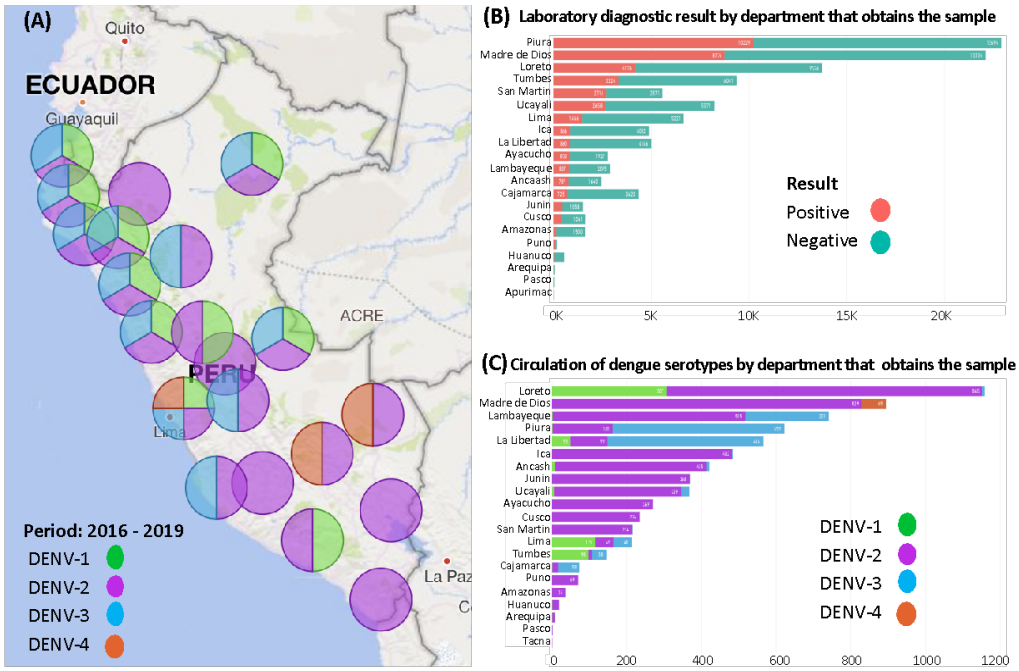


Figure 18. Dengue serotypes circulating in Peru from 2017 – 2020. (A). Cumulative number of dengue suspected cases analyzed by the reference laboratories and their results from 2017 – 2020. (B). Each horizontal bar corresponds to the region that obtains the sample for the analysis. Cumulative number of dengue confirmed samples serotyped by department that obtains the sample from 2017 – 2020. (C). Source: NET-LAB network, National Institute of Health (NIH). Accessed on November 2021. https://app.powerbi.com/

Information about the genotypes circulating in Peru is less accessible, this information is not available in the NetLab platform. Although there is a genomic surveillance network

for dengue called “ViGenDA” promoted by PAHO since 2014, the genotyping information is not open access. We can deduce that in Peru genotyping is performed mostly for research purposes, rather than as a routine procedure for molecular epidemiological surveillance of dengue by the reference laboratories. The information on the introduction of novel genotypes is only available through released reports or conferences, but unfortunately, the information is very limited.

Consequently, it is imperative to carry out genetic surveillance studies which in conjunction with antigenic maps could be used to quantify the extent to which emerging DENV strains could evade homotypic immunity in a specific population and thus trying to predict future epidemics associated with disease severity (60).

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1.2 A new genetic variant of dengue serotype 2 virus circulating in the Peruvian Amazon

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1.2.1 Highlights

- We report on a new dengue virus variant circulating in the Peruvian Amazon.
- Genotypic characterization of dengue virus is useful to monitor the distribution of circulating genotypes in endemic areas.
- Monitoring the introduction of new dengue virus strains helps to understand key drivers of dengue viral dynamics and epidemic patterns.

1.2.2 Abstract

We sequenced the envelope gene of dengue virus serotype 2 (DENV-2-E) in samples from an outbreak reported in 2018, in Yurimaguas, Peru. The strain belongs to lineage 2 of the American/Asian genotype. We report a variant with two novel mutations (I379T and V484I) located in domain III of DENV2-E.

Keywords: Dengue virus, Genotype, Lineage, Envelope protein, Domain III

1.2.3 Main text

In Peru, 80% of the population is at risk of dengue virus infection due to the spread of its main vector, *Aedes aegypti*, which is now present in 20 out of the 24 departments. The four dengue serotypes have been reported in Peru, with a different serotype typically dominating each epidemic season (Williams et al. 2014).

During October 2018 and March 2019, a DENV-2 outbreak was reported in Yurimaguas, a city located in the Loreto region of the Peruvian Amazon. Interestingly, the incidence rate during this outbreak reached around 30 per 100 000 inhabitants, three times the rate of previous years. 348 dengue cases were registered at the Hospital Santa Gema Yurimaguas (HSGY), of which 101 cases (~29%) required hospitalization. This number of hospitalizations is alarming when compared with the rates of 9% and 17% registered in 2016 and 2017, respectively (Unidad de Epidemiologia y Salud Ambiental, HSGY, personal communication) (Figure 2A). We hypothesized that, during this latest outbreak, aside from environmental, demographic, and host factors, viral determinants may have contributed to the increased viral spread and pathogenesis.

We sequenced the envelope gene (1485 bp) from 30 DENV-2 cases, collected between August 2018 and July 2019 in HSGY. Four additional samples collected in April 2018 in Iquitos, a city also located in the Loreto region, 397 km away from Yurimaguas, were also included. Sequences were aligned and edited with Codon Code Aligner and submitted to GenBank (accession No MT379579- MT379612).

Phylogenetic analysis based on nucleotide sequences showed that samples from the recent outbreak were clustered within the American/Asian (AM/AS) DENV-2 genotype, lineage II (Figure 1). The AM/AS lineage II displaced lineage I in Peru in 2000, and its appearance in Loreto resulted in the largest DHF epidemic in that region so far (Williams et al. 2014). Similarly, the AM/AS lineage I, displaced the American genotype in 2010, and coincided with the first reports of severe dengue in the country, with fatal cases.

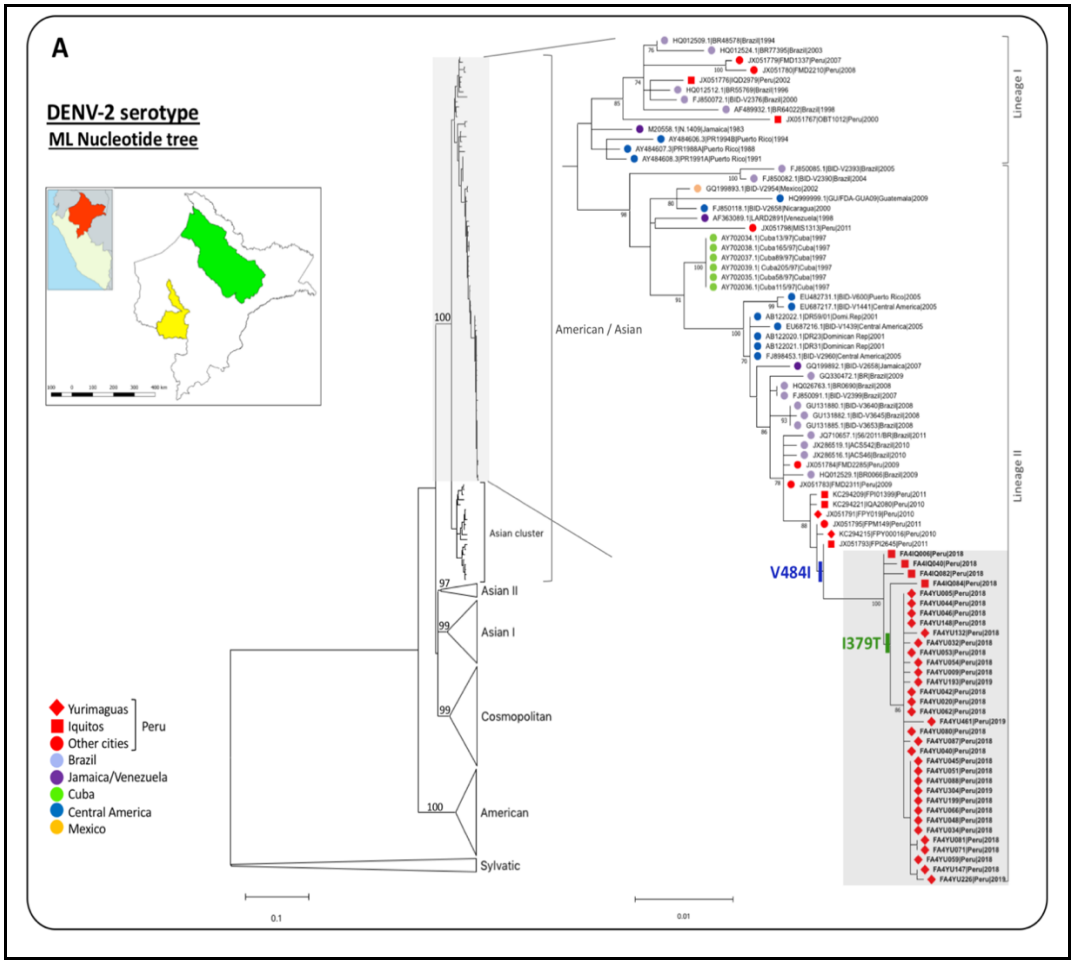


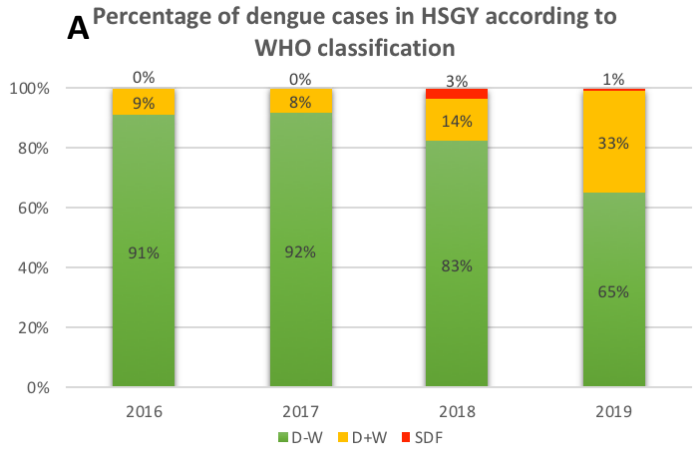
Figure 1. Phylogenetic relationship, based on the envelope gene of the Peruvian isolates, to previously characterized DENV-2 genotypes. The evolutionary relationship analysis was based on the maximum likelihood (ML) method using MEGAX and 1000 bootstrap repeat methods for statistical robustness. The best substitution model was also obtained from MEGA, based on the Bayesian information criterion (BIC). (A) Nucleotide tree. The Tamura-Nei model with gamma and invariable sites correction (TN93+G+ I) method was the model of nucleotide substitution that best fitted the data. Nucleotide (1485 bp) sequences of the DENV2-E gene from 151 reference strains, including all five genotypes – American/Asian, American, Asian I, Asian II and cosmopolitan – were retrieved from GenBank. The tree was rooted on a sylvatic genotype outgroup. Bootstrap supporting values greater than 70 are shown at the nodes. The American/Asian genotype clade containing lineages I and II is expanded. Taxa color tip markers indicate geographic location. The Peruvian DENV-2 samples sequenced in this study are in bold and highlighted in a grey box. Peruvian viruses from previous epidemics are represented with red markers. The green and blue bars in the branch of the Peruvian samples represents the amino acid changes at position I379 T and V484I, respectively. The taxon label is presented in the following format: GenBank accession number|strain name|country|year. (B) Map of the Loreto region, showing the provinces Alto Amazonas (yellow) and Maynas (green). Yurimaguas and Iquitos are the capitals of each province, respectively.

The flavivirus envelope gene is essential for viral attachment and membrane fusion (Bennett et al. 2006). It encodes the major type-specific epitopes targeted by neutralizing antibodies (NAbs) (Weaver and Vasilakis 2009; Hsieh et al. 2010; Wong et al. 2018), among which those directed against domain III (E-DIII) can be the most potent (Renner et al. 2018). Analysis of the DENV-E amino acid (AA) sequences of AM/AS strains (Fig. 2B) revealed that, aside from the single AA polymorphisms at positions 91, 129, 131, 170, 203, 340, and 380 relevant for phylogenetic classification (Bennett et al. 2006; Oliveira et al. 2010; Barcelos Figueiredo et al. 2014), viruses from the recent outbreak had a non-conservative and a conservative substitution at positions 379 and 484, respectively.

Mutation I379T is located in the β-strand F of the E-DIII region, against which the DENV 3H5, Zika ZV-67 and Zika Z006 NAbs have been reported (Dussupt et al. 2020), while mutation V484I is located in the transmembrane region of the E-protein, a highly conserved region among flaviviruses, playing an important role in the assembly of the protein (Hsieh et al. 2010).

Given that specific DENV genotypes have been associated with mild or severe disease (Bell et al. 2019), the introduction of the new genetic variant reported here might be

associated with an increase in the number of severe DENV cases currently reported in HSGY.



B

Serotype	Genotype	Lineage	Country	Strain name	Accession number	Year	City	Amino acid position respect to the envelope protein of DENV-2										
								II	II	II	I	I	II	III	III	III	TM	TM
								91	129	131	160	170	203	340	379	380	484	485
		1	Jamaica	N.1409	M20558	1983		L	V	L	K	I	D	M	I	I	V	V
DENV-2	DENV-2 American/Asian	1	Peru	OBT1012/PER/00	JX051767	2000	Piura	I	.	.	.	.	E	.	.	.	.	.
			Peru	IQD2979	JX051776	2002	Iquitos	I	.	.	E	.	E	.	.	.	.	.
			Peru	FMD1337	JX051779	2007	Madre de Dios	I	.	.	.	.	E	.	.	.	.	.
			Peru	FMD2210	JX051780	2008	Madre de Dios	I	.	.	.	.	E	.	.	.	.	.
		2	Peru	MIS1313	JX051798	2011	Tumbes	I	.	Q	.	T	.	T	.	.	.	.
			Peru	FMD2311	JX051783	2009	Madre de Dios	I	I	Q	.	T	.	T	.	V	.	.
			Peru	FMD2285	JX051784	2009	Madre de Dios	I	I	Q	.	T	.	T	.	V	.	.
			Peru	IQA 2080/10	KC294221	2010	Iquitos	I	I	Q	.	T	.	T	.	V	.	.
			Peru	FPI01399	KC294209	2011	Iquitos	I	I	Q	.	T	.	T	.	V	.	.
			Peru	FPY019	JX051791	2010	Yurimaguas	I	I	Q	.	T	.	T	.	V	.	.
			Peru	FPM149	JX051795	2011	Puerto Maldonado	I	I	Q	.	T	.	T	.	V	.	.
			Peru	FPY00016	KC294215	2010	Yurimaguas	I	I	Q	.	T	.	T	.	V	.	.
			Peru	FPI2645	JX051793	2011	Iquitos	I	I	Q	.	T	.	T	.	V	I	.
			Peru	FA4IQ|2018	--	2018	Iquitos	I	I	Q	.	T	.	T	.	V	I	.
			Peru	FA4IQ084	--	2018	Yurimaguas	I	I	Q	.	T	.	T	T	V	I	.
			Peru	FA4YU|2018	--	2018/2019	Yurimaguas	I	I	Q	.	T	.	T	T	V	I	.

Figure 2. (A) Percentage of DENV cases registered in Hospital Santa Gema Yurimaguas (HSGY). Percentages of cases are depicted in different colors according to the World Health Organization (WHO) classifications as follows: dengue without warning signs (D–W) in green, dengue with warning signs (D + W) in yellow, and severe dengue fever (SDF) in red. **(B) Amino acid sequence polymorphism of the American/Asian genotype DENV-2 envelope protein.** Partial alignment of the envelope protein shows the sites of amino acid polymorphism within the American/Asian genotype. Alignment of sequences is based on the Jamaica M20558 strain. Dots indicate residues identical to the reference strain. Isolates are arranged according to their genotype. Nucleotide alignment was carried out using ClustalX and the amino acid sequence was inferred from the nucleotide sequences using AliView v 1.26, following the standard genetic code.

Unfortunately, our study was limited by the scarcity of Peruvian sequences publicly available from 2011 onwards, as well as by our focus on only the envelope gene. Future functional studies and long-term follow-up are therefore needed to determine if these specific mutations correspond with functional antigenic changes, and to assess their possible implications for viral
fitness, as well as for the pathogenicity and dynamics of dengue disease in Yurimaguas.

1.2.4 Funding source

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1.2.5 Conflicts of interest

The authors report no conflicts of interest.

1.2.6 Ethical approval

Ethical approval was obtained from the Ethical Committee of the Universidad Peruana Cayetano Heredia (UPCH), the Research Ethics Committee (CEI) of the Hospital Regional de Lambayeque, the Institutional Review Board of the Institute of Tropical Medicine Antwerp and the Ethical Committee of the University Hospital Antwerp.

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Chapter 2: Diagnosis of dengue

Access to timely and accurate diagnostic tests that can detect DENV with high precision when and where it is needed is important not only for the clinical management of dengue cases, but they are also important for surveillance activities, outbreak control, vaccine development and clinical trials. Unfortunately, the poor deployment of laboratory-based diagnostic capacity in low-resource settings tackles the success of dengue surveillance programs, accounting as one of the major obstacles to understand the full burden of dengue (1).

The diagnosis of dengue poses a double challenge at the clinical and the laboratory level. Dengue symptomatology presents with nonspecific symptoms, especially at the early stage of the acute phase, which can be confused with the clinical manifestations of other febrile infectious diseases, many of which also overlap geographically with dengue, including those caused by parasites (Malaria), bacteria (Bartonelosis, Leptospirosis, Ricketsiosis) and viruses (Zika, chikungunya, other arboviral diseases, Covid-19, infectious mononucleosis, influenza, HIV seroconversion illness) (1,2). Therefore, because diagnosis cannot be done solely based on clinical and epidemiological data, the WHO strongly urges to confirm dengue suspected cases with laboratory techniques (1).

At the laboratory level, the decision of the method of choice for DENV diagnosis will depend on the timeframe between the onset of symptoms (normally fever) and the sampling day, and on the test available in the health center. The main methods used for the diagnosis of dengue with their advantages and disadvantages are shown in Figure 1.

To deeply address the current challenge of dengue diagnosis, with special emphasis on the available serological tests, their cross-reactivity problem and the potentiality of new tools for the discovering of better biomaterials, a review paper was written and published in Trends in Immunology (See next section 2.1).

Figure 1. Fundamental techniques employed for the confirmation of dengue virus infection. Direct detection methods target viral components such as viral proteins and genome. RT-PCR is the main technique used for viral genome detection; while ELISA and LFA are used for viral NS1 protein detection. A positive result indicates that the virus is present in the analyzed sample. Indirect detection methods are directed against anti-DENV antibodies produced by the host's immune cells after an infection event. This strategy uses whole viral lysates or recombinant viral proteins as bait to capture the host's antibodies. Due to the time needed for antibody seroconversion, a positive result means that the person was infected at some point in the past, but may not be currently infected. Antibodies can be detected by ELISAs and LFA; LFA: lateral flow

assay; ELISA: enzyme-linked immunosorbent assay; EIA: enzyme immunoassay; RT-qPCR: quantitative reverse transcription polymerase chain reaction.

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2.1 Reliable Serological Diagnostic Tests for Arboviruses: Feasible or Utopia?

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2.1.1 Abstract

Infections with arthropod-borne viruses are increasing globally as a result of climate and demographic changes, global dispersion of insect vectors, and increased air travel. The similar symptomatology of arboviral diseases and the cocirculation of different arboviruses in Africa, Asia, and South America complicate diagnosis. Despite the high sensitivity and specificity of molecular diagnostic tests, their utility is limited to the short viremic phase of arbovirus infections, and therefore the diagnosis of infection is frequently missed in clinical practice. Conversely, the duration of antibody responses provides a wider window of opportunity, making diagnosis more dependent on IgM/IgG detection. This review discusses the issues underlying the low specificity of antibody-detection assays, and addresses the challenges and strategies for discovering more specific biomarkers to enable a more accurate diagnosis.

Keywords: Diagnostics, serology, arbovirus, flavivirus, specificity

2.1.2 Highlights

Highly specific diagnostic tests for arbovirus infection are required that are easy to use, affordable, and applicable in low- and middle-income countries. When the acute phase is missed, diagnosis relies on detection of IgM, IgG, or both, in convalescent serum. This allows the diagnosis of past arboviral infections. Antigen-detection tests are useful as a first-line test in endemic areas. Currently, only a handful of validated commercial antigen-detection tests are available for dengue virus (DENV) and Zika virus (ZIKA), which suffer from sensitivity issues. Additionally, antibody detection methods lack good specificity because flaviviruses and other cocirculating pathogens share a degree of sequence and structural similarity, resulting in immunological cross reactivity. Being able to distinguish between type-specific and cross-reactive antibodies is important for clinical diagnosis (e.g., increased risk for severe disease upon secondary heterologous DENV infection, neurological complications, Zika-related developmental disorders), for serostatus determination related to DENV vaccination, and – once available – for specific antiviral treatment.

2.1.3 Outstanding Questions

What is the disease burden of arboviruses? Why is it important to differentiate between arboviruses and between recent and past infections? Are there consequences of a misdiagnosis?

What developmental strategy is required to improve serology-based clinical diagnosis/surveillance of arbovirus disease?

What is the importance of serotyping through serology? Is it important to be able to differentiate between serotype-specific and (non-neutralizing) cross-reactive antibodies? What are the knowledge gaps regarding specific antibody responses in arbovirus infections? How could resolving these gaps help to overcome antibody cross-reactivity?

To what extent are companies interested in marketing diagnostic tests for outbreak-prone infectious diseases? How willing are health authorities to use these tests for surveillance – and not only during epidemic situations, considering that they might only profit from these diagnostic tests during epidemics?

2.1.4 The Threat of (Re)emerging Arboviruses

Arthropod-borne viruses (arboviruses) are a unique, diverse, and fascinating group of viruses because they cycle between a wide variety of vertebrate hosts and arthropod species. More than 500 arboviruses have been described of which about 25% can cause human diseases [1]. Some of the most well-known arboviruses include dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV), Chikungunya virus (CHIKV), Tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), and Yellow Fever virus (YFV). This group of viruses gained significant public health importance because they inflict substantial illness, suffering, and death to human populations across the globe [2]. Together, these viruses threaten 3 billion people living in endemic areas

[3]. For dengue alone, the incidence increased nearly 400% between 1994 and 2017[i] (see Outstanding Questions).

The rapid dispersion and epidemic potential of these arboviruses is not a developing world problem alone, as developed nations are no longer spared from these diseases. This expansion is related to the increasing spread of competent insect vectors, mostly mosquitoes and ticks[ii] [4]. In the tropics, where Asia and Latin America contribute to more than 80% of the cases, the increase in the number and frequency of arbovirus outbreaks during the past decade is mainly due to demographic transformation in terms of growing populations and increased urbanization [3,5]. Conversely, in countries with a more temperate climate, the increase in international travel, from and to the tropics, and cargo transportation combined with climate change, has contributed to the expansion and adaptation of arthropod vectors to more temperate areas [3]. The gradual observation of arboviruses in these newly endemic regions shows a disconcerting trend, which started with the first major outbreak of WNV in Romania in 1996, followed by a dramatic geographic expansion into North America in 1999 [6], and a subsequent large outbreak in Greece in the summer of 2010 [7]. In Europe, ongoing WNV transmission is detected in Italy, Romania, Hungary, Spain, and the Balkans (Serbia and Greece) [7,8]. CHIKV occurred for the first time in Europe in Italy, as an outbreak with over 200 laboratory-confirmed cases in 2007 followed by an outbreak in 2017 [9]. In 2010 and 2014, CHIKV autochthonous cases were reported in the South of France [10,11], and more recently also in Spain. In contrast, DENV disappeared from Europe after the last outbreak in Greece between 1927 and 1928. Thereafter, in 2010, the first local cases of DENV were reported in France [10,12] and Croatia [10,13], and in 2012 an outbreak of sustained DENV transmission was observed in Madeira [10,14], followed by the detection of DENV in *Aedes albopictus* in Spain[iii] [15]. TBEV has occurred in Europe since 1931 and is endemic in 27 European countries, making it the most important arbovirus infection in Europe with an incidence of 10 000–15 000 clinical cases annually [16].

About 215 countries have suitable environmental conditions for the establishment of *Aedes aegypti* and/or *Ae. albopictus* mosquitoes [17]. With the presence of these vectors, a more frequent cocirculation of arboviruses, such as DENV, CHIKV, ZIKV, YFV, and other Aedes-borne viruses, may persist. In this regard, Letta et al. reported that more than

one arboviral disease was observed in 123 out of 250 countries (49.2%) [17]. This situation often leads to complications in the differential clinical diagnosis, especially in (sub)tropical areas, because the nonspecific symptoms (e.g., acute fever, rash, headache, nausea, fatigue, and malaise) are shared by different arbovirus infections and sometimes coincide with symptoms of diseases such as leptospirosis, malaria, and other (viral) diseases such as influenza [18]. Therefore, a diagnosis based on clinical symptoms alone is not feasible or is prone to error [19].

The principal measurements for the control and prevention of arboviral disease include personal protection by insect repellents and vector-control strategies. Advanced vector-control strategies with genetically modified mosquitoes and specific antiviral therapeutics are under development [5,20,21]. More than 30 potential drug candidates with an anti-arboviral activity have been reported and investigated in vitro, in animal models, or even in early clinical studies [22–24], but none of these have shown efficacy yet in large-scale clinical trials. In contrast to antiviral treatments, vaccines do exist for YFV, TBEV, JEV [20,25], and, although still somewhat controversial, also for DENV [26,27]. The first DENV vaccine (Dengvaxia, Sanofi Pasteur) was licensed in late 2015, and is nowadays approved in 19 countries, but has only been launched in 11 countries [28,29]. In 2018, Sanofi Pasteur released additional data from a Phase III clinical trial showing an increased risk of developing severe dengue in vaccine recipients without a history of DENV infection [3]. Therefore, the WHO advised that the serostatus of the vaccine recipient candidate should be known prior to vaccination and that the vaccine should be given only to individuals with a history of dengue exposure [30]. Although, confirming serostatus is currently challenging, due to the simultaneous cocirculation of other antigenically related flaviviruses in endemic areas, and false-positive results can jeopardize the intended recipients of this vaccine [30].

Although most arboviral infections are asymptomatic or self-limiting, correct diagnosis may be important because of the potential risk for complications such as ZIKV-congenital syndrome, DENV-hemorrhagic fever, and neurological manifestations [4,31]. Halstead et al. were the first to describe a mechanism that is associated with an increased risk of developing disease complications in DENV-symptomatic individuals. Antibody-dependent enhancement (ADE) is the phenomenon in which pre-existing subneutralizing

DENV antibodies (Abs) bind to the virus particles and subsequently increase infection by Fcγ receptor-mediated endocytosis [32,33]. A recent study by Katzelnick et al. on a long-term pediatric cohort in Nicaragua showed that the risk of severe dengue disease is highest within a narrow range of pre-existing anti-DENV Ab titers. Protection from symptomatic dengue disease is observed with high neutralizing Ab titers [34]. Recent reports have suggested that the presence of cross-neutralizing Abs induced against a prior ZIKV or DENV infection can prevent DENV ADE [35,36]; however, due to the lack of epidemiological studies on this matter, there is concern whether these cross-reactive non-neutralizing Abs might increase the risk for developing DENV ADE or ZIKV ADE in a secondary infection [37,38]. Accurate diagnosis is important because enhancement, and not just lack of protection, is of concern [4,19,39].

The incremental cocirculation of viruses causing clinically indistinguishable acute fever symptoms, the (re-)emergence of antigenically similar viruses (i.e., flaviviruses), the importance of dengue serostatus for vaccination, the lack of specific antiviral treatments, and the poor diagnostic armamentarium available have been extensively discussed in the scientific literature [27,39–43]. For the past 10 years diagnostic tools have been developed according to the ASSURED (affordable, sensitive, specific, user-friendly, rapid, equipment-free, delivered) criteria in order to be suitable for use in the developing world [44]. With the rapid advances in digital technology, these criteria have been revised to include real-time connectivity and ease of specimen collection (REASSURED) [44]. The updates to these criteria prompt us to begin an in-depth discussion regarding the virological /biological and technical issues underlying diagnostic developments for arboviruses, as we feel that this discussion has been missing from the existing literature. Here, we attempt to highlight the issue of cross-reactivity within current diagnostic assays and to address possible alternative strategies to find proper biomarkers that would allow for more accurate arbovirus diagnosis.

2.1.5 Properties of Currently Available Serology Tests for Arbovirus Diagnosis

The acute viremic phase of arbovirus infection is often missed, not only in patients from endemic regions where medical aid is limited or not accessible but also in returning travelers and incoming migrants as they often only visit a general practitioner when

symptoms persist or worsen beyond the febrile stage. At this stage of the disease, despite reports of the virus persisting for longer periods in sanctuary sites (i.e., the central nervous system, joints, and the urogenital tract), direct methods for the detection of the virus in the blood are no longer useful when the virus has been cleared by the immune system [45,46]. Further limitations associated with the challenges of implementing direct methods [nucleic acid amplification tests (NAATs) and viral isolation] are the costs and complexity associated with these tools, which are often compromised in resource-limited countries (Figure 1) [39,47]. Furthermore, viral antigens (Ags, e.g., DENV NS1) can be detected more easily, either by ELISA or by immunochromatographic assays, especially in low- or middle-income countries (LMICs). This is also the case for travelers returning from the tropics [48], but these tests suffer from limited sensitivity in secondary DENV infections [49]. With the exception of DENV and ZIKV, Ag detection methods are poorly commercially available for other flaviviruses and exist mainly as in-house laboratory-developed assays in specialized centers.

The detection of immunoglobulins (Igs) provides a wider window of opportunity for diagnosis because Abs remain detectable in the blood for longer periods of time (Figure 1) [50]. However, the presence of IgM and IgG should be carefully interpreted depending on the epidemiological context. During a typical primary infection with a flavivirus, IgM is usually detectable between 6 and 14 days from infection. After approximately 2 weeks of illness IgM reaches a peak and starts to decline during the following 2–3 months [51,52], although exceptional cases exist in which IgM levels persist for longer periods (e.g., DENV for 6 months [53], Zika for 12–19 months [54], TBEV for 32 months [55], and CHIKV for 1 year [56]). This persistence may complicate the clinical diagnosis on whether the case is an acute or recent infection. IgG rises, with a few days delay compared with IgM, and it can be detected for months, years, or even lifelong, depending on the virus [51]. In contrast, during a secondary infection the IgM response is often delayed, with low or even undetectable titers, while IgG rises rapidly within the first 2 days after the disease onset [52], making the diagnosis more complicated (Figure 1).

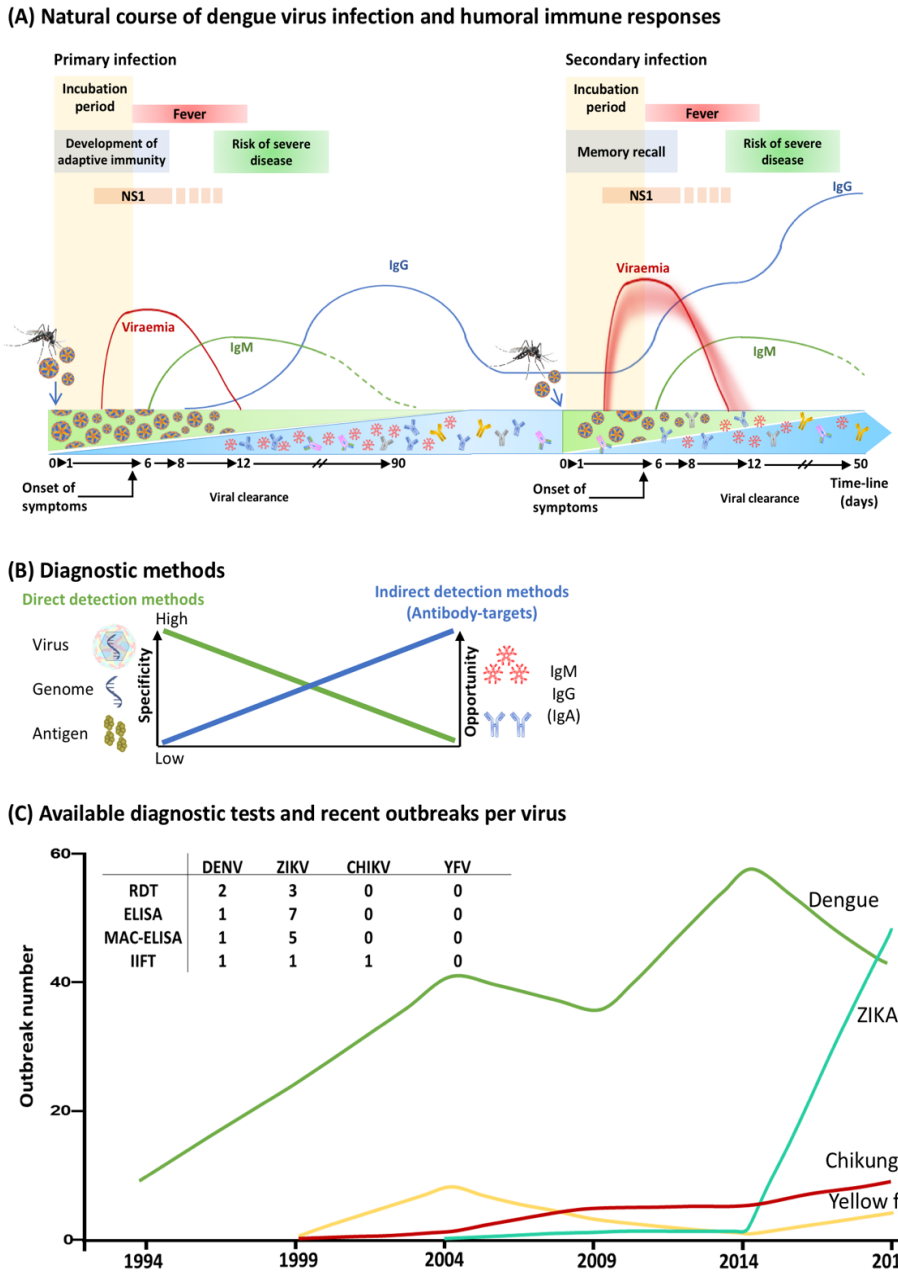

Figure 1. The Time Windows of Viremia, Antigenemia, and Antibody Responses against Arboviral Infections, and the Most Common Direct and Indirect Diagnostic Tools. (A) A typical arbovirus infection runs a biphasic course. The infection starts with an asymptomatic incubation period between 2 and 21 days, depending on the virus, followed by a relatively short viremic phase (5–7 days) which can initially cause an acute flu-like syndrome.

Typically, IgM antibodies (Abs) appear within a few days after infection and can persist for up to 6 months. Several days later IgG Abs develop, persisting for months, years, or even lifelong. (B) Laboratory diagnosis can be performed in various ways. During the acute/viremic phase, viral RNA or antigen (Ag) can be detected. In contrast to Ab detection, viral RNA detection has high specificity, whereas the test opportunity is low due to the short acute phase. The majority of arbovirus diagnostics rely on Ab detection. (C) Despite the increasing number of arbovirus outbreaks, the number of available diagnostic tests is limited to specific viruses. The diagnostic tests discussed are the ELISA, MAC-ELISA, and Indirect ImmunoFluorescent Test (IIFT)[v, vi] [105]. Abbreviation: CHIKV, Chikungunya virus; DENV, dengue virus; RDT, rapid diagnostic test; YFV, Yellow fever virus; ZIKV, Zika virus.

The types of test used for serology-based diagnosis of arboviruses are enzyme-linked immunosorbent assays (ELISAs), immunofluorescence assay (IFA), and the plaque-reduction neutralization test (PRNT). In contrast to the ELISA and IFA that capture antibodies directed to viral Ags from the wide diversity of antiviral antibodies raised in response to infection, the PRNT only looks at the subfraction of neutralizing antibodies. This test is currently considered as the gold standard [57]; however, PRNTs are expensive, labor-intensive, infrastructurally demanding and time-consuming (3–7 days per sample) [30,57–60].

A widely used serological test to diagnose the acute phases of arboviral infections is the IgM Ab capture immunoassay (MAC-ELISA). Traditionally, viral Ags used for these assays are derived from virus-infected cell culture supernatants or virus-infected suckling mouse brain preparations and thus contain a mixture of viral proteins [46,61]. From the MAC-ELISA over 50 commercial kits exist, but only one DENV and five ZIKV MAC-ELISAs are currently validated and approved by the FDA [39,62,63]. The remaining commercial kits are mainly available for DENV and ZIKV, but also for CHIKV, WNV, and JEV for which the sensitivity and specificity can vary significantly per test (Table 1, Key Table) [40,62,64]. One of the limitations of this type of assay is the high rate of false-positive results, especially in areas where flaviviruses cocirculate [61,65]. Therefore, in order to screen for possible misdiagnosis with the MAC-ELISA, there is a high need for confirmation by PRNTs [66,67].

Indirect ELISAs are also widely used for the detection of Abs to many arboviruses. So far, seven ZIKV [39,62,63] and one DENV [62] commercial ELISAs are validated and approved (Figure 1, Table 1) according to the WHO and FDA guidelines. Several studies

focused on testing and comparing a variety of ELISAs, measuring IgM and/or IgG, using either single or paired sera. Most of these tests showed a limited clinical specificity due to extensive cross-reactions between the different cocirculating flaviviruses [46,49,68].

Key table

Table 1. Approved Antibody-Detection Tests for DENV, ZIKV, and CHIKV[a]

NO°	Virus	Type of test	Molecule detected	Sensitivity/Specificity	Test name	Company	Approval	Refs
RDT								
1	Dengue	RDT	NS1, IgM, IgG	Sp: 86.8%-98.9%, Sec. Inf.: 100% ; Se: 39%-98.6%; Sec. Inf.: 52.1%	SD BIOLINE DENGUE DUO	Alere, USA	FDA EUA	[27,49,65,74]
2	ZIKA	RDT	IgM, IgG	PPA: 95.1 (83.9–98.2); NPA: 98.2 (96.7–99). No peer-review evidence	DPP® Zika IgM/IgG Assay	Chembio Diagnostic Systems Inc., USA	FDA UEA; WHO EUAL pipeline; CE-IVD; ANVISA	[39,49,106,107]
3	ZIKA	RDT	IgM, IgG	Se: 98%; Sp: 100. No peer-review evidence	STANDARD Q Zika IgM/IgG	SD Biosensor Inc., South Korea	WHO EUAL pipeline	[39,49; www.sdbiosensor.com/xe/product/2533]
4	ZIKA/ dengue	RDT	NS1, IgM	Not provided by the company. No peer-review evidence	STANDARD Q Zika/Dengue Trio	SD Biosensor Inc., South Korea	WHO EUAL pipeline	[https://idc-dx.org/resource/zika-virus-infection-diagnostics-landscape-2017/, 39]
IIFT								
1	ZIKV, CHIKV, dengue1-4	IIFT	IgM, IgG	Se: 95.7 - 98.5%; Sp: 93.4 - 99.6%	IIFT arbovirus fever mosaic 2	EUROIMMUN AG, Lübeck, Germany	CE-IVD; WHO EUAL pipeline, ANVISA	[www.eslbioscience.com/uploads/5/8/6/6/58661509/fi_2668_d_uk_a02.pdf, https://idc-dx.org; 39,64]
ELISA								
1	Dengue	ELISA	NS1	Se: 95.9% (95% C.I. 86.0–99.5%); Sp: 100.0% (95% C.I. 90.3–100%),	DENV Detect NS1 ELISA	InBios International, USA	FDA clearance for in vitro diagnostic use	[https://inbios.com/denv-detect-ns1-elisa-usa/; 107]
2	ZIKA	MAC-ELISA	IgM	Peer reviewed PPA: 83.3%–100%; NPA: 47.1%	MAC-ELISA[b]	CDC	FDA EUA	[54,63,106]

3	ZIKA	MAC-ELISA	IgM	No peer-review evidence	MAC-ELISA[c]	CDC	FDA EUA	[63]
4	ZIKA	MAC-ELISA	IgM	Peer reviewed PPA: 100%; NPA: 20–74%	Zika Detect IgM Capture ELISA	InBios International, USA	FDA EUA; WHO EUAL pipeline	[39,62,106]
5	ZIKA	MAC-ELISA	IgM	Peer reviewed PPA:85%; NPA: 56%	Liaison XL Zika Capture	DiaSorin S.p.A., Italy	FDA EUA	[39,63,106]
6	ZIKA	ELISA	IgM	Se: 49% (38–60); Sp: 99% (97–100)	Anti-ZIKA virus ELISA IgM	EUROIMMUN AG, Lübeck, Germany	CE-IVD (RUO); WHO EUAL approval; EUAL pipeline	[39,49,62,108]
7	ZIKA	ELISA	IgG	Se: 71% (61–81); Sp: 70% (61–79)	Anti-ZIKA virus ELISA IgG	EUROIMMUN AG, Lübeck, Germany	CE-IVD (RUO); WHO EUAL approval; EUAL pipeline	[39,49,62,108]
8	ZIKA	ELISA	IgM	Se: 69% (59–79); Sp: 96% (92–100).	ZIKV IgM ELISA kit	DIA.PRO Diagnostic Bioprobes Srl, Italy	EUAL pipeline	[39,49,108]
9	ZIKA	ELISA	IgG	Se: 79% (70–88); Sp: 62% (53–71).	ZIKV IgG ELISA kit	DIA.PRO Diagnostic Bioprobes Srl, Italy	EUAL pipeline	[39,49,108]
10	ZIKA	ELISA	IgM	No peer-review evidence PPA: 90.2% (87.5–94) NPA: 95.9% (91.6–98.2)	ADVIA Centaur Zika test	Siemens Healthcare Diagnostics, Berkeley, CA, USA	FDA EUA	[39, 106]
11	ZIKA	ELISA	IgM	Sp: 100% No peer-review evidence	STANDARD E Zika IgM ELISA	SD Biosensor Inc., South Korea	WHO EUAL pipeline	[www.sdbiosensor.com/xe/product/2449?category; 39,49]
12	ZIKA	ELISA	IgM	Se: 94.7%; Sp: 98.5%. No peer-review evidence	Recombi LISA Zika IgM ELISA kit	CTK biotech, USA	WHO EUAL pipeline	[https://ctkbiotech.com/product/zika-igm-elisa-test-ce/; 39]
13	ZIKA	MAC-ELISA	IgM	Se: 100%; Sp: 98.6%. No peer-review evidence	NovaLisa® ZIKV IgM µ-capture	NovaTec Immunodiagnostic GmbH, Germany	CE-IVD; EUAL pipeline	[www.novatec-id.com/products/novalisa/product/zika-virus-igmm-capture.html; 49]

[a]ANVISA, Brazilian Health Regulatory Agency; CDC, Centers for Disease Control; CE-IVD, CE-in vitro diagnostic; EUA, emergency use authorization; EUAL, emergency use authorization and listing; RUO, research use only; Se, sensitivity; Sp, specificity; PPA, positive percent agreement; NPA, negative percent agreement.

[b]Zika Vero E6 tissue culture antigen. [c]Zika COS-1 (MML), prME.

The use of recombinant flavivirus proteins, mainly the envelope (E) and NS1 proteins, as Ags in the ELISAs has demonstrated better performance compared with tests using purified viral particles [65]. The flavivirus NS1 protein is secreted from infected cells and accumulates abundantly in the serum, making it highly targeted by Abs, but it is not incorporated in viral particles [69]. The NS1-based blockade-of-binding assay (BOB-ELISA) measures the presence of serum Abs by using a ZIKV type-specific monoclonal Ab (mAb) that recognizes a conserved epitope on ZIKV NS1. This mAb competes with Abs present in the serum for the binding to the solid-phase ZIKV NS1 [70,71]. This assay was shown to be sensitive, specific, and robust [71]. The NS1 BOB assay can be used for ZIKV surveillance, seroprevalence studies, and intervention trials, and therefore, it could be useful to focus on a similar BOB approach using mAbs reacting with specific epitopes of DENV1–4 NS1 [68,71]. The E proteins are often the only viral Ags that the immune system can detect on intact virus particles and they were first used as a target for the development of neutralizing Abs [72]. Recently, ZIKA virus recombinant E protein has replaced whole viral lysates in the IgM capture ELISAs, showing a good sensitivity in accordance with the CDC MAC-ELISA; however, both assays lack good specificity [73].

In endemic settings in low- and middle-income countries, laboratory diagnostic resources are often limited. Simple rapid diagnostic tests (RDTs) provide opportunities for point-of-care (POC) diagnosis for both viral Ag and Ab capture because a result is available within 15–30 min [30]. Today, two DENV [39,74] and three ZIKV [39] RDTs approved by the FDA and WHO are available. Chembio Diagnostics, Inc. developed a POC multiplex test that simultaneously targets IgM and IgG Abs against ZIKV, DENV, and CHIKV which has the approval of Brazil's health regulatory agency ANVISA[iv]. With the cocirculation of several of these viruses, the simultaneous detection of multiple pathogens following a syndromic approach is the way forward, and with new technologies arising a higher degree of multiplexing becomes feasible (see Outstanding Questions) [75].

2.1.6 Knowledge Gaps and Challenges in Developing Next-Generation Serodiagnostics

As highlighted before, a major issue with the currently available serological tools is the lack of good specificity [46,49,67,68,71] as a result of the protein sequence identities and the structural and antigenic similarities of viruses within the families of flaviviruses and alphaviruses (Figures 2 and 3) [59,76]. The issue with the lack of specificity is mainly observed with the different flaviviruses, simply because, today, no other alphaviruses have spread geographically to a similar extent as CHIKV. However, in many regions around the globe the circumstances that could foster introduction and spread of alphaviruses, such as O'nyong-nyong virus (ONNV), Mayaro virus (MAYV), and Ross River virus (RRV), have been met.

The selection of the appropriate biomaterials to capture antiviral Abs is crucial for accurate diagnosis. In order to improve the reliability of Ab-based tests, there is a tendency to replace whole viral lysates by virus-specific fragments/biomaterials. Of the antigenic determinants (epitopes) targeted by human Abs that have been defined for members of the Flaviviridae, the majority comes from DENV, WNV, YFV, and JEV, with the bulk of data (90%) describing DENV epitopes. At the level of Ags, as expected, the envelope E protein predominates, comprising N80% of all Ab targets, followed by the nonstructural protein, NS1 (6%), more distantly by prM/M (3%), capsid (3%), and NS5 (1.8%). Little or no Ab-specific data are available for NS2A, NS2B, NS3, NS4A, and NS4B [77,78]. Nevertheless, the humoral immune response mounted against a specific virus will vary per individual, as well as be influenced by the genetic background and history of viral infections of the individual. Among members of the Alphaviridae it is known that viruses CHIKV, ONNV, MAYV, and RRV present a similar genetic structure. In humans, the dominant Ab responses appear to be directed against E2, the capsid, and nsP3 of which the E2 antigen is immunodominant across this family. Studies have shown that the highest amino acid similarity is observed between CHIKV and ONNV. Researchers found 14.1%of amino acids to be differentiating between these two viruses, whereas the difference with the other alphaviruses was in the range of 35–55% [79]. Due to the limited information available about the percentage identity of arboviruses, for this review, consensus sequences (Uniprot) of the flaviviruses DENV1-

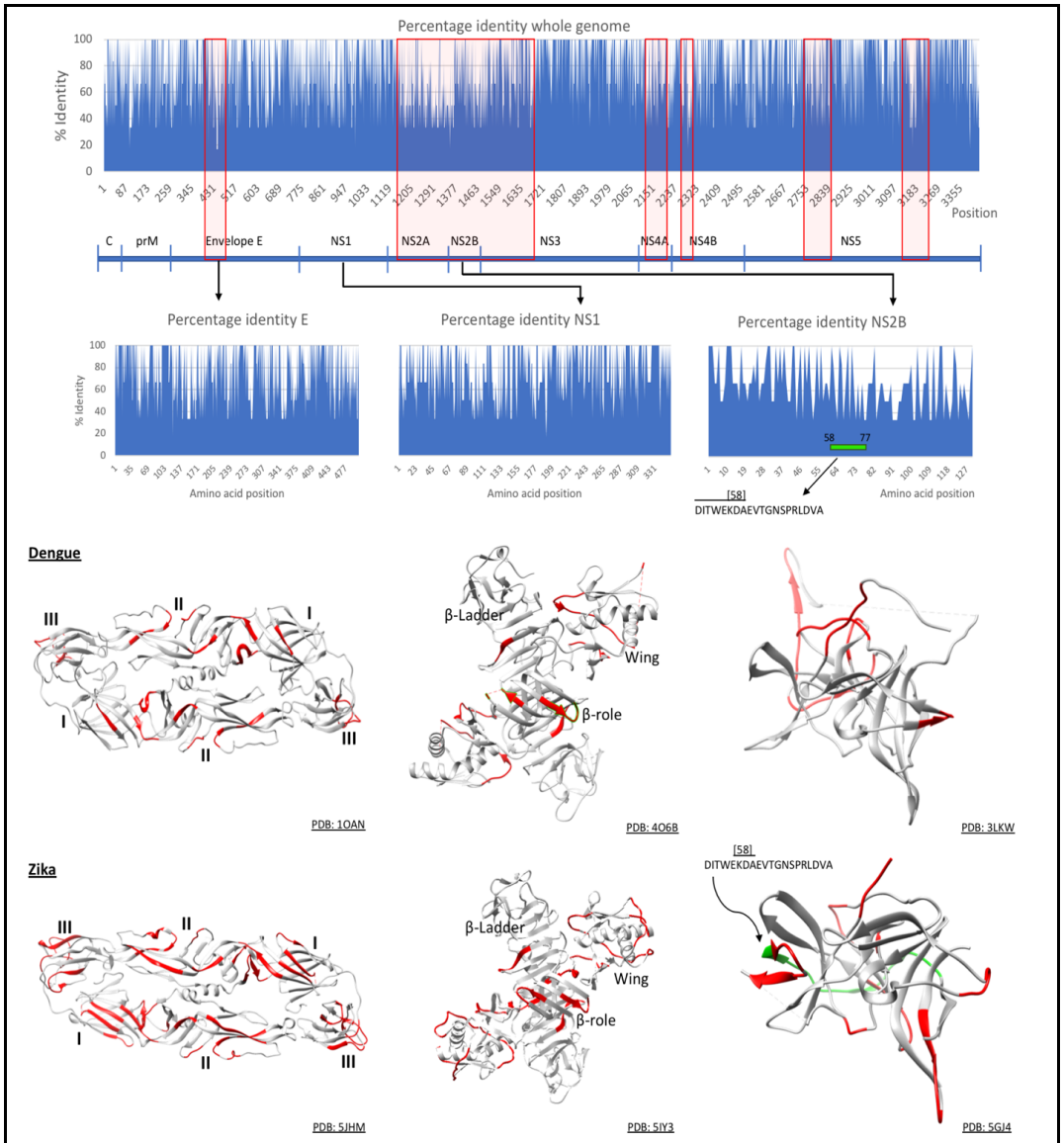

Figure 2. The Percentage Amino Acid Homology among the Flaviviruses Dengue (DENV1 to 4) and Zika. Barplots are made based on the conservation rate (percentage identity – blue bars) between the amino acid positions on the polyprotein, the envelope (E) protein, and non-structural proteins NS1 and NS2B. Red shadow areas highlight the regions where the lowest conservation was observed, which could indicate possible differentiating amino acid regions that can be used for biomarker discovery. The observed differentiating regions were then mapped on the 3D structures (red colored strains) of these viruses downloaded from the protein data bank (PDB), together with the peptides discovered with the microarray technology by Mishra et al. [58] that showed an overlap with the low-conserved regions in the NS2B protein in the barplot (red shadow area) and 3D structure PDB: 5GJ4 (green colored strain).

serotypes and the Asian and African ZIKV lineages (Figure 2), and the alphaviruses CHIKV, MAYV, ONNV, and RVV (Figure 3), were aligned in order to illustrate the percentage identity per protein. The flaviviruses shared approximately 60%amino acid sequence identity (C 55%, prM59%, E 61.4%, NS1 62.5%, NS2A 46.8%, NS2B 54.8%, NS3 67.7%, NS4A 58.4%, NS4B 64%, and NS5 67.3%), whereas the alphaviruses shared an 80% amino acid sequence identity (nsP1 86%, nsP2 84%, nsP3 65.4%, nsP4 88%, C 81.7%, E3 78%, E2 75.8%, E1 78.5%). Generally, a higher percentage identity is seen among the alphaviruses; however, there are regions of lower sequence identity that could be used for differential diagnosis for both virus families.

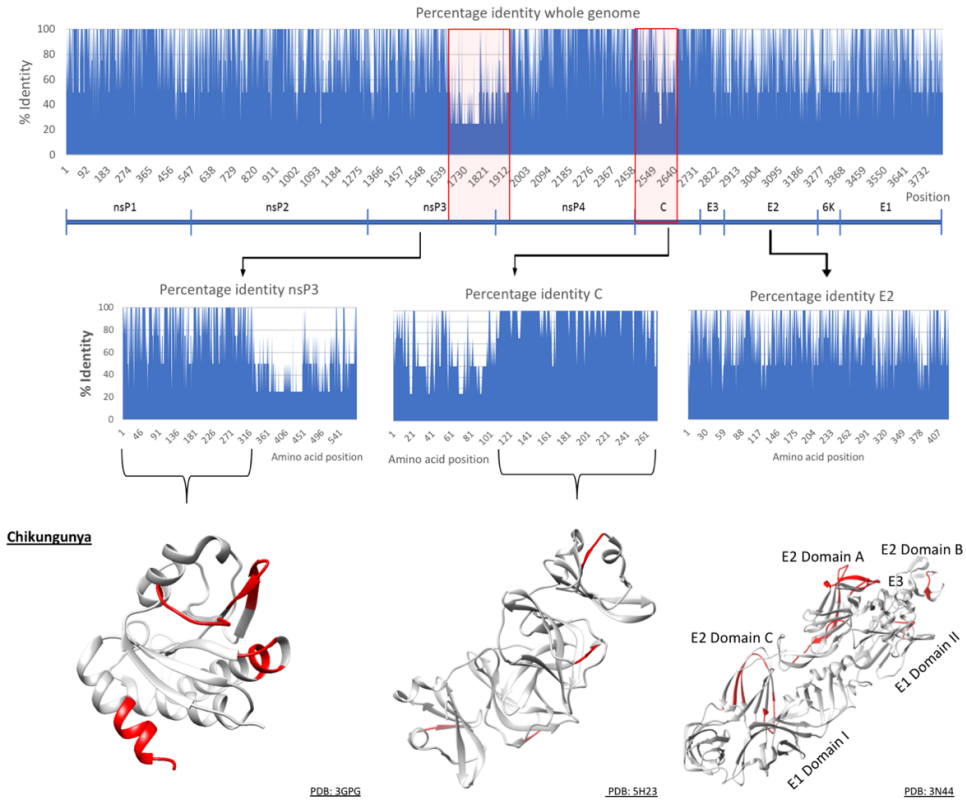

Figure 3. The Percentage Identity among Alphaviruses Chikungunya Virus (CHIKV), Mayaro Virus (MAYV), O'nyong-nyong Virus (ONNV), and Ross River Virus (RRV). Barplots are made based on the conservation rate (percentage identity – blue bars) between the amino acid positions on the polyprotein, the nonstructural protein nsP3, the capsid (C) protein, and the envelope (E) protein domain II. Red shadow areas highlight the regions where the lowest conservation was observed, which could indicate possible differentiating amino acid regions that can be used for biomarker discovery. The observed differentiating regions were then mapped on the 3D structures of these viruses downloaded from the protein data bank (PDB).

2.1.7 Technical Approaches towards More Specific Serodiagnostic Tests

New technologies are currently available for both the discovery of better protein biomarkers and the development of tests according to the REASSURED criteria mentioned earlier [44]. Because of geographic cocirculation, the ideal POC tests should be able to simultaneously detect different arboviruses [39,75]. Multipathogen tests will decrease the time to, and cost of, diagnosis and make them more fit for use in endemic settings [80] compared with the already commercially available tests (i.e., the lateral flow assay, ELISAs, or other immunoassays). These tests still pose challenges such as (i) poor specificity, (ii) lack of proper validation in areas were different arboviruses cocirculate, and (iii) difficulties with multiplexing. In this regard, the available recombinant proteins are good biomarkers; however, they do not completely resolve the issue of cross-reactivity (Figure 2). Therefore, other approaches are needed to identify antigenic regions at a higher amino acid resolution (Figure 4).

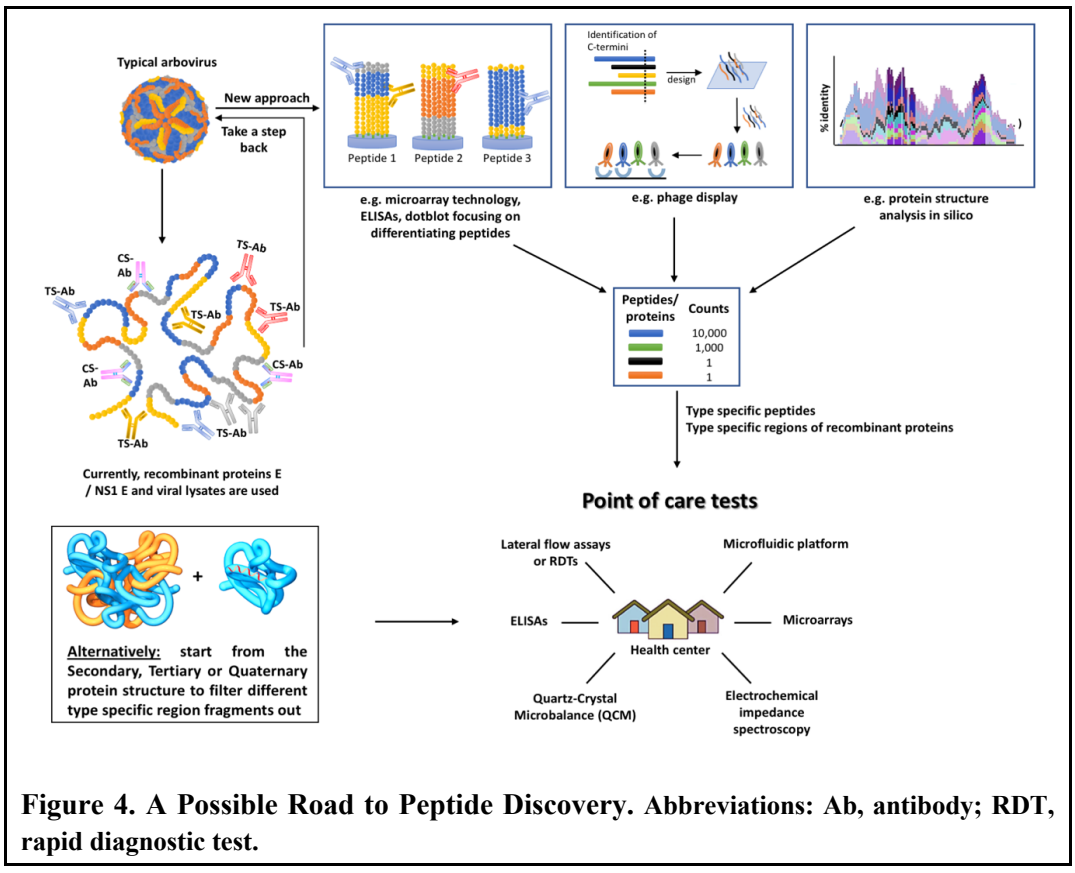

Figure 4. A Possible Road to Peptide Discovery. Abbreviations: Ab, antibody; RDT, rapid diagnostic test.

The current focus is on the identification of small protein regions that can be recognized by type-specific Abs, generally known as B cell epitope mapping. The vast majority of the neutralizing Ab epitopes found in proteins are conformational/quaternary, in which amino acids located in different regions are brought together during the protein folding process. Most reports in the literature focus on the identification of linear epitopes, which are continuous short stretches of amino acids [81,82]. Technical approaches for the identification of linear and conformational B cell epitopes can be divided into conventional 'wet' laboratory techniques, such as functional and structural methods, and computational tools using in silico analysis [83,84].

2.1.7.1 Virus Structure Studies

Conformational epitopes comprise approximately 90% of the B cell epitopes, while their identification is complicated due to the difficult isolation from the native Ag. X-ray crystallography, nuclear magnetic resonance, and cryo-electron microscopy are the technologies mainly used for obtaining high-resolution maps of Ab–Ag interactions, where the 3D structure of the epitope is retained. The use of X-ray crystallography for several flavivirus E proteins (JEV, DENV, WNV, ZIKV [85,86]) led to the identification of domain III as a potential candidate for differential serology [86]. Furthermore, Rouvinski et al. identified the epitopes located at the Edimer interface (EDE), which is the main target for broadly neutralizing Abs against dengue [87]. This approach could be extended not only to other proteins but also to other arboviruses. Unfortunately, obtaining a high-resolution structure of a protein is laborious, costly, technically difficult, and not applicable to all Ags. Hence, these structures are not available for all proteins and viruses (Table 2).

Table 2. The Availability of Protein Data Bank 3D Structures per Virus per Protein[a]

Flaviviridae		Availability of the 3D structure files per protein (Protein Data Bank)									
Group	Flavivirus	C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
Tick-borne encephalitis virus group	Langat virus			x							
	Omsk hemorrhagic fever virus			x							
	Tick-borne Encephalitis			x							
	Louping-ill virus			x							
Modoc virus group	Modoc virus										x
Yellow Fever virus group	Yellow fever			x	x			x			x
Dengue virus group	Dengue virus 1		x	x	x		x	x			
	Dengue virus 2		x	x	x	x	x	x			x
	Dengue virus 3			x			x	x			x
	Dengue virus 4		x	x			x	x			
Spondweni virus group	ZIKA virus	x	x	x	x		x	x			x
Japanese encephalitic virus group	Japanese encephalitis	x		x	x		x	x			x
	Saint-Louis encephalitis			x							
	West Nile virus	x		x	x		x	x			x
	Kunjin virus	x						x			
	Murray Valley encephalitis virus						x	x			x
	Usutu virus			x							

Togaviridae		Availability of the 3D structure files per protein (Protein Data Bank)								
Group	Alphaviruses	nsP1	nsP2	nsP3	nsP4	C	E3	E2	6K	E1
Semliki forest virus complex	Chikungunya virus		x	x		x	x	x		x
	Mayaro virus			x						
	Semliki Forest virus	x		x		x				x
Western equine encephalitis complex	Sinbis virus					x				
	Aura virus					x				
Venezuelian equine encephalitis complex	Venezuelian equine encephalitis		x	x		x				

aC, capsid protein; prM, pre-membrane protein; E, envelope protein; NS1–5. nonstructural proteins; nsP1–4, nonstructural proteins.

2.1.7.2 Computational Methods

Better understanding of immune responses has allowed computational modeling for the delineation and prediction of epitopes in pathogens. Major advances have been made for the discovery of T cell epitopes, while B cell epitope discovery is still under development due to the major complexity for prediction [83,88]. In silico analysis is extremely challenging because it predicts whether a patch in immunodominant proteins is a potential epitope that can be targeted by one or more of the many Abs potentially present in the host [86]. Many reports have focused on the use of bioinformatic tools for the prediction of epitopes that can be used as vaccine candidates for DENV [29], ZIKV [89], and CHIKV [90] as well as for the diagnosis of these and other arboviruses [29].

Using publicly available proteomic sequencing data, Chang et al. identified 294 ZIKV protein fragments [B cell (Ab) epitopes] with a low similarity compared with other flaviviruses (DENV, WNV, JEV, and YFV) and CHIKV. These fragments also showed a high conservation in ZIKV strains from other geographical regions, making them ideal candidates for use in diagnostic tests. Remarkably, 190 of these fragments are located in the NS4A protein of ZIKV [91]. Another study used the combination of comparative genomic analysis to predict diagnostic targets, together with the calculation of accessible surface analysis at the structure of these proteins and found 104 and 116 15-mer peptides in the E and NS1 proteins, respectively [92]. Further validation through functional assays of the predicted epitopes will be needed in order to determine the usefulness of bioinformatic tools for the discovery of better serological markers.

Bergamaschi et al. selected three potentially immunoreactive regions located in the DENV E protein using computational analysis combined with energetic and conformational dynamic profiling. These were further analyzed in a microarray with a panel of 20 DENV-infected individuals and 20 healthy controls. Eventually, only one linear peptide in E protein domain I (DI) was able to discriminate between both groups [93].

2.1.7.3 Functional Analysis

Individual characterization of the epitope candidate can be achieved through mutagenesis-based epitope mapping. This approach was successfully used to identify the amino acid position 106/107 located in DIII of the E protein, as a potential target for

type-specific anti-DENV and anti-ZIKV Abs [94,95], and allowed discrimination between ZIKV and DENV with an accuracy of 85% [95]. The limited number of mutant variants that can be screened simultaneously using this technique is a major disadvantage. Therefore, large-scale characterization of the viral proteome displaying high numbers of epitope candidates is needed to accelerate the discovery of serological biomarkers.

Phage display is a widely used technique that enables a high-throughput search for conformational epitopes. It was initially developed for the selection of specific Abs, although it can also be used to display complete Ags or fractions of proteins that can be recognized by Abs present in the serum. For example, libraries of the envelope glycoprotein DIII from DENV, screened with this technology, allowed the identification of a common conserved core functional epitope in DIII involving amino acid residues K310, L/I387, L389, and W391 that was recognized by two broadly neutralizing Abs, 4E11 and 4E5A [96]. A study using random peptide libraries displayed on phages identified 48 phages mimicking DENV epitopes. Further validation through binding and competitive inhibition assays, followed by the sequence analysis, revealed that two 9-mer peptides located at the NS3 and NS4B protein were the best candidates for diagnostic purposes [97]. Recently, a ZIKV whole-genome phage display library was also designed for the in-depth immune profiling of IgG and IgM Ab repertoires. Peptides derived from NS1, NS2, NS3, and NS5 showed high reactivity in acute and convalescent samples [98].

The peptide microarray technology was first developed by Geysen et al. in 1984 [99]. With this technique, short synthetic peptides are chemically synthesized and immobilized in discrete spatial locations on a solid phase, where the peptides are used as exposed probes in a parallel immunoassay format to specifically detect Abs (IgM and IgG) in serum of infected patients. Nowadays, this process is also available in a high-content peptide microarray format that enables screening of hundreds of thousands of immobilized peptides that cover entire proteomes for the identification of linear epitopes that are recognized by Abs [100,101]. This represents a robust platform for viral peptide discovery since the complete compilation of linear peptides derived from the viral proteome can be evaluated in a single assay.

The use of microarray technology was soon adapted for the identification of epitopes as potential biomarkers for diagnostic purposes in infectious diseases. This technology

shows remarkable advantages in terms of specificity, production, purity, and costs. The main limitations are seen in the complexity of the generation of libraries when many proteins are involved. The chemical synthesis process of linear peptides does not allow the inclusion of post-translational modifications, nor the study of quaternary epitopes. However, several successful studies have been conducted using the PEPSCAN technology to identify immunogens for diagnostic purposes, such as for Chagas' disease, tuberculosis, rotavirus, bovine leukemia virus, and toxoplasmosis [101–103].

Comparative epitope modelling and genome-wide peptide microarray analysis provide the opportunity to map discrete immunogenic regions within viral proteomes that can identify epitopes with sufficient specificity for differential diagnostics. Although challenging, finding conserved genomic regions and type-specific epitopes is feasible (as evidenced by Mishra et al. [58], Hansen et al. [104], and our own unpublished work). The difficulty might, however, lie in obtaining sufficiently high sensitivity with assays exploiting highly specific but perhaps less immunogenic epitopes. The epitope specificity and the abundance of antibody subsets directed against these epitopes might also differ between populations and individuals as a result of differences in genetics, epidemiological context, immunization, history of natural infection, and timing of sampling [30]. Assays combining type-specific peptides/epitopes might be required to capture as many as possible of the clinical cases. Finally, evaluation platforms and access to well characterized reference samples (such as from primo infections, people undergoing vaccination/immunization, multiple exposed individuals, and control sera from endemic regions) are urgently needed to accelerate test development and validation [41,42,49].

2.1.8 Concluding Remarks

During the past 40 years many promising approaches attempting to overcome the challenges in arboviral serodiagnostics have been developed mainly for ZIKV and DENV [41]. Due to the continuous geographical expansion of arboviruses and the growing numbers of infected people, a robust diagnostic assay could make sequential testing superfluous, and, with promising antivirals still in preclinical development, robust

diagnostic assays will become even more important once specific treatment options become available.

The primary focus should be on the discovery of high-quality biomaterials with strong discriminating potential between antigenically related viruses. Today, cross-reactivity is mostly an issue in flavivirus diagnostics, but future emergence of alphaviruses such as MAYV, ONNV, or RRV might extend this problem to the family of Alphaviridae. Emphasis should be put on multiplex testing in a syndromic approach to address the needs in areas where multiple arboviruses cocirculate. Finally, new assay development should be done in line with the REASSURED criteria and adapted for use in LMIC settings and outbreak contexts. It is equally important to carefully examine the calibration controls and appropriate screening panels, as well as the selection of well characterized reference samples for development, validation, and comparison of the performance of different assays.

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2.1.10 Resources

i http://ghdx.healthdata.org/gbd-2017
ii www.who.int/csr/don/archive/year/en/
iii www.who.int/csr/don/archive/year/2018/en/
iv http://portal.anvisa.gov.br/english
v www.who.int/csr/don/archive/year/en/
vi www.who.int/csr/don/archive/year/2018/en/

2.1.11 References

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2.2 Epidemiological surveillance of dengue in Peru

Fever is one of the major symptoms leading to health care seeking and hospital admission in Peru. Febrile surveillance in Peru is one of the strategies implemented by the Ministry of Health for the epidemiological surveillance of vector-borne diseases. In 2016, following the recommendation of the PAHO, a new technical standard was published by the Peruvian Ministry of Health aiming to strengthen epidemiological surveillance and diagnosis for the early detection of dengue, chikungunya, Zika and other emerging and re-emerging arboviruses, and at the same time to improve the monitoring of outbreaks and epidemics (1). This regulation applies to health establishments nationwide in the public and private sphere with a periodicity of daily monitoring and weekly reporting.

When a case of dengue is suspected in a healthcare facility, health workers must complete an epidemiological form where the suspected case should be classified according to the WHO guidelines as a probable case of dengue (i) without warning signs, (ii) with warning signs or (iii) severe dengue. The case is confirmed under two criteria (i) it has a positive result to one or more laboratory tests or (ii) through epidemiological link. While the case is discarded when (i) the sample taken from the patient is negative to one or more laboratory tests or (ii) another pathogen is identified as the causative agent of the febrile illness.

A case confirmed by epidemiological link applies when there is a verified dengue outbreak in the area of provenance of the patient – exposure to the bite of the vector- and when there is no sample for the laboratory.

2.2.1 Differential diagnosis at the clinical level

Dengue fever can be frequently misdiagnosed with other diseases that present with similar non-specific symptomatology, particularly early in disease progression and that are also endemic in Peru. Among the most frequent etiologies found to co-circulate with dengue in endemic regions causing undifferentiated febrile illness are *Plasmodium falciparum*, *P. vivax*, *Leptospira* sp*., Ricketsia* sp., *Bartonella* sp. and other arboviruses including Zika, Venezuelan equine encephalitis virus, yellow fever, chikungunya, mayaro, oropouche and Guaroa viruses (2).

Among the non-arboviral diseases, malaria, leptospirosis and COVID-19, are the major causes of acute undifferentiated fever in Peru (3). Before the implementation of the

malaria elimination plan “Zero Malaria Program” by the Peruvian Government in 2017, between 13% to 30% of febrile cases were caused by *Plasmodium* in malaria endemic regions, consequently, in these regions a negative thick blood smear result must be obtained in order to discard this disease (2,4). Once COVID-19, Malaria and Leptospirosis have been discarded, an investigation of possible arboviral etiology should be carried out. Unfortunately, the nonspecific symptoms and signs for most arboviral illnesses and their increasing co-circulation make it almost impossible to base the diagnosis solely on epidemiological and clinical data. Particularly dengue, zika, mayaro and chikungunya viruses tend to co-circulate, for which the term “ChikDenMaZika syndrome” has been suggested to indicate the clinical picture shared among them (5) (Figure 1)

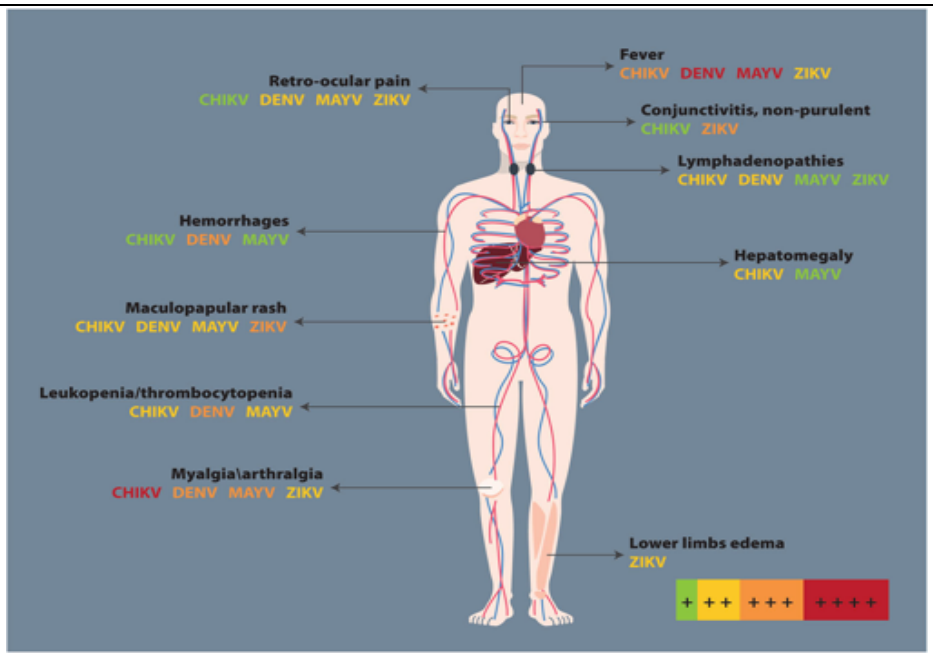

Figure 1. Clinical findings in symptomatic patients infected with chikungunya, dengue, mayaro, and Zika viruses. The figure includes a color code to indicate the symptom intensity produced by each arbovirus: red corresponds to ++++, orange to +++, yellow to ++, green to +, and the absence of the arbovirus name means no symptoms. Take from Acosta-Ampudia et al., 2018 (5)

Consequently, in light of the lack of distinct clinical presentation and the ample number of infectious etiologies, laboratory support is a critical component of effective

surveillance programs. It is estimated that in Peru approximately 30% of dengue suspected cases test positive for dengue using laboratory assays (2). This percentage tends to increase when there is an outbreak situation (e.g. 44% in the dengue epidemic in 2017) and reaches lowest valueswhen the number of cases decreases (e.g. 13% in 2018). From this, we can argue that the other 70% of cases meeting the clinical definition of suspected dengue may either correspond to false negatives due to intrinsic or extrinsic factors associated to the lab test used for the diagnosis or that the disease is caused by an infectious agent other than dengue.

Among the arboviruses that cause a clinical syndrome associated with dengue and that are reported in Peru are Zika, Venezuelan equine encephalitis virus, yellow fever, chikungunya, mayaro, oropouche and Guaroa viruses (2). Figure 2 shows the co-circulation of the most important arboviruses in Peru and that are reported by the Peruvian surveillance program. Unfortunately ruling out these arboviruses is not always possible due to the lack of resources and available tests. The NIH has an algorithm for the most prevalent arboviruses dengue, Zika and chikungunya, consisting in employing qRT-PCR assays, which due to its narrow diagnostic window can only be applicable for samples collected during the acute phase of the disease (1).

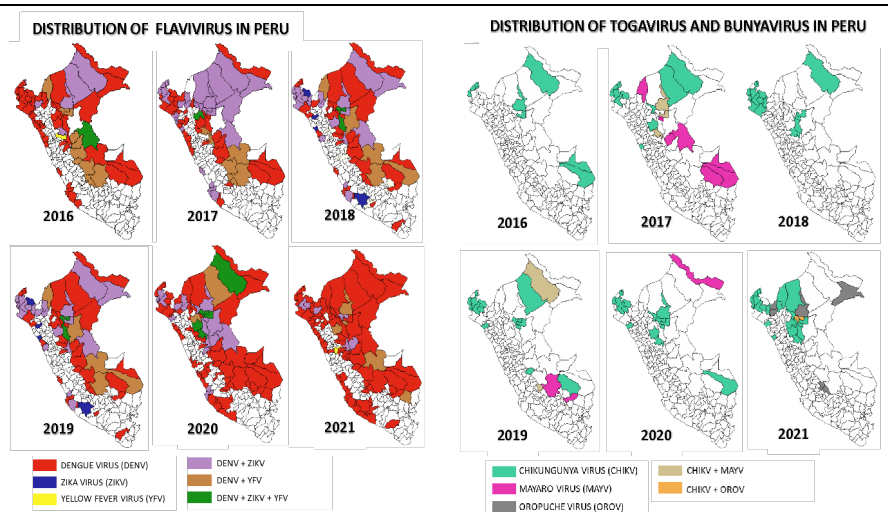

Figure 2. Distribution of arbovirus in Peru. Reports of arboviruses cases in the last six years. Figure created by Xiomara Merino using data from the Peruvian Center for Disease Control and Prevention (CDC). Data available at https://www.dge.gob.pe/salasituacional/sala/index/. Accessed in January 2022.

2.2.2 Algorithm for the diagnosis of dengue in Peru

The technical standard for arboviral diseases in Peru indicates that the recommended laboratory tests to confirm a probable case of dengue are viral isolation, real time RT-PCR, ELISA for the detection of NS1 antigen and ELISA for the detection of IgM antibodies against dengue. In the case of the ELISA IgM test, the case is confirmed either in a single sample taken from a patient that comes from an endemic area or when there is evidence of seroconversion in paired samples, where the second sample is taken more than 14 days AOS.

Peru has a network of 24 reference laboratories distributed in the different regions of the country and 1 national reference laboratory located in Lima and they operate under the supervision of the National Institute of Health (NIH). The information of the cases diagnosed by the reference laboratory network has recently being release on the Net-Lab platform implemented by the NIH, and has been updated for the years 2016 to 2019. From the available data it can be calculated that in the last two years there has been an effort to confirm dengue cases by laboratory diagnosis. Thus, the percentages went from 30% to 40% between 2016 and 2017 to approximately 67% in 2018 and 2019. The PAHO has updated data from the last two years, where the percentage of lab confirmed cases were 63% in 2020 and 74% in 2021 (Figure 3A, 3B).

Thirteen out of 24 regional reference labs have implemented the ELISA diagnostic test for the measurement of IgM and NS1, while only 4 out of the 24 are capable of performing the molecular RT-PCR test. Only the national reference laboratory has the capacity to provide all types of diagnosis, from serological, molecular, viral isolation, histopathology and plaque neutralization tests. Therefore, for those places where laboratory tests are not available, the samples need to be transported to Lima or the nearest reference lab for their respective analysis, and the diagnosis often reaches the patient late and even after the disease. This situation increases the costs associated to the diagnosis of dengue cases since samples must be transported under special temperature conditions and in many cases require air transportation (Table 1).

For those health centers lacking the necessary infrastructure and technical expertise to perform the tests at the local level, samples are shipped to the national reference laboratory, therefore results are not available in time for patient management and

frequently only provide with retrospective data for dengue statistics. The data regarding the number of samples performed in each regional reference laboratory is not available at the Net-Lab.

The poor deployment of reliable diagnostic tests in low-resource settings by the MoH has led clinicians to rely on rapid diagnostic tests (RDTs) frequently offered by private laboratories in order to have a prompt result for case management. The problem with these RDTs is that they have not been developed or tested enough resulting in varying reliability depending on the manufacturer or are not cost effective. Besides, the technical standard indicates that RDT results need confirmation with more reliable tests and therefore an additional sample from the patient should be send to a reference lab. In the dengue epidemic that occurred in 2017, RDTs were exceptionally accepted as official tests only for samples coming from Piura, the most affected region responsible for 60% of cases that year.

Table 1. Criteria for obtaining, preserving and transporting samples for the diagnosis of dengue.

Diagnostic method	Time since symptoms onset	Type of sample	Amount of sample	Shipment conditions	Time of result
ELISA NS1	< 5 days: viremic phase	Serum	2 ml	Sterile, < +8º C	3 days
qRT-PCR and Viral isolation	< 5 days: viremic phase	Serum	2 ml	Sterile, < +8º C	3 days for qRT-PCR; 30 days for viral isolation
		fatal cases, tissue: liver, brain, kidney, placent, umbilical cord	1 cm3	Sterile, < +8º C	
Histopatology or Immunohistochemistry	Fatal cases	Tissue: liver, brain, kidney, placent, umbilical cordon	1 cm3	RT fixed in 10% Neutral buffered formalin	7 days
ELISA IgM or IgG	Between 6 to 15 days	Serum	2 ml	Sterile, < +8º C	3 days

Source: "Normativa Técnica de Salud para la vigilancia epidemiológica y diagnostico de laboratorio de dengue, chikungunya, zika y otras arbovirosis". National Instiotute of Health, Ministerio de Salud, Peru. 2016. RM 982-2016 - NTS 125-MINSA/2016/CDC-INS.

The molecular test adopted by the NIH for the diagnosis of dengue is a 4-plex test that allows the simultaneous determination of the presence of genetic material of the virus and the serotype to which it belongs. For this purpose, it uses four pairs of specific

primers for each dengue serotype and 4-Taqman probes to determine the viral load present in the sample. The NIH has deployed this test to 4 regional reference laboratories using standardized and validated protocols. Unfortunately, there is no published data about the quality or performance of the adopted test or its clinical impact. The number of samples diagnosed by molecular tests has scaled-up in the last years, in 2017 only the 3.7% of positive cases were diagnosed using RT-PCR, this percentage increased to 37.3% in 2018 and dropped to 16.5% in 2019 (Figure 3C).

This decrease in the number of molecular tests performed in 2019, could be explained by the increase in the number of ELISA tests for the detection of NS1 employed by the NIH. The detection of NS1 for the diagnosis of dengue represented only 20% of the totality of tests performed in 2017, while in 2019 it represented 56% (Figure 3C). The rise in the number of these tests for the diagnosis of dengue is the result of the good diagnostic performance with sensitivities and specificities values higher than 90% (6) and because the test can be fairly easily implemented in low-resource laboratories with minimal equipment and trained personnel.

Viral isolation was the method of choice by the NIH until a few years ago mainly due to its high sensitivity and because once the virus was isolated, typing of the virus was possible through the indirect immunofluorescent assay using specific monoclonal antibodies. However, the great disadvantages are that it requires sophisticated laboratories and highly trained personnel to perform it and it is time consuming. From the network of laboratories, only the national reference laboratory located in Lima has the required infrastructure and personnel to perform this test. In the dengue epidemic of 2017, this test represented approximately 47% of the total tests performed for the diagnosis of suspected cases, while in 2019, this test only represented 3% of the total tests performed.

Regarding serology in Peru, the NIH developed a dengue IgM capture ELISA that was called Tariki-Dengue in collaboration with the US Center for Disease Control and the Pedro Kouri Institute of Cuba, this test was implemented in 2010 and could be transferred to 13 regional laboratories reference in 2011. Through an analysis carried out by the NIH with a total of 255 serum samples, the Tariki-Dengue test showed analytical sensitivity and specificity of 96% and 98%, respectively. Reports from the NIH mention that the kit

was validated in the field with clinical samples in an outbreak situation, unfortunately there is no published data available about this validation. According to the information available in recent years, the ELISA IgM test was used in approximately 27% of suspected dengue cases in 2017, while this percentage decreased to about to 20% in 2019 (Figure 3C). There is no recent information regarding the Zika epidemic, which could impact the specificity of the test, given the high genetic and antigenic similarities shared by both viruses.

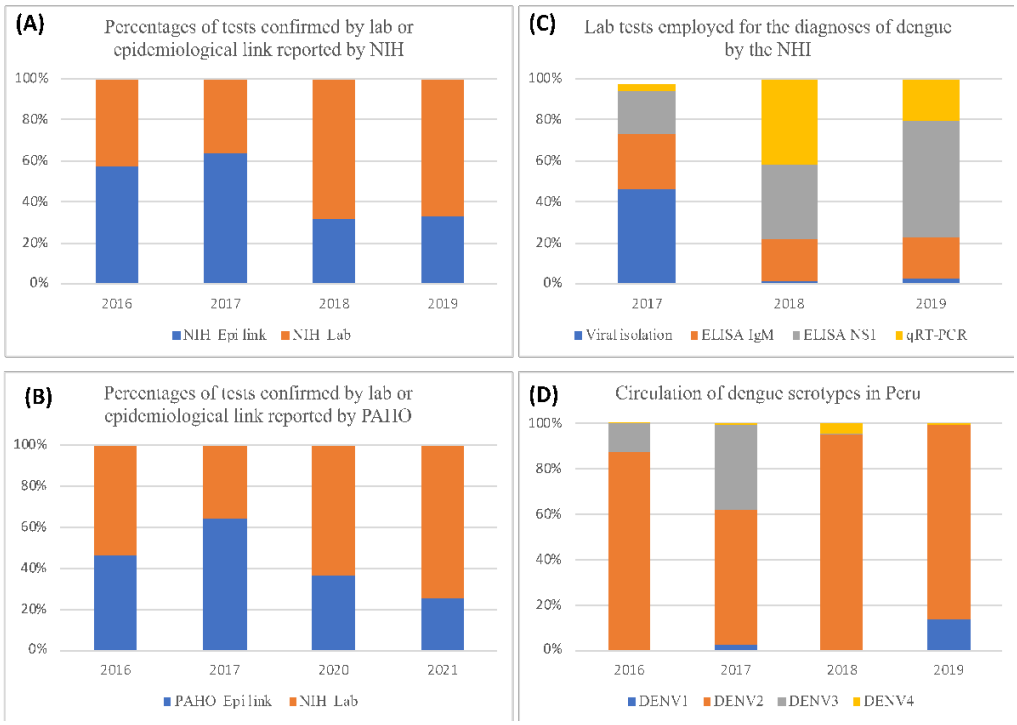

Figure 3. Percentages of dengue cases confirmed by laboratory tests or epidemiological link reported by the NIH (A) and the PAHO (B). Percentages of suspected cases performed by each of the four tests accepted by the technical standard (C). Percentage distribution of dengue serotypes among the positive samples (D). Source: NET-LAB network, National Institute of Health (NIH). Accessed on November 2021. https://app.powerbi.com/.

As previously described, dengue fever is a serious health problem in Peru. Several factors contribute to this situation, including the government's failure to implement proper public health policies to combat tropical diseases. Vulnerable populations are characterized by precarious economic conditions, limited access to drinking water, for living in difficult

to reach geographic areas, and by environmental conditions that contribute to the development of vectors. In addition, climate change has also led to the (re)-emergence and geographical expansion of other arboviruses that confound the diagnosis of dengue at the clinical and laboratory level.

Having access to better tools for detecting the virus, either through direct or indirect methods, would certainly not completely solve the dengue problem, but would help alleviate the burden of dengue in the country. Besides, the surveillance system can be improved when having access to more accurate and simpler diagnostic tools that are easier to use and implement in resource-constrained environments.

Confirmation of a suspected case of dengue through laboratory techniques would provide access to better management of clinical cases since the fatality rate of severe cases of dengue can be reduce from 20% to 1% when prompt diagnosis and proper treatment are available. In this regard, there have been an increase in the number of dengue cases confirmed by laboratories techniques in Peru in the last years, mainly due to the implementation of simpler tests such as the ELISA for the detection of the NS1 protein. This technique is more convenient for endemic areas in Peru because is easier to deploy in health centers with basic diagnostic facilities, but unfortunately its used is restricted to the viremic phase of the disease. If more accurate assays for the detection of DENV-type specific Abs are available, for instance ELISAs or RDT for the detection of IgM, it would provide a wider window for diagnosis when patients attend to the health center beyond the febrile stage.

Furthermore, the implementation of Ab-based disease surveillance methods, accompanied by genomic surveillance will aid in early detection of a potential outbreak, warning authorities from the public health system and allowing for a timely schedule to implement preparedness resources. This is especially important in countries with a fragile health system such as Peru.

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Chapter 3: Rationale, working hypothesis and objectives

3.1 Rationale

Surveillance is the foundation for disease control and prevention. The success of surveillance systems depends on the timely and accurate identification of clinical cases, but in order to count a case, it first needs to be diagnosed. It is estimated that in Peru, the percentage of dengue cases reported by the surveillance system is only 15% - 20%. Once there is access to quality screening and diagnostic tests, this will lead to major improvements in low-resource settings.

The diagnosis of dengue requires laboratory tests because the clinical features of dengue, especially in the early stages of infection, are insufficiently discriminatory from the other arboviruses of which the incidence and spread across the tropical and subtropical world has increased significantly.

Simple, affordable and high-quality antibody-based diagnostic tests that can accurately identify anti-dengue antibodies are in high demand, because molecular tests are difficult to deploy in low resource settings and have a narrow diagnostic window. The improvement of dengue serology can help alleviating the burden of the disease by (i) providing appropriate clinical management of dengue cases, (ii) determining serostatus for dengue pre-vaccination screening, because the unique dengue licensed vaccine can only be administered to people with a proven past dengue infection, and (iii) improving surveillance systems for a better support of control intervention programs based on more reliable evidence of the true dengue burden.

Unfortunately, dengue serology is hindered by low specificity, as a consequence of the high genetic and antigenic similarities between members of the *Flaviviridae* family that elicit cross-reactive antibodies upon infection. The selection of the appropriate antigens to capture specific anti-dengue antibodies in the serologic assays is crucial for accurate diagnosis. Consequently, there is a tendency to replace unspecific whole viral lysates by recombinant proteins or protein-derived oligopeptides. Two main characteristics are kept in mind when selecting such biomaterials; i.e. these must be highly immunogenic to elicit detectable levels of antibodies (determines assay sensitivity) and sufficiently virus-specific to limit binding of cross-reactive antibodies (determines assay specificity).

The use of DENV structural proteins in the form of recombinant proteins in enzyme immunoassays or lateral flow assays has partly alleviated the low-specificity problem from DENV serology tests, yet, they still display flavivirus-common motifs that can capture cross-reactive antibodies and lead to potential misinterpretations in the diagnostic setting. In addition, full-length proteins pose difficulties regarding chemical instability and batch-to-batch production reproducibility.

Short linear peptides are attractive biomarkers for infectious disease diagnostic development because of (i) their ability to display discrete epitopes allowing a fine-tuned mapping of the linear immunodominant regions of an antigen, (ii) their capacity to retain the immunoreactivity of the continuous epitope acting as molecular bioprobes to bind to the antigen-binding pocket (paratope) of the immunoglobulin, and (ii) their synthetic accessibility through solid-phase methods.

In the attempt to delineate DENV-specific epitopes at the proteome level, peptide microarrays are appealing tools because they can display simultaneously hundreds to thousands of different peptides on solid supports in a spatially discrete pattern. Therefore, the ease and speed of production make peptides ideal probes and peptide microarrays an efficient screening platform for deciphering the immunodominant regions of the dengue proteome at the epitope level.

The main goal of this PhD research is to delineate the dynamics of the antibody response against DENV at the epitope level using a high throughput flavivirus peptide microarray with the ultimate goal to identify/discover novel biomarkers for serological diagnostic purposes.

3.2 Working hypothesis

Linear epitopes represented by short overlapping peptides covering the entire proteomes of the most prevalent flaviviruses DENV, ZIKV and YFV can be used as bioprobes that maintain their immunogenicity and offer the possibility to delineate the dynamics in the humoral response against DENV and simultaneously offer an efficient platform for the discovery of virus-specific peptides for diagnostic purposes.

3.3 Objectives

Specific objective 1:

To characterize the temporal evolution of the IgG and IgM antibody responses at the epitope level in a cohort of dengue infected individuals from Peru and from overseas travelers returning to Belgium, as representatives of secondary/multiple and primary infections, respectively.

To accomplish this, a custom-designed high-density peptide microarray containing 9,072 linear epitopes spanning the entire proteome and diversity of DENV, ZIKV, YFV and the co-circulating alphavirus CHIKV was used.

Specific objective 2:

To identify DENV-specific and flavivirus immunodominant regions of antibody reactivity for diagnostic purposes.

Specific objective 3

To evaluate the diagnostic potential of synthetic peptides using a multiplex peptide immunoassay based on the xMAP Luminex technology on a large panel of sera from individuals infected with DENV, ZIKV, YFV, WNV, CHIKV and HIV or individuals that received vaccination against YFV, TBEV or JEV.

Chapter 4: Dynamics of the Magnitude, Breadth and Depth of the Antibody Response at Epitope Level Following Dengue Infection

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4.1 Abstract

Dengue is a major public health problem in tropical and sub-tropical regions worldwide. Since the Zika epidemic and the increased co-circulation of other arboviruses, the serology-based diagnosis of dengue has become more problematic due to the high antigenic resemblance, especially among the flavivirus family. Therefore, a more comprehensive understanding of the diversity, specificity and temporal evolution of the antibody response following dengue infection is needed. In order to close this knowledge gap, we used a high-density peptide microarray of 9,072 linear peptides covering the entire proteome diversity of dengue, Zika, yellow fever and chikungunya viruses. The IgM and IgG antibody responses were measured against the designed microarray in symptomatic dengue infected individuals from an arbovirus endemic area in Peru and in overseas travelers returning to Belgium, as representatives of multiple-exposed and primary infections, respectively. Serum samples were collected longitudinally across four time points over the period of six months in Peru and over two time points in travelers. We show that epitopes eliciting the strongest flavivirus cross-reactive antibodies, in both primary and secondary infections were concentrated in the capsid, E, NS1, NS3 and NS5 proteins. The IgG antibody responses against NS1 and NS3 followed a rise-and-fall pattern, with peak titers between two to four weeks after onset of illness. The response to the E and NS5 proteins increased rapidly in the acute phase and was maintained at stable levels until at least 6 months after illness. A more scattered IgM antibody reactivity across the viral proteome was observed in the acute phase of the disease and that persisted through the 6-month window. The magnitude, breadth (i.e. number of unique epitopes targeted) and depth (i.e. number of epitope variants recognized) of the IgG response was higher in secondary infections compared to primary infections. For IgM antibodies, the magnitude of the response was higher in primary infected individuals whereas the breadth and depth of the response was lower in this group compared with the endemic subjects. Finally, through this arboviral proteome-wide epitope mapping, we were able to identify IgM and IgG dengue-specific epitopes which can be useful serological markers for dengue diagnosis and serostatus determination.

Keywords: peptide microarray, dengue, antibody epitopes, antibody evolution, flavivirus.

4.2 Introduction

Arboviruses represent a large group of viruses transmitted by arthropod vectors, predominantly mosquitoes and ticks. Given their worldwide (re-)emergence in the last decade, they have gained a high priority for global public health (1). In Peru and other tropical regions around the globe, the genera Flavivirus, in particular dengue virus (DENV), Zika virus (ZIKV), and yellow fever virus (YFV), and Alphavirus, in particular chikungunya virus (CHIKV), are seasonally and geographically widely distributed (2, 3). Most of the arboviral infections are either asymptomatic or with presentation of mild symptoms including fever, rash or malaise, followed by a rapid resolution of symptoms. However, some patients develop complicated illness after the non-specific phase. These complications include: (i) hemorrhagic fever with DENV and YFV; (ii) congenital disorders associated with ZIKV infection during pregnancy; (iii) encephalitis associated with neuroinvasive viruses such as DENV, West Nile virus (WNV), Saint Louis encephalitis (SLEV); and (iv) severe arthritis following CHIKV infections (1, 4). An early diagnosis and timely management of cases are crucial for disease outcome.

Determining factors for a poor prognosis in the clinical outcome of arboviral diseases are not fully understood. However, it has been well established that pre-existing heterotypic immunity plays a critical role in dengue disease enhancement. For instance, a subsequent infection with a different DENV serotype represents the greatest risk factor for dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (4, 5). Lately, it was also found that prior ZIKV infection enhances subsequent DENV2 infection and increases disease severity (6).

Recent insights have underpinned a critical role played by antibody-dependent enhancement (ADE) as the mechanism leading to the observed higher risk to develop severe forms of disease in subsequent flavivirus infections (4, 6–8). The antigenically related flaviviruses can induce protective type-specific antibodies (TS-Abs) in naïve individuals, but also broadly flavivirus cross-reactive antibodies (CR-Abs). It is presumed that during a subsequent exposure with a different flavivirus, instead of

mounting a specific immune response, the infected individuals could elicit an anamnestic humoral response as consequence of the "original antigenic sin" (9, 10). This phenomenon occurs when the immunological memory to highly genetic and structural similar immunogens dominates the response in a subsequent infection, then rendering cross-reactive non-neutralizing Abs. Consequently, exacerbated illness occurs when these pre-existing antibodies instead of neutralizing enhance the active transportation of virus particles into antigen-presenting cells, mainly through fragment crystallizable (Fc) gamma receptors (FcgRs) leading to an increased replication of the virus that finally translates into more severe clinical manifestations of the disease.

Lately, several in vitro studies on ADE also reported that cross-reactive West Nile Virus (WNV) Abs enhance ZIKV infection (11, 12) and that ZIKV Abs increase WNV, SLEV, Rocio virus (ROCV) and Ilheus virus (ILHV) infection (13). However, larger clinical studies in flavivirus endemic human populations are needed to establish the role of pre-existing flavivirus humoral responses with either a protective or detrimental role in subsequent flavivirus infections.

This enhancement in developing severe symptoms due to pre-existing heterotypic immunity also extends to vaccines. The only licensed dengue vaccine Dengvaxia® (Sanofi) (CYDTDV), registered an excess of hospitalizations among seronegative vaccine recipients (14). The main hypothesis for the excess of hospitalized cases is that the vaccine could mimic a primary infection placing the recipient at an increased risk to develop severe dengue in a subsequent infection. In light of this evidence, the World Health Organization (WHO) only recommends its administration to dengue seropositive individuals (15).

Collectively, these studies outline the importance of an early identification of the causative agent of a suspected arboviral case and the need for discerning naïve from experienced flavivirus individuals due to their relevance in a prompt case management and for vaccination strategies (16–19).

In endemic regions the confirmation of arboviral cases relies heavily on the presence of clinical symptoms and the epidemiological context, and not on laboratory results. In the period of 2017-2020, between 20.3%-44.7% of the total dengue cases reported by the Pan-American Health Organization were confirmed by laboratory assays (20).

Unfortunately, these reports are often inaccurate due to an increasing co-circulation of flaviviruses in endemic areas and the non-discriminating symptoms developed by patients during the acute phase of the disease (1, 3).

Consequently, reliable diagnosis of arboviral infections require accurate laboratory techniques. The real time RT-PCR (reverse transcriptase) test and/or virus isolation is the most sensitive and specific (21); nevertheless, the limited diagnostic capacity in endemic areas make these tools often unavailable in primary health care centers in Low- and Middle-Income Countries (LMIC). Instead, the detection of antibodies (Abs) or abundantly expressed antigens (e.g. NS1 in DENV and ZIKV) by ELISA or rapid tests is frequently used in resource-limited settings (21, 22). However, several criteria should be taken into consideration when interpreting Ab-based assays.

Firstly, the dynamics of the humoral immune response to arboviruses varies between a primary and secondary infection and this will impact the interpretation of the test results. IgM detection has proved to be useful in diagnosing primo-infected individuals, given its rapid appearance in the acute phase of the disease. However, because IgM can persist longer than 2-3 months after the onset of symptoms (AOS), its detection can be confounded with a recent infection. Therefore, seroconversion in paired samples at different timepoints is often required. In addition, IgM levels in flavivirus-experienced patients are frequently negligible leading to false-negative results (23). IgG Abs, following a different kinetics, become detectable a few days later compared to IgM in primary infections and they can persist for life, while in secondary infections the IgG levels increase rapidly AOS and with higher titers. However, as a consequence of the "original antigenic sin" the rapid raise in IgG does not always correspond to a specific humoral response against the latest infection.

Secondly, the accuracy of serological tests depends on the nature of the antigen used in the assay. Highly similar epitopes known to elicit CR-Abs can confound diagnosis (24). However, given that the genetic and structural similarities in the Flaviviridae family varies across the genome depending on the protein region and the viruses in comparison (25), the detection of CR-Abs can be overcome if diagnostic antigens are properly chosen. Therefore, in the last years there has been an effort to replace conventional antigens (e.g. infected cell lines or fixed virus particles), by recombinant viral proteins

or protein domains [e.g. non-structural 1 (NS1), E protein, domain III of E (EDIII)]. These second-generation assays show promising results because of improved specificity (26–28), but do not completely resolve the issue of cross-reactivity (25, 29). Probably, this is because individual homologous proteins from different flaviviruses still present discrete conserved immunodominant regions with high similarity that are recognized by CR-Abs (5, 25, 30). Epitope level analysis therefore could have the potential to add several layers of information through the detailed mapping of regions responsible for eliciting type-specific (TS) or CR-Abs.

Synthetic peptide microarrays are an attractive tool to address the cross-reactivity problems seen in the current available diagnostic assays for Flaviviruses. Peptide microarrays allow a high-throughput screening of the Ab reactivities at the epitope resolution across proteins and even entire viral proteomes. When using spatially spotted microarrays with immobilized overlapping peptides, the Ab profile can be better stratified and information about the precise epitopes, that are potentially relevant for diagnosis, prognosis, and surveillance can be obtained (31–34). Moreover, the multiplexing feature of the microarray technology allows the simultaneous displaying of entire peptide libraries from different arboviruses that can be used in longitudinal studies to determine the Ab-response dynamics and the temporal evolution of TS and CR Abs. Especially in the context of co-circulation of arboviruses, the depiction of the Ab response at epitope resolution, would identify seromarker candidates for a more accurate diagnosis of the virus responsible for the most recent infection in individuals with a previous flavivirus exposure either by natural infection or by vaccination.

Here, we designed a high-density peptide microarray containing 9,072 linear epitopes spanning the entire proteome and diversity of DENV, ZIKV, YFV and the co-circulating alphavirus CHIKV. The CHIKV was included in the design due to its local transmission in the Americas, but for purposes of this study, the analysis was focused on the measurement of the Ab diversity against the flaviviruses only. Using this microarray, we characterized the temporal evolution of the IgG and IgM Ab responses in a cohort of dengue infected individuals from Peru and from overseas travelers returning to Belgium, as representatives of secondary/multiple and primary infections, respectively. Through

our analysis we were able to identify DENV-specific and flavivirus immunodominant regions of Ab reactivity.

4.3 Materials and methods

4.3.1 Study Subjects

Endemic Patients

A prospective longitudinal study was carried out between July 2018 and March 2019 in "Santa Gema Hospital (SGH)" in Yurimaguas, a city located at the Alto Amazonas province in the Peruvian Amazon. Patients with acute, undifferentiated febrile illness, with a temperature ≥ 37°C for 7 days or less, together with at least one of the following symptoms: arthralgia, myalgia, head ache or rash, aged between 5 to 65 years old, attending the SGH were included for the study, regardless of gender and ethnicity. Exclusion criteria included febrile patients admitted in the hospital with an identifiable disease (malaria or leptospirosis) or with a fever for more than seven days and individuals younger than 5 and older than 65 years of age. DENV
infection was confirmed by RT-PCR as previously described (35) and subsequently serotyped as DENV-2 using an RT-PCR protocol previously reported (36, 37) and confirmed by genome sequencing (38). PCR positive subjects were followed up during a six-months period in which three additional serum samples were obtained. Ten subjects with four follow-up samples were selected to be included in the microarray analysis. Acute samples were obtained between day 1 to 5 AOS and three convalescent samples: early, between day 10 to 28 AOS; mid, between day 65-126 AOS; and late, day 142 to 203 AOS were also obtained. Immunoreactivity was further characterized by measuring neutralizing Abs (NAbs) against the four DENV serotypes, ZIKV, and CHIKV using in house whole virus neutralization tests (39, 40), and against YFV using PRNT (Table 1). The endemic samples are representative for secondary/multiple arboviral infections based on: (i) Circulation of DENV1-4, ZIKV, CHIKV and Mayaro virus (MAYV) in Yurimaguas; (ii) YFV vaccination is recommended to persons living in the Peruvian Amazon; and (iii) detection of pre-existing NAbs against all DENV serotypes, ZIKV and YFV in most of the samples (Table 1).

Travelers

These samples were stored samples from travelers consulting the travel clinic at the Institute of Tropical Medicine Antwerp, Belgium, hosting the national reference center for arboviruses. Three patients with a dengue infection confirmed by PCR (two DENV1 and one DENV2) with two follow-up serum samples from the acute (days 5 to 8 AOS) and convalescent (days 10 to 18 AOS) phase of the disease, were selected for the microarray analysis. Samples were negative for the presence of NAbs against YFV using PRNT, besides they were collected in a period where ZIKV was not circulating in the country where the infection was acquired (Table 1).

The traveler samples are representative for primary infections since these patients claimed visiting only once an area where arboviruses are prevalent.

Negative Samples

Sixteen samples from Antwerp citizens, with no register of visiting arbovirus endemic areas, testing negative for DENV, ZIKV, YFV, tick borne encephalitis virus and CHIKV by virus neutralization tests, were pooled and included in the microarray assay as a non-endemic negative control. One serum sample from a Lima citizen with no register of visiting endemic areas or receiving the yellow fever vaccine, who tested negative to Flavivirus IFAT (Euroimmun, Lübeck, Germany) was included in the microarray assay as an endemic negative control.

4.3.2 Ethical Clearance

The study was approved by the ethical review boards of the Peruvian University Cayetano Heredia, Peru (Protocol N° 101480), the Institute of Tropical Medicine Antwerp, Belgium (Protocol N° ITG 1304/19) and the University of Antwerp, Belgium (Protocol N° 19/42/477). The study involved analysis of samples collected in the FA4 – AC4 (ITM-DGD) Framework Agreement 4 (2017–2021). Written informed consent was obtained from adults or–in case of minors–from their caretaker. This study was conducted in compliance with the ethical standards of the latest amended Declaration of Helsinki and of the International Conference Harmonization (ICH) guidelines, plus adhering to local laws and regulations.

TABLE 1| Samples included in the study.

Group	Number	Age (y)/ Sex	YFV vaccine	H	DENV serotyp	Country*	Sampling days AOS				NT90 DENV				NT90 ZIKV	NT90 CHIKV	PRNT90 YFV
							1st	2nd	3rd	4th	DENV1	DENV2	DENV3	DENV4			
Endemic	1	46/F	Y	N	DENV2	Peru	2	17	126	203	1392.9	819.81	>1600	201.84	> 1.600	< 50	< 20
	2	16/M	Y	Y	DENV2	Peru	4	16	118	196	<50	203.19	138.19	< 50	< 50	< 50	51
	3	31/F	–	Y	DENV2	Peru	0	20	65	142	246.23	451.98	527.8	220.82	81.09	< 50	50
	4	20/M	N	Y	DENV2	Peru	5	28	104	181	< 50	358.94	125.99	< 50	< 50	< 50	46
	5	28/F	Y	Y	DENV2	Peru	3	21	97	179	210.61	340.11	552.76	257.87	64.47	< 50	88
	6	43/F	Y	N	DENV2	Peru	2	19	91	171	1392.9	493.94	962.42	53.41	492.46	< 50	98
	7	17/M	N	N	DENV2	Peru	3	13	82	164	116.58	1365.6	224.02	<50	226.93	< 50	92
	8	27/M	N	N	DENV2	Peru	3	24	80	159	808.8	266.91	649.8	218.03	> 1.600	< 50	22
	9	27/M	Y	N	DENV2	Peru	2	10	78	155	< 50	129.64	54.05	<50	< 50	< 50	>640
	10	44/F	Y	N	DENV2	Peru	4	12	80	157	115.13	928.1	585.63	< 50	73	< 50	106
Traveler	1	34/F	N	N	DENV1	Thailand	2	10	--	--	–	--	–	--	–	--	<10
	2	45/M	N	N	DENV2	Cuba	6	18	--	--	–	--	–	--	–	--	<10
	3	54/M	N	N	DENV1	Thailand	8	17	--	--	–	--	–	--	–	--	<10

DENV, dengue virus; ZIKV, Zika virus; YFV, yellow fever virus; CHIKV: chikungunya virus
H, hospitalization; AOS, After onset of symptoms
Y, yes; N, no, -, no data available.
** In the traveller group it refers to the country where the infection was contracted.*

4.3.3 Bioinformatic Analysis of DENV, ZIKV, YFV and CHIKV Proteomes to Create the Peptide Library

We developed an arbovirus peptide microarray covering the proteomes of DENV-1, DENV-2, DENV-3, DENV-4, ZIKV, YFV and CHIKV to perform a precise epitope dissection of the immunodominant regions targeted by IgG and IgM Abs. DENV and ZIKV were selected based on the seasonal outbreaks of these viruses in endemic areas, while YFV was included because vaccination is recommended but not compulsory for people traveling or living in the Amazon. The broad antigenic similarities between these viruses could imply a considerable problem for differential diagnosis. As already mentioned, the CHIKV alphavirus was included in the design but its analysis is part of a larger study beyond the focus of this paper.

The peptide library was generated with the help of BISC Global, Ghent, Belgium. The Uniprot resource (https://www.uniprot.org) was used to confirm the correct length and position of the proteins from dengue (DENV1, DENV2, DENV3 and DENV4), ZIKV (Asian, African) and YFV (ECA, SA, WA) viruses. Fasta files available before September 2018 per virus, lineage and protein were retrieved from the ViPR database https://www.viprbrc.org. The retrieved data represented a wide geographical coverage including countries from Southeast Asia, East Asia, America and Africa.

The variable and conserved regions were compared among the downloaded isolates after aligning the sequences using Jalview (https://www.jalview.org). Based on the conservation degree of >75% per AA of the analysed sequences and the AA properties, one or multiple unique sequences per virus, lineage and protein were obtained with optimal global coverage. Finally, a total of 284 unique consensus sequences (135 for DENV, 28 for ZIKV, 72 for YFV and 49 for CHIKV) were retrieved from this analysis to generate the peptide library.

The unique sequences were cut into pentadecapeptides (15-mer) with a consecutive overlap of 11 residues in order to create unique short peptides. The resulting 9,023 peptides covered the proteomes from DENV, ZIKV, YFV and CHIKV completely: 8,963 were unique peptides (4,444 for DENV; 1,134 for ZIKV; 1,913 for YFV; 1,472 for CHIKV) and 60 were non-unique peptides (present in more than one flavivirus). Additionally, 49 pan-flavivirus reactive peptides identified previously using another microarray (unpublished) were also included in the peptide library. Peptides were mapped to the Uniprot reference strains for DENV (P33478), ZIKV (A0A142I5B9), YFV (P03314) and CHIKV (Non-Structural Proteins: Q5WQY5, and Structural Proteins: A0A1I7PCZ2).

The peptide library covered more than 70% of the global diversity of available sequences from DENV, ZIKV, YFV and CHIKV sequence identities available in the ViPR database. The density of peptides in the library varied across the proteome (Supplementary Figure S1). For DENV, the array included on average 7 peptide variants per each location with a maximum of 10 variants for the most variable regions in the proteome.

4.3.4 Microarray Peptide Synthesis, Immunolabeling and Pre-Processing

The peptide microarray synthesis, immunolabeling assay and image processing were done in collaboration with Schafer-N (Copenhagen, Denmark) and the AIT Austrian Institute of Technology GmbH (Vienna, Austria). A random position was assigned to each peptide on the array slide to minimize the impact of locational bias. The peptide set (interest and control peptides) was deposited in twelve identical sub-arrays per slide, enabling the analysis of 12 samples simultaneously. The 48 serum samples included in

the analysis were chemically inactivated with Triton X-100 0.1% before performing the immunolabeling. The non-specific binding sites in the slides were blocked with 0.1% Bovine Serum Albumin in PBST (blocking buffer) for 1 h at room temperature. After removing the blocking buffer, the serum, diluted 1:100 in blocking buffer, was applied and incubated on a rotator for 1 h at room temperature. Slides were then washed with blocking buffer and incubated for 1 h at room temperature with polyclonal Alexa Fluor 647 conjugated goat anti-human IgM (1 µg/ml) and Cy3 conjugated goat anti-human IgG (1 µg/ml). The slides were then washed with blocking buffer, spin dried and scanned. The images were analyzed using the PepArray program (Schafer-N). This process included the subtraction of the local background surrounding the peptide fields. The background corrected intensities were converted into arbitrary units of fluorescence intensity in 8-bit format. The data were finally transformed to 16bit for further analysis.

4.3.5 Data Processing

The array data (MFI values) was quantile normalized using the add-in tool from JMP® Pro, Version 14.0. SAS Institute Inc., Cary, NC, 1989-2019. Results from each peptide were categorized to their respective protein, lineage and virus using a custom-designed R script. Another custom designed R script was also created for the calculation of the threshold value using the expectation–maximization (E-M) algorithm, in which a bimodal curve is created per sample based on the spread of the noise- and signal distribution. The mean of the noise distribution +2SD (Standard Deviation; based on a $p < 0.01$ in order to have <1% chance to false positives) was defined as the cutoff value for a positive signal. Then, the calculated threshold was subtracted from the MFI to every peptide signal in each sample. Each value below the threshold was considered negative and changed to “0”.

4.3.6 Mapping the Magnitude, Depth and Breadth of the IgG and IgM Antibody Response to Linear Peptides

First, the number of hits, i.e., the peptides above the cut-off (IgG or IgM reactive peptides) at each time point in every sample were normalized to the number of peptides

included in the microarray per virus and per protein. Heatmaps were created based on the calculated percentages.

Second, the humoral responses against DENV, ZIKV and YFV were visualized independently by plotting the magnitude of IgG and IgM Ab binding, measured in arbitrary fluorescence intensities by peptide location (starting amino acid position). The R packages pepStat, pepDat, ggplot2, Pviz and Gviz were used to plot the magnitude of the Ab response in terms of fluorescent intensities to individual peptides, categorized by protein and amino acid start position as aligned to the proteome of the Uniprot reference strains for DENV (P33478), ZIKV (A0A142I5B9) and YFV (P03314). Individual plots per time point and per virus were generated.

Third, antibody target regions (ATR) were calculated to represent the breadth and depth of the humoral response. We first identified peptides above the cut-off (IgG or IgM reactive peptides) in at least 5 or more patients for the endemic group and 2 or 3 in the traveler group, for further analysis. If two reactive peptides shared an identical sequence of 5 or more contiguous AAs, peptides were considered as a single positive binding site and called an ATR. This approach is based on established methods to analyze Ab breadth as described in Stephenson et al. (66). Then, the breadth of the Ab response was defined as the number of non-overlapping ATRs across the polyprotein in each virus. Because ATRs are calculated based on reactive overlapping peptides, the breadth of the response should be interpreted considering not only the number of ATRs but also their width (length in terms of number of AAs). Special attention was given when comparing the breadth of Ab responses between viruses. The depth of the Ab response was evaluated based on the number of unique sequence variations per ATR (classified by serotype for DENV and lineage for DENV, ZIKV, YFV) recognized by each sample among the groups. The selected ATRs were plotted using a custom-designed R script.

Fourth, the fraction of patients (> 5 patients for the endemic group and 2 or 3 in the traveler group) that recognized a 15-mer peptide at each position across the virus polyprotein, were compared for IgG and IgM responses. Plots for each protein and for each time point were created using JMP®, Version 15. SAS Institute Inc., Cary, NC, 1989-2019. software. Finally, a longitudinal humoral response analysis based on the Ab fold change in the early-, mid- and late-convalescent samples, in respect to the acute

sample, was done per each patient included in the study. When the fluorescence intensity was "0" in the acute sample, the FI value from the convalescent samples was used for plotting. Plots for each patient were created using JMP®, Version 15. SAS Institute Inc., Cary, NC, 1989-2019. software.

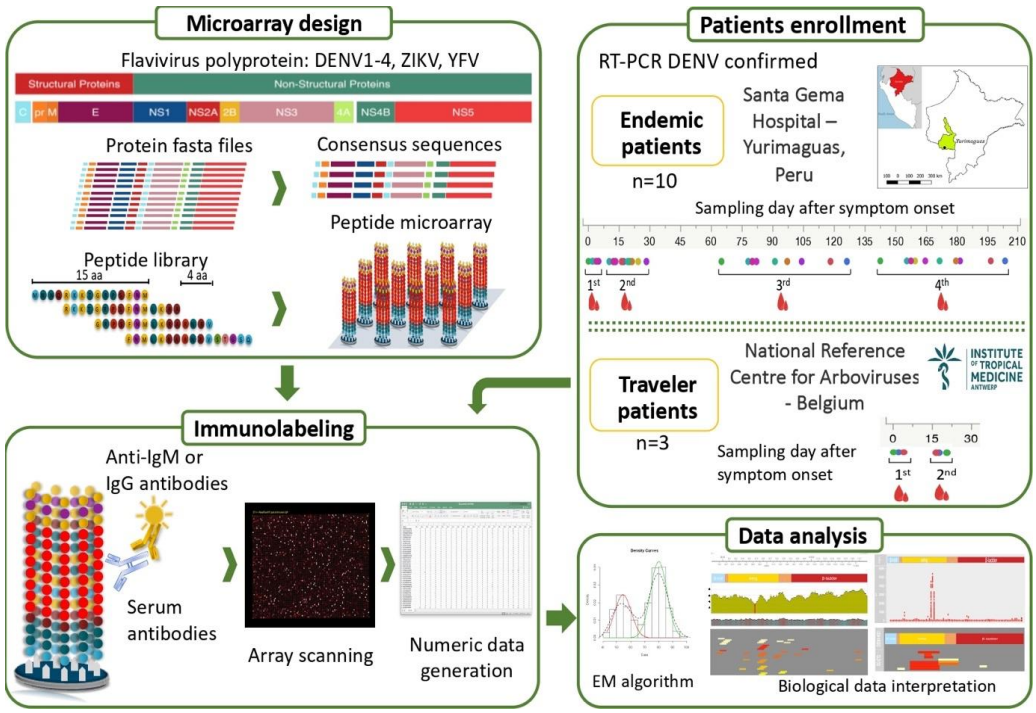

FIGURE 1 | Overview of the study design. Two groups of febrile patients: (i) endemic individuals coming from the Peruvian Amazon and (ii) Belgian returning travellers were enrolled. DENV infection was confirmed by RT-PCR. Four blood samples were collected over six months after symptom onset in the endemic group and two samples within a month after onset of symptoms in the traveler group. The bioinformatic analysis to design the peptide microarray included downloading fasta files from the ViPR database https://www.viprbrc.org, representing a wide geographical coverage of DENV, ZIKV, YFV and CHIKV. Based on the conservation degree of the analysed sequences and the AA properties, one or multiple sequences per virus, lineage and protein were obtained with optimal global coverage, which were finally used to generate the peptide library. The 284 unique sequences were broken up into 9,023 peptides, each of 15 amino acids (aa) length tiling every 4 AA across the DENV, ZIKV, YFV and CHIKV proteomes. The synthetized peptide library was mixed with sera containing antibodies that bind to their cognate epitope on the peptide. Bound antibodies were stained with either anti-IgG– or anti-IgM–secondary antibodies. Lastly, the peptide microarrays were scanned and the image files were converted to numeric data. Cut-offs were calculated per sample using the expectation maximization (EM) algorithm and the data was used for further analysis.

4.3.7 Identification of Flavivirus Cross-Reactive and DENV-Specific Peptides

We created individual alignments for each flavivirus protein using the sequences retrieved for the microarray design. The conservation rates (percentage identity) between the amino acid positions were represented in bar plots using a sliding window size of 15 amino acid positions. Conservation plots were created using Geneious Prime 2020.2.4. Based on these alignments, the DENV, ZIKV, YFV peptides were aligned onto the DENV polyprotein (Uniprot: P33478). Reactive peptides from DENV1-4, ZIKV and YFV targeted by IgG and IgM Abs from at least five out of the 10 endemic patients and two or three traveler patients were plotted together at each time-point. Individual graphs per each protein, mapping the aligned flavivirus peptides were created using a custom-designed R script.

Based on this analysis, PanFlavi (overlapping DENV and ZIKV and/or YFV peptides) and DENV-specific (DENV peptides showing non-overlapping reactivity with ZIKV or YFV peptides) regions, that were reactive at any time-point of the follow-up, were schematized.

Finally, individual heatmaps were created for the more representative selected peptides using JMP®, Version 15. SAS Institute Inc., Cary, NC, 1989-2019. software.

4.4 Results

4.4.1 Antibodies From DENV-Infected Individuals Target Epitopes in Linear Peptides From DENV, ZIKV and YFV

To measure the humoral response, we compared IgG and IgM reactivity profiles in our custom-designed microarray from a cohort of DENV2-infected individuals from an endemic area in Peru (representative for arbovirus-experienced individuals) to those from infected individuals with DENV after visiting endemic areas (Belgian travelers representative for a primary infection) (Figure 1). This comparison facilitated the identification of peptides that elicited specific and CR-Abs as well as the mapping of conserved immunodominant regions among the flaviviruses recognized by endemic and traveller individuals after a symptomatic DENV infection.

Interestingly, similar percentages of DENV, ZIKV and YFV peptides were targeted by Abs from DENV infected subjects at individual level (Figure 2A). In the endemic group,

the relative number of IgG-targeted peptides together with the onset of the Ab responses differed markedly between individuals, whereas the percentage of peptides targeted by IgM Abs was more homogeneous between subjects and constant over time. For the travelers, the percentages of IgG-targeted peptides increased from acute to convalescent samples, while for IgM the reactive peptides remained constant with low percentages in both time-points.

When analyzing the percentages of reactive peptides from each protein of the DENV polyprotein targeted by IgG Abs, the capsid (C), envelope (E), NS1, NS3 and NS5 proteins showed the highest values. This reactivity was consistent between individuals for IgG, whereas for IgM only the C protein was homogeneously and constantly targeted, while the response against the other DENV proteins differed per individual (Figure 2B). Given that all endemic patients were infected with DENV2, we would expect an increased reactivity against DENV2 peptides, however when we stratified the response into the four DENV serotypes plus those peptides with shared sequences by more than one serotype (PanDENV), no skewed response was observed towards the DENV2 serotype. When the Ab responses against ZIKV and YFV peptides were also stratified into the ten proteins, a similar preference towards the most immunogenic proteins was observed (Supplementary Figure S2).

We then plotted the absolute number of DENV peptides targeted by IgG and IgM Abs. We observed that in the endemic group, the number of IgG-targeted peptides increases from the acute to the early-convalescent samples and then declines over time, while for IgM the number remains quite constant across time (Figure 2C). Also noticeable were the high number of ZIKV and YFV peptides targeted by IgG Abs early in the acute phase of the disease by the endemic group, in contrast to the low number observed in the travelers.

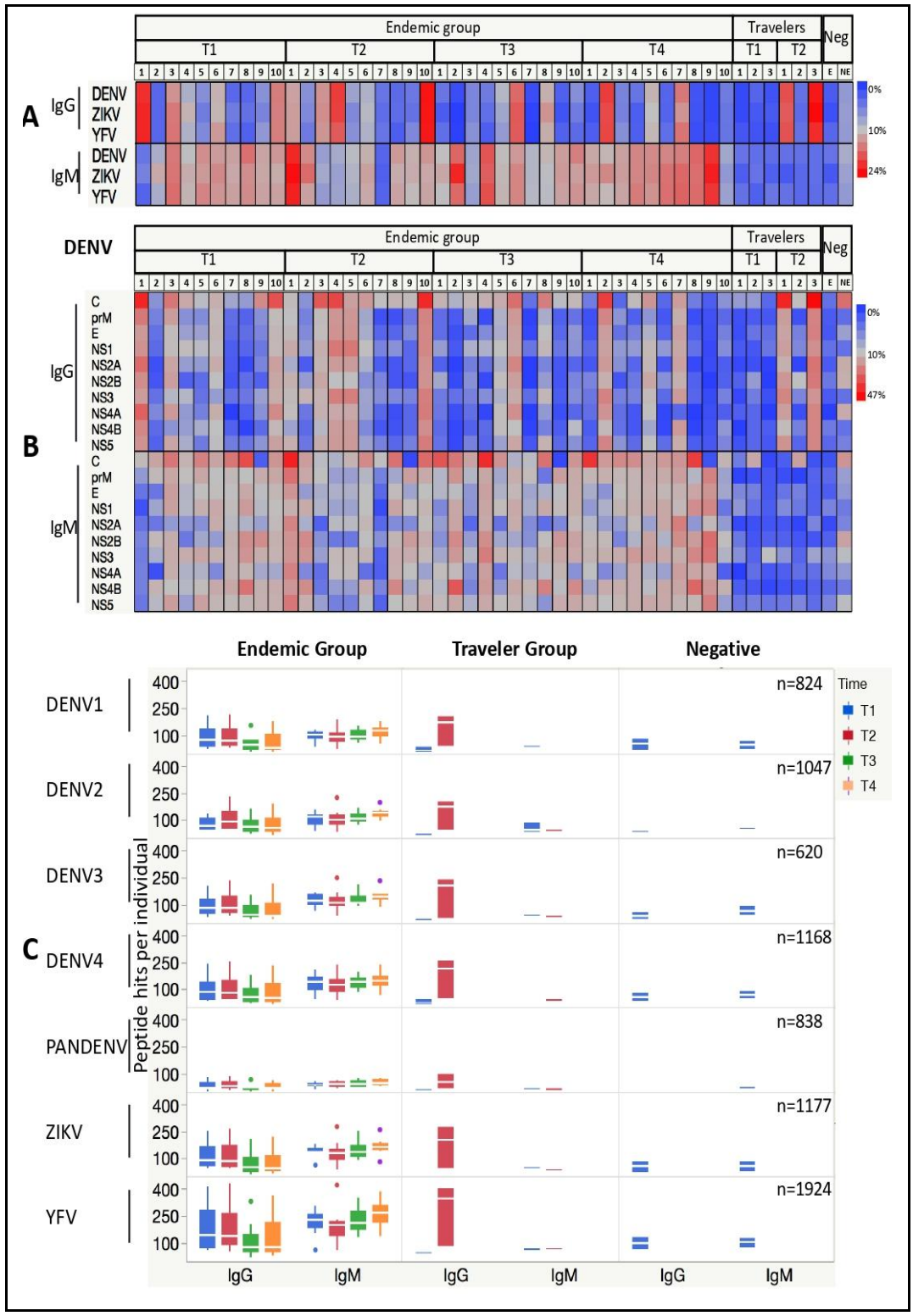
A
Endemic group
Travelers
Neg
T1
T2
T3
T4
IgG
IgM
DENV
ZIKV
YFV
0%
10%
24%
B
DENV
C
prM
E
NS1
NS2A
NS2B
NS3
NS4A
NS4B
NS5
47%
C
Endemic Group
Traveler Group
Negative
Peptide hits per individual
DENV1
DENV2
DENV3
DENV4
PANDENV
ZIKV
YFV
n=824
n=1047
n=620
n=1168
n=838
n=1177
n=1924
Time
T1
T2
T3
T4

FIGURE 2 | Antibodies from DENV-infected individuals target epitopes in linear peptides from DENV, ZIKV and YFV. (A) Heatmaps depicting the relative number of peptides targeted by IgG and IgM Abs present in the sera from endemic (n=10) and travelers (n=3) at the time samples were collected: T1 (acute), T2 (early convalescent), T3 (mid convalescent) and T4 (late convalescent). Each column represents one sample. Samples from the negative subjects are the two last columns (E: endemic, NE: non-endemic). The color intensity indicates the relative number of 15-mer peptides from the indicated arbovirus against which IgG and IgM antibodies are reactive. (B) Heatmaps (as in A) of the relative number of peptides from each DENV protein targeted by IgG and IgM Abs. Each column represents a sample, and each row represents a DENV protein. (C) Box plots illustrating the number of peptide hits targeted by IgG and IgM Abs present in the sera from DENV patients and negative samples. The box indicates the interquartile range, with the line at the median. The time points are color coded as follows: acute (blue box), early convalescent (red box), mid convalescent (green box) and late convalescent (orange box) samples. The boxes for the negatives are in blue. The numbers in the right corner in each row indicate the total number of peptides included in the microarray for each virus.

4.4.2 Humoral Responses to Linear Epitopes Covering the DENV, ZIKV and YFV Proteome

The IgG and IgM binding pattern against each peptide spanning the DENV proteome is shown in Figures 3A, B, respectively. What stands out in this figure is the different reactivity pattern seen between IgG and IgM Abs. While the IgG Ab responses were directed towards discrete regions preferentially located at the C, E, NS1, NS3 and NS5 proteins, the IgM responses were characterized by being scattered across the DENV polyprotein, making it difficult to identify regions preferentially targeted by these Abs.

IgG Abs from the endemic and traveler groups targeted regions directed towards the N-terminal of C, the DI/DII hinge region of E, the wing domain of NS1, the N-and C-terminal protease domain and DIII of NS3, and the finger and palm domains of NS5. The highest Ab signal intensities were directed towards peptides located in the wing domain of NS1. Differences between both groups were seen in the high IgG levels directed against regions located in the C (C-terminal), the E (DI and TM) and the NS3 (protease domain, the DI and DIII) in the sera from endemic samples, compared to low or absent reactivity against the same regions in the travelers. Through this visual analysis, we were not able to identify regions that are exclusively targeted by Abs in sera from primary infections.

As expected, the IgG and IgM reactivities from the traveller and endemic groups showed patterns consistent with the described humoral responses for primary and secondary infections, respectively. On one side, the traveler group showed a rapid rise of IgM Abs AOS and a gradual increase of IgG levels, while the humoral response in the endemic patients was characterized for presenting low and steady levels of IgM and a rapid increase of the IgG response with high Ab levels.

In the endemic group the peptide reactivities changed over time and differed between IgG and IgM Abs. By visual analysis, the IgG response followed a rise and fall pattern, where fluorescence intensity values peaked in the early convalescent samples, waned towards the mid convalescent sample and reached the lowest levels by month six AOS (Figure 3A). By contrast, the IgM response remained steady throughout the time interval studied (Figure 3B). Sera from the negative controls showed reactivity against some peptides in the DENV proteome, although the recognized peptides were fewer in number and lower in magnitude compared to the responses from DENV infected individuals.

Next, we analyzed the breadth and depth of the Ab responses towards the DENV proteome over time based on the calculated ATRs. In this study, the depth of the IgG and IgM responses in the travelers was lower compared to the endemic group, however, the IgG depth increased in the convalescent samples in both groups (Figure 4A), while the IgM depth remained stable (Figure 4B). The IgG depth was greater in specific regions of the polyprotein mainly in the C, E, NS1, NS3 and NS5 proteins, meaning that the IgG Abs recognized a broader sequence diversity in these immunodominant regions. For the negative controls, despite the low Ab levels observed in the intensity maps, a strikingly similar pattern in depth and breadth with respect to the DENV-infected individuals was observed. This may be explained by (i) a general background of epitopes probably targeted by natural Abs, (ii) the use of a Log2 scale for the fluorescence intensity values in the representation of the color intensities of the ATRs in Figure 3 and that (iii) calculation of the ATRs is based on a unique result: one serum sample in the endemic control and a pool of sixteen sera in the nonendemic control.

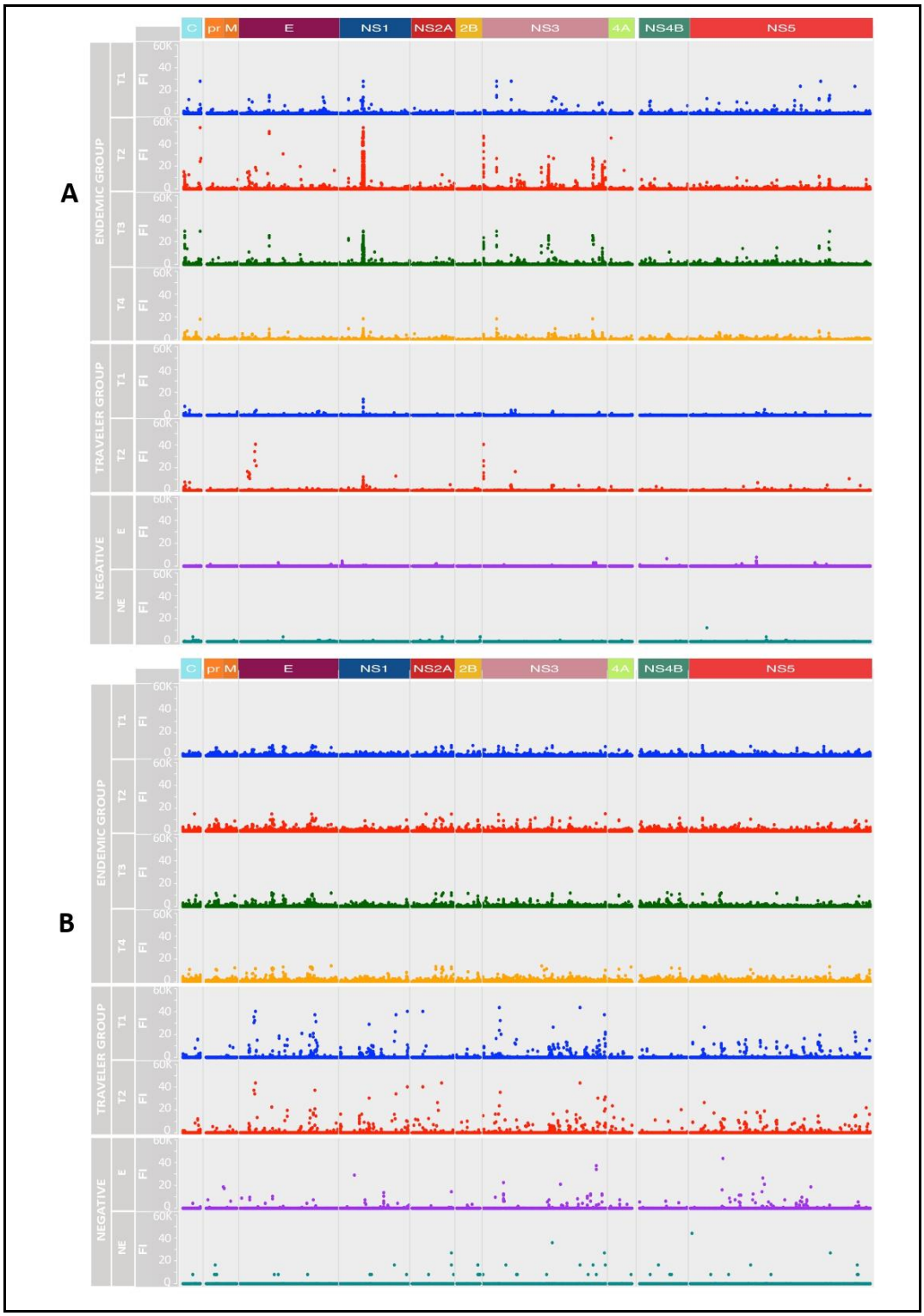
A
B
C
pr
M
E
NS1
NS2A
2B
NS3
4A
NS4B
NS5
ENDEMIC GROUP
TRAVELER GROUP
NEGATIVE
T1
T2
T3
T4
E
NE
FI
60K
40
20
0

FIGURE 3 | Longitudinal humoral responses to linear epitopes covering the DENV proteome. The plots show the level of IgG (A) and IgM (B) Ab reactivity measured in fluorescence units relative to proteomic coordinates for DENV (Uniprot ref: P33478). Structural: Capsid (C), Membrane (pr, M) and Envelope (E), and non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are color represented; each dot represents the Ab binding to a single peptide in the microarray. In both A and B plots, the endemic group is at the top (four-time points), the traveler group at the center (two-time points) and the negatives at the bottom (one-time point). T1: acute (blue dots), T2: early convalescent (red dots), T3: mid convalescent (green dots) and T4: late convalescent (orange dots) samples. Responses from the endemic (E) and non-endemic (NE) negatives are represented in purple and cyan dots, respectively.

Despite the small number of variants recognized by IgM Abs in the travelers, the magnitude of the IgM response towards these ATRs was higher compared to the response elicited by endemic patients that showed an IgM response higher in depth but lower in magnitude. In the endemic and traveler groups, the breadth of the IgG Ab response was lower in terms of ATR numbers across the polyprotein and also these ATRs were characterized for being shorter in length compared to the IgM response. This is in accordance with the findings mentioned above that the IgG Abs were directed towards discrete regions of the polyprotein, while IgM Abs targeted regions scattered across the entire polyprotein. The Ab breadth differs between groups over time, for the endemic group the breadth remained constant during the follow-up period and in the traveler group the breadth was higher in the convalescent samples compared to the acute time points.

Taken together, these results show that for the endemic group, the IgG response increased from the acute to the early convalescent time point in terms of depth and magnitude, while the breadth remained stable and, in the travelers, these three features increased over time. For the IgM response, the depth, magnitude and breadth were similar in both groups, they remained stable over the studied time points in terms of the magnitude and breadth, but the depth increased over time.

Overall, the IgG and IgM Ab responses against peptides from ZIKV and YFV in the endemic and traveler groups followed a similar pattern as for DENV, both for the targeted antigens and for the temporal evolution of Ab responses (Supplementary Figures S3). However, the major differences in the response against these viruses were found in the magnitude and breadth of the response. Thus, in the endemic group, the magnitude together with the breadth of Ab responses against ZIKV and YFV was lower compared

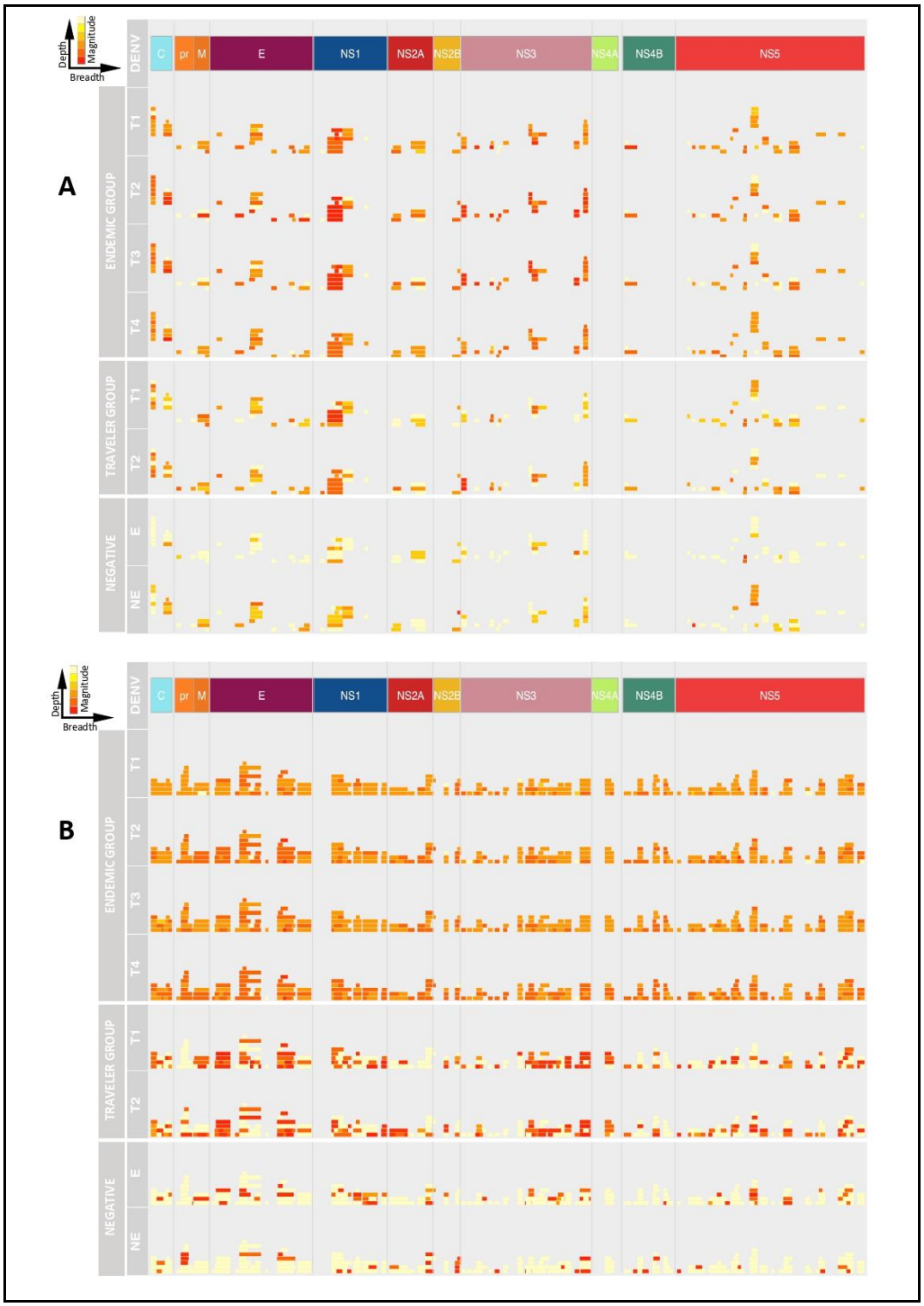
A
Depth
Magnitude
Breadth
DENV
C
pr
M
E
NS1
NS2A
NS2B
NS3
NS4A
NS4B
NS5
ENDEMIC GROUP
T1
T2
T3
T4
TRAVELER GROUP
T1
T2
NEGATIVE
E
NE
B
Depth
Magnitude
Breadth
DENV
C
pr
M
E
NS1
NS2A
NS2B
NS3
NS4A
NS4B
NS5
ENDEMIC GROUP
T1
T2
T3
T4
TRAVELER GROUP
T1
T2
NEGATIVE
E
NE

FIGURE 4 | Depth and breadth of the humoral response against DENV peptides. The plots show the Ab target regions (ATRs) for IgG (A) and IgM (B) aligned to the proteomic coordinates for DENV (Uniprot ref: P33478). Structural: Capsid (C), Membrane (pr, M) and Envelope (E), and non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are color represented. Each bar in the plot represents an ATR. The depth (different epitope variants recognized by the sera) of the Ab response is read vertically, the breadth (binding sites across the polyprotein) of the Ab response is read horizontally and the color intensity of each ATR indicates the magnitude of the Ab response, in terms of arbitrary fluorescence intensity units after Log2 transformation (ranging from 0 to 16). T1: acute, T2: early convalescent, T3: mid convalescent and T4: late convalescent samples. E: endemic and NE: non-endemic negative samples.

to the responses seen against DENV (Figure S4).

To determine if the responses against ZIKV or YFV in the endemic group was higher in those patients that have NAbs against these viruses, we divided the samples into those with presence or absence of NAbs and plotted heatmaps based on the IgG and IgM signal intensities of the ATRs. No difference was observed between the split groups (Supplementary Figure S14).

For the travelers, despite the fact that the number of ZIKV and YFV peptides targeted by IgG Abs were similar to the DENV peptides, the fluorescence intensity values were lower (Supplementary Figures S3A). This was not the case for the IgM response, of which the levels were remarkably high in this group, especially against peptides located in the E, NS1, NS3 and NS5 proteins (Supplementary Figures S3B) with similar fluorescence intensity values as the ones detected against DENV peptides, but with fewer ATRs (Supplementary Figures S4). Since individuals from the traveler group experienced a primary infection, it is likely that this response is a measure of CR-Abs elicited towards conserved flavivirus immunodominant epitopes.

4.4.3 Identification of the Most Frequently Targeted Regions Recognized by IgG and IgM Abs

Next, we compared the fraction of patients that patients that have IgG and IgM antibodies against immunodominant regions. Overall for DENV, few regions were concurrently targeted by both antibody isotypes. These regions could be identified in domain II of the

envelope (EDII), the wing domain of NS1, the protease domain of NS3 and the finger domain of NS5.

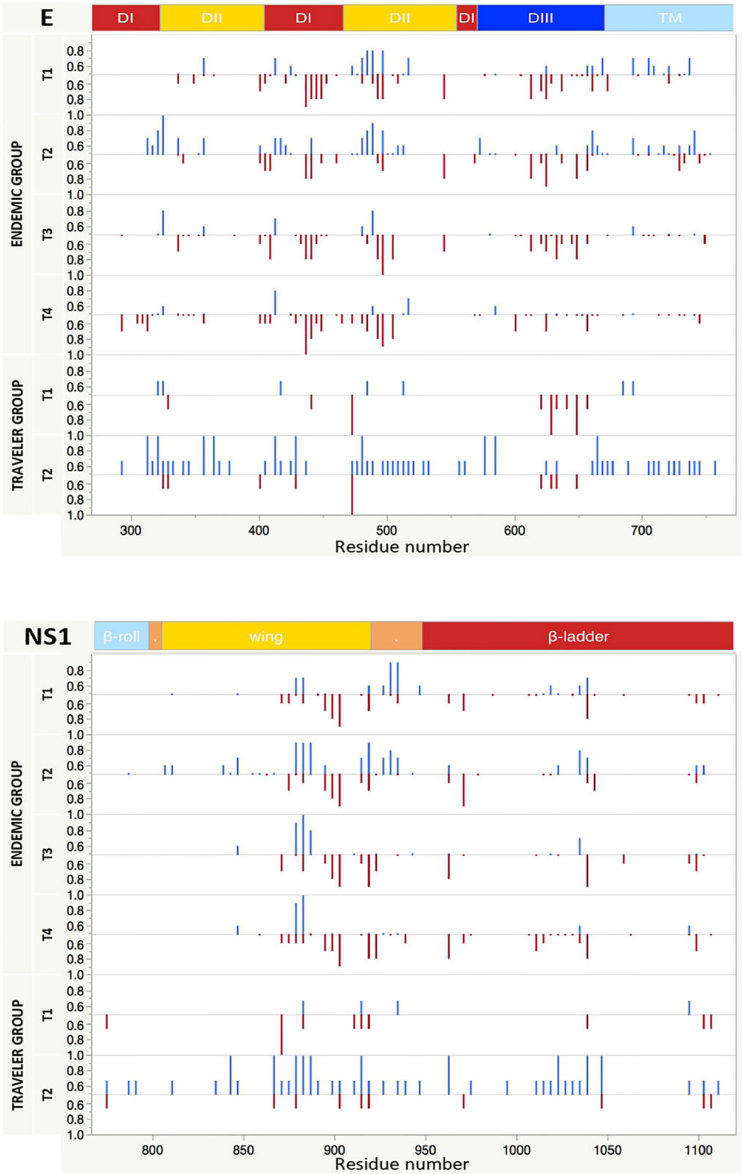

FIGURE 5 | Longitudinal IgG and IgM reactivity against E and NS1 proteins from DENV. The y axis indicates the fraction of endemic (n = 10) or traveler (n=3) patients reacting against peptides from E (A) and NS1 (B) proteins with either IgG (top, blue bars) or IgM (bottom, red bars) antibodies at the time points samples were collected. T1: acute, T2: early convalescent, T3: mid convalescent and T4: late convalescent samples. The x axis indicates the proteomic coordinates (amino acid start position) according to DENV Uniprot ref: P33478. The protein domains for E and NS1 are color represented at the top of each plot.

There were also regions preferentially targeted by one specific isotype, such as the regions located in the DI from E, the C-terminal of the NS1 wing domain, the domain I of NS3 and the MTase and the thumb domains of NS5, which were more frequently targeted by IgM Abs; or the regions at the DI/DII hinge of the E, the greasy finger of the wing domain of NS1 and the C-terminal of the finger domain of NS5 that were mainly targeted by IgG Abs (Figure 5 and Supplementary Figures S5).

We also observed that the levels of Ab reactivity did not always correspond with the frequency of patients responding against a specific region. This is the case of NS2A and the C-terminal part of NS5 that showed low IgG levels (Figure 3), but were frequently recognized by the DENV-infected individuals (Supplementary Figures S5).

When we compared the response against DENV peptides with the response against ZIKV and YFV, we noticed a low frequency of patients responding against ZIKV and YFV peptides (Supplementary Figure S6). In general, the Ab reactivity in the endemic and traveler groups against non-DENV flaviviruses was lower in magnitude and in numbers of reactive individuals. However, there were also regions such as the hinge of EDII-ZIKV, C-terminal of prM-ZIKV, NS4B-ZIKV, C-terminal of NS2B-YFV and NS5-YFV proteins with low IgG levels but with a high fraction of patients reacting against them.

4.4.4 Longitudinal Analysis of the Ab Response

A longitudinal analysis of the IgG and IgM responses per each patient was performed by calculating the fold change of the fluorescence intensity levels in the convalescent samples (early, mid and late) in respect to their acute samples per each peptide. This analysis revealed two patterns of IgG seroconversion in patients from the endemic group (Figure 6). One pattern, for patients 1, 2, 3 and 5 was characterized for a rapid increase of IgG levels towards NS3 peptides from the acute to the early convalescent sample that later declined over time. Interestingly, subjects 2, 3, and 5 developed severe symptoms of dengue and needed hospitalization (Table 1). The second pattern, for patients 4, 6, 7, 8 and 10 was mostly characterized by high fold change values towards peptides from NS1 protein in the early convalescent sample (Figure 6). The fold change values for the E and NS5 peptides were lower and sustained over time. For the IgM response, the

seroconversion patterns were slightly divergent between patients, but overall showed higher scores for peptides from the prM and the NS5 proteins. The fold change values for prM peptides remained constant over time, while for NS5 peptides differed markedly between individuals (Figure 6).

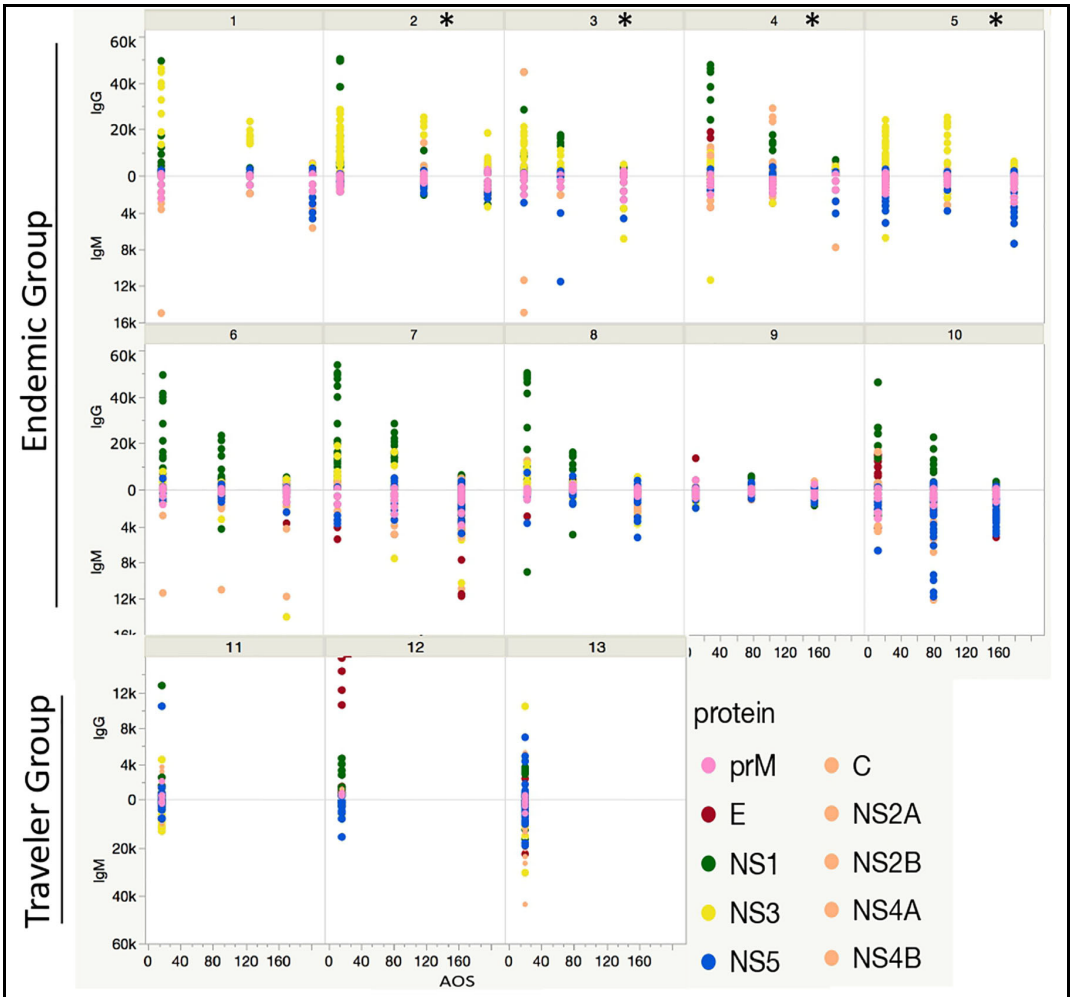

FIGURE 6 | Longitudinal humoral response to DENV proteins. Each box represents the individual response of each patient through time in the endemic (from 1 to 10) and the traveler (11 - 13) groups. The y axis indicates the Ab fold change per patient at the early-, mid- and late convalescent samples, relative to the acute sample, colored by protein target. The x axis indicates the days when the convalescent samples were collected. The * symbol refers to patients presenting severe symptoms of dengue and needed hospitalization.

For the travelers the considerable divergence in the fold change IgG and IgM values towards peptides between individuals made it difficult to identify a preferential Ab pattern against a particular protein.

We also performed the same longitudinal analysis for ZIKV and YFV peptides. The seroconversion patterns for IgG and IgM Abs were similar as those described for DENV, with the main difference that the fold change values were significantly lower for ZIKV and YFV (Supplementary Figures S7). For the endemic group, contrasting with the high IgG seroconversion values against NS3 peptides from DENV and ZIKV, the seroconversion values for YFV-NS3 were negligible. The seroconversion patterns in the traveler group were different between individuals and also between ZIKV and YFV and at the same time, they differed in pattern compared to DENV.

4.4.5 Identification of DENV Specific and Flavivirus Broadly Immunoreactive Epitopes

Next, we mapped the flavivirus peptides from DENV, ZIKV and YFV that were recognized by >50% of DENV-infected patients onto the individual coordinates of the structural and non-structural DENV proteins (DENV1, Uniprot: P33478) at the different time-points of the follow-up.

The simultaneous mapping of flavivirus peptides targeted by the traveler individuals revealed that together with a high number of DENV peptides recognized by IgG Abs in the early convalescence samples, an extensive number of ZIKV and YFV peptides were also targeted; while fewer peptides were targeted by IgM Abs, most of them corresponding to DENV peptides, with small reactivity towards ZIKV and YFV peptides. For the endemic group, the opposite was observed, a larger number of co-localized DENV, ZIKV and YFV peptides were targeted by IgM Abs, while for IgG the reactivity was directed towards fewer overlapping regions for the three flaviviruses (Supplementary Figures S8–S10). Abs from the traveler group

that simultaneously targeted DENV and ZIKV or YFV colocalized peptides are likely to be CR-Abs, while those Abs from either the endemic and the traveler group that mainly targeted DENV peptides, with low or absent reactivity towards ZIKV or YFV peptides

are most likely TS-Abs. Then, these immunodominant regions likely to raise CR-Abs are called here PanFlavi and those able to elicit TS-Abs called DENV-specific.

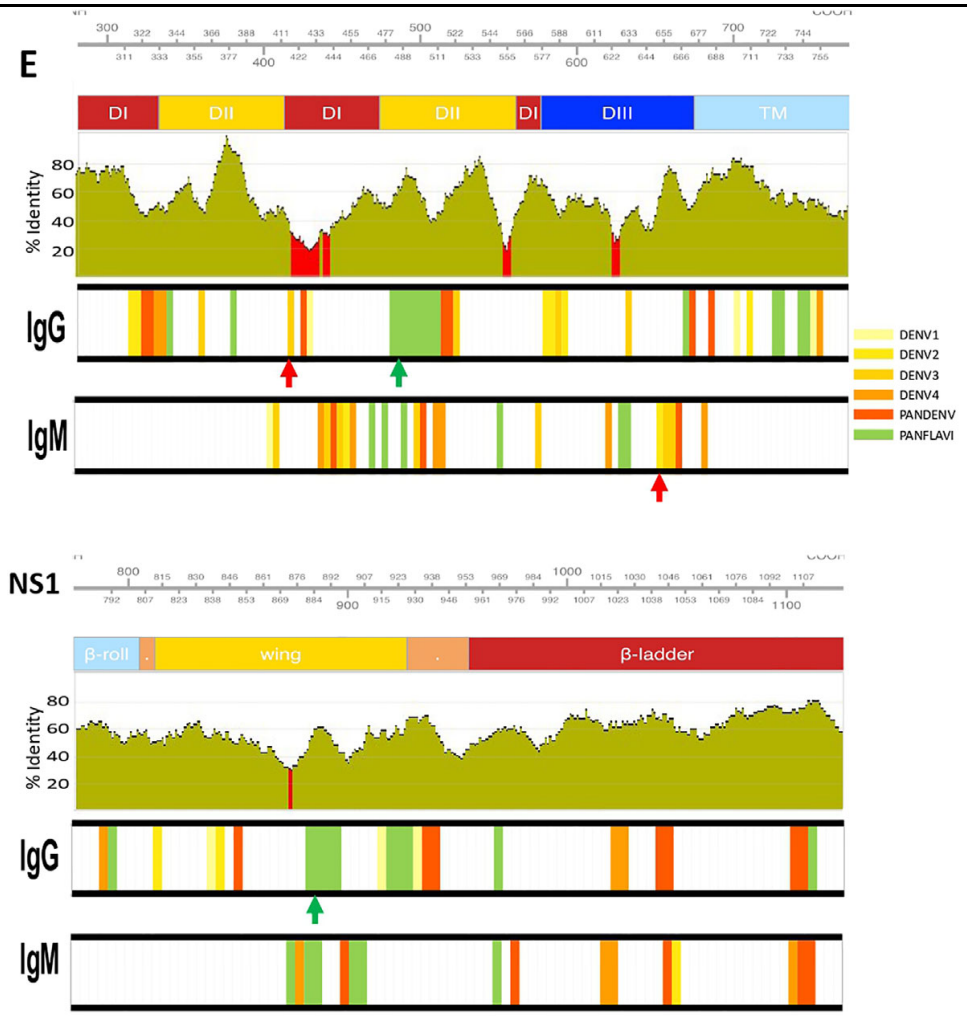

FIGURE 7 | Identification of DENV specific and flavivirus broadly immunoreactive epitopes. IgM and IgG targeted epitopes at the E (A) and NS1 (B) proteins from DENV, ZIKV and YFV viruses, recognized by DENV patients were filtered out and mapped to the proteomic coordinates of DENV (Uniprot ref: P33478). Colored bars from light-yellow to orange represent immunodominant regions belonging to one DENV serotype. Red bars represent immunodominant regions belonging to more than one serotype (here called PanDENV). Green bars represent immunodominant regions belonging to DENV and ZIKV or/and YFV here called PanFlavi. E and NS1 sequences from DENV1-4, ZIKV and YFV were aligned and the conservation rate (percentage identity) between the amino acid positions is represented as green bar plots using a sliding window size of 15 amino acid positions. Red regions correspond to percentage identities below 30%. Red and green arrows are DENV-specific and PanFlavi peptides, respectively, and for which the Ab responses are further detailed in Supplementary Figure S13.

In order to simplify the schematization of the PanFlavi and DENV-specific regions, the peptides that were positive at any time point of the follow up were filtered out and mapped based on their start amino acid position onto the E and NS1 (Figure 7) and C, prM, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Supplementary Figures S11, S12) sequences. The identified PanFlavi and DENV-specific peptides, targeted by IgG and IgM Abs are listed in Tables 2, 3, respectively.

For the E protein, this analysis revealed five immunodominant regions recognized by IgG Abs (Figure 7A). One located at$EDII_{474-511}$ of the DENV proteome is likely to be PanFlavi, since the same regions in E-ZIKV and E-YFV were recognized not only by endemic patients but also by travelers. Another three regions are likely to be DENV-specific. These regions located in $EDI/II_{311-338}$, $EDI/II_{411-443}$ and $EDIII_{622-637}$ were frequently recognized by Abs in the endemic and traveler groups but their homologous regions in ZIKV and YFV showed minimal or no reactivity. The last immunodominant region was identified at $EDII_{356-371}$ and is likely to be DENV-specific with the difference that it was targeted preferentially by the endemic group but not by the travelers. Coincidentally, the PanFlavi regions correspond with regions that are highly conserved among DENV serotypes, ZIKV and YFV, which could explain the high reactivity among them. In contrast, the DENV-specific epitope sequences were located in regions with low identity percentages among these viruses.

For E regions targeted by IgM Abs, we identified five immunodominant regions, the PanFlavi epitopes were located in $EDI/DII_{455-499}$ and $EDIII_{625-639}$, while the DENV-specific sequences were identified in $EDI_{434-465}$, $EDII_{500-522}$ and $EDIII_{649-675}$ (Figure 7A). The highly conserved fusion loop (FL) was recognized by IgG Abs in three out of ten endemic patients and in two out of three travelers, and by IgM Abs in only one out of the ten endemic patients (Supplementary Figure S11).

For NS1, five immunodominant regions were identified to be most frequently targeted by IgG Abs (Figure 7B). One region, highly conserved among the flaviviruses was located in the wing domain of $NS1_{877-906}$ and it was targeted by IgG Abs from individuals of the endemic and traveler group. These Abs are likely to be cross-reactive, since not only the endemic but also the traveler individuals recognized the same region in the ZIKV and YFV proteome. Another three regions, one spanning the wing domain $NS1_{838-861}$ and

the other two in the ß-ladder at positions $NS1_{1033-1053}$ and $NS1_{1093-1114}$ were also targeted by IgG Abs from endemic and traveler individuals, however sera from the traveller group recognized not only DENV peptides, but also ZIKV and YFV peptides located in the same regions, meaning that these were PanFlavi regions for the travelers but DENV specific for the endemic group (Supplementary Figure S11).

A PanFlavi region in the NS1 protein spanning the wing domain from residues 859-915 was frequently recognized by IgM Abs from endemic samples. Three other regions in NS1 were likely to be DENV specific, since IgM Abs from the endemic and the traveler individuals recognized mainly DENV peptides in this region, with little or no reactivity towards ZIKV or YFV peptides (Figure 7B).

The more representative PanFlavi and DENV-specific peptides targeted by IgMand IgG Abs that could represent good serological markers are shown in Supplementary Figure S13. Notably, the DENV-specific peptides located at $E_{654-668}$, $NS4B_{2396-2410}$, $E_{413-427}$, $NS2B_{1433-1447}$ and $NS4B_{2312-2326}$, belong to a unique DENV serotype, while regions located at $NS4B_{2324-2338}$ and $NS3_{2059-2073}$ belong to multiple DENV serotypes.

TABLE 2 | Most reactive IgG peptides selected by the high-density microarray.

	Type	Protein	Protein position		Amino acid length
			start	end	
IgG	PanFlavi	C	1	23	23
		C	61	100	40
		prM	117	155	39
		E	197	239	43
		NS1	105	131	27
		NS2A	85	99	15
		NS2B	109	130	22
		NS3	1	31	31
		NS3	321	335	15
		NS3	337	351	15
		NS5	369	391	23
		NS5	465	491	27
	DENV-Specific	prM	29	47	19
		E	33	67	35
		E	133	147	15
		E	293	319	27
		NS1	61	87	27
		NS2A	141	179	39
		NS2B	89	103	15
		NS3	477	491	15
		NS3	585	603	19
		NS4A	29	47	19
		NS4B	204	218	15
		NS5	321	339	19

TABLE 3 | Most reactive IgM peptides selected by the high-density microarray.

	Type	Protein	Protein position		Amino acid length
			start	end	
IgM	PanFlavi	C	61	100	40
		prM	33	67	35
		prM	97	163	67
		E	181	219	39
		E	337	359	23
		NS1	93	143	51
		NS3	57	135	79
		NS3	309	355	47
		NS4B	5	43	39
		NS5	89	123	35
		NS5	177	223	47
		NS5	269	299	31
		NS5	509	547	39
	DENV-specific	E	153	187	35
		E	213	243	31
		E	365	391	27
		NS1	265	283	19
		NS1	321	343	23
		NS2B	109	130	22
		NS3	201	227	27
		NS3	465	491	27
		NS3	593	611	19
		NS4A	89	107	19
		NS4B	73	107	35
		NS4B	193	218	26
		NS5	469	483	15
		NS5	869	883	15
		NS5	881	899	19

4.5 Discussion

The negative impact of the increasing cocirculation of different arboviruses in endemic areas on the reliability of current serological assays highlights the necessity for the discovery of better serological markers that can solve the cross-reactivity problem. These improvements may be of benefit to the clinical management of arboviral suspected cases given the risk of severe clinical manifestations in secondary infections, but will also help to better define DENV serostatus required for vaccination strategies with Dengvaxia. In this work, we conducted for the first time a high throughput longitudinal scanning of the diversity of IgM and IgG Abs using a proteome-wide microarray containing overlapping linear peptides from DENV, ZIKV, YFV and CHIKV in sera from individuals infected with DENV.

A fundamental aspect of this work is the comparison of the longitudinal IgM and IgG Ab profiles to the entire viral proteome in individuals from endemic areas, which most likely

experienced previous arboviral infections, in contrast to the Ab response from individuals that experienced a first DENV infection after visiting an endemic area. Our results demonstrated that IgM and IgG responses towards DENV linear peptides in primary infected travelers and in individuals living in endemic areas were in agreement with the dynamics of primary and secondary infections, respectively.

We found that the IgG response differed in magnitude across time and across the proteome, with the strongest responses in the early convalescent phase and with the most immunodominant peptides located in proteins C, prM, E and NS1. This is in accordance with previous work showing that these proteins are the main targets of the Ab responses (41). Although NS3 and NS5 proteins are known to contain T cell epitopes mainly targeted by CD8+ T cells (42, 43), our results showed that these proteins also have epitopes targeted by IgG antibodies.

The observed low levels of IgM Abs in the endemic samples compared to the response in the travelers has been previously reported, indicating that secondary infections with homologous viruses are characterized by the production of lower levels of IgM compared to the response in primary infections (44, 45). This has been linked to the "original antigenic sin" phenomenon, stating that prior exposure induces an ineffective response or even absence of response to a related antigen upon re-exposure. Although IgM Ab response in primary viral infections is usually transient in nature (23, 45), other studies have reported the long persistence of IgM in DENV-primary infected individuals (46). Here, in agreement with these studies, we found that IgM Abs persisted at sustained levels and even 6-months AOS in the endemic group. Unfortunately, we were unable to follow the evolution of the IgM response in the traveller group because we only had an acute and early convalescent sample for this group. Taken together, these studies imply that using IgM could be problematic in providing a differential and accurate diagnosis because it could render: (i) a false-positive result due to the reaction to related viruses by CR-Abs (47–49), (ii) a false positive result of a current infection as a consequence of TS- or CR- long-lived IgM Abs (50), or (iii) a false negative result due to the absence of IgM in secondary flavivirus infections (46).

The high IgG titers appearing very early AOS in the endemic group, compared with the magnitude of the response in the traveler group is consistent with an anamnestic humoral

response reported previously as the result of a “boost” in Ab titers when homologous reinfection or a low-level heterotypic infection takes place (1, 25, 51). With yearly DENV outbreaks occurring during the rainy seasons in Peru, it is very likely that the high IgG levels detected early AOS are the result of re-exposure with a homotypic virus in the past. Secondary flavivirus exposures are common in endemic areas where there is cocirculation of multiple dengue serotypes plus other flaviviruses with an increasing prevalence such as ZIKV, and programs of YFV vaccination (2), resulting in a complex multiply primed immune system. As a consequence, the observed Ab repertoire of DENV-infected individuals from an endemic area could include re-evoked homotypic or heterotypic antibodies, new DENV2-specific Abs developed against the current infection, or long-lived specific Abs from a previous exposure towards ZIKV after a natural infection or towards YFV as a consequence of vaccination.

An unexpected finding in this study was the overall more extensive recognition of ZIKV and YFV peptides by IgG Abs compared to IgM in the convalescent samples from the travelers. Most of these regions overlapped with reactive DENV peptides, with the difference that the magnitude of the response against DENV peptides was substantially higher as compared to the response against the other viruses. We identified the Abs recognizing these regions as cross-reactive which also happened to correspond with regions of high genetic and antigenic similarity between these viruses. We were able to map the immunodominant regions across the proteome that evoked CR-Abs.

Severe manifestations of dengue are more commonly seen in secondary infections (6). The pathogenesis of dengue is multifactorial with contributions from the virus and the host, and the degree to which the humoral responses may influence the balance between protective and detrimental effects of DENV-specific immune responses is still not fully understood. However, in secondary infections suboptimal levels of CR-Abs, mainly directed against structural proteins, could drive ADE, resulting in more severe pathology (21). We found that in a sub-set of endemic patients that developed severe dengue disease, the seroconversion in the early convalescent samples was mainly dominated by high IgG scores againstNS3 peptides. The role of Abs against non-structural proteins other than NS1 have been poorly studied. Because NS3 is an intracellular protein, most studies have focused on identifying T-cell immunodominant regions, reporting that

epitopes located in the NTPase and helicase of NS3 are the most immunodominant in the cellular response against dengue (52, 53) and that this response towards NS3 is associated with dengue hemorrhagic fever (54). However, the mechanisms leading to this exacerbation of symptoms are not fully understood. Whether Abs directed towards peptides from NS3 protein could also play a role in the pathogenesis of the disease remains unclear and could be the focus of future investigation.

The E protein together with the PrM protein elicit most of the Ab responses against the flaviviruses. In our study, IgG and IgM Abs from endemic people and travelers recognized peptides in the prM protein fromDENV1-4, ZIKV and YFV. This is in agreement with previous reports indicating that Abs directed against this protein are a major component of a cross-reactive response and are characterized by being non-neutralizing and able to induce ADE in DENV in secondary infections (6, 44, 55, 56).

Epitopes covering the highly conserved fusion loop (FL) of the E protein were not frequently recognized by the DENV patients that we studied. However, a large proportion of anti-DENV Abs appears to be cross-reactive and to target the FL. These CR-Abs are also considered to be non-neutralizing and able to induce ADE in vivo and in vitro (57, 58). The lack of reactivity against this peptide in our study can be explained by the fact that this is a conformational epitope which is buried in the immature form of the E protein and becomes accessible during the "dynamic" breathing of the E dimers at the virion surface. The native folding of this epitope might not be present in the linear peptides covered in our microarray.

The characterization of NAbs against the E protein has revealed that the most potent DENV NAbs are directed against the E-DIII in mice while in human the NAbs seem to preferentially recognize quaternary epitopes present only in virions. Although we cannot refer to the detected Abs as neutralizing, we were able to identify a DENV-specific peptide located in the $EDIII_{622-637}$ which region was previously reported as being targeted by NAbs (59).

High levels IgG Abs were detected and they reacted against regions from DENV1-4, ZIKV and YFV which underlies the cross reactivity in various serological assays. For example, our data showed that a highly conserved region among the flaviviruses, targeted by IgG Abs from the endemic group, is located in the wing domain of the NS1 protein

which is currently used as target for the differentiation between DENV and ZIKV infections.

Some of the DENV peptides that we have identified in this study, have also been reported by previous studies to be recognized by monoclonal Abs and polyclonal sera from DENV infected individuals, especially those located in the E (60, 61) and NS1 protein (32, 62–65). However, a limitation of our linear peptide array is that conformational epitopes are lacking and thus we likely missed these epitopes in our analysis.

In summary, our study provides novel insights in the proteome-wide antibody response against dengue virus and demonstrates that peptide microarrays represent a powerful tool for mapping type-specific and cross-reactive Abs following natural infection, allowing the identification of peptides that exhibit a great potential for diagnostic purposes. To overcome the limitation of the relatively low number of samples used in this study, the diagnostic performance of the identified biomarker candidates is part of an ongoing work using a bead-based multiplex peptide immunoassay with a large panel of samples originating from individuals with different flavivirus infections. Our findings present potentially valuable biomarkers for a future generation of peptide-based diagnostics that would be more specific and easier to manufacture compared to the conventional full-length recombinant protein assays.

4.5 Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

4.6 Ethics statement

The studies involving human participants were reviewed and approved by Ethical review boards of the Peruvian University Cayetano Heredia, Lima, Peru (Protocol N° 101480); the Institute of Tropical Medicine Antwerp, Belgium (Protocol N° ITG1304/19) and the University of Antwerp, Belgium (Protocol N° 19/42/477). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

4.7 Author contributions

FF-A wrote the manuscript text. KK and FF-A implemented the analysis. KA, FF-A, KK, and KB conceived the study. FF-A, XM, and JM processed samples. FF-A analyzed the

data. MT, FF-A, XM, and ME wrote study protocols and coordinated sample collection. KA, KK, and KB have verified the underlying data. All authors contributed to the article and approved the
submitted version.

4.8 Funding

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4.9 Acknowledgments

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The microarray design was created by KK in collaboration with BISC Global (Ghent, Belgium) and is submitted in parallel with this manuscript. We also thank the staff at the Molecular Diagnostics Center for Health & Bioresources at AIT Austrian Institute of Technology GmbH (Vienna, Austria) and the staff at Schafer-N ApS (Copenhagen, Denmark). We are grateful to Anne Hauner for critically reading this manuscript. Finally, we are grateful to the study participants and their families.

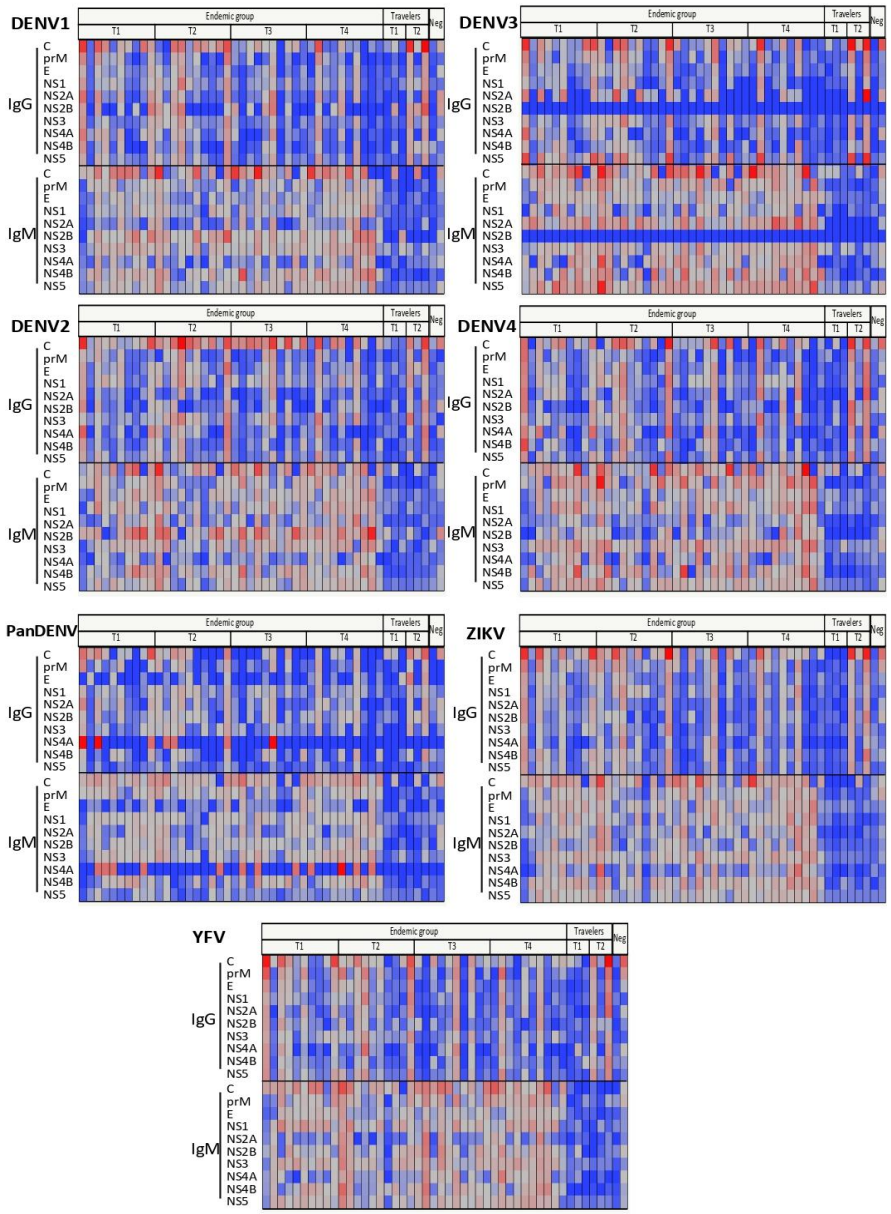
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.10 Supplementary material

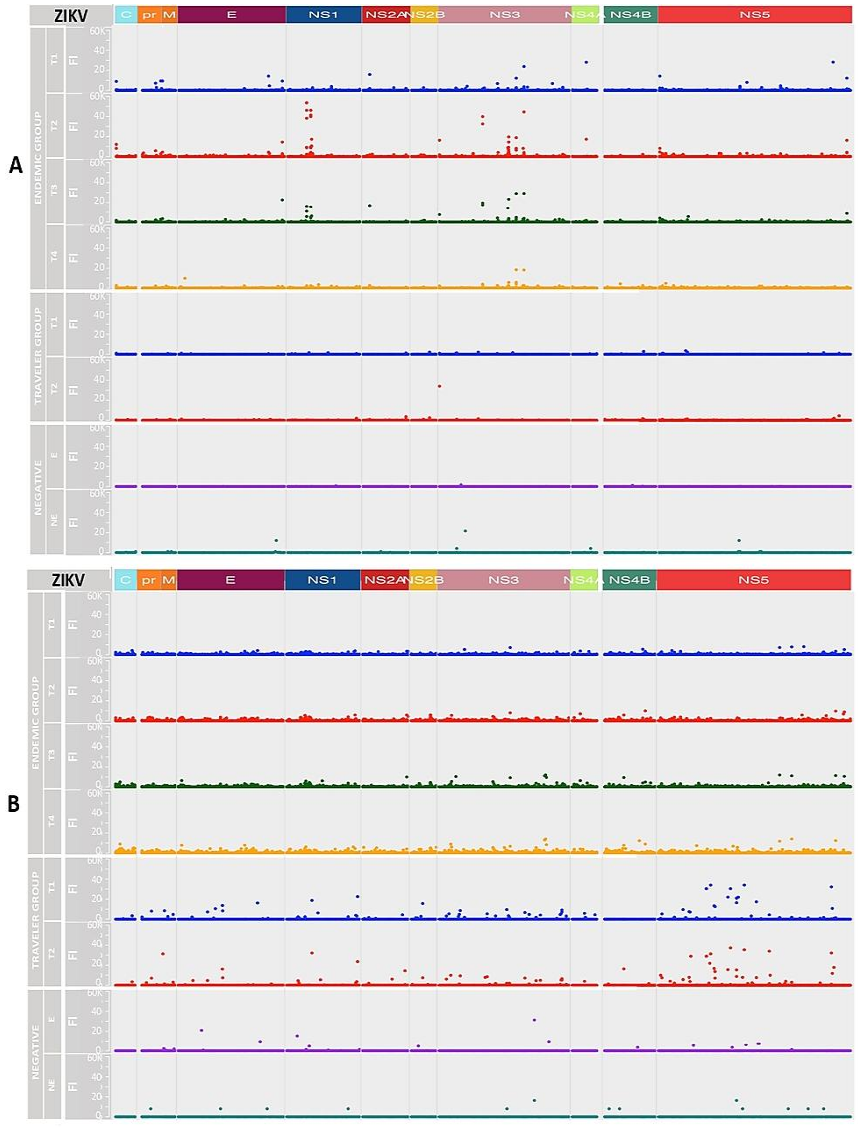
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.686691/full#supplementary-material

Supplementary Figure 1 | Coverage of peptide library per virus. Peptides were plotted relative to polyprotein coordinates (start amino acid position) for DENV (P33478), ZIKV (A0A142I5B9) and YFV (P03314). Structural proteins: Capsid (C), Membrane (pr, M) and Envelope (E), and non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are color represented; each yellow dot represents a single unique peptide in the microarray.

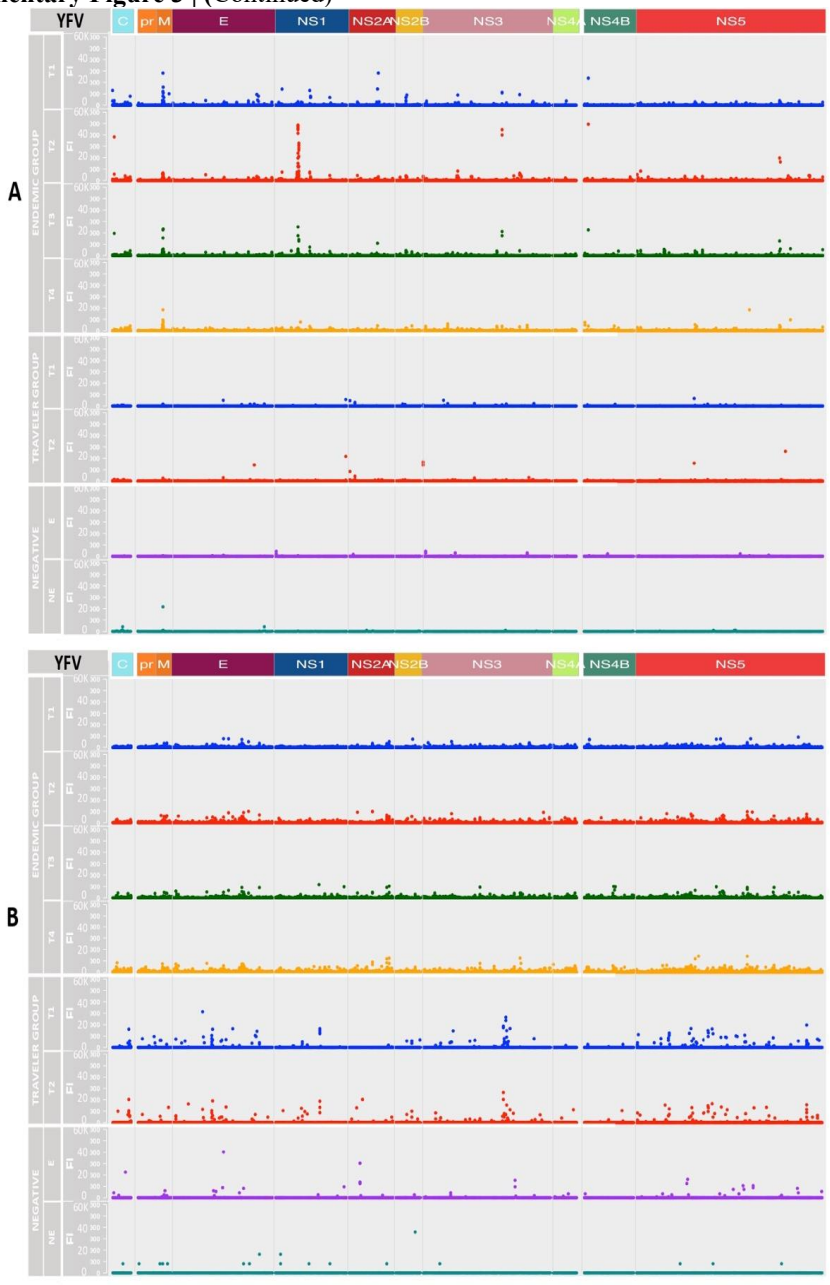
Supplementary Figure 2 | Antibodies from DENV-infected individuals target epitopes in linear peptides from DENV, ZIKV and YFV. Heatmaps depicting the relative number of peptides targeted by IgG and IgM Abs present in individual serum from endemic (n=10) and traveler patients (n=3) at the time samples were collected: T1 (acute), T2 (early convalescent), T3 (mid convalescent) and T4 (late convalescent). Each column represents a sample and each row represents a protein from the correspondent arbovirus. Samples from the negative subjects are the two last columns. The color intensity indicates the relative number of 15-mer peptides from the indicated arbovirus reacting against IgG and IgM antibodies.



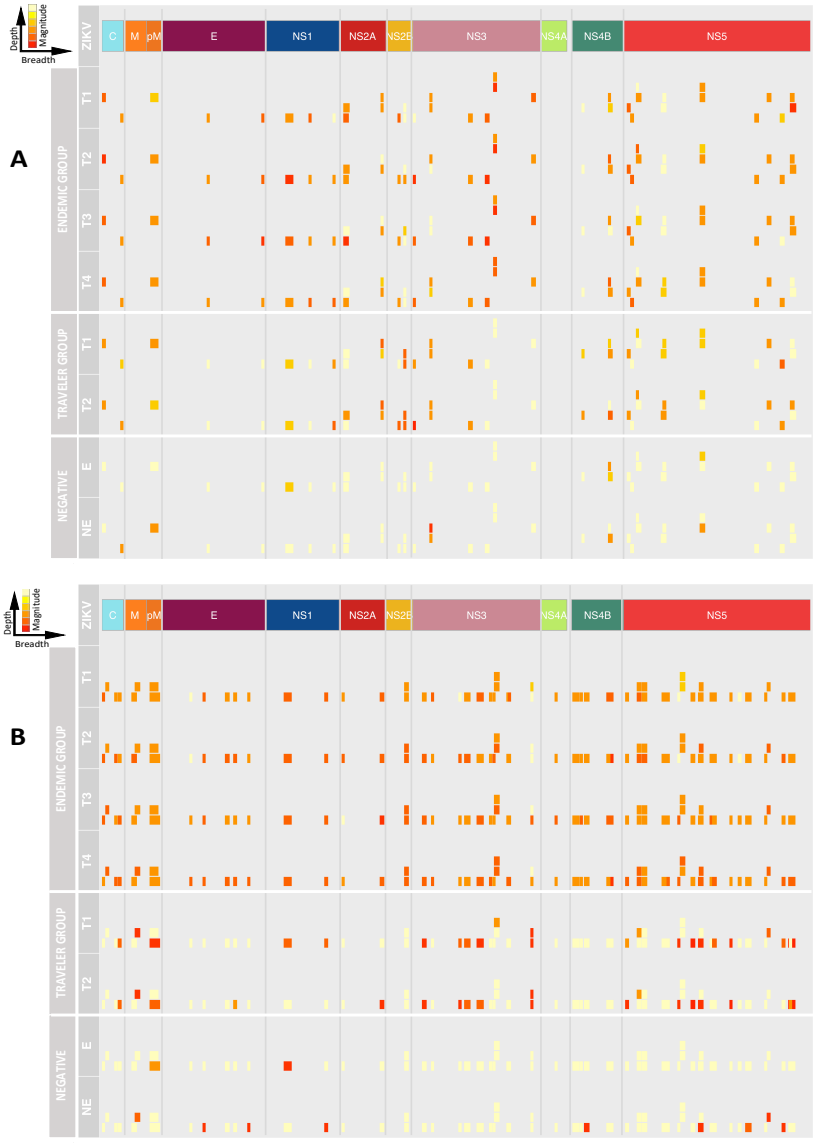
Supplementary Figure 3 | Longitudinal humoral responses to linear epitopes covering the ZIKV and YFV proteomes. The plots show the level of IgG (A) and IgM (B) Ab reactivity measured in fluorescence units relative to proteomic coordinates for ZIKV (Uniprot ref. A0A142I5B9) and YFV (Uniprot ref. P03314). Structural: Capsid (C), Membrane (pr, M) and Envelope (E), and non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are color represented; each dot represents the Ab binding to a single peptide in the microarray. In both (A, B) plots, the endemic group is at the top (four-time points), the traveler group at the center (two-time points) and the negatives at the bottom (one-time point). T1: acute (blue dots), T2: early convalescent (red dots), T3: mid convalescent (green dots) and T4: late convalescent (orange dots) samples. Responses from the endemic (E) and non-endemic (NE) negatives are represented in purple and cyan dots, respectively.



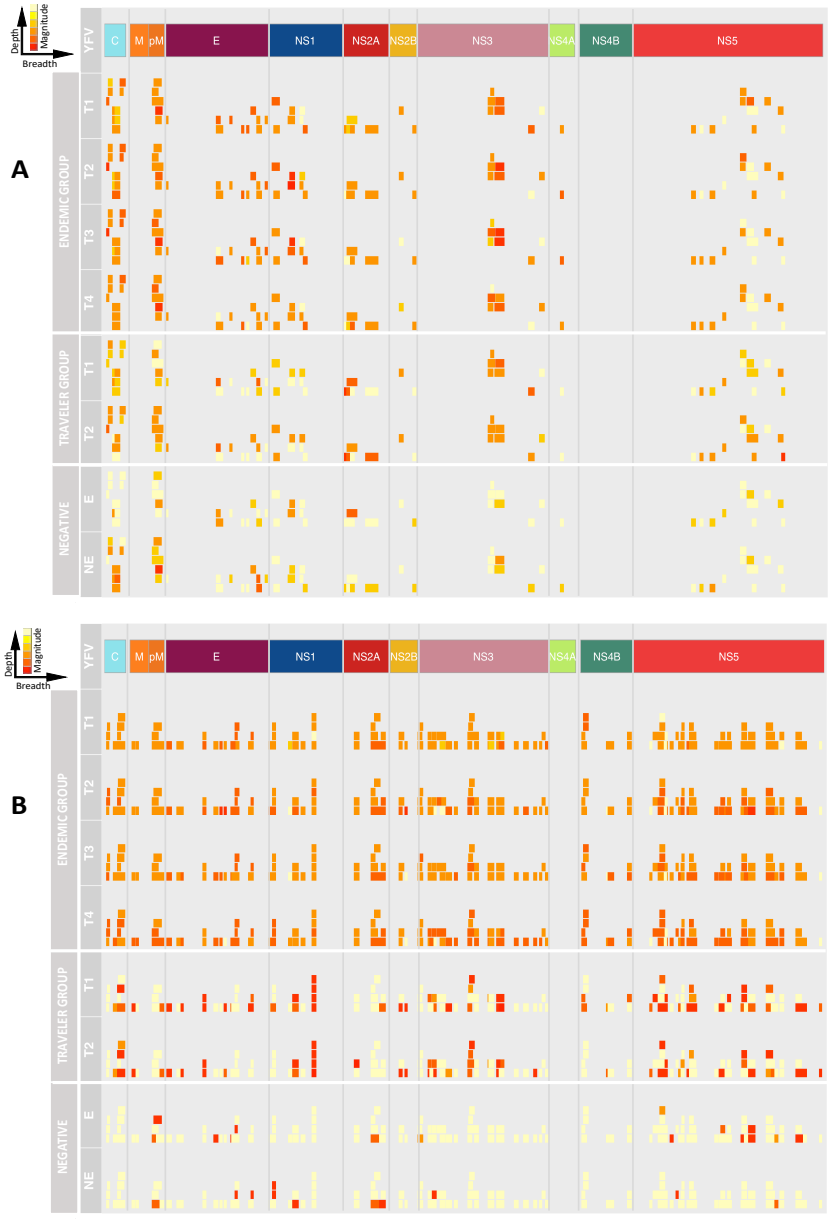
Supplementary Figure 3 | (Continued)



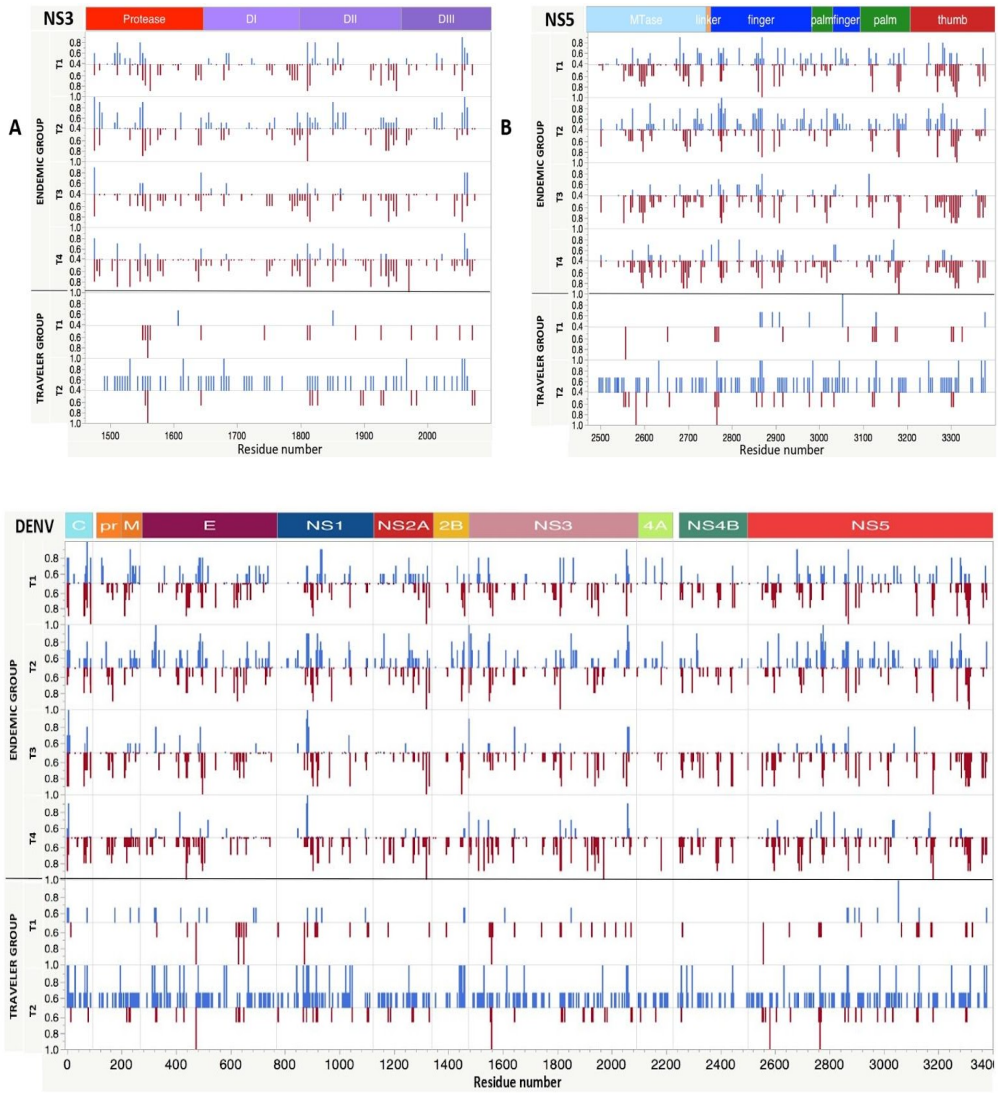
Supplementary Figure 4 | Depth and breadth of the humoral response against ZIKV and YFV peptides. The plots show the Ab target regions (ATRs) for IgG (A) and IgM (B) aligned to the proteomic coordinates for ZIKV (Uniprot ref. A0A142I5B9) and YFV (Uniprot ref. P03314). Structural: Capsid (C), Membrane (pr, M) and Envelope (E), and non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are color represented. Each bar in the plot represents an ATR. The depth (different epitope variants recognized by the sera) of the Ab response is read vertically, the breadth (binding sites across the polyprotein) of the Ab response is read horizontally and the color intensity of each ATR indicates the magnitude of the Ab response, in terms of arbitrary fluorescence intensity units after Log2 transformation (ranging from 0 to 16). T1: acute, T2: early convalescent, T3: mid convalescent and T4: late convalescent (orange dots) samples. E: endemic and NE: non-endemic negative samples.



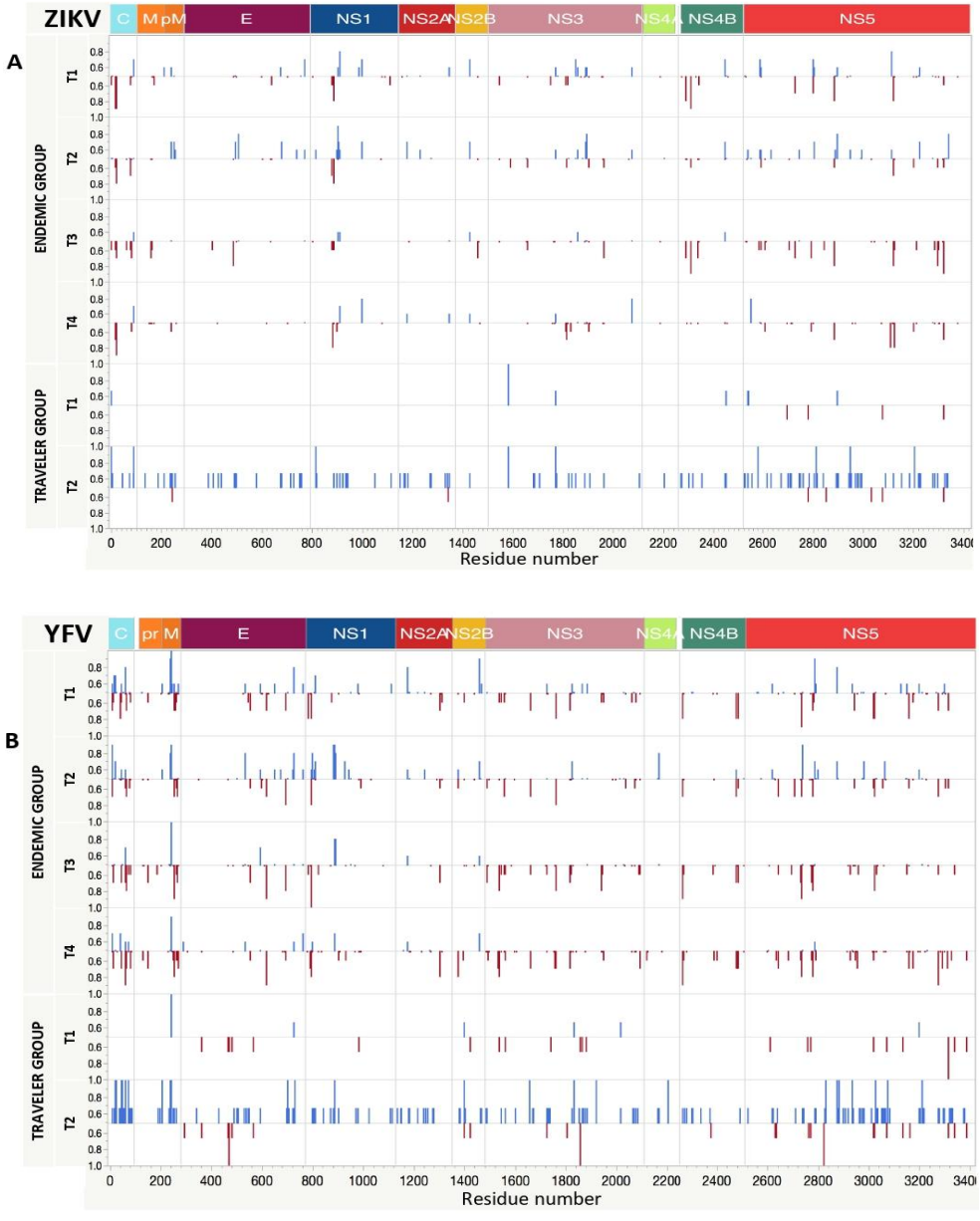
Supplementary Figure 4 | (Continued)



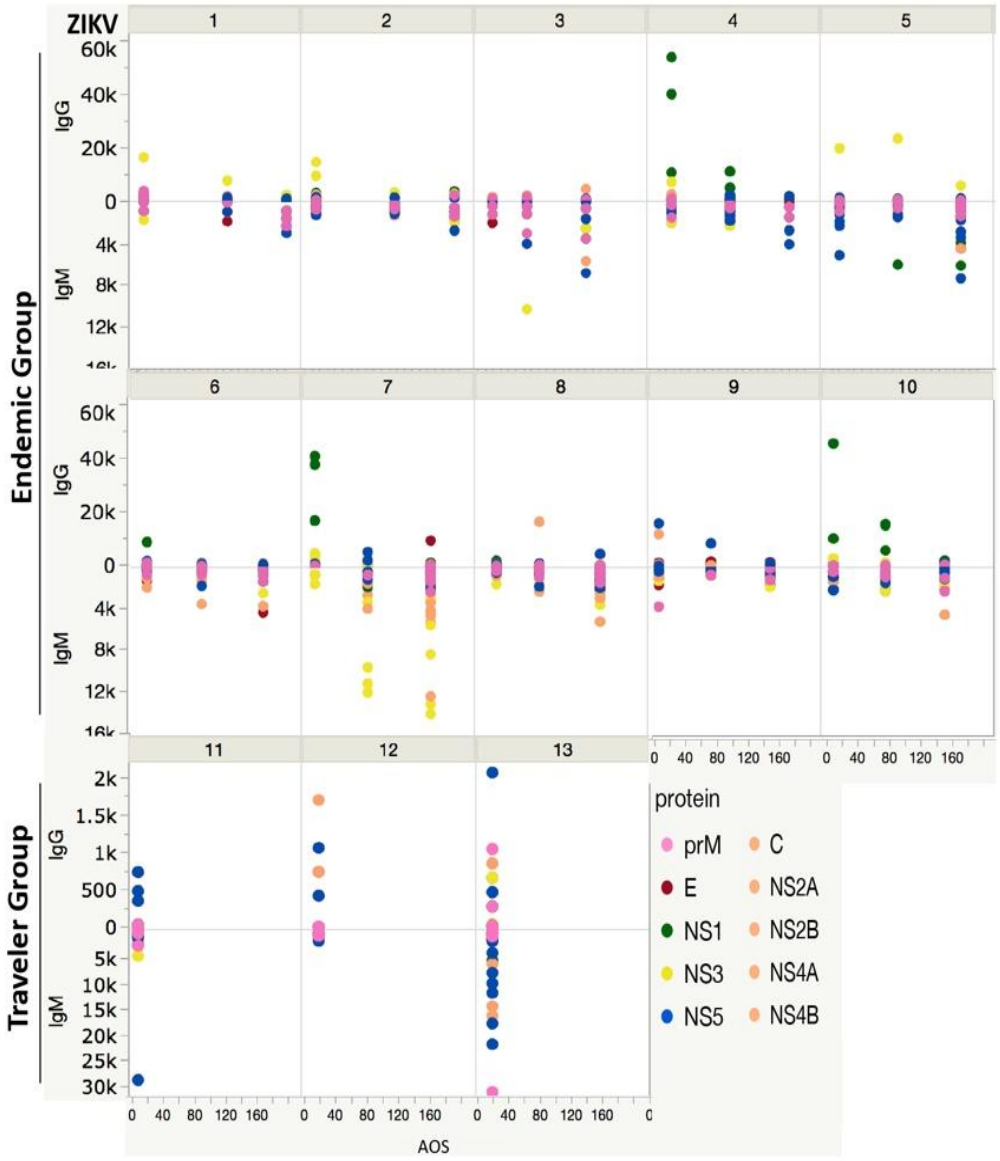
Supplementary Figure 5 | Longitudinal IgG and IgM reactivity against NS3, NS5 DENV proteins and complete DENV proteome. The y axis indicates the fraction of endemic (n = 10) or traveler (n=3) patients reacting against peptides from NS3 (A), NS5 (B) and DENV proteome (C) with either IgG (top, blue bars) or IgM (bottom, red bars) antibodies at the time points samples were collected. T1: acute, T2: early convalescent, T3: mid convalescent and T4: late convalescent (orange dots) samples. The x axis indicates the proteomic coordinates (amino acid start position) according to DENV Uniprot ref: P33478. The protein domains for NS3 and NS5 are color represented at the top of each plot.



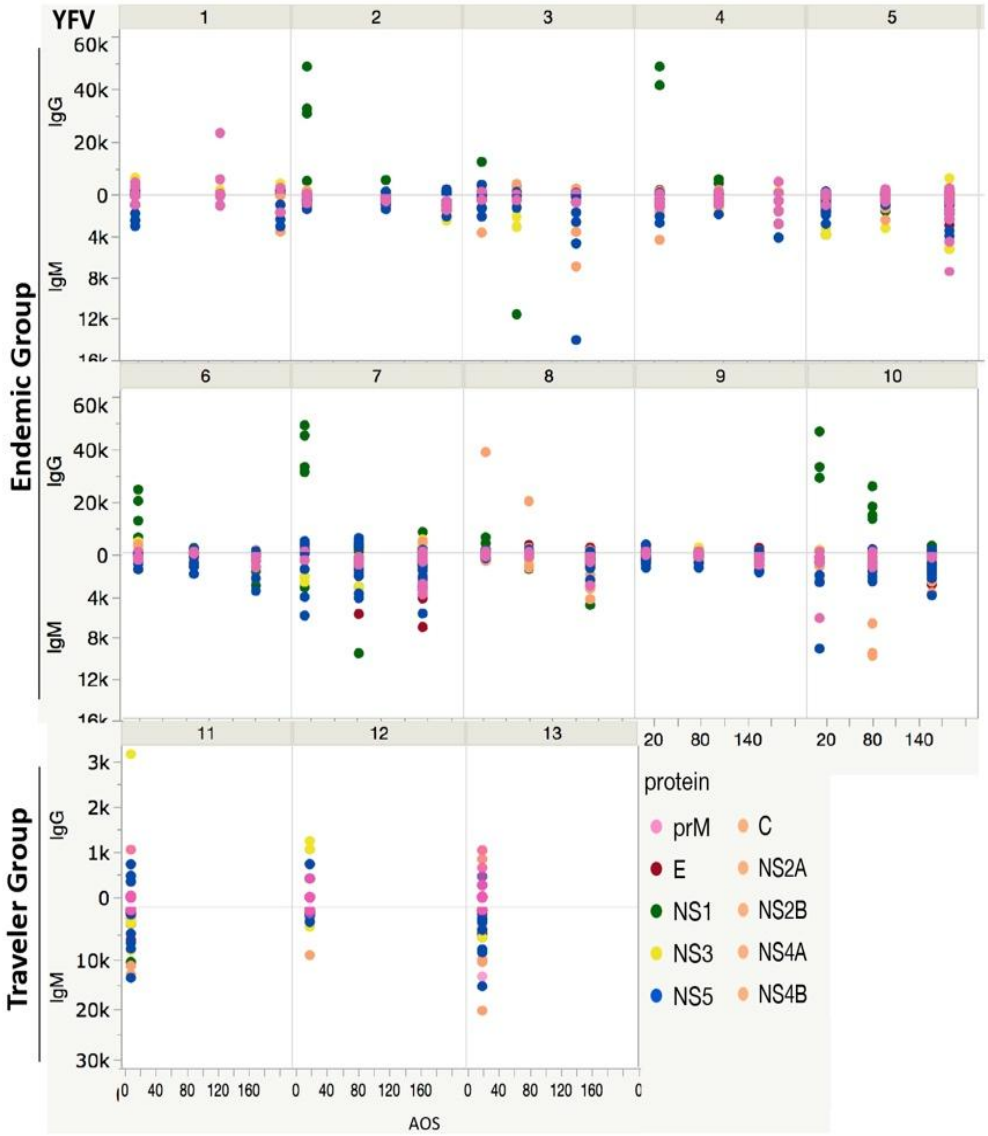
Supplementary Figure 6 | Frequency of IgG and IgM reactivity against ZIKV and YFV proteome. The y axis indicates the fraction of endemic (n = 10) or traveler (n=3) patients reacting against peptides from ZIKV (A) and YFV (B) proteomes with either IgG (top, blue bars) or IgM (bottom, red bars) antibodies at the time points samples were collected. T1: acute, T2: early convalescent, T3: mid convalescent and T4: late convalescent samples. The X axis indicates the proteomic coordinates (amino acid start position) according to ZIKV Uniprot ref: A0A142I5B9 and YFV Uniprot ref: P03314. The structural and non-structural proteins are color represented at the top of each plot.



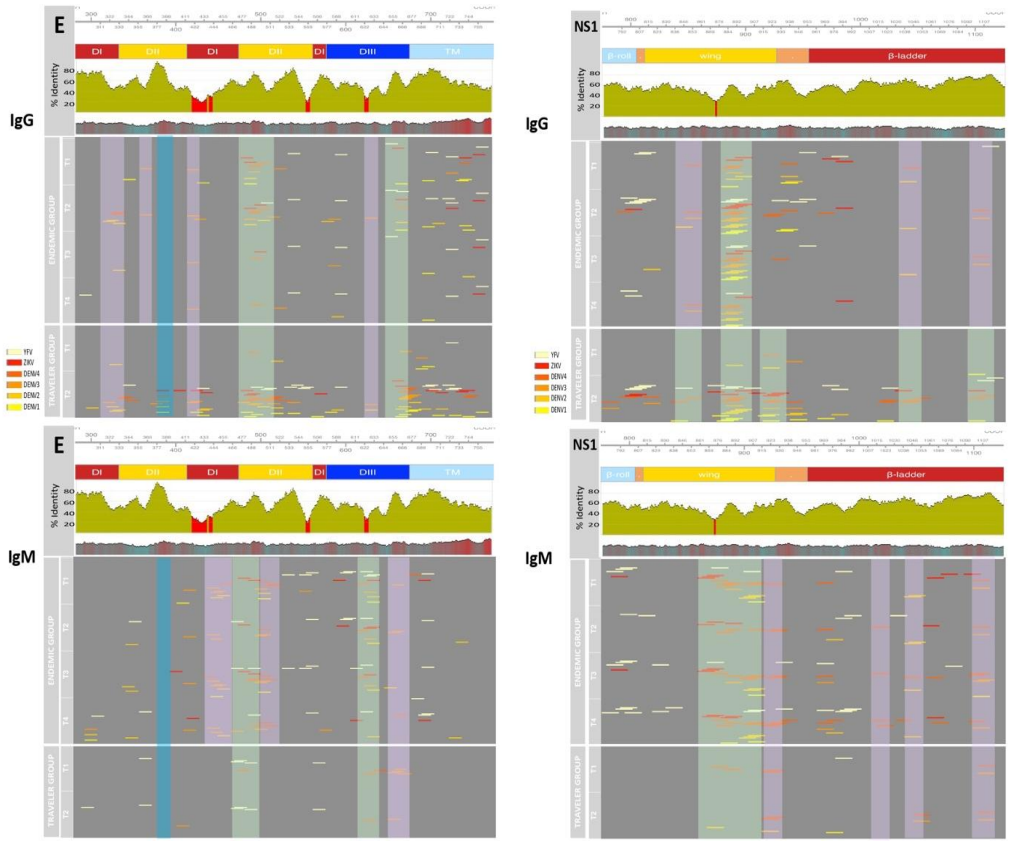
Supplementary Figure 7 | Longitudinal humoral response to ZIKV and YFV proteins. Each box represents the individual response of each patient through time in the endemic (from 1 to 10) and traveler (11 - 13) groups. The y axis indicates the Ab fold change per patient at the early-, mid- and late-convalescent samples, respect to the acute sample, colored by protein target. The x axis indicates the days when the convalescent samples were collected.



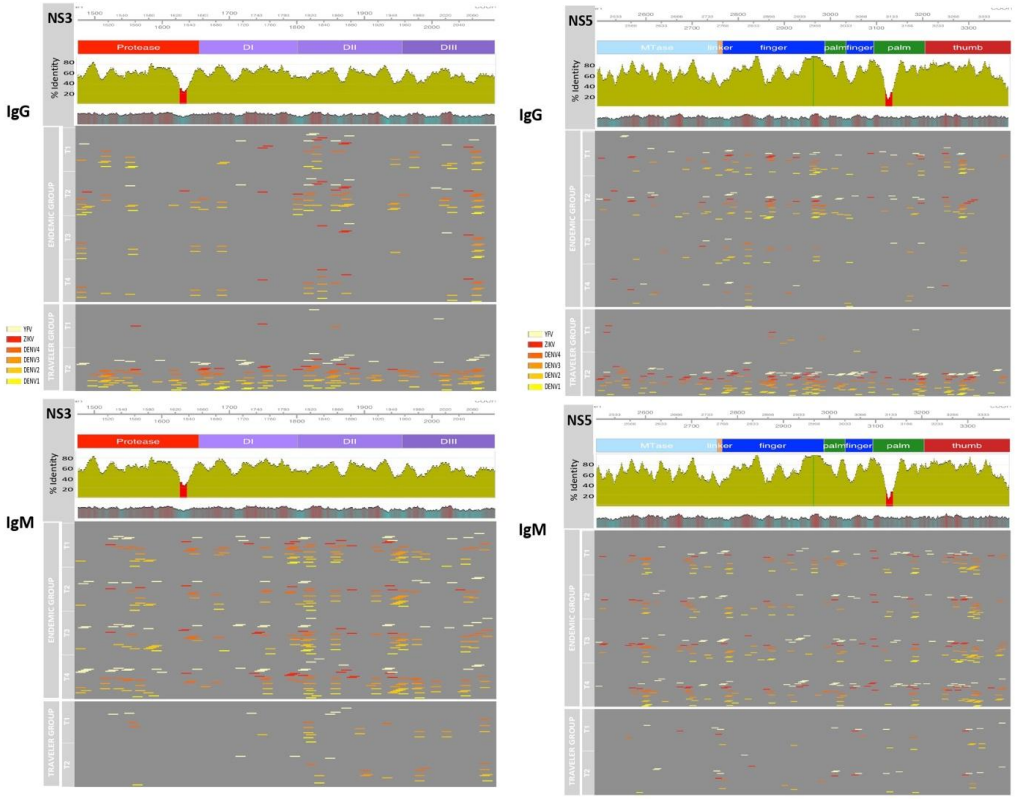
Supplementary Figure 7 | (Continued)



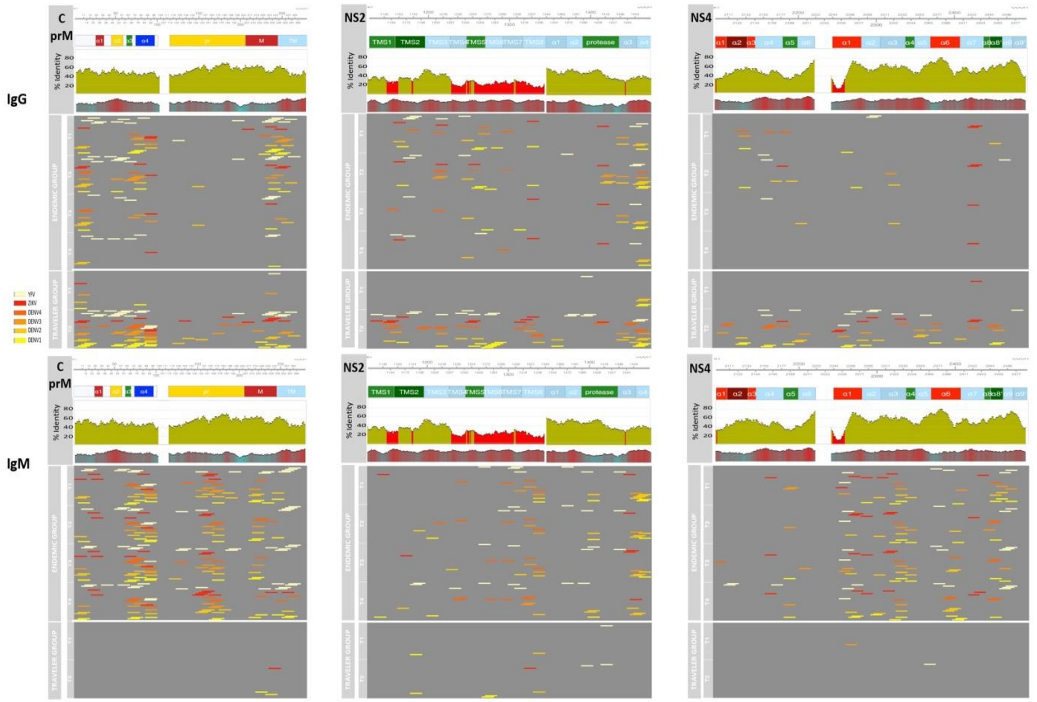
Supplementary Figure 8 | Reactive epitopes from E and NS1 proteins among DENV, ZIKV and YFV flaviviruses. 15-mer peptides from DENV1-4, ZIKV and YFV targeted by IgG and IgM recognized by >5 endemic patients (or > two traveller patients) are indicated by horizontal colored lines. The upper row in each panel shows the proteomic coordinates according to the DENV Uniprot ref: P33478 and the domains of E and NS1 are color represented. E and NS1 amino acid sequences from DENV1-4, ZIKV and YFV were aligned and the conservation rate (percentage identity) between the amino acid positions is represented as green bar plots using a sliding window size of 15 amino acid positions. Red regions correspond to percentage identities below 30%. T1: acute, T2: early convalescent, T3: mid convalescent and T4: late convalescent samples. Regions highlighted in purple and green correspond to the most representative DENV-specific and Panflavi peptides, respectively. Cyan box corresponds to the location of the fusion loop.



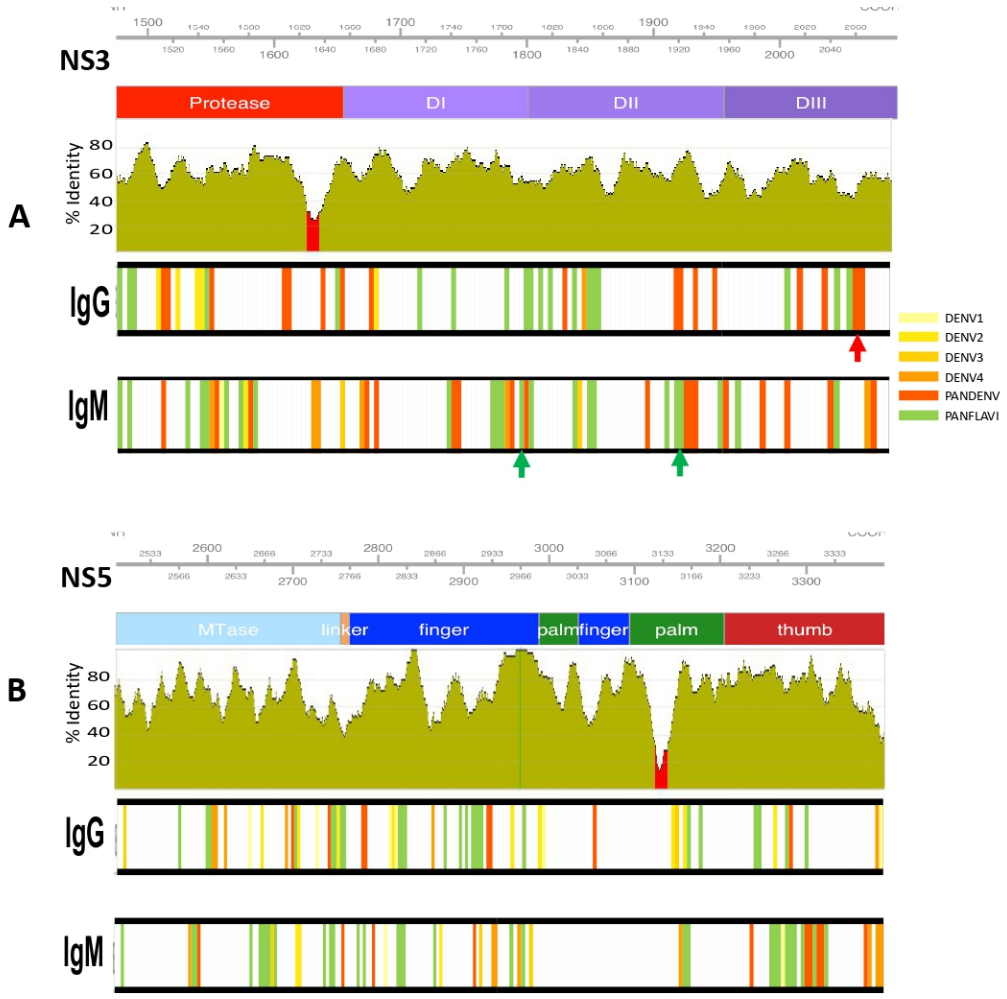
Supplementary Figure 9 | Reactive epitopes from NS3 and NS5 proteins among DENV, ZIKV and YFV flaviviruses. 15-mer peptides from DENV1-4, ZIKV and YFV targeted by IgG (A) and IgM (B) recognized by >5 endemic patients (or > two traveler patients) are indicated by horizontal colored lines. Proteomic coordinates and protein domains percentage identities are as in Supplementary Figure 8. T1: acute, T2: early convalescent, T3: mid convalescent and T4: late convalescent (orange dots) samples.



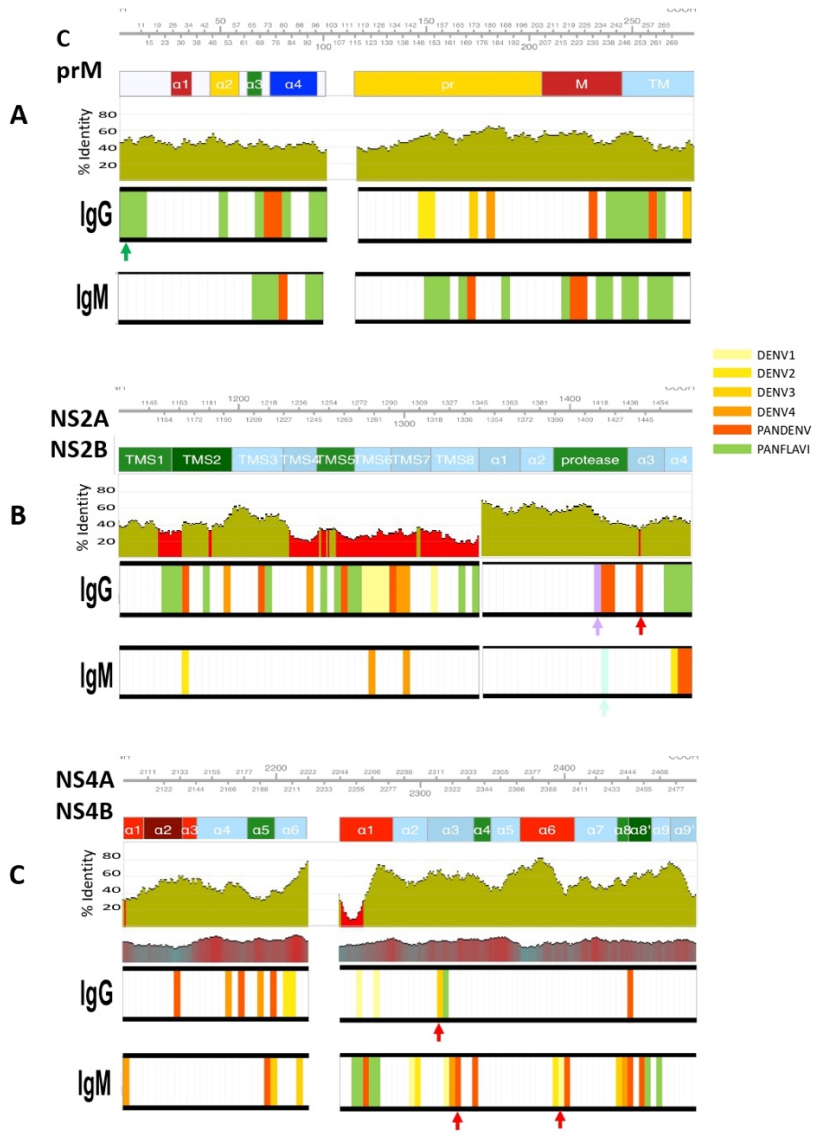
Supplementary Figure 10 | Reactive epitopes from C, prM, NS2A, NS2B, NS4A and NS4B proteins among DENV, ZIKV and YFV flaviviruses. 15-mer peptides from DENV1-4, ZIKV and YFV targeted by IgG (A) and IgM (B) recognized by >5 endemic patients (or > two traveler patients) are indicated by horizontal colored lines. Proteomic coordinates and protein domains percentage identities are as in Supplementary Figure S8. T1: acute, T2: early convalescent, T3: mid convalescent and T4: late convalescent (orange dots) samples.



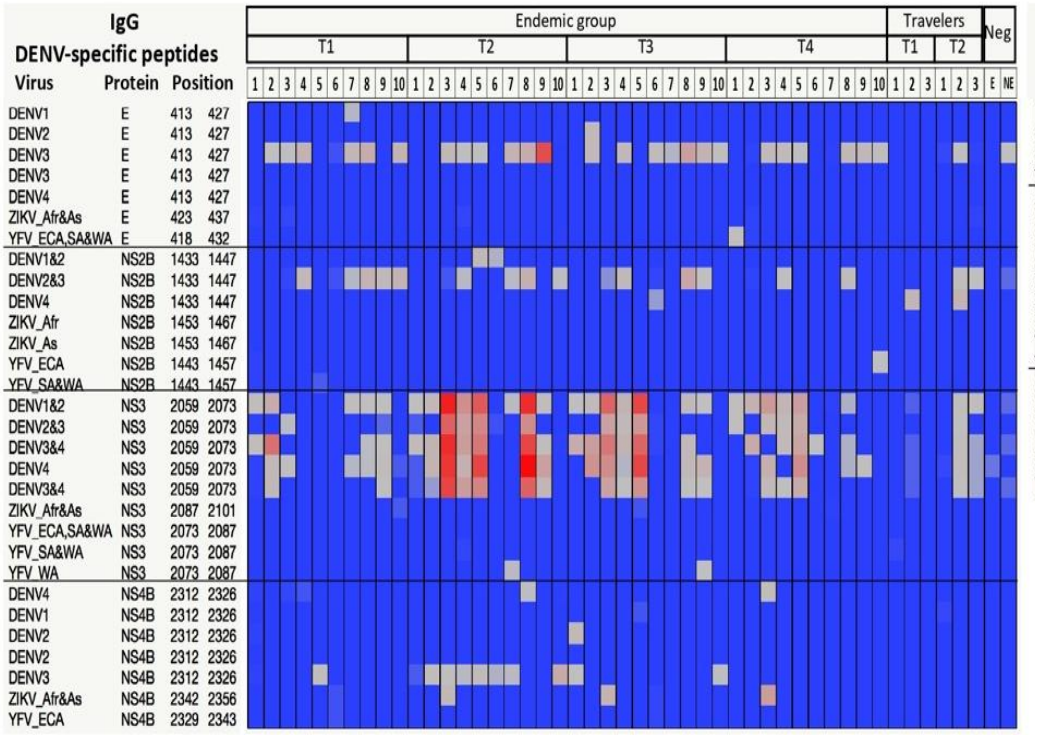
Supplementary Figure 11 | Identification of DENV specific and flavivirus broadly immunogenic epitopes in NS3 and NS5 proteins. IgM and IgG targeted epitopes at the NS3 (A) and NS5 (B) proteins from DENV, ZIKV and YFV viruses, recognized by DENV patients were filtered out and mapped to the proteomic coordinates of DENV (Uniprot ref: P33478). Colored bars from light-yellow to orange represent immunodominant regions belonging to one DENV serotype. Red bars represent immunodominant regions belonging to more than one serotype here called PanDENV. Green bars represent immunodominant regions belonging to DENV and ZIKV or/and YFV here called PanFlavi. For both panels the protein domains and the percentage identities are as in Supplementary Figure S8. Red and green arrows are DENV specific and PanFlavi peptides, respectively, which Abs responses are further detailed in Supplementary Figure S13.

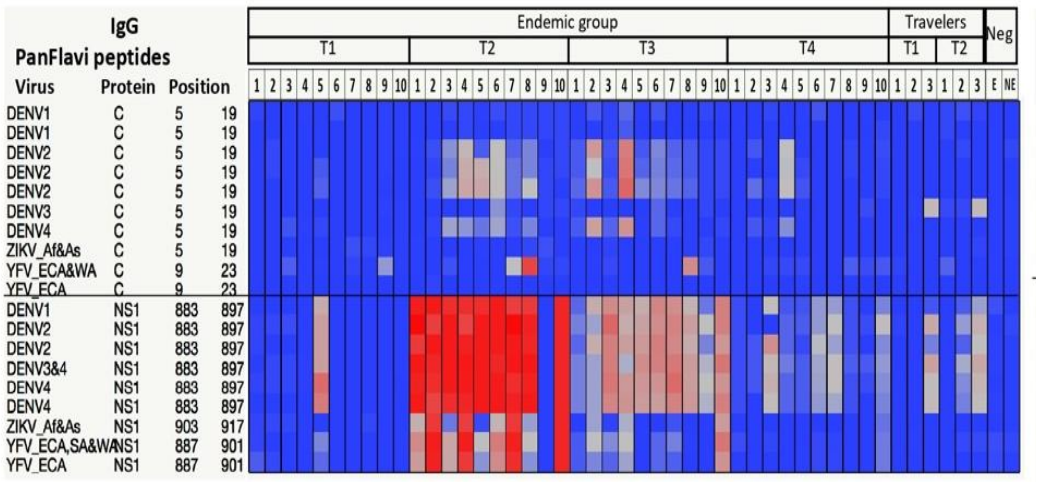


Supplementary Figure 12 | Identification of DENV specific and flavivirus broadly immunogenic epitopes in C, NS2A, NS2B, NS4A amd NS4B. IgM and IgG targeted epitopes at the E (A) and NS1 (B) proteins from DENV, ZIKV and YFV viruses, recognized by DENV patients were filtered out and mapped to the proteomic coordinates of DENV (Uniprot ref: P33478). Colored bars from light-yellow to orange represent immunodominant regions belonging to one DENV serotype. Red bars represent immunodominant regions belonging to more than one serotype here called PanDENV. Green bars represent immunodominant regions belonging to DENV and ZIKV or/and YFV here called PanFlavi. For both panels the protein domains and the percentage identities are as in Supplementary Figure S8. Red arrows are peptides which Abs responses are further detailed in Supplementary Figure S13. Purple and cyan arrows correspond to YFV and ZIKV peptides previously identified as ZIKV specific by Mishra et al. (31).

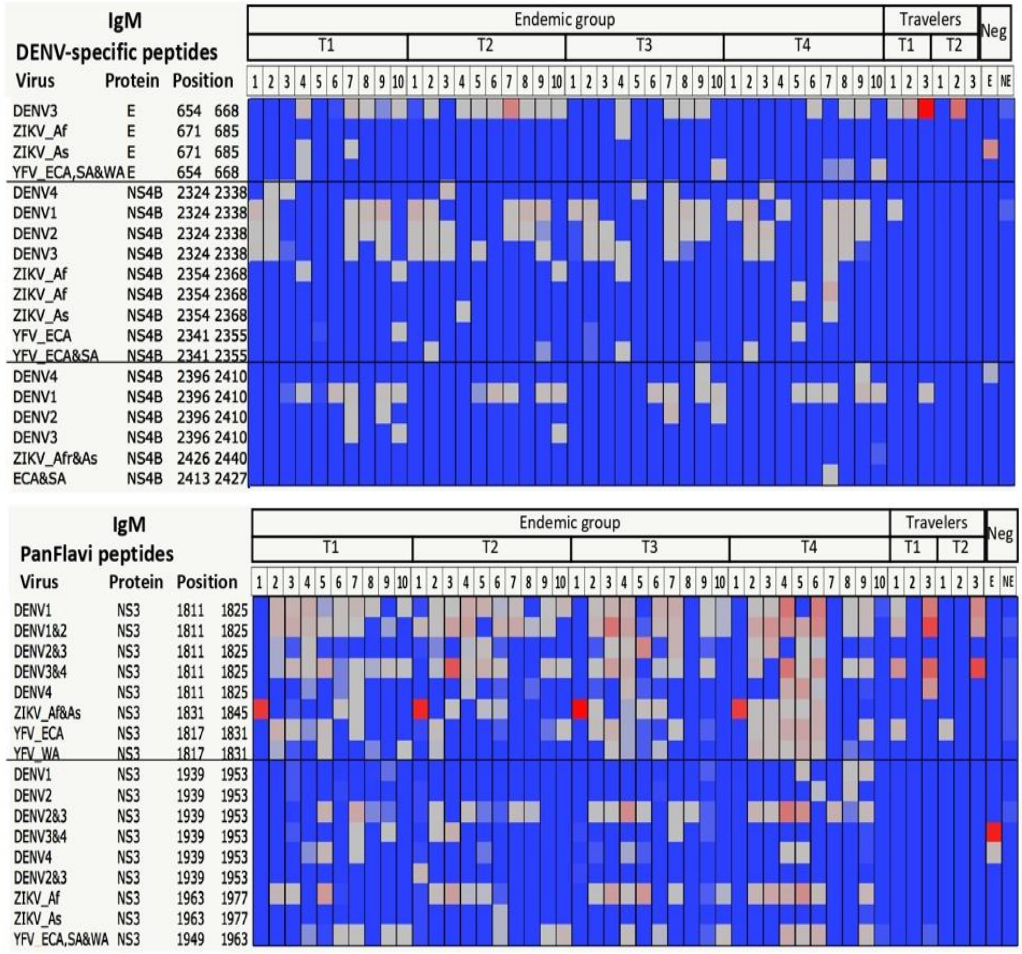


Supplementary Figure 13 | Heat map showing DENV-specific and PanFlavi peptides targeted by IgM and IgG Abs. Heatmaps depicting the Ab reactivity from endemic patients, traveler patients and negative controls against each peptide. The left panel correspond to IgG selected peptides while the right panel to IgM selected peptides. Each column represents a sample. The heatmap color scale indicates the fluorescence intensity going from low(blue) to high (red). Each row represents a peptide from the correspondent protein and the coordinates of DENV (Uniprot ref: P33478).

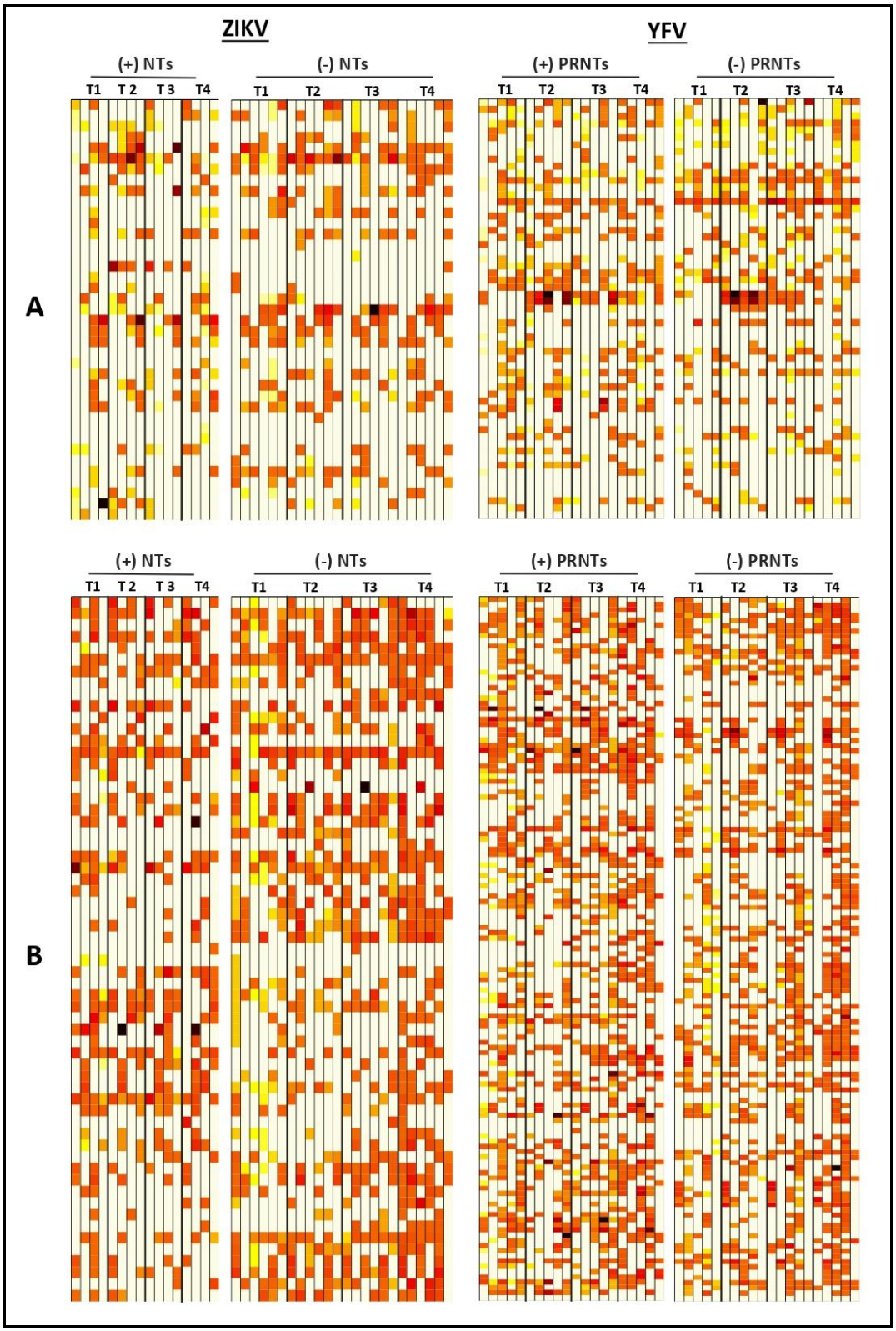




Supplementary Figure 13 | (Continued)



Supplementary Figure 14 | Heatmaps showing the humoral response against ZIKV and YFV ATRs. IgG (A) and IgM (B) responses from the endemic group against ZIKV and YFV ATRs were divided into patients with high neutralizing Abs (NAbs): (+) NTs, (+) PRNTs) titers or absent/low NAbs titers against ZIKV and YFV (see Table 1). T1 (acute), T2 (early convalescent), T3 (mid convalescent) and T4 (late convalescent) samples. Each column represents a sample. The heatmap color scale indicates the fluorescence intensity going from low (light yellow) to high (red). Each row represents an ATR.


ZIKV
YFV
(+) NTs
(-) NTs
(+) PRNTs
(-) PRNTs
T1
T 2
T 3
T2
T3
T4
A
B

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Chapter 5: Peptide biomarkers for the diagnosis of dengue infection

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5.1 Abstract

In a world with an increasing population at risk of exposure to arthropod-borne flaviviruses, access to timely and accurate diagnostic tests would impact profoundly on the management of cases. Twenty peptides previously identified using a flavivirus proteome-wide microarray were evaluated to determine their discriminatory potential to detect dengue virus (DENV) infection. This included nine peptides recognized by IgM antibodies (PM peptides) and 11 peptides recognized by IgG antibodies (PG peptides). A bead-based multiplex peptide immunoassay (MPIA) using the Luminex technology was set-up to determine Ab binding levels to each of these peptides in a panel of 323 carefully selected human serum samples. Sera are derived from individuals either infected with different viruses, namely, the four DENV serotypes, Zika virus (ZIKV), yellow fever virus (YFV), chikungunya virus (CHIKV), West Nile virus (WNV) and Human immunodeficiency virus (HIV), or receiving vaccination against YFV, tick-borne encephalitis (TBEV), and Japanese encephalitis virus (JEV). Additionally, a set of healthy controls were included. We targeted a minimum specificity of 80% for all the analysis. The PG-9 peptide had the best sensitivity (73%) when testing DENV sera from acute patients (A-DENV; <8 days since symptom onset). With sera from convalescent DENV patients (C-DENV; >10 days since symptom onset) the FPG-1 peptide was the best seromarker with a sensitivity of 86%. When combining all A-DENV and C-DENV samples, peptides PM-22 and FPG-1 had the best-diagnostic performance with a sensitivity of 60 and 61.1%, and areas under the curve (AUC) of 0.7865 and 0.8131, respectively. A Random forest (RF) algorithm was used to select the best combination of peptides to classify DENV infection at a targeted specificity >80%. The best RF model for PM peptides that included A-DENV and C-DENV samples, reached a sensitivity of 72.3%, while for PG peptides, the best RF models for A DENV only, C-DENV only and A-DENV + C-DENV reached a sensitivity of 88.9%, 89.1%, and 88.3%, respectively. In conclusion, the combination of multiple peptides constitutes a founding set of seromarkers for the discrimination of DENV infected individuals from other flavivirus infections.

5.2 Introduction

The World Health Organization (WHO) reported an increase from 3.2 million dengue symptomatic infections in 2015 to 5.2 million in 2019 (1). These numbers however, do not reflect the actual burden of the dengue virus (DENV), because in 2013 a report estimated that the number of dengue infections could reach 390 million annually worldwide (2). The difficulties to spot-on true dengue numbers are mainly attributed to the failure in the surveillance systems unable to capture cases that do not seek healthcare (under-ascertainment); and to report cases that do seek healthcare (underreporting). Among the underreported infections, the under-diagnosis is of specific concern in resource limited setting (RLS) where insufficient testing, poor deployment of diagnostic tools, and misdiagnosis with other febrile infectious diseases take place. In the Americas in 2020, the vast majority of the North American dengue cases reported to the Pan American Health Organization (PAHO) were lab confirmed, whereas in the Andean sub-region only 25% of the reported dengue cases were confirmed by a lab test (3).

Molecular techniques are preferred for the diagnosis of DENV because of their high sensitivity and specificity, however they are not the most widely applied due to the constraints to deploy them in RLS and the mostly short viremic window during which viral RNA can be detected in the blood. Serological tests on the other hand are more suitable for identifying infected individuals in RLS and tackle the problem of the narrow diagnostic window because anti-DENV antibodies (Abs) remain in the serum for much longer periods. The main concern with Ab-based detection techniques is cross-reactivity by Abs towards antigens (Ags) of other antigenically related flaviviruses, of which proteins can share approximately 60% or higher amino acid sequence identity (4). The detrimental implications of cross-reactivity on the accuracy of the serological tests leads to false-positive test results (5, 6).

The current increasing incidence of epidemics and spread across the tropical and subtropical world of different flaviviruses (7, 8) and immunization against different flaviviruses with vaccines for yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), and Japanese encephalitis virus (JEV) (9, 10), reinforce the importance for developing high-quality serological tests not only to accurately identify DENV infections, but also in distinguishing past from current infections to determine serostatus

for DENV pre-vaccination screening (11, 12). The accurate discrimination of anti-DENV Abs from Abs raised against related flaviviruses, with tests that can be properly deployed to LRS providing same-day results would significant impact on (i) offering opportune clinical management of DENV cases, (ii) determining suitability for vaccination, and (iii) improving surveillance systems for a better support of control intervention programs based on more reliable evidence-based decision making.

DENV serological diagnosis includes a wide spectrum of different formats and Ag designs (13), however low specificity has constantly been reported for these tests due to the presence of impurities in Ags widely used in commercial tests such as whole viral lysates. Recombinant proteins such as NS1 and Envelope proteins have helped in reducing false positivity and were rapidly adapted to different formats such as indirect ELISA, MAC-ELISA, and lateral flow devices for their commercial application (4, 14–17). Nevertheless, despite the progress made with the operational characteristics and good sensitivity, challenges with false positivity remain especially with the increasing co-circulation of arthropod-borne flaviviruses across the globe (4, 18–21). Therefore, more appropriate biomaterials that involves the selection of fragments with low sequence identity to proteins other than target are needed to circumvent the issues of cross-reactivity.

In previous work, we screened a 15-mer peptide microarray library covering the entire proteomes of the four DENV serotypes, ZIKV and YFV and identified 20 immunodominant peptides that were recognized by the sera of DENV infected individuals (22). Using a bead-based multiplex peptide immunoassay (MPIA), we report here the diagnostic potential of the selected synthetic peptides on a larger panel of carefully selected sera from individuals previously infected with DENV, ZIKV, YFV, WNV, CHIKV, and HIV or receiving vaccination against YFV, TBEV or JEV. This study validates our initial findings with respect to the specificity of the selected peptides for detecting anti-DENV Abs in clinical specimens and offers strong supportive evidence for the application of specific peptide combinations for next-generation DENV diagnostic tests.

5.3 Materials and methods

5.3.1 Human serum samples

Endemic samples: From a prospective longitudinal study carried out between July 2018 and March 2019 in the Santa Gema Hospital (SGH) in Yurimaguas, Peru, 136 patients with acute undifferentiated febrile illness, with a temperature ≥37°C for 7 days or less, together with at least one of the following symptoms: arthralgia, myalgia, head ache or rash, aged between 5 and 65 years old, were enrolled regardless of gender and ethnicity. DENV infection was confirmed by RT-PCR (n= 49 patients), as previously described (23). Four additional patients recruited in Iquitos, Peru in April 2018, positive for DENV by RT-PCR were also included. Samples were subsequently serotyped as DENV-2 using a multiplex RT-PCR protocol previously reported (24, 25).

A subset of 32 DENV patients were followed up and serum samples were collected up to 217 days after symptom onset (DASO). During this time, one, two or three additional serum samples were obtained making a total of 119 samples. Follow-up samples were collected depending on the willingness of the patient to donate additional samples for the study.

Two serum samples from Peruvian individuals with a YFV infection collected in 2007 were included in the set of DENV negative samples. YFV infection was confirmed by clinical symptomatology and the presence of IgM Abs by using an inhouse IgM capture ELISA developed by the National Institute of Health from Peru. Both patients had not received yellow fever vaccination at the time the samples were collected.

Non-endemic samples: Biobanked samples from travelers consulting the travel clinic at the Institute of Tropical Medicine Antwerp, Belgium, hosting the national reference center for arboviruses were selected. Returning travelers with a recent DENV infection (n = 18) confirmed by RT-PCR or IgM and/or IgG detection were included. A panel of 65 serum samples from Belgian citizens receiving vaccination against the flaviviruses TBEV (n = 16), JEV (n = 10), and YFV (n = 22) were included in the analysis. For YFV, follow-up serum samples were obtained from some individuals up to 1 year after receiving vaccination,

making a total of 39 serum samples. Belgian travelers returning from arbovirus endemic areas with a RT-PCR or a serology positive test for ZIKV (n = 58), WNV (n = 8) or CHIKV (n = 18) infection were also included. A set of 18 serum samples from HIV infected individuals were included as controls (**Table 1**).

Table 1. Overview of samples used in the study

Place	Virus	Infection/ Vaccination	Number of individuals	Number of samples	Age, years	Confirmatory test
Yurimaguas, Peru	DENV	Infection	49	110	6 - 64	RT-PCR (Ref. 36)
Iquitos, Peru	DENV	Infection	4	9	8 - 65	RT-PCR (Ref. 36)
Peru	YFV	Infection	2	2	>18	In-house capture IgM ELISA (NIH, Peru)
Lima, Peru	Healthy donor		1	1	>18	N.A.
Institute of Tropical Medicine (Antwerp, Belgium)	DENV	Infection	18	18	>18	RT-PCR/ELISA (IgM-IgG) (Ref. 39)
	ZIKV	Infection	58	58	>18	RT-PCR/ELISA (IgM-IgG) /IFA (IgM-IgG) / In-house VNT (Ref. 40)
	YFV	Vaccination	22	39	>18	In-house PRNT90 adapted from (Ref 41); Anti-Yellow fever IIFT (IgM / IgG) - Euroimmun
	TBEV	Vaccination	16	16	>18	In-house VNT90
	JEV	Vaccination	10	10	>18	In-house VNT90
	WNV	Infection	8	8	>18	RT-PCR / ELISA (IgM) Capture DxSelectTM / ELISA IgG DxSelectTM; Focus Diagnostics (Ref 42)
	CHIKV	Infection	18	18	>18	RT-PCR / IFA (IgM-IgG) Euroimmun (Ref 42)
	HIV	Infection	18	18	>18	Enzygnost Anti-HIV 1/2 Plus Vironostika HIV Uniform II Ag/Ab; Innotest HIV Ag mAb; InnoLIA HIV I/II Score
	Healthy donors		16	16	>18	N.A.

DENV: dengue virus, YFV: yellow fever virus, ZIKV: Zika virus, TBEV: tick-borne encephalitis virus, JEV: Japanese encephalitis virus

WNV: West Nile virus, CHIKV: chikungunya virus, HIV: Human immunodeficiency virus

RT-PCR: real time PCR; VNT90: virus neutralization test; PRNT90: plaque reduction neutralization test

IFA: immunofluorescence assay, IIFT: indirect immunofluorescence test; NA: not applicable

Negative samples from healthy blood donors: Sixteen samples from healthy citizens of the city of Antwerp were selected from a panel of sera with ethical approval for broad Ab testing. One serum sample from a citizen of the city of Lima with no register of visiting endemic areas or receiving the yellow fever vaccine, who tested negative to flavivirus IFAT (Euroimmun, Lübeck, Germany) was included in the analysis (Table 1).

For the assessment of the potential use of the peptides for the diagnosis of DENV, we considered as positive samples those sera from patients with a DENV infection confirmed by RT-PCR (endemic individuals), and/or by IgM or IgG detection (returning travelers). The negative samples included sera from individuals with history of flavivirus exposure either by infection or vaccination, sera from CHIKV patients, sera from HIV patients, and sera from healthy donors. The presence of anti-DENV antibodies was not ruled out in the negative sample set.

All serum samples were heat inactivated at 56°C for 30 min before serological analyses.

5.3.2 Peptides

Nine peptides recognized by IgM Abs (PM peptides) located in the E, NS1, NS2a, NS2b, NS3, and NS4b proteins, and 11 peptides recognized by IgG Abs (PG peptides) located in the prM, E, NS1, NS2b, NS3, NS4b, and NS5 proteins and that were previously identified to be highly immunogenic (22) were selected for further analysis. Figure 1 shows the location of the peptides in the DENV proteome.

Peptide synthesis was done according to standard protocol by using solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with automated synthesizers (Genecust, Boynes, France). A cysteine was added to the N-terminus to facilitate conjugation of BSA. The purity of peptides was confirmed to be >95% by Mass spectrum analysis and HPLC. Peptide information is detailed in Table 2. Additionally, the viral lysates (VL) from the four DENV serotypes (D1–4) (ZeptoMetrix®, NY, USA) were included as positive controls in the analysis.

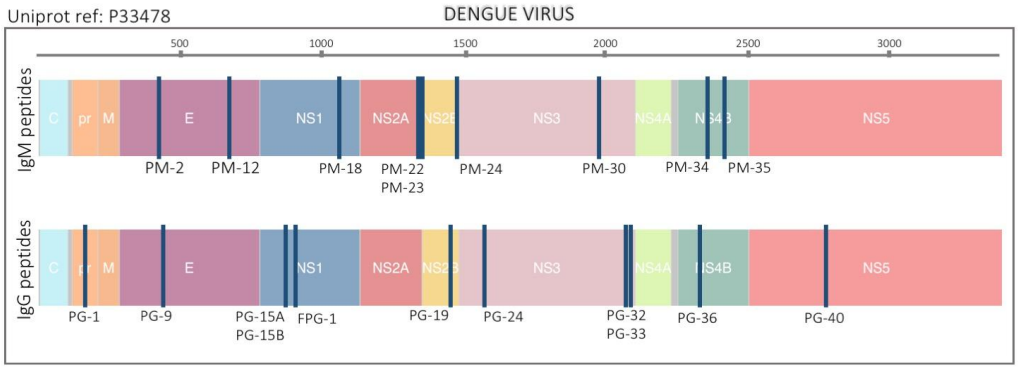

Figure 1. Location of peptide biomarkers in the DENV proteome.
Location of peptide biomarkers in the DENV proteome. Selected peptides are plotted relative to the polyprotein coordinates (start amino acid position) for DENV (P33478). Each blue vertical bar represents a single peptide. Top and bottom plots are IgM and IgG peptides respectively. Structural proteins: Capsid (C), Membrane (pr, M) and Envelope (E), and non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 are color coded. The peptide names are also indicated.

5.3.3 Multiplex peptide immunoassay (MPIA)

We used a serological assay based on the Luminex technique to measure IgM and IgG immunoglobulin levels. The covalent coupling of the nine IgM peptides, eleven IgG peptides and the four VL-D1-4 Ags to paramagnetic MagPlex 6.5 µm COOH microspheres from Luminex Corporation (Austin, TX) was carried out as previously described (30, 31). In brief, all peptides and the four VLs were coupled at a concentration of 5 µg/ml for 106 beads/µl. IgG and IgM Abs were measured in separate assays. A mixture of the antigen-coupled microspheres was prepared in a hypertonic phosphate buffered saline, 1% BSA, 0.05% Sodium Azide solution (PBS-BN) to a final concentration of 1,000 beads/antigen/well. A mixture of 25 µl of coupled microspheres was added to a 96-well µClear® flat bottom microplate (Falcon™ 353072). Serum diluted 1:100 was found to be the optimal serum concentration to measure both IgM and IgG Abs based on serial dilution standardization. For the detection of IgM Abs, GullSORB™ IgG inactivation reagent (Meridian Bioscience™) in 1:10 dilution was added to the sera to remove the rheumatoid factor prior to testing. The wells were then incubated with 50 µl of human serum diluted 1:100 in blocking solution (PBS-TBN:

PBS-BN + 0.05% Tween). Microplates were incubated for 1 h at room temperature (RT) on a plate shaker (600 rpm) in the dark. The plates were washed three times with PBS-TBN. Following the washing step, the PE conjugated secondary goat anti-human IgG or anti-human μ- chain IgM (Jackson Immuno Research, PA, USA) Ab diluted 1:125 in 100 μl PBS-TBN per well was added for 45 min at RT on a plate shaker (600 rpm) in the dark. After a second washing step, beads were resuspended in 150 μl of PBS-BN. Plates were put on the plate shaker (900 rpm) in the dark for 5 min before reading. Data was acquired using a Luminex® 100/200 analyzer. Results were expressed as median fluorescent intensities (MFI). All tests were performed in duplicates and in two independent experiments. Raw data with the MFI for each of the peptides is detailed in Supplementary Table 1.

5.3.4 Diagnostic performance of the DENV peptides

The Receiver operating characteristic (ROC) curves, their corresponding area under the curve (AUC), and the specificity and sensitivity of the IgM and IgG assays, were established using all 137 DENV-positive samples (acute and convalescent), and 186 DENV-negative samples (ZIKV, TBEV, JEV, WNV, CHIKV, HIV, and negative control sera). Acute samples from endemic patients were also included for the classification performance because most of these were IgG positive in DENV-ELISA probably as a result of previous flavivirus infections. The serum samples coming from DENV positive cases were stratified into two groups based on the time since onset of symptoms: (i) acute samples (A-DENV), ≤8 DASO, N = 54 and (ii) early convalescent samples (E-DENV), ≥10 and ≤70 DASO, N = 46. A third group (iii) was made that contains all samples: A-DENV, E-DENV and late convalescent (L-DENV), ≥71 up to 230 DASO, N = 38. The trade-off between sensitivity and specificity was graphically displayed with the ROC curve analyses. Based on this analysis cut-off values were assigned to each peptide in single-plex. We calculated the AUC and selected three targets: a sensitivity of at least 80%, and with this constraint, (i) the specificity is maximized, ii) the sensitivity and specificity are equally weighted, so that a combination that maximizes sensitivity and specificity is selected, and iii) when a specificity of at least 80% is enforced, the sensitivity is maximized.

Table 2. Overview of the peptides used in the study

Antibody class	Peptide	Sequence (N-terminal to C-terminal)	g/mol	Isolelectric point	Length	DENV Serotype	DENV Protein	Polyprotein position	
								Start	End
IgM	PM-2	CVTKLEGKIVQYENL	1737.03	5.71	15	DENV1	E	401	415
	PM-12	CPPFGESNIVIGIGDK	1645.88	4.18	16	DENV3	E	654	668
	PM-18	CAGPWHLGRLEMDFDF	1894.15	4.36	16	DENV2	NS1	1039	1053
	PM-22	CPMAVAAMGVPPLPLF	1614.06	5.28	16	DENV3, DENV4	NS2A	1319	1333
	PM-23	CTAIFLTTLSRTSKKR	1826.18	11.65	16	DENV2	NS2A	1330	1344
	PM-24	CVFPVSIPITAAAWYL	1751.1	5.28	16	DENV2	NS2B	1453	1467
	PM-30	CEPLENDEDCAHWKEA	1888.99	3.84	16	DENV2, DENV3	NS3	1951	1965
	PM-34	CLLAIGCYSQVNPITL	1708.06	5.28	16	DENV2	NS4B	2336	2350
	PM-35	CAIDLDPVVYDAKFEK	1826.08	4.06	16	DENV1	NS4B	2396	2410
IgG	PG-1	CDGVNMCTLMAMDLGE	1703.02	3.38	16	DENV2	prM	143	157
	PG-9	CENLKYTVIITVHTGD	1806.05	5.39	16	DENV3	E	413	427
	PG-15A	CNELNYILWENNIKLT	1980.25	4.26	16	DENV3, DENV4	NS1	847	861
	PG-15B	CNELNYVLWEGGHDLT	1863.02	3.93	16	DENV4	NS1	847	861
	FPG-1	CMELKYSWKTWGKAKI	1972.39	10.02	16	DENV3, DENV4	NS1	883	897
	PG-19	CEEEEQTLTILIRTGL	1848.09	3.82	16	DENV2, DENV3	NS2B	1433	1447
	PG-24	CISYGGGWRFQGSWNT	1818.97	7.74	16	DENV1, DENV2	NS3	1551	1565
	PG-32	CKEGERKKLRPRWLDAR	2141.51	11.02	17	DENV1, DENV2	NS3	2059	2073
	PG-33	CRKKLRPRWLDARTYSD	2164.5	10.86	17	DENV1, DENV2	NS3	2063	2077
	PG-36	CANQAVVLMGLDKGWP	1702.02	5.55	26	DENV1	NS4B	2312	2326
	PG-40	CTRHVAVEPEVANLDI	1765.99	4.42	16	DENV1	NS5	2753	2767

To assess the prediction capacity of peptide combinations, ROC curves, their correspondent AUC, and the specificity and sensitivity of the IgM and IgG assays were calculated using the predicted values estimated by supervised machine learning Random Forest (RF) algorithm models as implemented in the R-package 'randomForest' (32). Samples were stratified and randomly spliced into a training and a test set. The training samples were used to fit a random forest classifier which then predicted the negative vs. positive category of unseen test samples (2/3 of total samples). This was repeated n =

50,000 times. Variable (each antigen) importance was assessed using the 'varImplot' function of the same package and was ranked according to the 'mean decrease in accuracy' and 'mean decrease in Gini'. The peptides ranking in the top six of the 'mean decrease in accuracy' were selected for the training and cross-validation analysis. Three RF models were built for PM peptides (RFM) and PG peptides (RFG), namely, (i) A-DENV samples (RFM1 and RFG1), (ii) E-DENV samples (RFM2 and RFG2), and (iii) A-DENV+E-DENV+L-DENV (RFM3 and RFG3). Each of these positive control sets was analyzed with the negative samples (n = 185). For each model, we calculated the AUC and enforced a specificity target of at least 80%, and based on these constraints, sensitivity was maximized. Classification algorithms implemented in R (version 3.6.3) were adapted from Rosado et al. (33).

The R Stats package was used to perform this analysis and Spearman's rho statistics was used to estimate the rank-based measure of association. Heatmaps based on the calculated rho scores were created for the analyzed IgM and IgG peptides. For all analyses, differences with probabilities of $p < 0.05$ were considered statistically significant. Differences in measured Ab responses were assessed using the non-parametric (Steel-Dwass) tests for independent pair-wise comparison with Bonferroni adjustment. Differences in classification performance were assessed by pairwise comparison using McNemar's test.

5.3.5 Ethical clearance

The study was approved by the ethical review boards of the Peruvian University Cayetano Heredia, Peru (Protocol No. 101480), the Institute of Tropical Medicine Antwerp, Belgium (Protocol No. ITG 1304/19) and the University of Antwerp, Belgium (Protocol No. 19/42/477). This study was conducted in compliance with the ethical standards of the latest amended Declaration of Helsinki and of the International Conference Harmonization (ICH) guidelines, plus adhering to local laws and regulations.

5.4 Results

5.4.1 Antibody levels against DENV peptides

In a previous study, using a high throughput 15-mer peptide microarray, immunogenic epitopes recognized by IgM and IgG Abs present in the sera of confirmed DENV infected individuals were identified. In order to further evaluate their diagnostic capacity, two multi-antigen assays were developed, one with the nine PM peptides recognized by IgM Abs and a second with ten PG peptides out of the eleven peptides recognized by IgG Abs. The PG-36 peptide was unable to couple to the microspheres and therefore was not included in the multiplexing. For each assay, the peptides plus the VLs (containing the entire viral proteome) of the four DENV serotypes were immobilized on microspheres to allow the detection of IgM and IgG Abs in a panel of 323 serum samples of DENV, ZIKV, YFV, TBEV, JEV, WNV, CHIKV, HIV, and negative healthy controls.

When assessing the levels of IgMand IgG Abs directed towards the peptides, the MFI values for most of the peptides were significantly higher in DENV-infected individuals compared to the levels in the DENV-negative samples that included sera from individuals with exposure to other flavivirus (Supplementary Table 2; Positive vs Negative, $P < .05$), however in four out of the 19 peptides evaluated (i.e., PG-9, PG-15A, PG-19, and PG-24) this difference was not significant ($P > .05$).

Figure 2 shows the comparison between the Ab response against each peptide for the different groups of samples. In general, the measured IgG levels against the evaluated Ags were higher than the IgM levels. From the graph, it can be seen that IgM levels against PM-22 and IgG levels against FPG-1 were clearly higher in the DENV group than in the other groups of samples. Sera from individuals with a flavivirus history of infection (ZIKV, WNV) or vaccination (YFV, TBEV, JEV), showed lower IgM titers against PM peptides, but similar IgG levels against the PG peptides when compared with the Ab levels from DENV infected individuals. Of note, high IgM Ab levels against peptides PM-18, PM-22, PM-23, PM-24, PM-34, and PM-35 were observed in CHIKV infected individuals.

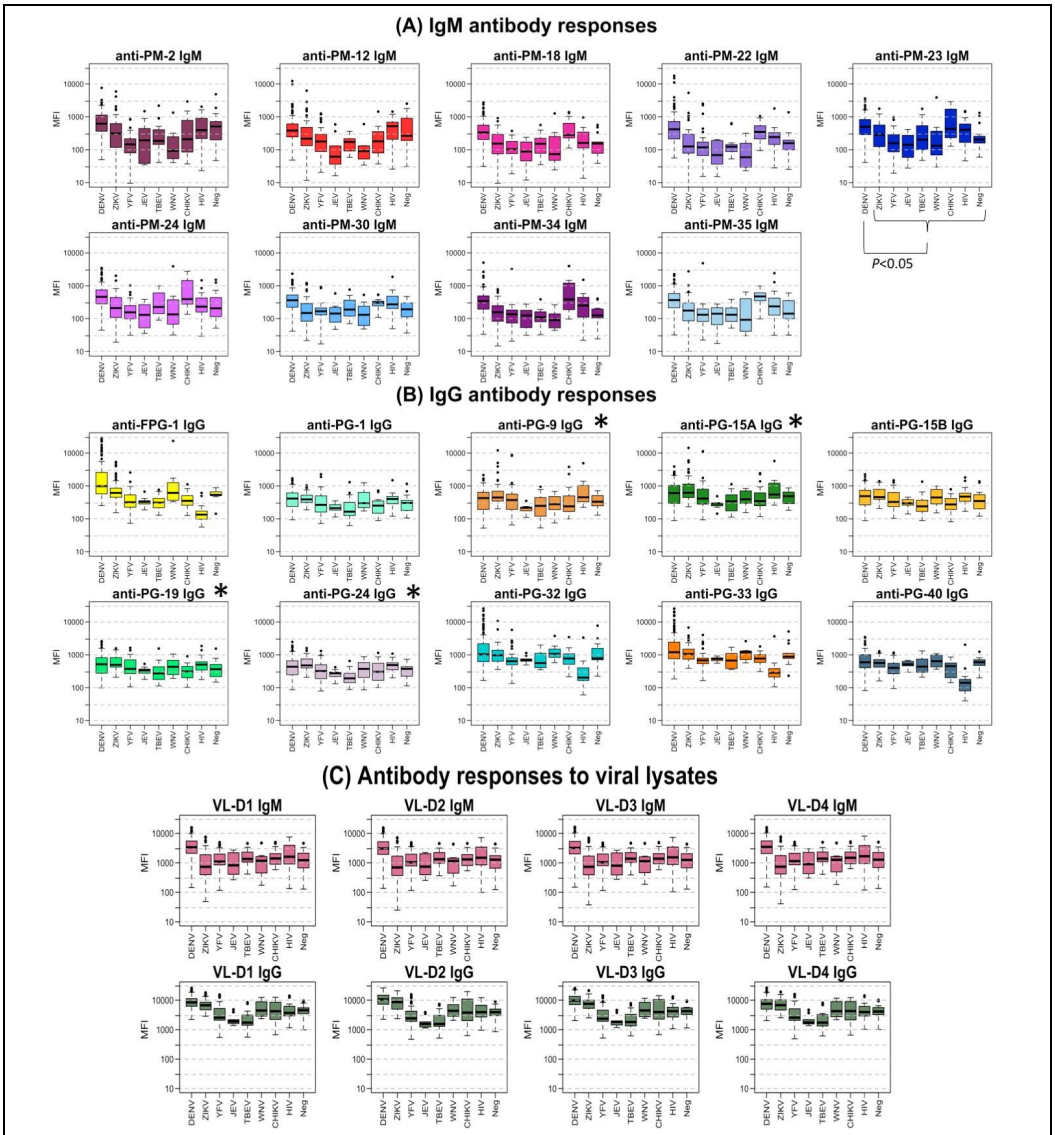

Figure 2. Antibody responses to peptide biomarkers. (A) Antibody responses to peptide biomarkers. (A) Boxplot display of the IgM response against the peptides in median fluorescence intensity (MFI) values in serum samples for each virus group. Positive DENV samples (n = 137) where confirmed by RT-PCR in endemic patients and RT-PCR and/or IgM/IgG seroconversion in travelers. DENV-negative samples (n = 185) corresponded to ZIKV, YFV, TBEV, JEV, WNV, CHIKV, HIV, and negative controls from non-endemic healthy donors. Horizontal box boundaries and midline denote sample quartiles. (B) Boxplot display of the measured IgG against the biomarkers in MFI values in serum samples from each group. (C) Boxplot display of the measured IgM and IgG (MFI) against the viral lysates (entire proteome) of the four DENV serotypes in serum samples from each group. Significant differences (P<.05) between MFI values in DENV-infected individuals compared to the response from people with exposure to other flaviviruses was found for all peptides, except for those labeled with *.

When analyzing only the dengue positive samples, we stratified the samples into different groups in an attempt to determine if the biomarkers could differentiate between: (i) endemic vs. nonendemic samples, (ii) hospitalized vs. non-hospitalized, and (iii) acute vs. convalescent samples (Supplementary Table 2). The Ab levels against peptides PM-2, PM-35, PG-1, PG-15B, PG-19, and PG-40 were significantly higher in samples coming from endemic individuals in comparison to the Ab levels measured in nonendemic samples ($P < .05$). DENV patients that were hospitalized after presenting severe symptoms of DENV showed significantly higher IgG levels against PG-33 compared to the Ab levels in non-hospitalized patients ($P = .0087$). The IgG levels against FPG-1 were higher in E-DENV compared to A-DENV samples ($P < .001$) and to L-DENV ($P < .05$) (Supplementary Figure 1).

Next, we represented the Ab-binding data of the PM and PG peptides using a cell plot. The FPG-1 peptide showed a clear discriminatory response, higher titers (red cells) are observed in DENV positive samples, while sera from individuals with exposure to arboviruses other than DENV show low MFI values against this peptide (blue cells) (Supplementary Figure 2).

5.4.2 Performance assessment of the DENV peptide biomarkers

ROC curves were used to compare the diagnostic value of the 19 peptides individually (Figure 3). High AUCs mean high specificity and high sensitivity and, therefore, a greater predictive capacity of the test. For the PM peptides, when DENV positive samples were analyzed separately into acute and convalescent samples, their diagnostic performance in terms of AUCs were lower compared to the analysis of all samples (acute and convalescent) together (Figures 3A, C and Supplementary Table 3). On the other hand, when only acute samples were included in the analysis, the AUC of peptides PG-1, PG9, PG15A, PG15B, PG-19, and PG-24 increased, while if only convalescent samples were included, FPG1, PG-32, and PG-33 showed an improvement in their diagnostic performance (Figures 3B, C). When specificity was targeted to at least 80%, PM-22 and FPG-1 were the best biomarkers to diagnose DENV infections regardless of the time after symptom onset, with specificity, sensitivity and AUC values of 80.4%, 60.3%, and

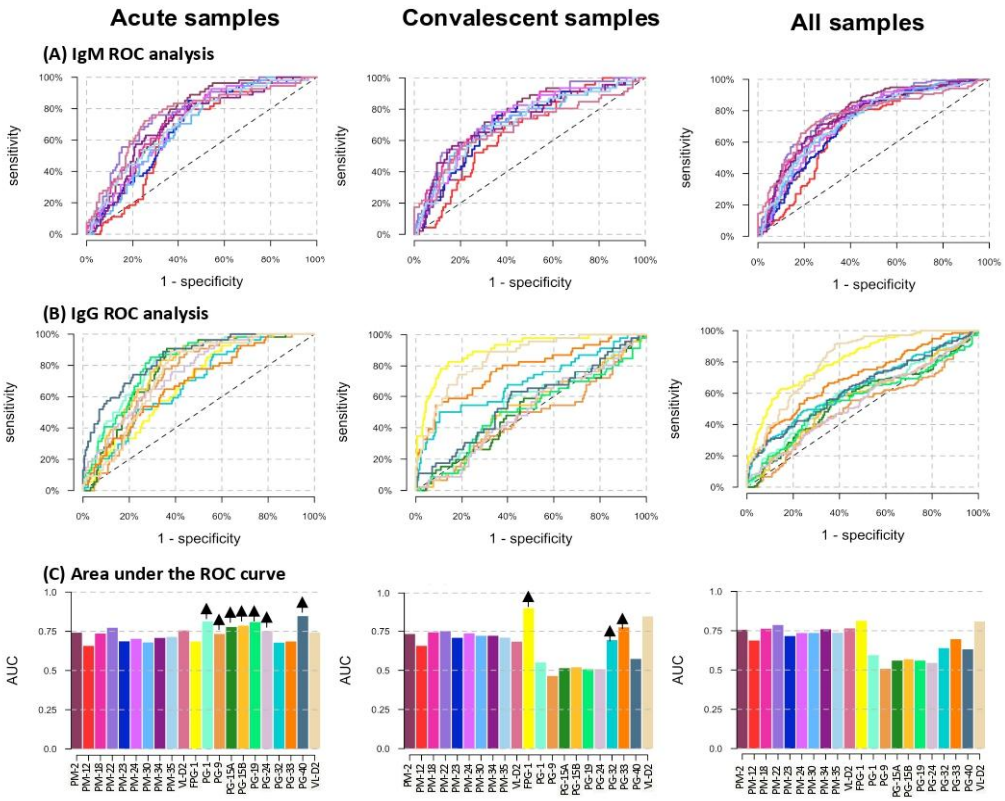

Figure 3. Diagnostic performance of the peptide biomarkers. (A) ROC curves for IgM peptides and (B) IgG peptides. The three columns correspond to the separate analysis of the data considering as positive DENV samples: acute samples only (left graphs), convalescent samples only (center graphs) and acute + convalescent (right graphs). Colors correspond to Abs against the different antigens as shown in panel C. (C) Area under the ROC curve (AUC) for individual biomarkers. Arrows in the top of each bar indicates an increase in the AUC of the peptide when only acute DENV or convalescent DENV samples are included as positive controls in the analysis.

0.7865 for PM-22 and 80.5%, 61.1%, and 0.8131 for FPG-1, respectively (Supplementary Table 3). When only A-DENV samples were included in the analysis, the best PG biomarkers to detect DENV infection are PG-1, PG-19, and PG-40 with 69.7%, and 0.8114, 69.7%, and 0.8114, and 69.7%, and 0.8114, of sensitivity and AUC respectively and minimally 80% specificity. The diagnostic performance of FPG-1 improved when only E-DENV samples were included in the analysis, reaching a sensitivity of 86.5% and an AUC of 0.9031 (Supplementary Table 3). For a targeted 80% specificity, cut-off values were calculated and the classification outcomes (positive or

negative) were obtained for each peptide. Based on this classification, discriminatory test performance was performed for the peptides using the McNemar's and Cohen's kappa tests. When the classification outcome of each peptide was pairwise compared with the reference classification (DENV-positive and DENV-negative, Table 1), FPG-1 and PM-22 rendered the best discriminatory test performance (McNemar's test: Supplementary Table 4) and the highest agreement with respect to the reference (Cohen's kappa test: Supplementary Table 5).

We then evaluated the correlation between the Ab levels against the different biomarkers using the Spearman's rank correlation test (Figure 4). The Ab levels between the nine PM peptides were strongly correlated (Figure 4A). In the case of the PG peptides, two groups were observed: (i) a group comprising PG-1, PG-9, PG-15A, PG-15B, PG-19, and PG-24 peptides with a strong correlation between them, that at the same time showed low correlation with the VL-D2, and (ii) a second group of FPG-1, PG-32, PG-33, and PG-40 peptides that showed weak correlation between them, while only FPG-1 showed good correlation with the VL-D2 (Figure 4B).

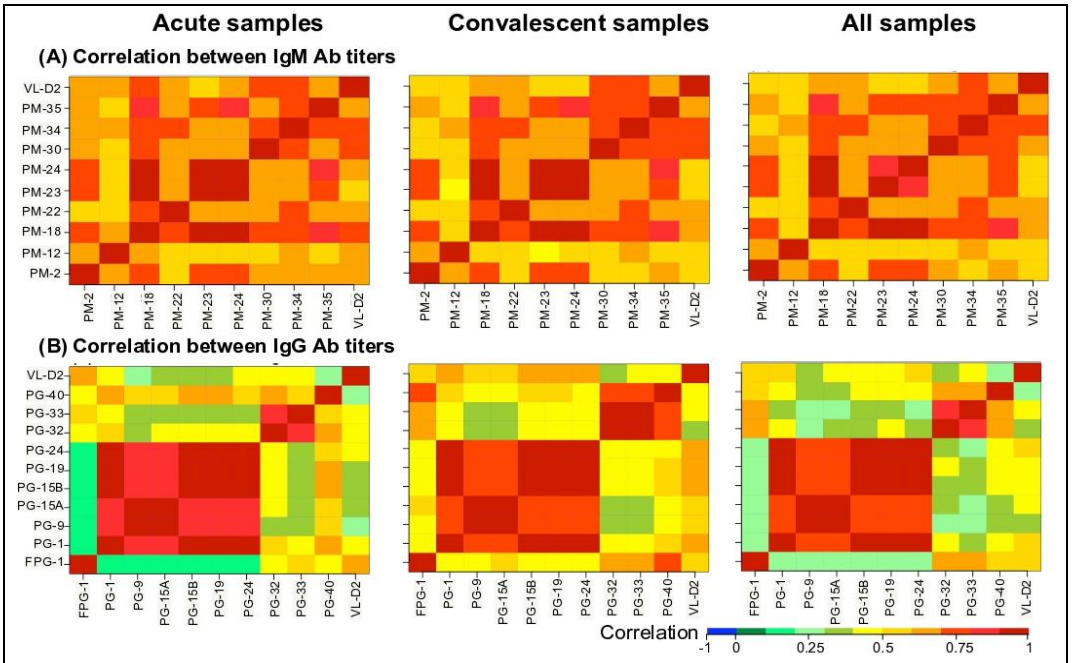

Figure 4. Heatmap of the Spearman's correlation coefficient between biomarkers. (A) IgM and (B) IgG Ab responses against the peptides were correlated. Correlation coefficients are indicated by the color scale. Blue indicates a negative correlation; red indicates a positive correlation.

5.4.3 ROC performance analysis combining multiple peptides

To determine if the combination of peptides could improve the overall diagnostic performance in terms of sensitivity, specificity and AUC to differentiate DENV infected from non-infected individuals, three RF models were built for each of the PM and PG peptides.

The importance of each biomarker in the outcome variable was ranked by RFs, targeting a minimal specificity of 80%. The variable importance plots for the three RF models are detailed in Supplementary Figure 3. A higher Mean Decrease in Accuracy (MDA) indicates higher importance of the variable in the model. The top six peptides ranked in the MDA plots were selected to be included in the training and validation analysis. For PM peptides, PM-22 was ranked first in the three RFM models and PM-2 second for RFM1 and RFM3; while for PG peptides, FPG-1 ranked first for RFG2 and RFG3 and PG-40 had the highest importance in the classification algorithm for the RFG1 model.

The combination of multiple peptides in the RFM and RFG models showed to be superior in terms of sensitivity, specificity and AUC compared to single peptides. For the RFM3 model that included peptides PM-22, PM-2, PM-30, PM-34, PM-23, and PM-12, the sensitivity reached 72.3% (95%CI, 64.2–79.1) (Figure 5B). Using the RFM1 and RFM2 models did not improve the diagnostic performance (Supplementary Table 6). For the PG peptides, the RFG3 model that included peptides FPG-1, PG-40, PG-15A, PG-33, PG-1, and PG-9 rendered a sensitivity of 88.3% (95%CI, 81.9–92.7) (Figure 5D), while higher sensitivities were obtained for the RFG1 model that contained PG-40, PG-15A, FPG-1, PG-19, PG-1, and PG-24 peptides (88.9%, 95%CI, 77.8–94.8) and for the RFG2 model comprising FPG-1, PG-33, PG-9, PG-32, PG-15A, and PG-1 peptides (89.1%, 95%CI, 77–95.3) (Supplementary Figure 4). The sensitivity of the RFG3 model was 84.7% (95%CI, 77.7-89.8) and 81% (95%CI, 73.6-86.7) when the targeted specificity was set to a minimum of 85% or 90%, respectively (Supplementary Figures 4E, F). The ROC curves for the RFM3 and RFG3 models are shown in Figures 5A, C, and for the RFG1 and RFG2 models in Supplementary Figures 4A, C. The sensitivity values, targeting a minimal specificity of 80% for the multiple combinations of peptides are shown in Supplementary Table 6.

The corresponding curves of the relationship between DENV prevalence and the positive (PPV) and negative (NPV) predictive value for the RFM3 and RFG3 models at the calculated sensitivity and specificity are shown in Figure 6. For an assumed DENV prevalence of 15% the PPV and NPV will be 40 and 94.6% for the RFM3 model, and 44.4 and 97.5% for the RFG3 model, respectively.

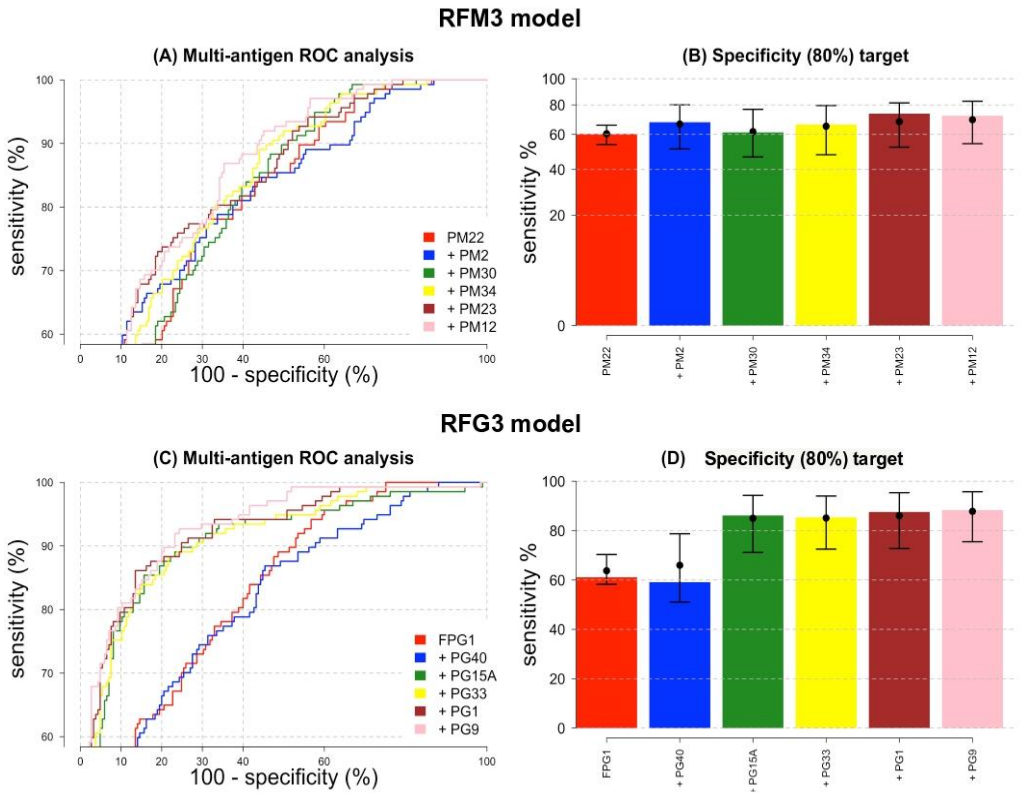

Figure 5. ROC performance analysis combining multiple peptides using a random forest algorithm. ROC curves for (A) IgM and (C) IgG peptides. The peptides are added sequentially based on their classification accuracy. The axes have been rescaled to better differentiate between high values of sensitivity and specificity. (B) For a pre-defined assay specificity of 80%, the assay sensitivity increases with sequentially adding additional peptide biomarkers for detecting IgM (B) and IgG (D). Sensitivity was estimated using a random forest classifier. Points and whiskers denote the median and 95% CIs from repeat cross-validation.

5.4.4 Comparison of MPIA with commercial Dengue diagnostic tests

We compared the results from the Luminex MPIA with two commercial test kits, i.e., DENV ELISA for IgM and IgG (Euroimmun, Lübeck, Germany) and a rapid diagnostic

test (RDT) for combined detection of NS1 antigen, IgM and IgG (Standard diagnostics (SD), Yongin-Si, Korea) in a subset of 41 patients from an endemic region and with a confirmed DENV infection (n = 41 acute samples, n = 27 early convalescent samples, n = 14 late convalescent samples). There was a weak but significant positive correlation between the IgG OD ratios from the DENV ELISA and the MFI values for the FPG-1 peptide in the MPIA (r = 0.51, P <.001). Also, weak but significant positive correlations were observed between the IgM OD ratios from the DENV ELISA and the MFI values for the peptides PM-18 (r = 0.28, P = .0.0099), PM-23 (r = 0.32, P = .0037) and PM-24 (r = 0.31, P = .0047) in the MPIA (Supplementary Figure 5A).

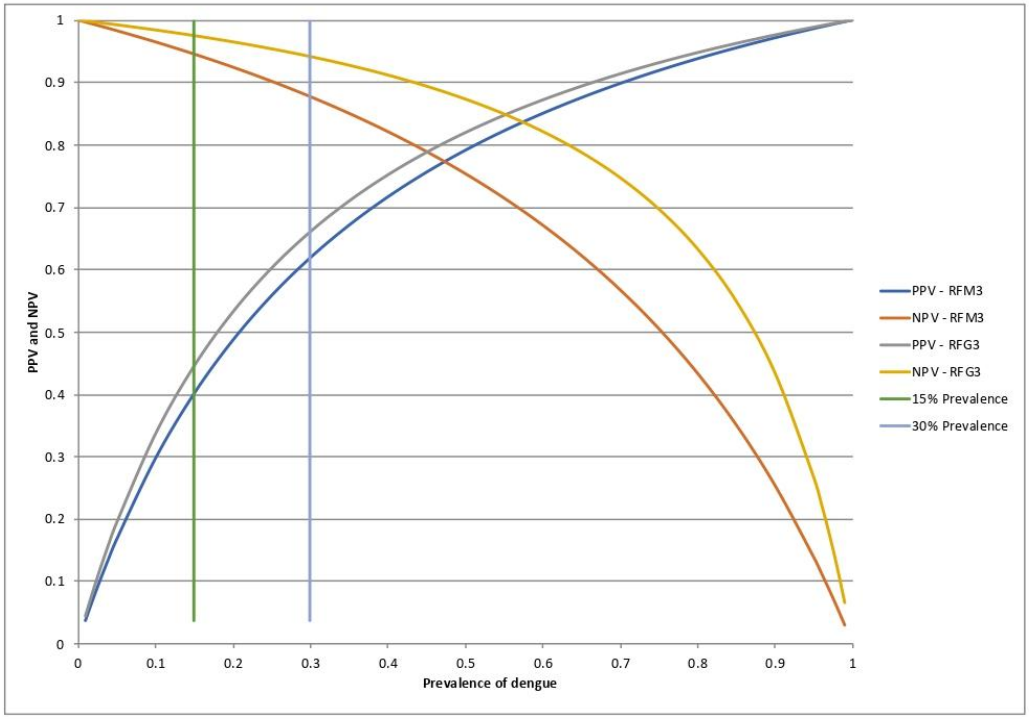

Figure 6. Positive predictive values (PPV) and negative predictive values (NPV) for the RFM3 and RFG3 models. According to the Random Forest analysis for the combination of multiple peptides, the calculated specificity and sensitivity values were fixed at 80% and 74% for the RFM3 model, and 80% and 88% for the RFG3 model, respectively. The RFM3 model included the following peptides: PM-22, PM-2, PM30, PM-34, PM-23, and PM-12. The RFG3 model included the FPG-1, PG-40, PG15A, PG-33, PG-1, and PG-9 peptides. PPV and NPV calculated at a pre-set DENV prevalence of 15% and 30%, which corresponds prevalence which corresponds to the intersection of the horizontal bars with the curves.

We also performed pairwise comparisons between the commercial DENV ELISA and the evaluated peptides. For the PM peptides, we observed that less than 26% of the evaluated samples were positive in both assays and for those peptides that showed significant correlation with the commercial IgM ELISA, acute and early convalescent samples were involved in this correlation (Supplementary Figure 5B). While for PG peptides, about 60% of the samples were positive for FPG1 and the IgG ELISA, of which approximately 71% of the samples were convalescent. For the other PG peptides, the percentage of samples that were positive in both assays was less than 45% and the samples were mostly acute phase samples (>75%), except for the PG-32 and PG-33 peptides for which 56 and 62% (respectively) of the samples that were positive in both assays were categorized as convalescent (Supplementary Figure 5C).

A more detailed comparison of the MPIA with the DENV ELISA and RDT commercial tests is shown in Supplementary Figure 6. The cell plot indicates the classification of each sample as positive or negative for each seromarker used. The classification of the samples as positive or negative when using the RFM 1, 2, and 3 models was the same. Thus, the three RFM models were able to equally identify 32 out of the 41 DENV patients (78%) and they matched in 21 out of the 41 (51%) ADENV samples and in 12 out of the 27 (44%) E-DENV samples with the DENV IgM ELISA, and in 19 out of 40 A-DENV (47.5%) with the IgM component of the DENV RDT. The RFG1 model identified as positive 36 out of the 41 (88%) DENV acute samples, convalescent sera were available for three out of the five samples classified as negative in the acute sample, but none of them showed antibodies against the combination of peptides included in the RFG1 model. For the RFG3 model, samples seroconverted against peptides multiplexed in this model, for which the positivity percent went from 80% (33/41) in A-DENV samples to 88% (36/41) in E-DENV samples.

5.5 Discussion

Diagnostic testing has a central position in outbreak control. Without diagnostic tests, it is impossible to trace whether people with the disease have infected others, whether the virus persists in survivors, or to investigate the cause of deaths. These objectives can only be accomplished when tests are available with an excellent diagnostic performance.

Unfortunately, the current tests available for the detection of Abs against arboviruses in general, and DENV in particular, do not meet these criteria. Specificity represents a major problem given that most of them are based on the use of whole (recombinant) proteins. DENV proteins contain epitopes that are unique to DENV, and also epitopes with high amino acid identity to those present in other flaviviruses (4), therefore in the current context of increasing global circulation of flaviviruses, the use of whole-protein Ags, either natural or recombinant, has intrinsic problems because they can capture cross-reactive Abs.

The use of synthetic peptides as Ags in seroassays present a promising alternative to whole-protein Ags, since immunodominant regions with low sequence identity to proteins other than target can be selected, reducing the risk of capturing cross-reactive Abs in the immunoassay. In this regard, extensive analysis has been performed on the characterization of immunodominant epitopes present in the flavivirus structural proteins Capsid, prM and Envelope and the non-structural protein NS1, which are main targets of the humoral immune response (19). Important epitopes present in the other non-structural proteins have also been described to be targeted by Abs (22, 34) and they represent potential Ags to be used in immunoassays. The peptides evaluated in this work span the entire DENV proteome and they were based on their ability to be recognized by Abs present in the serum from DENV infected individuals (22). Among the evaluated peptides, PM-22 from Envelope, PM-30 from NS3, FPG-1 from NS1, PG-15B from NS1, PG-19 from NS2B, PG-24 from NS3 and PG-40 from NS5 were able to classify DENV samples in the positive category and sera from individuals with other flavivirus history in the negative category. Particularly FPG-1 and PM-22 were the most promising biomarkers for application as Ags in new serological tests.

Remarkably, sera from CHIKV-infected returning travelers presented high IgM Abs against some PM peptides evaluated in this study. Since DENV and CHIKV belong to different genus and no Ab cross-reactivity is expected, a possible explanation for this observation is that these CHIKV-infected patients underwent a previous infection with another microorganism or virus different to CHIKV able to induce polyclonal B-cell reactivity B cell activation, producing antibodies that cross-react with the dengue

epitopes. This ability to induce poly reactive B-cells has been described for *Plasmodium* spp., for instance it was shown that Malaria-positive sera can react against ZIKV-antigens present in commercial ZIKV-ELISA tests (27) and spike and RBD antigens from SARS-CoV2 (35, 36). We were not able to rule out the possibility that these CHIKV-positive patients had a past exposure to *Plasmodium* parasites. However, these results highlight the need to include sera from more diverse exposure background during the evaluation or validation of serological tests.

Of note, we have used the peptide sequences as they came off the microarray and thus the amino acid sequences have not been optimized to further enhance recognition and binding affinity. Given the individual variability of the Ab-response towards viral Ags (22), also observed in this study against the evaluated peptides, it is evident that a single unique 15-mer peptide is unlikely to offer sufficiently high sensitivity and specificity. Part of the sequence of FPG-1 peptide has been previously reported to be an immunodominant targeted by the immune system following DENV vaccination and natural infection (37), while there are no reports in scientific literature describing the PM-22 peptide sequence located in NS2A as a potential diagnostic antigen.

Interestingly, when comparing the longitudinal Ab responses against FPG-1 in DENV positive patients, it was shown that the responses in the early convalescent phase were higher than in the acute phase, and that this response waned over time (>70 days after symptom onset), which is in agreement with results obtained in our previous study for the Ab response targeting immudominant regions located in the NS1 protein using the microarray platform (22). The low reactivity towards FPG-1 in A-DENV samples from endemic-area patients contrasted with the high IgG titers measured against the antigens present in the commercial ELISA corresponding to virus particles of DENV-2 (data not shown). These results suggest that this biomarker could be useful to diagnose DENV infection based on IgG seroconversion using paired sera in DENV endemic regions where secondary/multiple DENV infections occurs. Despite the fact that NS1 is highly conserved among flaviviruses (38), a good specificity was observed for FPG-1 peptide, since samples from individuals exposed to other flavivirus showed low reactivity.

When the Ab titers from endemic and non-endemic ADENV sera were compared, we noticed that the magnitude of the Ab response against PG-40 was significantly higher in the endemic group. These results suggest that this peptide could be useful for the differentiation of primary from secondary infection, under the assumption that the rapid rise of IgG titers against PG-40 detected in A-DENV samples from endemic patients corresponded with an anamnestic response from a previous DENV infection. Unfortunately, no documented history of previous DENV infection is available for these samples. PG-40 peptide could also be of special interest as a biomarker for serostatus determination, given that a positive test confirming prior DENV infection is crucial to guide vaccination with Dengvaxia (11). According to a recent review, the current available DENV RDTs are highly specific (100%), but the sensitivity is lower than 41% for the detection of prior infection in endemic samples (12).

We found that hospitalized DENV patients showed significantly higher IgG titers against the PG-33 peptide located in NS3 compare to the titers observed in non-hospitalized individuals, making this peptide a potentially attractive biomarker for disease severity. However, more samples are need to be tested to confirm this finding. No linear continuous immunoreactive peptides from flaviviruses located in the NS3 protein have been previously reported (39).

A fundamental aspect of this work is the use of machine learning classification algorithms to evaluate the diagnostic performance of peptides in order to select the best possible combination that results in the highest possible sensitivity and specificity. For this purpose, the specificity was prioritized over sensitivity given the impact that specificity has on flavivirus diagnosis. These findings revealed that the combination of six different peptides for RFM and RFG models showed an improvement in the sensitivity compared to the observed sensitivity when single peptides were evaluated. Despite that specificity was targeted at minimally 80%, this constitutes a clear progress in respect to the performance of currently available commercial tests for DENV serology.

Our work adds important insights to the growing number of studies that seek for biomarkers for the improved serological diagnosis of flavivirus infections (12, 39, 40). Further modification and subsequent functional analysis of these peptides with a larger

number of samples from DENV confirmed cases and from patients with undifferentiated fever is required to further evaluate and prioritize these biomarkers for future DENV test development.

5.6 Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

5.7 Ethics statement

The studies involving human participants were reviewed and approved by the ethical review boards of the Universidad Peruana Cayetano Heredia, Lima, Peru (Protocol No. 101480), the Institute of Tropical Medicine Antwerp, Belgium (Protocol No. ITG 1304/19) and the University of Antwerp, Belgium (Protocol No. 19/42/477). This study was conducted in compliance with the ethical standards of the latest amended Declaration of Helsinki and of the International Conference Harmonization (ICH) guidelines, plus adhering to local laws and regulations. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

5.8 Author contributions

FF-A and KKA wrote the manuscript text. FF-A and KK implemented the analysis. KKA, FF-A, and KK conceived the study. FF-A, XM, and DB processed samples. FF-A analyzed the data. MT, FF-A, XM, and ME wrote study protocols and coordinated sample collection. All authors contributed to the article and approved the submitted version.

5.9 Funding

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5.10 Acknowledgments

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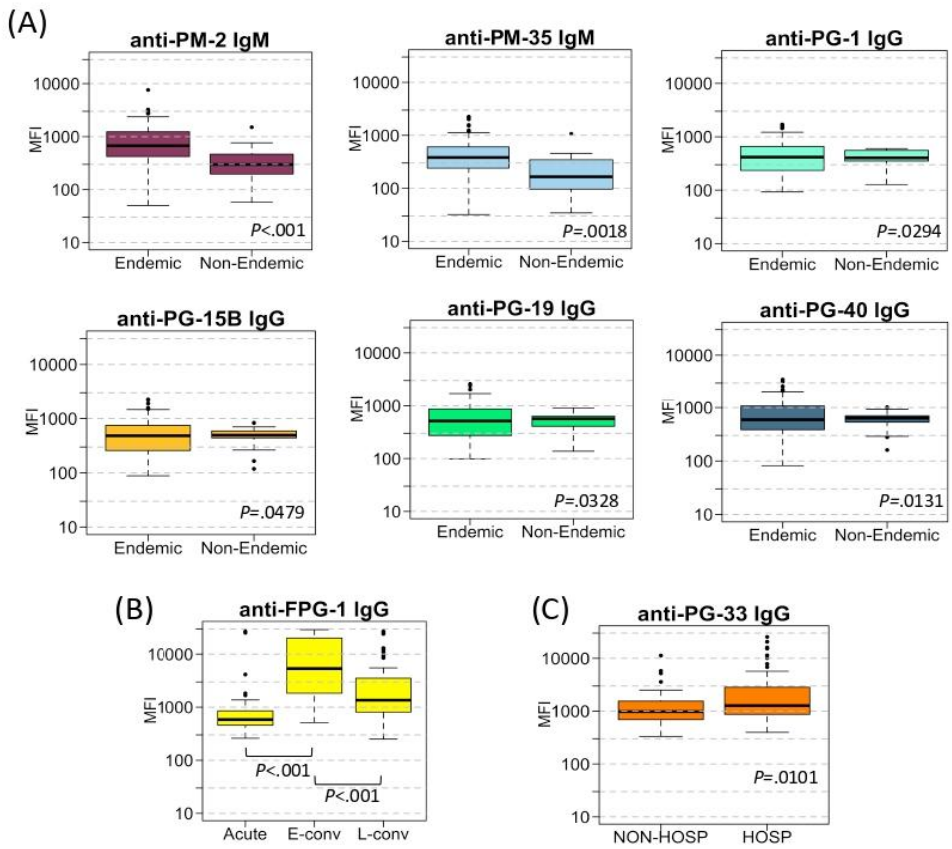
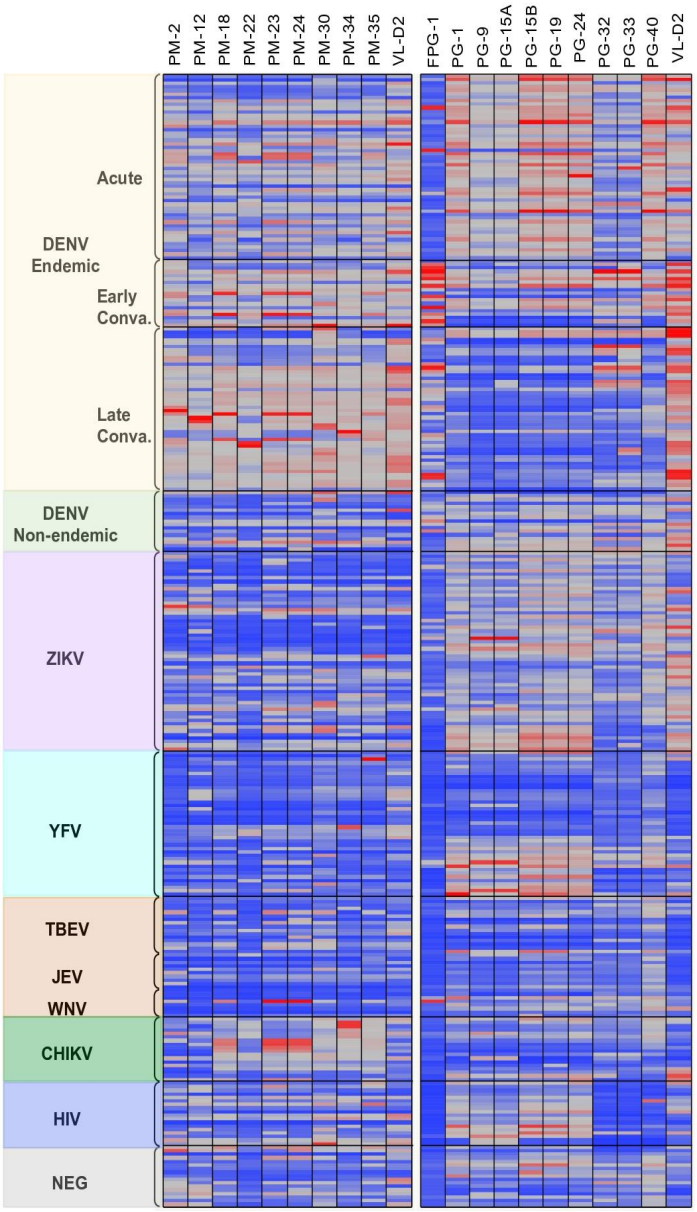
5.11 Supplementary material

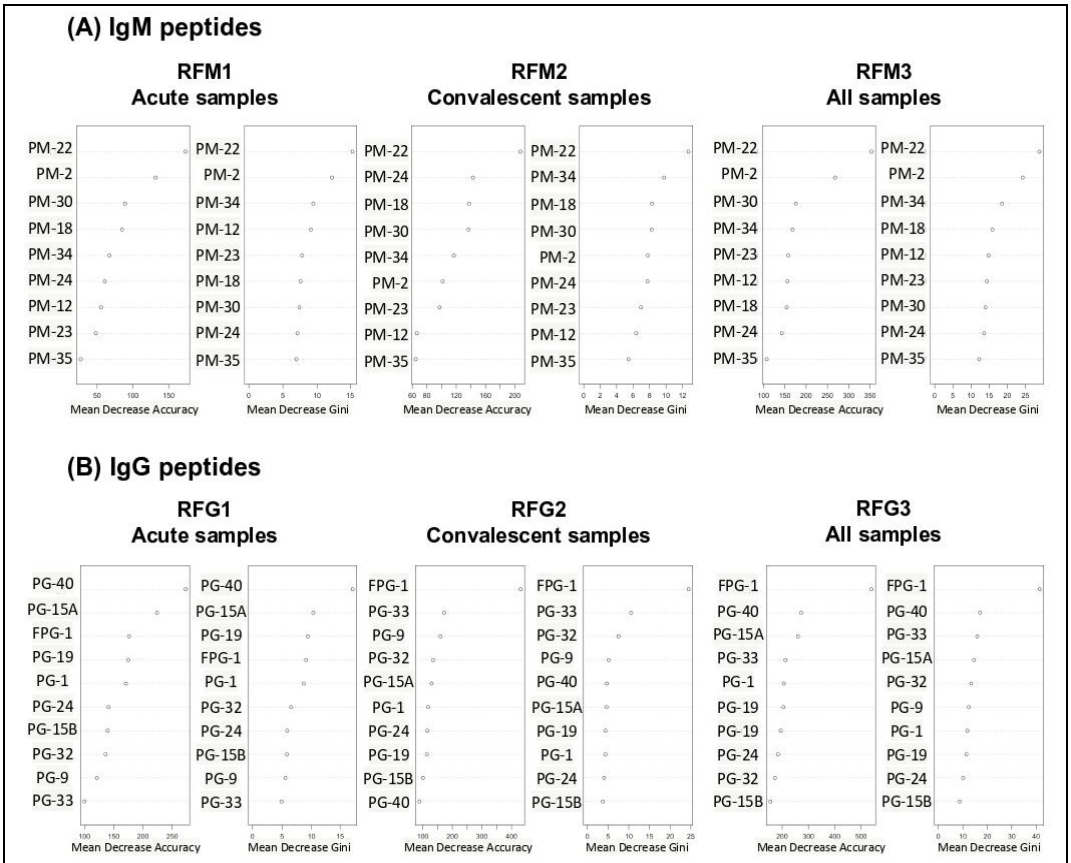
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.793882/full#supplementary-material



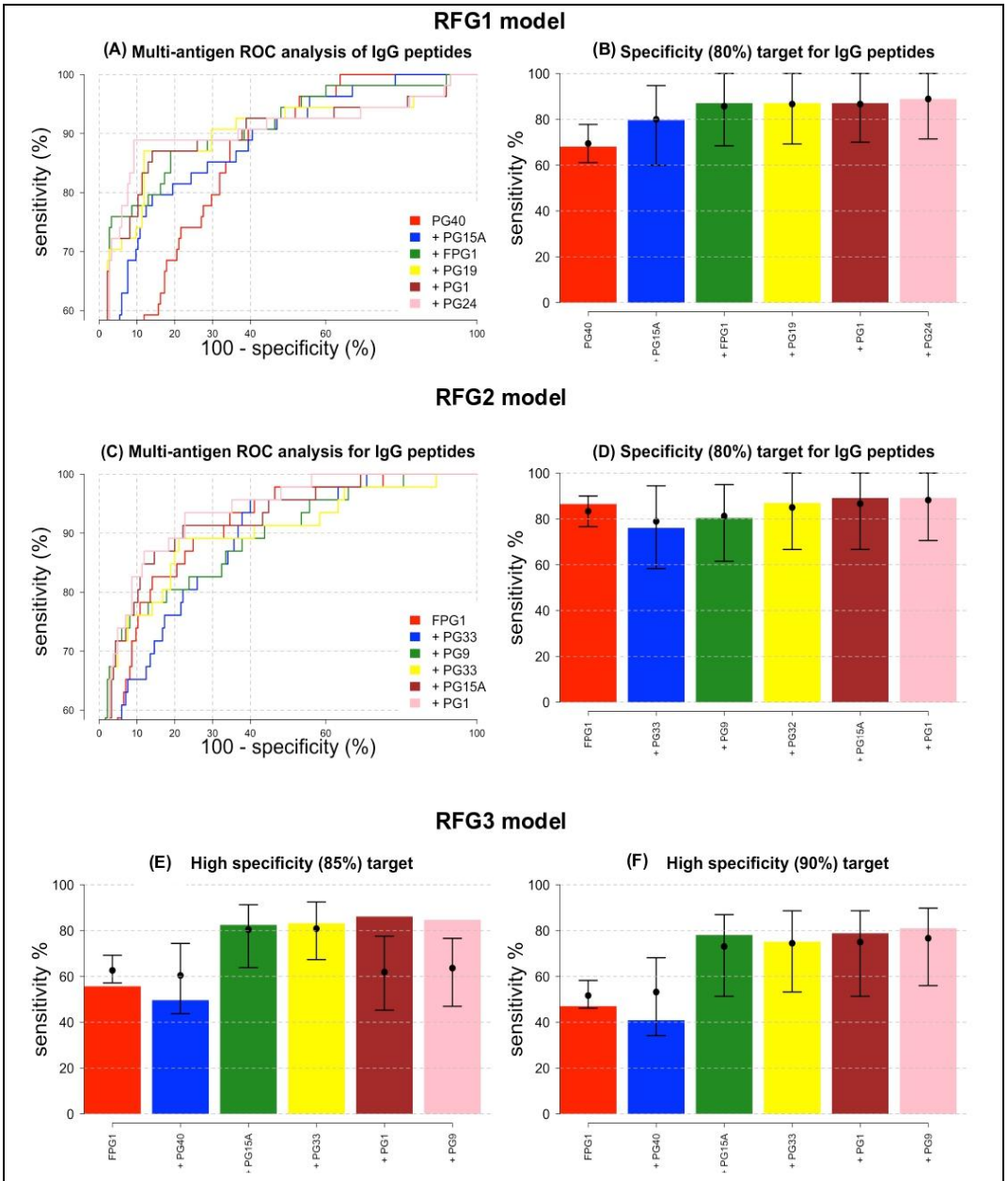
Supplementary Figure 1. Antibody responses to single peptides among DENV positive samples. Boxplots represent the comparison of the measured Ab response (MFI levels) in DENV positive samples against the detailed peptides between (A) endemic and non-endemic samples, (B) acute and early convalescent samples, and between early and late convalescent samples, and (C) hospitalized versus non-hospitalized samples. Positive DENV samples were confirmed by RT-PCR and/or IgM/IgG seroconversion. Horizontal box boundaries and midline denote sample quartiles. The analysis was performed for all the peptides, but only those showing statistically significant differences between groups are plotted (P <.05).



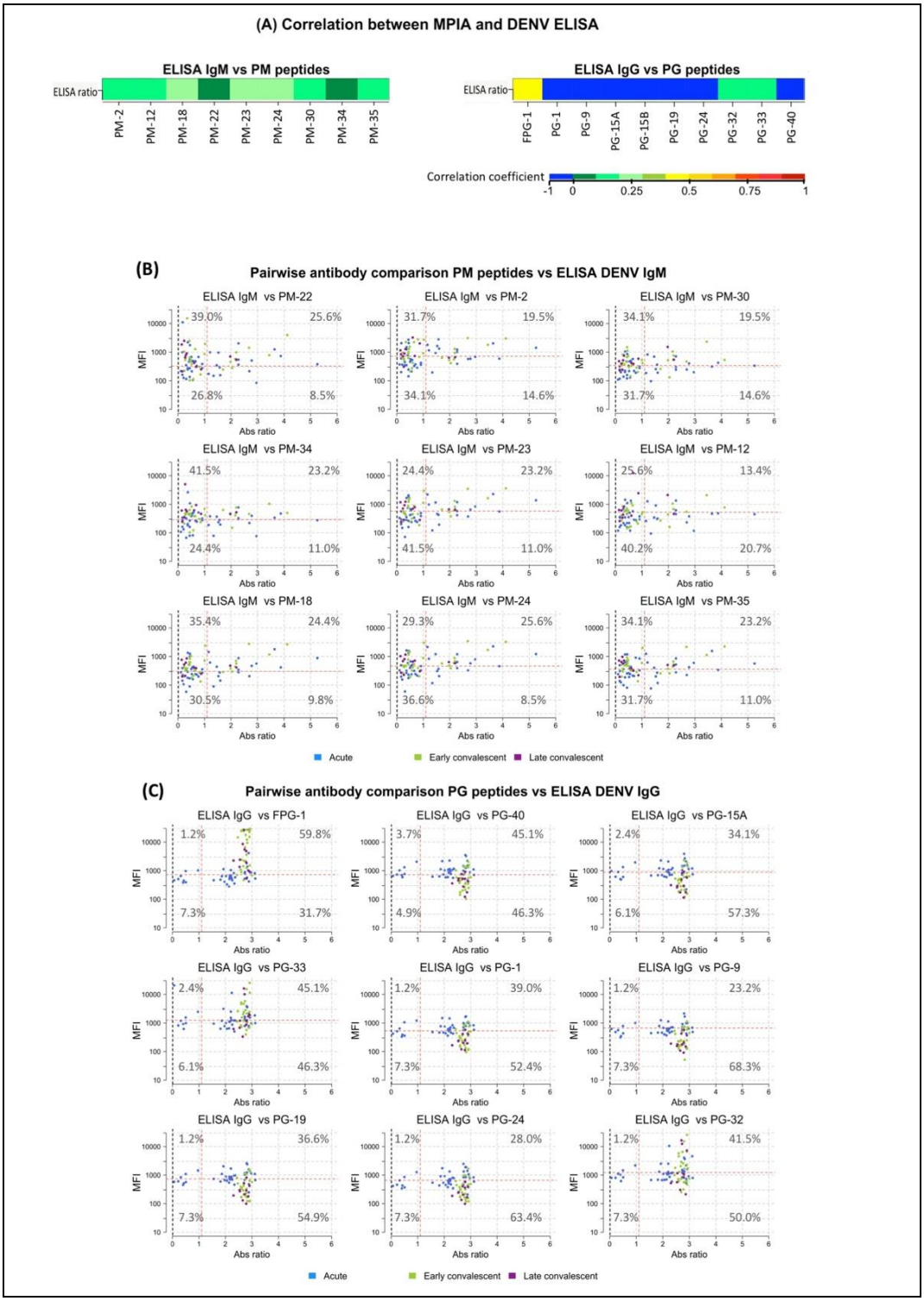
Supplementary Figure 2. Heat map representing antibody levels against DENV peptides. The heatmap depicts the intensity of the IgM and IgG antibodies measured in MFI. Each column corresponds to one peptide and each row represents a sample. The color intensity indicates the measured MFI values going from low (light yellow) to high (red) levels. DENV, dengue virus; ZIKV, Zika virus; YFV, yellow fever virus; TBEV, Tick borne encephalitis virus; JEV, Japanese encephalitis virus; WNV, West Nile virus; CHIKV, chikungunya virus; HIV, Human immunodeficiency virus; NEG, healthy donors; Conva, convalescent sample.



Supplementary Figure 3. Contribution of each peptide to classification accuracy. Random forest machine learning technique ranked the contribution of each peptide to the classification accuracy using the mean decrease accuracy (MDA) and the mean decrease gini (MDG) index. Ranking of (A) IgM peptides, and (B) IgG peptides. Three different RF models were implemented based on the period since onset of symptoms: acute (≤8 days after symptoms onset), early convalescent (≥10 - ≤70 days after symptoms onset) and all samples (acute + convalescent). The same panel of negative samples were used for the three models.

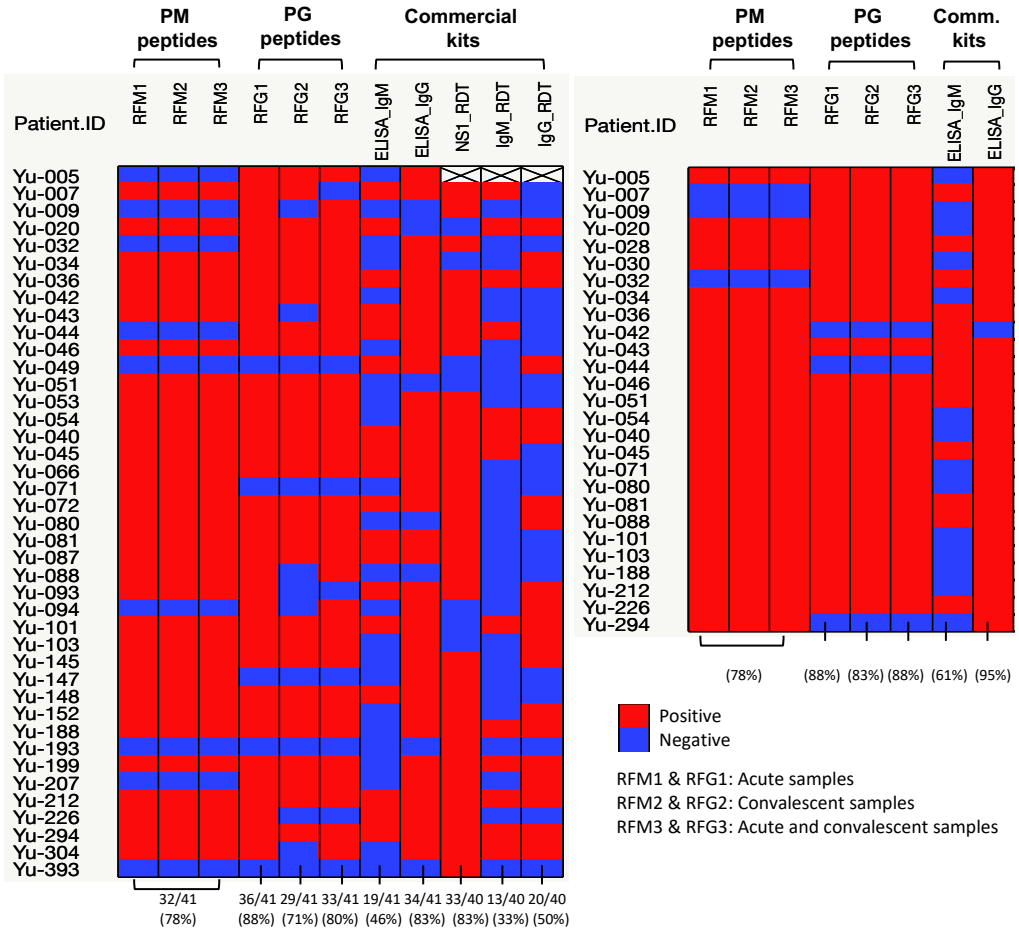


Supplementary Figure 4. ROC performance analysis combining multiple IgG peptides using a random forest algorithm. ROC curves for IgG peptides in acute RFG1 (A) and convalescent RFG2 (C) samples. The peptides were added sequentially based on their classification accuracy. The axes have been rescaled to better differentiate between high values of sensitivity and specificity. For a specificity set at 80% (B, D), 85% (E) and 90% (F), we plotted the respective sensitivity. Sensitivity was estimated using a random forests classifier and peptide biomarkers were added sequentially. Points and whiskers denote the median and 95% CIs from repeat cross-validation.


(A) Correlation between MPIA and DENV ELISA
ELISA IgM vs PM peptides
ELISA ratio
PM-2
PM-12
PM-18
PM-22
PM-23
PM-24
PM-30
PM-34
PM-35
ELISA IgG vs PG peptides
ELISA ratio
FPG-1
PG-1
PG-9
PG-15A
PG-15B
PG-19
PG-24
PG-32
PG-33
PG-40
Correlation coefficient
-1
0
0.25
0.5
0.75
1
(B)
Pairwise antibody comparison PM peptides vs ELISA DENV IgM
ELISA IgM vs PM-22
39.0%
25.6%
26.8%
8.5%
ELISA IgM vs PM-2
31.7%
19.5%
34.1%
14.6%
ELISA IgM vs PM-30
34.1%
19.5%
31.7%
14.6%
ELISA IgM vs PM-34
41.5%
23.2%
24.4%
11.0%
ELISA IgM vs PM-23
24.4%
23.2%
41.5%
11.0%
ELISA IgM vs PM-12
25.6%
13.4%
40.2%
20.7%
ELISA IgM vs PM-18
35.4%
24.4%
30.5%
9.8%
ELISA IgM vs PM-24
29.3%
25.6%
36.6%
8.5%
ELISA IgM vs PM-35
34.1%
23.2%
31.7%
11.0%
MFI
Abs ratio
Acute
Early convalescent
Late convalescent
(C)
Pairwise antibody comparison PG peptides vs ELISA DENV IgG
ELISA IgG vs FPG-1
1.2%
59.8%
7.3%
31.7%
ELISA IgG vs PG-40
3.7%
45.1%
4.9%
46.3%
ELISA IgG vs PG-15A
2.4%
34.1%
6.1%
57.3%
ELISA IgG vs PG-33
2.4%
45.1%
6.1%
46.3%
ELISA IgG vs PG-1
1.2%
39.0%
7.3%
52.4%
ELISA IgG vs PG-9
1.2%
23.2%
7.3%
68.3%
ELISA IgG vs PG-19
1.2%
36.6%
7.3%
54.9%
ELISA IgG vs PG-24
1.2%
28.0%
7.3%
63.4%
ELISA IgG vs PG-32
1.2%
41.5%
7.3%
50.0%
MFI
Abs ratio
Acute
Early convalescent
Late convalescent

Supplementary Figure 5. Correlation of antibody titers between individual peptides and the commercial ELISA for dengue. (A) Heatmap of the Spearman's correlation coefficient between the antibody titers against the synthetic peptides and a commercial DENV ELISA. For the commercial kit, the OD (optical density measured at 450 nm wavelength) and the OD ratio (OD values of the sample and the calibrator provided in the kit) were used to calculate the correlation. Correlation coefficients are indicating by the color scale. Blue indicates a negative correlation; red indicates a positive correlation. (B) Pairwise correlation between each IgM peptide and the commercial DENV ELISA IgM kit. (C) Pairwise correlation between each IgG peptide and the commercial DENV ELISA IgG kit. The antibody response was measured in MFI for the synthetic peptides and OD (Absorbance at 450 nm) for the commercial kit. Each dot represents a sample. Dashed red lines indicate the cut-off values for the commercial kit according the manufacturer instructions (vertical line) and for the peptide based on the ROC curves enforcing a minimum specificity of 80% (horizontal line).

(A)



(B)

Peptide	RFM1	RFM2	RFM3
PM-2			
PM-12			
PM-18			
PM-22			
PM-23			
PM-24			
PM-30			
PM-34			
PM-35			

Peptide	RFG1	RFG2	RFG3
FPG-1			
PG-1			
PG-9			
PG-15A			
PG-15B			
PG-19			
PG-24			
PG-32			
PG-33			
PG-40			

(C)

Cohen's kappa	RFM1 / RFM2/RFM3	RFG1	RFG2	RFG3	ELISA_IgM	ELISA_IgG
RFM1/RFM2 /RFM3		0.183 (-0.091 - 0.458)	0.248 (-0.013 - 0.509)	0.141 (-0.121-0.402)	0.043 (-0.089-0.174)	0.207 (-0.074-0.488)
RFG1	0.0463*		0.614 (0.375-0.852)	0.773 (0.559-0.986)	0.031 (-0.083-0.146)	0.278 (-0.034-0.591)
RFG2	0.0117*	<.0001*		0.727 (0.522-0.932)	0.01 (-0.139-0.158)	0.352 (0.083-0.622)
RFG3	0.101	<.0001*	<.0001*		-0.062 (-0.197-0.072)	0.229 (-0.063-0.52)
ELISA_IgM	0.268	0.300	0.449	0.1720		0.056 (-0.049-0.162)
ELISA_IgG	0.0270*	0.0058*	0.0003*	0.0177*	0.1599	

McNemar's test	RFM1 / RFM2/RFM3	RFG1	RFG2	RFG3	ELISA_IgM	ELISA_IgG
RFM1/RFM2 /RFM3		3.556 (0.753-16.779)	4.286 (1.135-16.182)	2.583 (0.577-11.576)	1.5 (0.413-5.45)	4.333 (0.882-21.294)
RFG1	0.439		75.429 (8.192-694.531)	186.667 (17.303-2013.783)	1.476 (0.342-6.366)	6.8 (1.297-35.655)
RFG2	0.467	0.0339*		132 (13.945-1249.489)	1.077 (0.344-3.372)	10.667 (2.199-51.73)
RFG3	0.808	0.317	0.103		0.543 (0.151-1.949)	4.95 (0.991-24.726)
ELISA_IgM	<.0001*	<.0001*	<.0001*	<.0001*		2.286 (0.432-12.082)
ELISA_IgG	0.285	0.763	0.052	0.4054	<.0001*	

Supplementary Figure 6. Comparison of result outcomes between the random forest models and DENV commercial diagnostic kits. The analysis was performed in a subset of 41 endemic DENV positive samples. (A) Cell plot where each column represents a serologic test and each row represents a sample. The analysis for the DENV peptides was done with the combination of peptides based on the random forest analysis. (B) Peptide composition of each model for IgM peptides (RFM) and IgG peptides (RFG). (C) Differences in classification were assessed by pairwise comparison using Cohen's kappa and McNemar's test. The values above the diagonal indicates the kappa coefficient with the 95% CI range for the Cohen's test while for the MacNemar's test they represent the Odds ratio. The values below the diagonal in each table corresponds to the p value.

Supplementary Table 1: Measurements of antibody responses from the multiplex peptide immunoassay using a Luminex® 100/200 analyzer.

Supplementary Table 2: Comparison between groups using non parametric tests. The median of the antibody titers measured for each of the peptides evaluated in the study were compared using the non-parametric test Steel-Dwass to determine if the values were significantly different between DENV-positive (dengue infected individuals) vs DENV-negative (individuals with exposure to other flavivirus(es), CHIKV, HIV and negative healthy controls). Non-parametric tests were also performed on DENV-positive samples after categorizing them into: (i) DENV-endemic (samples from patients living in areas where DENV causes period outbreaks) vs DENV-non endemic (samples collected from overseas travelers returning to Belgium from an area endemic for DENV), (ii) Hospitalized vs non-hospitalized samples, (iii) Acute samples (A-DENV, ≤ 8 days after symptoms onset (DASO)) vs Early convalescent (E-DENV, > 8,<70 DASO), (iv)Acute samples vs Late convalescent (L-DENV, ≥71, up to 230 DASO) and (v) E-DENV vs L-DENV. P values <0.05 were considered different.

Supplementary Table 3: Area under the curves for individual peptides

The diagnostic performance for each individual peptide was evaluated in terms of sensitivity, specificity and area under the curve (AUC). This analysis was separately performed for the three groups: (i) A-DENV, (ii) E-DENV and (iii) A-DENV+E-DENV+L-DENV. To calculate the sensitivity and specificity, three targets were assessed: (i) a sensitivity >80% is enforced in order to maximize specificity, (ii) equally weighted sensitivity and specificity and (iii) a specificity >80% is enforced in order to maximize sensitivity.

Supplementary Table 4: McNemar's test for pairwise comparisons

Differences in classification performance were assessed by pairwise comparison using McNemar's test. The above diagonal element shows the odds ratio with 95% confidence intervals. The corresponding element below the diagonal presents the P value. P values <.05 indicate that there is significant disagreement in the outcome between the compared tests. The standard of comparison refers to the test used to diagnose the DENV-samples according to Table 1. The above diagonal element shows the estimated odds ratio (OR) for the seromarker in the row in detecting sera with a positive result for the seromarker in the column. For each peptide in the row, OR>1 indicates better discriminatory test performance respect to the peptide in the column.

When the comparison of the peptides was done with the reference standard, the estimated OR for peptide FPG-1 in detecting dengue infection is 7.18. This means that for peptide FPG-1 the odds for positivity among subjects with dengue infection is 7.18 times higher than the odds for positivity among subjects without dengue infection. Similarly, peptides PM-18, PM-22 and PM-34 have the highest OR values among the PM peptides, the estimated OR for these peptides in detecting dengue infection is 5.4, 6.12 and 5.91, respectively. FPG-1 has the highest OR in detecting dengue infection compared to the other evaluated peptides.

Supplementary Table 5: Cohen's kappa test for pairwise comparisons

Cohen's kappa coefficient was calculated to estimate the degree of agreement in the data. The Cohen's kappa coefficient goes on a scale from -1 to 1. Positive values of kappa mean that there is agreement; negative values mean that there is disagreement. The above diagonal element in the table shows the $\hat{\kappa}$ (kappa) with 95% confidence intervals. The corresponding element below the diagonal presents the two-sided P value. P values <.05 indicate that that degree of agreement or disagreement in the outcome between the compared tests is statistically significant. All peptides showed positive kappa values with respect to the reference standard, with the exception of PG-9, PG-15B, PG-19 and PG-24 peptides. PM-22, PM-34 and FPG-1 have the highest agreement with the outcome of the reference standard.

Supplementary Table 6: Sensitivity values for specificity target >80% in multiple combinations of peptides

Antigen combinations were selected to optimize sensitivity while enforcing a specificity > 80%. The calculated sensitivity after the multiple combination of peptides up to size six is shown. Confidence intervals were calculated using Wilson's method (95% binomial confidence interval).

Supplementary Table 2. Comparison between groups using non parametric tests

Antigen	Dengue (positive, N=137) vs other samples (negative, N=185)			DENV-Endemic (N=80) vs DENV-Non-Endemic (N=16)			Hospitalized (N=51) vs Non-hospitalized (N=54)			Acute (1, N=54) vs Early convalescent (2, N=27) vs Late convalescent (3, N=56)					
	Positive MFI-Median	Negative MFI-Median	p-value*	Endemic MFI-Median	Non-end. MFI-Median	p-value*	Hospitalized MFI-Median	Non-Hospi MFI-Median	p-value*	Acute MFI-Median	E-conv MFI-Median	L-conv MFI-Median	p-value* 1,2	1,3	2,3
PM-2	621.13	210.31	<.0001	594.19	297.25	0.0009	665.13	810.69	0.5736	550.31	602.00	717.88	0.9173	0.2235	0.6285
PM-12	383.00	197.50	<.0001	349.25	258.00	0.0198	457.25	411.13	0.8148	342.63	383.00	482.13	0.9590	0.0179	0.2588
PM-18	334.75	149.94	<.0001	320.44	150.38	0.0082	415.00	372.38	0.2300	282.38	315.63	397.63	0.8962	0.1243	0.5853
PM-22	417.88	138.25	<.0001	408.25	133.13	0.0012	626.38	419.44	0.1059	362.94	417.88	527.88	0.8850	0.3429	0.8187
PM-23	498.00	220.81	<.0001	463.69	345.38	0.0448	586.75	503.13	0.5736	414.13	525.63	612.00	0.7358	0.1275	0.7664
PM-24	460.50	206.25	<.0001	447.75	325.25	0.0885	491.88	498.13	0.9016	380.00	451.25	571.13	0.8485	0.0732	0.5090
PM-30	361.88	187.13	<.0001	310.13	271.50	0.1506	438.88	348.50	0.5451	291.81	339.50	467.50	0.2422	0.0010	0.3856
PM-34	340.75	149.88	<.0001	304.13	148.50	0.0069	447.75	371.13	0.2287	277.00	356.50	464.50	0.3395	0.0007	0.1104
PM-35	366.25	163.06	<.0001	352.81	165.63	0.0018	481.19	377.44	0.1812	301.44	345.63	436.19	0.9575	0.3320	0.6867
VL-D2_IgM	3058.13	1106.13	<.0001	2931.63	705.75	0.0009	3958.13	3441.88	0.4682	2796.38	3007.50	3999.56	0.9997	0.0769	0.1685
FPG-1	985.63	430.50	<.0001	812.56	1027.13	0.4172	1480.13	819.88	0.0562	586.25	5363.50	1358.75	<.0001	<.0001	0.0076
PG-1	413.25	324.00	0.0037	518.25	401.50	0.0294	374.50	405.63	0.7499	625.25	401.50	240.50	0.0007	<.0001	0.0804
PG-9	426.38	370.38	0.7998	522.00	552.88	0.8162	369.75	373.00	0.8682	626.38	414.75	198.88	0.0024	<.0001	0.0832
PG-15A	606.25	482.50	0.0639	759.38	590.00	0.4550	594.25	608.13	0.8199	970.63	525.75	308.38	0.0001	<.0001	0.3137
PG-15B	488.75	391.50	0.0361	626.38	497.00	0.0479	471.75	437.88	0.8836	737.88	461.25	280.38	0.0008	<.0001	0.1128
PG-19	515.75	428.00	0.0632	675.75	568.00	0.0328	473.63	470.88	0.7425	795.38	509.00	290.00	0.0003	<.0001	0.0832
PG-24	433.75	399.25	0.1668	524.50	498.25	0.2052	391.63	405.25	0.7872	646.50	435.00	273.88	0.0033	<.0001	0.1104
PG-32	1033.88	725.00	<.0001	1147.19	1252.75	0.6117	1122.06	849.00	0.3438	1104.25	1794.63	885.00	0.4137	0.5683	0.4103
PG.33	1198.75	763.25	<.0001	1223.00	1510.75	0.7220	1274.25	982.25	0.0087	1110.88	1875.00	1192.63	0.0713	0.9609	0.3643
PG-40	593.25	474.38	<.0001	769.44	630.38	0.0131	530.25	620.75	0.3995	1000.50	578.25	444.38	0.0012	<.0001	0.0950
VL-D2_IgG	10590.00	4162.00	<.0001	9993.25	11023.00	0.5595	11187.88	9958.63	0.3211	8441.13	14033.50	13080.00	0.0315	0.0002	0.9994

* Non-parametric Steel-Dwass test

DENV positive (n=137): acute and convalescent samples from DENV infected individuals confirmed by RT-PCR and/or Ab seroconversion

DENV negative (n=186): Serum samples from individuals either infected or vaccinated with arboviruses (ZIKV, YFV, TBEV, JEV, WNV) + HIV positive samples, Negative pre-pandemic samples

DENV-endemic (n=80) refers to samples from patients living in areas where DENV causes period outbreaks. Including only acute and early convalescent samples

DENV-non endemic (n=16) refers to samples collected from overseas travelers returning to Belgium from an area endemic for DENV.

Acute samples (1): ≤ 8 days after symptoms onset; Early convalescent (2): > 8,<70 days after symptoms onset; Late convalescent (3) ≥71, up to 230 days after symptoms onset

In red statistical significant differences (p-values<0.05).

Supplementary Table 3: Area under the curves for individual peptides

Antigens	Acute+ convalescent						Acute						Convalescent					
	AUC	> 80% target sensitivity		balanced sens/spec %	> 80% target specificity		AUC	> 80% target sensitivity		balanced sens/spec	> 80% target specificity		AUC	> 80% target sensitivity		balanced sens/spec	> 80% target specificity	
		Sens %	Spec %		Sens %	Spec %		sensitivity	specificity		sensitivity	specificity		sensitivity	specificity		sensitivity	specificity
PM-2	0.7562	80.3	43.1	69.6	63.6	80.4	0.7413	81.5	35.2	66.7	60.9	80.4	0.7322	80.4	34.8	69.6	59.8	80.4
PM-12	0.6879	81.0	32.1	66.3	55.4	80.4	0.6568	81.5	18.5	64.8	46.2	80.4	0.6567	80.4	34.8	63.0	38.6	80.4
PM-18	0.7631	80.3	56.9	68.5	63.0	80.4	0.7349	81.5	44.4	66.7	57.1	80.4	0.7426	80.4	56.5	67.4	50.5	80.4
PM-22	0.7865	80.3	59.1	72.8	60.3	80.4	0.7728	81.5	57.4	72.2	58.2	80.4	0.7509	80.4	54.3	69.6	47.3	80.4
PM-23	0.7158	80.3	43.1	65.8	57.1	80.4	0.6861	81.5	33.3	64.8	56.0	80.4	0.7082	80.4	41.3	65.2	56.0	80.4
PM-24	0.7357	80.3	49.6	66.3	58.2	80.4	0.7024	81.5	37.0	64.8	55.4	80.4	0.7365	80.4	54.3	69.6	58.7	80.4
PM-30	0.7361	80.3	51.8	67.4	54.3	80.4	0.6792	81.5	31.5	61.1	49.5	80.4	0.7210	80.4	54.3	69.6	51.1	80.4
PM-34	0.7587	80.3	59.1	71.7	57.6	80.4	0.7080	81.5	46.3	66.7	54.9	80.4	0.7211	80.4	58.7	65.2	44.6	80.4
PM-35	0.7372	80.3	51.8	66.8	54.3	80.4	0.7140	81.5	44.4	64.8	53.3	80.4	0.7092	80.4	50.0	67.4	51.1	80.4
VL-D2_IgM	0.7650	80.3	65.7	73.4	57.1	81.0	0.7557	81.5	59.3	72.2	64.7	81.0	0.6832	80.4	54.3	65.2	38.6	81.0
FPG-1	0.8131	80.3	64.2	71.4	61.1	80.5	0.6846	81.5	33.3	61.1	47.0	80.5	0.9031	80.4	82.6	82.7	86.5	80.5
PG-1	0.5945	80.3	34.3	60.0	25.4	80.5	0.8114	81.5	61.1	73.5	69.7	80.5	0.5504	80.4	23.9	58.9	18.9	80.5
PG-9	0.5083	80.3	24.1	55.1	14.6	80.5	0.7329	81.5	42.6	68.1	58.9	80.5	0.4645	80.4	17.4	49.7	14.6	80.5
PG-15A	0.5604	80.3	28.5	58.4	18.4	80.5	0.7773	81.5	51.9	70.3	66.5	80.5	0.5144	80.4	19.6	54.6	18.4	80.5
PG-15B	0.5683	80.3	27.0	58.4	20.5	80.5	0.7862	81.5	53.7	70.8	66.5	80.5	0.5200	80.4	13.0	54.6	20.0	80.5
PG-19	0.5605	80.3	28.5	55.7	17.8	80.5	0.8091	81.5	55.6	75.1	73.0	80.5	0.5072	80.4	13.0	52.4	14.6	80.5
PG-24	0.5452	80.3	24.1	51.9	20.0	80.5	0.7512	81.5	48.1	65.9	53.5	80.5	0.5073	80.4	10.9	51.4	18.4	80.5
PG-32	0.6400	80.3	40.9	58.4	30.8	80.5	0.6779	81.5	35.2	59.5	44.9	80.5	0.6932	80.4	54.3	61.6	36.8	80.5
PG.33	0.6964	80.3	45.3	64.9	38.4	80.5	0.6854	81.5	37.0	64.9	41.1	80.5	0.7752	80.4	58.7	69.7	62.2	80.5
PG-40	0.6316	81.0	38.7	59.5	30.8	80.5	0.8472	81.5	68.5	74.1	68.1	80.5	0.5730	80.4	26.1	58.9	23.2	80.5
VL-D2_IgG	0.8088	80.3	59.1	70.8	68.1	80.5	0.7426	81.5	40.7	69.2	64.3	80.5	0.8501	80.4	73.9	76.2	70.8	80.5

- DENV positive: acute + convalescent (n=137); DENV infected individuals confirmed by RT-PCR and/or Ab seroconversion
- DENV negative: (n=186); Serum samples from individuals either infected or vaccinated with arboviruses (ZIKV, YFV, TBEV, JEV, WNV, CHIKV), HIV, Negative pre-pandemic samples
- Acute samples: (N=54); <8 days after symptoms onset
- Convalescent: (N=46); convalescent samples collected between 10 and 60 days after symptoms onset
- In red biomarkers with highest AUC values, > 0.75 for IgM peptides; > 0.8 for IgG peptides
- VL-D2: viral lysate from DENV serotype 2. This antigen is included in the table as a comparison

Supplementary Table 4. McNemar's test for pairwise comparisons

		PM2	PM12	PM18	PM22	PM23	PM24	PM30	PM34	PM35	VL-D2	FPG1
		IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgG
PM2	IgM		5.7 (3.3-9.84)	8.39 (4.89-14.41)	3.95 (2.38-6.54)	14.08 (7.9-25.12)	10.33 (5.94-17.97)	6.04 (3.58-10.2)	4.13 (2.49-6.84)	5.63 (3.35-9.47)	4.62 (2.77-7.71)	2.27 (1.39-3.7)
PM12	IgM	0.0956		5.76 (3.33-9.95)	4.08 (2.4-6.94)	4.53 (2.64-7.76)	4.25 (2.49-7.23)	6.76 (3.89-11.76)	6.32 (3.63-10.99)	3.2 (1.89-5.4)	8.46 (4.7-15.23)	1.86 (1.11-3.1)
PM18	IgM	0.0304	0.0003		8.91 (5.27-15.06)	43.28 (21.19-88.41)	67.61 (32.07-142.53)	20.3 (11.22-36.73)	20.66 (11.48-37.19)	33.21 (17.5-63.4)	10.61 (6.21-18.13)	2.61 (1.63-4.19)
PM22	IgM	0.0348	0.0003	0.8185		4.22 (2.54-7)	5.39 (3.25-8.95)	8.49 (5.01-14.38)	22.69 (12.51-41.16)	11.45 (6.63-19.78)	12.3 (7.14-21.19)	2.48 (1.55-3.97)
PM23	IgM	1.0000	0.1078	0.0038	0.0330		109.89 (47.5-254.22)	11.07 (6.33-19.34)	7.21 (4.23-12.27)	20.5 (11.15-37.71)	4.62 (2.77-7.71)	2.57 (1.57-4.2)
PM24	IgM	0.2786	0.0123	0.0863	0.2059	0.0833		12.61 (7.22-22.01)	10.71 (6.21-18.48)	31.62 (16.68-59.95)	5.74 (3.45-9.56)	2.87 (1.77-4.65)
PM30	IgM	0.1904	0.0027	0.3452	0.3051	0.1456	0.7098		12.99 (7.45-22.65)	9.7 (5.66-16.6)	10.46 (6.08-18.02)	2.08 (1.29-3.35)
PM34	IgM	0.0263	<.0001	0.6858	0.8907	0.0151	0.1229	0.2184		18.21 (10.16-32.64)	17.66 (9.96-31.32)	3.05 (1.9-4.9)
PM35	IgM	0.1957	0.0072	0.2967	0.2786	0.1025	0.6547	1.0000	0.1892		7.79 (4.62-13.14)	2.64 (1.63-4.25)
VL-D2	IgM	0.0012	<.0001	0.1281	0.1854	0.0012	0.0159	0.0201	0.1967	0.0272		2.25 (1.42-3.58)
FPG1	IgG	0.0079	<.0001	0.3532	0.4615	0.0068	0.0588	0.1253	0.5064	0.1129	0.7868	
PG1	IgG	0.3035	0.7932	0.0095	0.0051	0.2926	0.0583	0.0411	0.0033	0.0339	0.0003	0.0002
PG9	IgG	0.0216	0.3133	0.0001	<.0001	0.0196	0.0017	0.0009	<.0001	0.0008	<.0001	<.0001
PG15A	IgG	0.1540	0.9276	0.0028	0.0013	0.1508	0.0242	0.0156	0.0009	0.0119	<.0001	<.0001
PG15B	IgG	0.0500	0.5312	0.0005	0.0002	0.0446	0.0042	0.0038	0.0002	0.0027	<.0001	<.0001
PG19	IgG	0.0944	0.7194	0.0012	0.0005	0.0867	0.0106	0.0076	0.0004	0.0056	<.0001	<.0001
PG24	IgG	0.0298	0.4208	0.0002	0.0002	0.0246	0.0019	0.0016	<.0001	0.0011	<.0001	<.0001
PG32	IgG	0.8638	0.2632	0.0686	0.0511	0.8563	0.3252	0.2265	0.0411	0.2195	0.0043	0.0008
PG33	IgG	0.6646	0.0725	0.2230	0.1669	0.6573	0.7216	0.5377	0.1345	0.5377	0.0192	0.0059
PG40	IgG	0.6146	0.4317	0.0366	0.0201	0.6042	0.1900	0.1282	0.0164	0.1144	0.0018	0.0008
VL-D2	IgG	0.0365	0.0008	0.7103	0.8514	0.0381	0.1975	0.3092	0.9251	0.3050	0.4208	0.5176
Reference standard		<.0001	<.0001	0.0183	0.0277	<.0001	0.0013	0.0030	0.0371	0.0030	0.2752	0.1957

The above diagonal element shows the odds ratio with 95% confidence intervals. The corresponding element below the diagonal presents the P value. P values <.05 indicate that there is significant disagreement in the outcome between the compared tests.

Continuation **Supplementary Table 4. McNemar's test for pairwise comparisons**

		PG1	PG9	PG15A	PG15B	PG19	PG24	PG32	PG33	PG40	VL-D2	Reference
		IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	standard
PM2	**IgM**	0.75 (0.42-1.31)	0.8 (0.44-1.45)	1.04 (0.6-1.82)	0.94 (0.53-1.68)	0.88 (0.5-1.55)	1.08 (0.61-1.92)	0.89 (0.52-1.52)	1.01 (0.65-1.83)	0.66 (0.37-1.15)	1.99 (1.22-3.26)	3.09 (1.88-5.08)
PM12	**IgM**	0.65 (0.35-1.2)	0.79 (0.42-1.5)	0.93 (0.52-1.69)	0.65 (0.34-1.24)	0.75 (0.4-1.4)	0.61 (0.31-1.18)	0.7 (0.39-1.25)	1.26 (0.74-2.15)	0.76 (0.42-1.36)	1.47 (0.88-2.46)	1.93 (1.16-3.22)
PM18	**IgM**	0.83 (0.49-1.41)	0.88 (0.5-1.54)	0.99 (0.58-1.68)	0.92 (0.54-1.6)	0.92 (0.54-1.58)	1.14 (0.66-1.97)	1.29 (0.78-2.12)	1.4 (0.86-2.28)	0.89 (0.53-1.49)	2.52 (1.57-4.05)	5.4 (3.28-8.88)
PM22	**IgM**	0.99 (0.59-1.67)	0.85 (0.48-1.49)	1.19 (0.7-2)	0.97 (0.56-1.66)	1.11 (0.65-1.9)	0.8 (0.46-1.41)	1.09 (0.66-1.79)	1.34 (0.83-2.18)	1.2 (0.73-1.99)	2.7 (1.68-4.33)	6.12 (3.7-10.1)
PM23	**IgM**	0.95 (0.55-1.65)	0.96 (0.53-1.72)	1.13 (0.65-1.96)	1.21 (0.69-2.13)	1.12 (0.64-1.96)	1.51 (0.86-2.63)	1.46 (0.87-2.45)	1.32 (0.81-2.23)	0.9 (0.52-1.54)	1.87 (1.15-3.06)	3.09 (1.88-5.08)
PM24	**IgM**	1.44 (0.85-2.42)	1.14 (0.65-2)	1.38 (0.81-2.35)	1.63 (0.95-2.8)	1.5 (0.88-2.56)	1.87 (1.09-3.21)	1.48 (0.89-2.45)	1.52 (0.93-2.5)	1.16 (0.69-1.94)	2.27 (1.4-3.68)	4.02 (2.45-6.6)
PM30	**IgM**	0.88 (0.51-1.5)	0.99 (0.56-1.75)	0.97 (0.56-1.66)	0.76 (0.43-1.34)	0.9 (0.52-1.55)	1.02 (0.58-1.78)	1.14 (0.68-1.89)	1.43 (0.87-2.34)	0.88 (0.52-1.49)	2.37 (1.47-3.83)	4.39 (2.68-7.21)
PM34	**IgM**	1.2 (0.72-2.01)	1.06 (0.61-1.84)	1.34 (0.8-2.25)	1.02 (0.6-1.76)	1.18 (0.69-2)	1.08 (0.63-1.87)	1.14 (0.69-1.87)	1.58 (0.97-2.57)	1.18 (0.71-1.95)	2.79 (1.74-4.48)	5.91 (3.59-9.73)
PM35	**IgM**	1.26 (0.75-2.13)	1.08 (0.62-1.89)	1.4 (0.83-2.38)	1.13 (0.66-1.96)	1.31 (0.77-2.24)	1.4 (0.81-2.42)	1.3 (0.78-2.15)	1.43 (0.87-2.34)	1.25 (0.75-2.08)	2.52 (1.56-4.07)	4.39 (2.68-7.21)
VL-D2	**IgM**	0.94 (0.56-1.57)	0.83 (0.48-1.44)	1.12 (0.67-1.88)	0.8 (0.47-1.38)	0.92 (0.54-1.56)	0.85 (0.49-1.46)	1.11 (0.7-1.86)	1.55 (0.96-2.51)	0.92 (0.56-1.52)	2.08 (1.31-3.32)	7.56 (4.56-12.51)
FPG1	**IgG**	2 (1.2-3.3)	3.05 (1.77-5.27)	2.12 (1.27-3.54)	2.1 (1.24-3.55)	1.9 (1.13-3.19)	2.09 (1.23-3.56)	7.27 (4.25-12.44)	9.87 (5.71-17.03)	3.18 (1.92-5.27)	6.74 (4.08-11.14)	7.18 (4.35-11.87)
PG1	**IgG**		28.2 (14.15-56.19)	39 (19.4-78.38)	130.06 (51.77-326.77)	279.6 (95.31-820.22)	76.33 (33.41-174.91)	5.16 (3.02-8.82)	4.03 (2.38-6.81)	19.11 (10.31-35.43)	2.24 (1.35-3.71)	2.09 (1.26-3.46)
PG9	**IgG**	0.0348		100.75 (41.85-242.56)	26.73 (13.55-52.71)	23.33 (12-45.37)	33.51 (16.57-67.77)	4.89 (2.79-8.57)	4.17 (2.4-7.27)	5.86 (3.32-10.34)	2 (1.17-3.42)	1.27 (0.75-2.16)
PG15A	**IgG**	0.5164	0.0499		37.24 (18.39-75.41)	39.71 (19.59-80.48)	37.5 (18.4-76.46)	4.35 (2.54-7.45)	3.69 (2.17-6.26)	7.14 (4.09-12.47)	2.92 (1.74-4.91)	1.83 (1.1-3.05)
PG15B	**IgG**	0.0412	0.5371	0.3304		602.5 (171.42-2117.63)	84.29 (37.07-191.65)	5.81 (3.32-10.15)	4.54 (2.63-7.86)	15.9 (8.53-29.64)	2.02 (1.2-1.2)	1.48 (0.88-2.49)
PG19	**IgG**	0.0896	0.2967	0.6219	0.3657		97.13 (41.61-226.73)	5.63 (3.24-9.76)	4.75 (2.76-8.16)	18.31 (9.77-34.3)	1.97 (1.17-3.31)	1.65 (0.99-2.76)
PG24	**IgG**	0.0285	0.7456	0.1944	0.6949	0.3173		4.92 (2.82-8.58)	3.87 (2.24-6.69)	9.05 (5.04-16.26)	2.71 (1.58-4.62)	1.37 (0.81-2.32)
PG32	**IgG**	0.2752	0.0088	0.1356	0.0253	0.0589	0.0164		125.1389 (53.24-294.11)	4.62 (2.73-7.81)	4.41 (2.65-7.34)	2.85 (1.74-4.68)
PG33	**IgG**	0.0779	0.0012	0.0312	0.0042	0.0105	0.0026	0.1615		5.11 (3.03-8.63)	4.02 (2.45-6.6)	3.29 (2.02-5.38)
PG40	**IgG**	0.4054	0.0235	0.2450	0.0325	0.0741	0.0290	0.6698	0.2383		1.65 (1.01-2.71)	2.52 (1.53-4.15)
VL-D2	**IgG**	0.0012	<.0001	0.0002	<.0001	0.0001	<.0001	0.0111	0.0719	0.0101		5.96 (3.63-9.8)
Reference standard		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0007	<.0001	0.0606	

The above diagonal element shows the odds ratio with 95% confidence intervals. The corresponding element below the diagonal presents the P value. P values <.05 indicate that there is significant disagreement in the outcome between the compared tests.

Supplementary Table 5. Cohen's kappa test for pairwise comparisons

		PM2	PM12	PM18	PM22	PM23	PM24	PM30	PM34	PM35	VL-D2	FPG1
		IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgG
PM2	IgM		0.36 (0.25-0.48)	0.46 (0.35-0.56)	0.3 (0.2-0.41)	0.55 (0.45-0.65)	0.5 (0.39-0.5997)	0.39 (0.29-0.5)	0.31 (0.2-0.42)	0.38 (0.27-0.49)	0.33 (0.23-0.44)	0.18 (0.07-0.29)
PM12	IgM	<.0001		0.36 (0.25-0.46)	0.29 (0.18-0.4)	0.32 (0.2-0.43)	0.3 (0.19-0.414238)	0.39 (0.29-0.5)	0.37 (0.27-0.48)	0.24 (0.13-0.35)	0.41 (0.31-0.51)	0.13 (0.02-0.23)
PM18	IgM	<.0001	<.0001		0.48 (0.38-0.58)	0.7 (0.61-0.78)	0.76 (0.69-0.838409)	0.62 (0.53-0.71)	0.63 (0.54-0.72)	0.69 (0.61-0.77)	0.51 (0.42-0.61)	0.22 (0.12-0.33)
PM22	IgM	<.0001	<.0001	<.0001		0.32 (0.21-0.43)	0.38 (0.27-0.48341)	0.47 (0.37-0.57)	0.64 (0.55-0.73)	0.53 (0.43-0.62)	0.54 (0.45-0.64)	0.21 (0.1-0.32)
PM23	IgM	<.0001	<.0001	<.0001	<.0001		0.8 (0.73-0.873939)	0.51 (0.41-0.61)	0.42 (0.32-0.53)	0.61 (0.52-0.7)	0.33 (0.23-0.44)	0.21 (0.1-0.32)
PM24	IgM	<.0001	<.0001	<.0001	<.0001	<.0001		0.54 (0.44-0.64)	0.51 (0.41-0.61)	0.68 (0.6-0.77)	0.39 (0.28-0.49)	0.24 (0.13-0.35)
PM30	IgM	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.55 (0.45-0.64)	0.49 (0.39-0.6)	0.5 (0.41-0.6)	0.17 (0.06-0.28)
PM34	IgM	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.6 (0.51-0.69)	0.6 (0.51-0.69)	0.26 (0.15-0.37)
PM35	IgM	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.45 (0.35-0.55)	0.22 (0.11-0.33)
VL-D2	IgM	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.19 (0.08-0.3)
FPG1	IgG	0.0009	0.0171	<.0001	<.0001	<.0001	<.0002	0.0023	<.0001	<.0001	<.0001	
PG1	IgG	0.3095	0.1618	0.4939	0.9815	0.8581	0.1712	<.0001	0.4823	0.3801	0.8063	0.0068
PG9	IgG	0.4598	0.48	0.6536	0.5693	0.8854	0.6476	0.9835	0.8275	0.7891	0.5077	<.0001
PG15A	IgG	0.8767	0.8223	0.969	0.524	0.6607	0.2313	0.9091	0.2679	0.2077	0.6625	0.0038
PG15B	IgG	0.8447	0.191	<.0001	0.898	0.4972	0.0743	0.3357	0.9318	0.6533	0.4184	0.0052
PG19	IgG	0.6532	0.3628	<.0001	0.6919	0.6794	0.1372	0.6941	0.5462	0.3178	0.7549	0.0146
PG24	IgG	0.786	0.14	<.0001	0.4436	0.1472	0.0228	0.941	0.7713	0.2245	0.5492	0.0058
PG32	IgG	0.6645	0.2269	<.0001	0.7416	0.1463	0.129	0.6194	0.6097	0.3084	0.5988	<.0001
PG33	IgG	0.729	0.4034	<.0001	0.2334	0.255	0.0942	0.155	0.0625	0.155	0.0715	<.0001
PG40	IgG	0.1387	0.3491	<.0001	0.4774	0.6978	0.5805	0.6417	0.5241	0.3915	0.7361	<.0001
VL-D2	IgG	0.0054	0.1411	<.0001	<.0001	0.0115	0.0007	0.0004	<.0001	<.0001	0.0018	<.0001
Reference standard		<.0001	0.011	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

The above diagonal element shows the κ̂ (kappa) with 95% confidence intervals. The corresponding element below the diagonal presents the two-sided P value. P values <.05 indicate that that degree of agreement or disagreement in the outcome between the compared tests is statistically significant.

Continuation **Supplementary Table 5. Cohen's kappa test for pairwise comparisons**

		PG1	PG9	PG15A	PG15B	PG19	PG24	PG32	PG33	PG40	VL-D2	Reference
		IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	IgG	standard
PM2	**IgM**	-0.06 (-0.16-0.05)	-0.04 (-0.14-0.06)	0.01 (-0.10-0.12)	-0.01 (-0.12-0.1)	-0.02 (-0.13-0.08)	0.01 (-0.09-0.12)	-0.02 (-0.13-0.08)	0.02 (-0.09-0.13)	-0.08 (-0.19-0.02)	0.15 (0.04-0.26)	0.24 (0.14-0.35)
PM12	**IgM**	-0.08 (-0.18-0.02)	-0.04 (-0.14-0.07)	-0.01 (-0.12-0.1)	-0.07 (-0.18-0.03)	-0.05 (-0.156-0.05)	-0.08 (-0.18-0.02)	-0.07 (-0.17-0.04)	0.05 (-0.06-0.16)	-0.05 (-0.16-0.05)	0.08 (-0.03-0.19)	0.13 (0.03-0.23)
PM18	**IgM**	-0.04 (-0.14-0.07)	-0.02 (-0.13-0.08)	-0.002 (-0.11-0.1)	-0.01 (-0.12-0.09)	-0.02 (-0.12-0.09)	0.03 (-0.08-0.13)	0.05 (-0.05-0.16)	0.08 (-0.04-0.19)	-0.02 (-0.13-0.08)	0.22 (0.11-0.33)	0.38 (0.28-0.48)
PM22	**IgM**	-0.001 (-0.11-0.11)	-0.03 (-0.13-0.07)	0.03 (-0.07-0.14)	-0.01 (-0.11-0.1)	0.02 (-0.08-0.13)	-0.04 (-0.14-0.06)	0.02 (-0.09-0.13)	0.07 (-0.04-0.18)	0.04 (-0.07-0.15)	0.23 (0.12-0.34)	0.41 (0.13-0.51)
PM23	**IgM**	-0.01 (-0.12-0.1)	-0.01 (-0.11-0.1)	0.02 (-0.09-0.13)	0.04 (-0.07-0.15)	0.02 (-0.09-0.13)	0.08 (-0.03-0.19)	0.08 (-0.03-0.19)	0.06 (-0.05-0.18)	-0.02 (-0.13-0.09)	0.14 (0.03-0.25)	0.24 (0.14-0.35)
PM24	**IgM**	0.08 (-0.04-0.19)	0.02 (-0.08-0.13)	0.07 (-0.04-0.178)	0.1 (-0.01-0.21)	0.08 (-0.03-0.19)	0.12 (0.01-0.23)	0.08 (-0.03-0.2)	0.09 (-0.02-0.21)	0.03 (-0.08-0.14)	0.19 (0.08-0.3)	0.31 (0.21-0.41)
PM30	**IgM**	-0.03 (-0.13-0.08)	-0.001 (-0.11-0.1)	-0.01 (-0.113-0.1)	-0.05 (-0.15-0.05)	-0.02 (-0.13-0.08)	0.004 (-0.1-0.11)	0.03 (-0.08-0.14)	0.08 (-0.03-0.19)	-0.03 (-0.13-0.08)	0.2 (0.09-0.31)	0.33 (0.23-0.43)
PM34	**IgM**	0.04 (-0.07-0.15)	0.01 (-0.09-0.11)	0.06 (-0.05-0.17)	0.005 (-0.10-0.11)	0.03 (-0.07-0.14)	0.02 (-0.09-0.12)	0.03 (-0.08-0.14)	0.1 (-0.01-0.21)	0.03 (-0.07-0.14)	0.24 (0.13-0.35)	0.4 (0.3-0.5)
PM35	**IgM**	0.05 (-0.06-0.16)	0.01 (-0.09-0.12)	0.07 (-0.04-0.18)	0.02 (-0.08-0.13)	0.05 (-0.05-0.16)	0.07 (-0.04-0.17)	0.06 (-0.05-0.17)	0.08 (-0.03-0.19)	0.05 (-0.06-0.16)	0.21 (0.1-0.32)	0.33 (0.23-0.43)
VL-D2	**IgM**	-0.01 (-0.12-0.09)	-0.03 (-0.13-0.06)	0.02 (-0.08-0.13)	-0.04 (-0.14-0.06)	-0.02 (-0.112-0.09)	-0.03 (-0.13-0.07)	0.03 (-0.08-0.14)	0.1 (-0.01-0.21)	-0.02 (-0.12-0.09)	0.17 (0.06-0.28)	0.46 (0.36-0.56)
FPG1	**IgG**	0.14 (0.04-0.25)	0.21 (0.11-0.31)	0.15 (0.05-0.26)	0.15 (0.04-0.25)	0.13 (0.023-0.23)	0.14 (0.04-0.25)	0.42 (0.32-0.52)	0.49 (0.39-0.58)	0.25 (0.14-0.36)	0.43 (0.33-0.53)	0.45 (0.35-0.55)
PG1	**IgG**		0.63 (0.53-0.73)	0.69 (0.6-0.78)	0.8 (0.72-0.88)	0.86 (0.79-0.92)	0.75 (0.66-0.83)	0.35 (0.24-0.46)	0.3 (0.19-0.41)	0.59 (0.49-0.69)	0.17 (0.06-0.28)	0.15 (0.05-0.25)
PG9	**IgG**	<.0001		0.77 (0.69-0.86)	0.62 (0.52-0.73)	0.6 (0.45-0.71)	0.66 (0.56-0.76)	0.32 (0.2-0.43)	0.28 (0.17-0.4)	0.35 (0.24-0.47)	0.13 (0.03-0.24)	0.04 (-0.05-0.14)
PG15A	**IgG**	<.0001	<.0001		0.68 (0.58-0.77)	0.69 (0.6-0.78)	0.67 (0.58-0.77)	0.31 (0.19-0.42)	0.27 (0.16-0.39)	0.41 (0.3-0.52)	0.22 (0.11-0.33)	0.12 (0.02-0.22)
PG15B	**IgG**	<.0001	<.0001	<.0001		0.9 (0.85-0.96)	0.77 (0.69-0.85)	0.36 (0.25-0.47)	0.31 (0.2-0.42)	0.54 (0.44-0.65)	0.14 (0.03-0.25)	0.07 (-0.03-0.17)
PG19	**IgG**	<.0001	<.0001	<.0001	<.0001		0.78 (0.7-0.86)	0.36 (0.24-0.47)	0.32 (0.21-0.43)	0.57 (0.47-0.67)	0.14 (0.03-0.24)	0.1 (0-0.2)
PG24	**IgG**	<.0001	<.0001	<.0001	<.0001	<.0001		0.32 (0.21-0.44)	0.27 (0.16-0.38)	0.44 (0.33-0.55)	0.19 (0.09-0.3)	0.06 (-0.04-0.16)
PG32	**IgG**	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.82 (0.75-0.89)	0.33 (0.22-0.44)	0.32 (0.22-0.43)	0.23 (0.12-0.33)
PG33	**IgG**	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.35 (0.24-0.46)	0.32 (0.2-0.42)	0.26 (0.16-0.37)
PG40	**IgG**	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.11 (0-0.22)	0.2 (0.09-0.3)
VL-D2	**IgG**	0.0017	0.0106	<.0001	0.0081	0.0098	0.0002	<.0001	<.0001	0.0465		0.41 (0.3-0.51)
Reference standard		0.0038	0.3793	0.0194	0.1395	0.0558	0.2375	<.0001	<.0001	0.0002	<.0001	

The above diagonal element shows the κ (kappa) with 95% confidence intervals. The corresponding element below the diagonal presents the two-sided P value. P values <.05 indicate that that degree of agreement or disagreement in the outcome between the compared tests is statistically significant.

Supplementary Table 6: Sensitivity values for specificity target >80% in multiple combinations of peptides

Samples included	IgM peptides				IgG peptides			
	RF model	Peptide	sensitivity	95% confidence interval	RF model	Peptide	sensitivity	95% confidence interval
Acute DENV pos: N=54 DENV neg: N=185	RFM1	PM22	0.4950	(0.4240 - 0.5660)	RFG1	PG40	0.6810	(0.6110 - 0.7440)
		+PM2	0.2037	(0.1177 - 0.329)		+PG15A	0.8333	(0.7126 - 0.9098)
		+PM30	0.5741	(0.4416 - 0.6967)		+FPG1	0.8704	(0.7558 - 0.9358)
		+PM18	0.5000	(0.3711 - 0.6288)		+PG19	0.8704	(0.7558 - 0.9358)
		+PM34	0.5370	(0.4061 - 0.6631)		+PG1	0.8704	(0.7558 - 0.9358)
		+PM24	0.5741	(0.4416 - 0.6967)		+PG24	0.8889	(0.7781 - 0.9481)
Convalescent DENV pos: N=46 DENV neg: N=185	RFM2	PM22	0.4730	(0.4020 - 0.5440)	RFG2	FPG1	0.8650	(0.8080 - 0.9070)
		+PM24	0.5652	(0.4225 - 0.6979)		+PG33	0.7609	(0.6206 - 0.8609)
		+PM18	0.5435	(0.4018 - 0.6784)		+PG9	0.8043	(0.6683 - 0.8935)
		+PM30	0.5217	(0.3814 - 0.6587)		+PG32	0.8696	(0.7433 - 0.9388)
		+PM34	0.5217	(0.3814 - 0.6587)		+PG15A	0.8913	(0.7696 - 0.9527)
		+PM2	0.5652	(0.4225 - 0.6979)		+PG1	0.8913	(0.7696 - 0.9527)
Acute + convalescent DENV pos: N=137 DENV neg: N=185	RFM3	PM22	0.6030	(0.5320 - 0.6710)	RFG3	FPG1	0.6110	(0.5390 - 0.6780)
		+PM2	0.6788	(0.5967 - 0.7512)		+PG40	0.5912	(0.5075 - 0.671)
		+PM30	0.6131	(0.5296 - 0.6905)		+PG15A	0.8613	(0.7935 - 0.9094)
		+PM34	0.6642	(0.5816 - 0.7379)		+PG33	0.8540	(0.7853 - 0.9035)
		+PM23	0.7372	(0.6578 - 0.8037)		+PG1	0.8759	(0.8103 - 0.9211)
		+PM12	0.7226	(0.6424 - 0.7907)		+PG9	0.8832	(0.8187 - 0.9268)

Confidence intervals were calculated using Wilson's method (95% binomial confidence interval). Antigen combinations were selected to optimize sensitivity while enforcing specificity > 80%.

5.12 References

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Chapter 6: General discussion

The antibody response against a pathogen is dynamic, complex and influenced by different intrinsic and extrinsic factors in the host and the virus. For the members of the *Flavivirus* family, their increased spread across the tropical and sub-tropical world has added an extra layer of complexity at both clinical and laboratory level. At the clinical level acute dengue disease presents similar clinical symptomatology compared to the different arboviruses that circulate in the same endemic areas. At the laboratory level, the similarity is particularly observed at epitope level, because the *Flavivirus* family shares approximately 60% of amino acid identity that elicits cross-reactive antibodies upon infection and could confound an accurate diagnosis. This problem emphasizes the need for a better insight in the antibody response, and in particular the viral epitopes targeted by the immune response.

In this study, a custom designed high-density proteome-wide peptide microarray was synthetized for DENV1-4, ZIKV and YFV and subsequently used to profile the IgM and IgG antibody response in DENV-infected individuals at a single epitope resolution. Highly reactive peptides were identified as seromarker candidates that could improve dengue serology testing. These were finally evaluated in a large panel of patient sera infected with DENV, ZIKV, YFV, WNV and HIV or immunized against TBEV, YFV and JEV, as well as healthy individuals, to further assess their diagnostic potential.

At the start of this study in 2018, a patient cohort study was set up in Yurimaguas, in the Peruvian Amazon region, for the recruiting of acute febrile patients to study the etiology of acute tropical fever. During this time, a dengue outbreak occurred in this region between October 2018 and March 2019, which has led to the identification of a new dengue virus variant through the sequencing of the envelope gene (chapter 1, Section 1.2 of this thesis). The phylogenetic analysis revealed that the circulating strain belongs to serotype 2 and the American/Asian genotype. The circulating variant was characterized by the presence of two novel mutations (I379T and V484I), located at the DIII and transmembrane region of the E protein, respectively.

Coincidentally, the dengue outbreak was associated with an increase in the number of hospitalized cases (~29%) in the region, in comparison with previous years (9% in 2016 and 17% in 2017). This finding contributes to the molecular epidemiological understanding of the DENV circulation in Peru. Therefore, active long-term molecular

surveillance and genomic characterization of DENV and other arboviruses in Peru are necessary to better understand the evolutionary basis of the genetic changes, determine its association with antigenic variation and the possible implication in the population dynamics of arboviral diseases.

6.1 Short synthetic peptides resemble the immunoreactivity of linear peptides present in whole protein antigens

After encountering a pathogen, the immune system develops an antibody response produced by B cells for the development of short- and long-lived plasma cells as well as B-memory cells. The long-lived plasma cells secretion antibodies that can last from a few months to a lifetime. The objective of serological assays is to capture antibodies that bind to antigens of the pathogen with high sensitivity and specificity. The performance of the test strongly depends on the nature of the antigen used. A rapid and straightforward way to capture antibodies reactive against a pathogen is to utilize the entire proteome of the pathogen by growing it *in vitro* and to display either the entire purified pathogen or disrupted fractions of it. Newer, recombinant protein production platforms have led to diagnostic tests that contain one or more recombinant proteins of a pathogen.

The difficulties associated with the production of complete organisms and the cross-reactivity frequently found (1–3), led to the search for more discrete antigens that can provide more specific results and with greater sensitivity. The rapid scale-up and ease in the production of recombinant proteins through different recombinant expression systems have led serology to replace the use of entire pathogens for these less-complex biomaterials. Thus, the use of recombinant proteins resulted in increased specificity of the tests by eliminating undesirable content from cell cultures. The drawback of this approach is that recombinant proteins, depending on the expression system in which they are produced, do not always completely mimic the native protein.

For dengue and other flaviviruses, mammalian expression systems (HEK-293 cells) are preferred for the expression of recombinant proteins to prokaryotic hosts since they yield properly folded and assembled proteins, with native-like post-translational modifications needed to maintain their structural and immunological properties present in the native protein. For dengue serology, NS1 protein has been rapidly adopted in dengue

diagnostics for its direct detection in the bloodstream (circulate at high quantities during the acute phase) and also given its high immunogenicity it is used as an antigen for the detection of anti-NS1 IgM and IgG antibodies. Since NS1 requires glycosylation for its correct folding and dimerization, HEK-293 cells are the preferred system for its production (4,5).

The Envelope protein is also considered as a good diagnostic marker because it is a structural protein that contains the highest number of immunodominant epitopes and thus not only the specificity but also the sensitivity could be improved if it is implemented in diagnostic tests (6). The domain III (EDIII) is the preferred region for diagnostic purposes because it is highly immunogenic and able to elicit type-specific antibodies. Recombinant proteins of the EDIII have shown promising results and have been postulated as an alternative simpler and cheaper option to the gold standard neutralization test for serotype classification (7–9).

Over time, the increasing co-circulation of other members of the *Flavivirus* family in previously predominantly solely dengue regions resulted in a loss of specificity for the available assays with significant cross-reactivity. As a result of the high antigenic similarity shared among flaviviruses, pre-existing antibodies and the original antigenic sin phenomenon (re-call of antibodies with higher affinity to previous infection than current infection) increase the difficulties of an accurate diagnosis of dengue. Therefore, relevant antigens with higher epitope resolution able to capture specific antibodies are needed to overcome the challenge for differential diagnosis among these highly similar pathogens.

In this work, it was demonstrated that linear epitopes from the most prevalent flaviviruses DENV, ZIKV and YFV in the Americas, presented in a high-content microarray library can be recognized by IgM and IgG antibodies present in the sera of DENV-infected individuals. Unlike previous works in the field of arboviruses that used this technology on: (i) selected peptides from different flaviviruses that were reported as immunogenic epitopes, (ii) overlapping peptides covering single proteins such as ZIKV-NS1 (10,11), DENV-NS4B (12), ZIKV-Envelope (13), DENV-NS1 (10,14), (iii) overlapping peptides covering single proteomes of TBEV (15), ZIKV (16) and CHIKV (17), this work together with the one published by Mishra *et al.* (18) are the only ones that use a microarray library

covering the entire proteomes of DENV, ZIKV and YFV with overlapping peptides using human samples. Unlike Mishra *et al*. (18) that focused their work on the search of peptides for the differential diagnosis of ZIKV, this study was not only focusing on the selection of discriminatory peptides for DENV diagnosis, but also in elucidating the dynamics of the antibody responses through time by including a large batch of follow-up samples from DENV-infected individuals and targeting IgM and IgG antibodies. This study confirms previous studies stating that short synthetic peptides can act as surrogates of true peptides, mimicking the immunoreactivity of continuous epitopes present in whole proteins (19) and acting as molecular bioprobes to capture the antibody diversity present in naturally-infected DENV patients (20,21).

The mapping resolution of the microarray used in this study consisted of 15-mer peptides with an overlapping of 11 residues. To increase this resolution and identify the contribution of each position to the antibody binding, a useful approach could be to design custom-peptide microarray libraries covering the peptides of interest with an offset resolution of one amino acid. Moreover, despite the fact that an epitope could range from 5 to 22 amino acids in size (22) not all the residues are in close contact with the paratope in the antibody, the inclusion of peptides with different sizes will add another layer of information regarding the specific length of the epitope involved in the antibody binding.

The main concern with the peptide microarray technology employed in this study is that it only contains linear epitopes. This is because it has been described that nearly 90-95% of the epitopes targeted by B cells that are present in an intact protein are known to be structural or discontinuous (23).

Mapping conformational epitopes through the structure of antigen-antibody crystals is a demanding task not only for the intrinsic difficulties associated to crystallographic and nuclear magnetic resonance methods, but also because for many arboviral proteins the detailed 3-dimensional structures are not resolved (24). Unfortunately, obtaining a high-resolution structure of a protein is laborious, costly, technically difficult, and not applicable to all proteins. Alternatively, phage (25), bacteria and yeast cells (26) have been used to display libraries of DENV antigens in an attempt to screen discontinuous epitopes. Using these technologies, thousands to millions of peptides can be displayed;

however, the main difficulty associated with these methods is the lack of tracking the exact peptide sequences expressed and the subsequent need to sequence positive clones, making the identification of the conformational time-consuming.

In addition, most studies have focused on mapping structural epitopes as a basis for the identification of immunogens that can elicit neutralizing antibodies that can be used for example as vaccine candidates (27–29) or for therapeutic purposes (30). However, for diagnostics the main criteria for identifying a good biomarker is that its cognate epitope is recognized by the antibody likely with high affinity and in a specific way, independently of its conformation (continuous or discontinuous) or its role in disease control.

Therefore, the peptide microarray technology has led to a revolution in the field of biomarker discovery. Especially, since it is able to screen and map thousands of continuous amino acid sequences simultaneously to define linear epitopes present in the protein that are recognized by antibodies.

Linear epitopes only represent approximately 5-10% of the epitopes in a protein (23); however, after the encounter with a pathogen, an almost unlimited repertoire/diversity of antibodies are produced. These antibodies react not only against continuous residues present in native protein, but also against linear epitopes that can become available in unfolded or processed proteins. Therefore, continuous epitopes although not abundant in whole-proteins, represent an important part of the antigenicity of the viral proteins, which in combination with a high-content presentation in the microarray platform can be exploited for the discovery of diagnostic, therapeutic or as vaccine candidates.

Recently, new peptide microarray libraries containing conformational epitopes are available. To create these three-dimensional libraries, peptides are linearly synthetized using high-throughput microarray synthesis technology, later the peptides conformational structures such as loops, beta sheets or alpha-helices are created using chemical linkages such as insertion of cysteines at specific positions. This technology, known as CLIPS for Chemical Linkage of Peptides onto Scaffolds, is being currently used for the discovery of drugs or for the identification of enzyme-recognition motifs (31)

In this study, the analysis was centered at finding peptides for their potential application in dengue serology, for which only samples from patients experiencing a recent dengue infection using follow-up samples were included to understand the dynamic of the antibody responses. In a parallel study, we have used the same microarray library to screen ZIKV, YFV and CHIKV samples from primo-infected individuals with the objective to select differentiating peptides that could lead to the improvement of the current serological methods for these highly co-circulating viruses (manuscript in preparation).

6.2. Highly immunodominant linear epitopes are present in structural and non-structural proteins of DENV

Another fundamental part of this work is that the proteome-wide linear epitope screening allowed both the reiteration of widely known immunological relevance of epitopes present in structural proteins, i.e. NS1 and E, and has led to the discovery of novel highly immunodominant epitopes present in the less characterized non-structural proteins. Because peptides were synthetized in equimolar amounts in each spot of the microarray, the magnitude of the antibody response against each peptide was quantified and it was possible to determine that despite the magnitude of the response against NS1 protein, peptides located in NS3 and NS5 were also targeted with high reactivity.

Shortly after our results of the screening of linear peptides using the peptide microarray were published, Fumagali *et al*. published a review describing the linear and continuous epitopes available in the literature for Flaviviruses for which immunoreactivity was confirmed in infected humans (21). In this review it is highlighted that the vast majority of described epitopes are biased towards structural proteins that comprise 63% (96/153) of the total epitopes, and among the epitopes described in the non-structural proteins, 88% (50/57) belong to the NS1 protein. Our work added important and detailed information regarding immunodominant epitopes targeted not only by IgG antibodies, but also by IgM antibodies. We were able to identify novel linear epitopes in structural proteins and also described important peptides with high reactivity and broad recognition by DENV-infected individuals located in non-structural proteins.

In our study, important immunodominant regions were mapped to domain I of NS3 protein, to the MTase and thumb domains of NS5 and also highly reactive regions on smaller proteins such as the NS2, NS4a and NS4b were targeted by IgM and IgG antibodies. These findings provide evidence that sequences present in non-structural proteins can also contain linear epitopes recognized by antibodies. The approach in this study using peptide microarrays has provided proof of principle that it allows for in depth epitope mapping at proteome level and that it holds promise for the discovery of unique peptide biomarkers for other emerging and re-emerging pathogens.

The role that antibodies targeting linear epitopes might have in the response against dengue will also require further extensive analysis. In order to better understand the antibody response against dengue, research on the role of antibodies directed against far less studied non-structural proteins such as NS2A, NS2B, NS4A, NS4B is important to be carried out. The high immunogenicity from regions located in these proteins targeted by IgM and IgG antibodies implies their value as attractive targets for diagnosis given the lower amino acid identity shared among flavivirus compared to the structural proteins (24).

6.3. The dynamics of the antibody response

Despite the limited number of samples included in the screening of the microarray library, differences in the humoral response of patients experiencing a primary infection (travelers) were found in contrast to patients from endemic areas, who most likely underwent a past infection with other arboviruses, thus experiencing a secondary (or multiple) infection. Consistent with previous studies that aimed to characterize the dynamics of the antibody response against flaviviruses, it was observed that the reactivity against linear epitopes is higher in patients experiencing secondary infections compared to the response in individuals with a primary infection (32,33), while the opposite was observed for the IgM response, higher levels in primary infections in comparison to the response in secondary infections (34–36). The dynamics of the humoral response was also dependent on the class of immunoglobulins (IgG vs IgM antibodies). In general, the IgG antibody response from the evaluated individuals was characterized by a high depth, recognizing peptide sequences from different genotypes, serotypes or genus, especially

for peptides located in regions with higher amino acid identity. The IgG response followed a rise and fall pattern, reaching peak levels between 10 and 30 days after symptoms onset. The IgM antibody response on the other hand exhibited a strong breadth and was highly variable among patients. IgM-targeted peptides were scattered across the polyprotein and serotype- or genotype-specific sequences were frequently uniquely distinctively targeted by different patients. The magnitude of the IgM response was lower compared to IgG, but its levels of reactivity remained quite constant for the observed period (approx. 6 months).

6.4. Single peptides offer acceptable diagnostic performance but will be improved by combining multiple peptides using a machine learning algorithm

The results obtained from the evaluation of the selected peptides using the multiplex immunoassay are promising for the development of efficient diagnostic methods for the differential diagnosis of dengue. These results confirm the feasibility of the use of synthetic peptides as substitutes of full antigens in serological tests with comparable sensitivity and specificity.

In addition to the potential use of the selected peptides to identify dengue infected individuals, peptides PM-2, PM-35, PG-1, PG-15B, PG-19 and PG-40 showed potential to discriminate between endemic (most likely secondary infections) and non-endemic (primary infections) individuals. Endemic individuals showed significant higher values against these peptides, which could be of potential utility to determine the serostatus for vaccine-candidate recipients of the Dengvaxia vaccine.

6.5. General conclusion

The enormous potential of peptide microarrays to detect, differentiate and characterize antibodies in a manner unique to this high-content, high-throughput technology makes it an attractive approach to discover novel biomarkers. The work presented here used a peptide microarray as a targeted immune-based approach to provide a deeper insight into the humoral immune response against flavivirus at the epitope level and laid the foundation for the development of specific serological tests for DENV infection. The study is based on four pillars: (i) a peptide microarray based analysis of the IgM and IgG

immune response against the entire DENV proteome and other Flavivirus proteomes, (ii) the identification of linear immunodominant epitopes at high resolution at the proteome level, (iii) the selection of DENV peptides that are recognized by serum antibodies of DENV patients but not by serum antibodies from healthy controls, and whose homologous immudominant sequences present in other Flavivirus are not (or weakly) recognized by serum antibodies from DENV patients (reduce cross-reactivity), and (v) the selected DENV peptides showed promising diagnostic performance after its further evaluation using a diverse sera panel.

Finally, this study present with sufficient proof that going from high- to low-complexity in the number of peptides evaluated and from low to high number of samples, represent a promising profiling workflow towards the discovery of novel seromarkers.

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Chapter 7: Future perspectives

The peptide microarray approach used in this study can serve as a reference for similar discovery expeditions for other arboviruses, such as the emerging togaviruses CHIKV, O'nyong-nyong (ONNV), Mayaro virus (MAYV) and flaviviruses Rocio and Ilheus. The co-circulation of CHIKV and MAYV, and Rocio, Ilheus, Zika and dengue in Southern and Central America and CHIKV and ONNV in the African continent (1), imposes a similar problem of cross-reactivity as observed today with dengue and Zika: a reliable diagnosis cannot be based nor on geographical information nor in the clinical symptomatology because the clinical picture is similar to those of other arboviruses with a “dengue-like” syndrome.

Because accurate laboratory diagnostic tools are also needed to ensure the timely detection and proper management of alphavirus cases and the few available tests are also hindered by cross-reactivity of antibodies (2) as a result of the high percentage identity across alphavirus antigens (3), the application of a high-content overlapping-peptide microarray covering the proteomes of important (re-)emerging alphaviruses would allow the antibody profiling and identification of discriminative epitopes for the development of differential diagnostic tests that could tackle future cross-reactivity issues associated with the increase co-circulation of these viruses across tropical and sub-tropical areas.

With several arboviruses spreading more globally and presenting a growing public health concern, the interest to develop vaccines for these viruses has consequently gained traction. The successful application of mRNA-based vaccines for SARS-CoV-2 will likely lead to similar developments of vaccines for dengue, Zika and Chikungunya viruses (4–6). Therefore, the development of antibody-based diagnostic tools with sufficient discriminatory potential between flaviviruses is important, not only for the proper management of clinical cases but also for the monitoring of the humoral response following vaccination and to distinguish between a current infection, a past infection or vaccine-induced responses. The antigen used in the vaccine will determine which peptides/antigens can be used in diagnostic tests to enable differentiation between antibodies induced by infection versus those induced by vaccination. Our proteome-wide mapping of peptides is a useful resource to select biomarkers that are specific for dengue infection or vaccination. In this work we identified these epitopes targeted by dengue-infected individuals, but a similar approach can be applied to other flaviviruses.

Defining the antigenicity of pathogens is not an easy task, especially because this process is more accurately done by mapping structural epitopes using monoclonal antibodies that bind 3D-structures of proteins. Unfortunately, high-resolution structures are not available for many flavivirus proteins (3). While the peptide microarray approach implies the simplification of complex structural regions (discontinuous epitopes) present in intact proteins into their primary amino acidic sequences (continuous epitopes) it allows to determine the immunodominance of the antibody response in the entire proteome of the virus, through the quantification of the magnitude of the antibody response directed against short overlapping peptides.

Further characterization will be needed to determine if the peptides found to be targeted by IgM and IgG antibodies in this study are part of epitopes present in either accessible surface or less accessible regions of the proteins. For those proteins for which the 3-D structures are available, the modelling of the peptides could be done relatively straightforward using bioinformatics resources (3).

Additionally, peptides that are recognized by antibodies early after infection and that are generated only in patients experiencing severe forms of the disease are of higher interest because they can be used as possible diagnostic biomarker candidates for the prediction of disease severity. Thus, further evaluation including a separate validation in cohort of patients which span mild and severe disease states would be required to confirm the value of those peptides that were more highly reactive in hospitalized patients respect to the patients that were not, such as PG-32 and PG-33 localized in the NS3 protein.

For future studies it would be also interesting to see how the levels of IgM and IgG antibodies correlates with the elicited B-cell repertoire after infection. In this study we have evaluated the antibody response on soluble immunoglobulins. However, to better understand the humoral response against DENV, it will be interesting to characterize as well if the immunogenic regions identified in this work are also targeted by B-cell receptors and if their hierarchy against immunodominant regions is similar to the profile found in this work. This will help to determine if the reactivity against highly similar sequences is due to the recognition of the same antibody that cross-reacts with these sequences because critical residues are kept in the epitope or if each sequence is recognized by a particular antibody of the vast diverse antibody repertoire.

It will also be interesting to see how the dynamics of the antibody responses correlate with antigen accessibility. Further characterization is needed to see if higher antibody titers and duration of the antibodies in the sera after infection are related with: (i) higher accessibility of the epitopes, (ii) longer presence of the antigen in the system (longer exposure), (iii) high affinity of the antibody for its cognate epitope, and (iv) the IgG subclasses involved in the antibody response.

Although the peptides selected in this study did not show sufficient sensitivity or specificity values (specificities below 81%) to be immediately used in less complex tests or POC tests, they have the potential to be improved with currently available engineering techniques. This would allow the following improvements: (i) determine the contribution of each amino acid to the antibody binding, (ii) replace those amino acids that provide little reactivity with others that could increase it, (iii) synthesized them with post-translational modifications in order to more closely resemble the native epitope, or (iv) synthesize them together with others peptides to create a microenvironment that enhances the presentation of the peptide to the antibody.

Therefore, to gain finer specificity on the critical residues involved in the antibody recognition towards the selected peptides, a systematic single substitution at each of the 15 positions with all 20 naturally occurring amino acids can be done. Buus *et al.* used peptide libraries to map the specificity of polyclonal antibodies to linear epitopes through single amino-acid substitutions, thus identifying the length and most dominating residues contained in the epitope of each 15-mer peptide (7). Similarly, peptides identified in this study could follow a similar approach, which together with the development of the ArrayPitope application, it could allow the identification of the critical residues comprising the targeted epitope by visualizing the contribution of each single amino-acid substitutions through the visualization of epitope logos (8). Comparably, epitope substitutions scans to determine critical amino acids are also commercially available (Pepperprint, Germany) which apart from substituting each position of the peptide with the 20 natural amino acids, non-natural amino acids can also be included in the analysis. Another powerful approach to evaluate the role of specific amino acids within the epitope is alanine scanning mutagenesis. This method involves the single substitution of non-alanine residues by alanine and later the mutant libraries are evaluated by functional

testing. The substitution with a small residue such as alanine could result in loss of effective interactions, but it also could reduce the steric hindrance impose by large residues in the binding, which results in an overall affinity enhanced interaction of the epitope with the paratope in the desired antibody (9), therefore determining the relevance of specific amino acid sidechains within the peptide of interest. Through this scanning non-critical amino acids present in the epitope could be replaced to improve its binding strength to the antibody. This controlled randomized library has been widely used for functional analysis of DENV proteins to identify important residues involved in the function of the protein (10,11) or to map neutralizing epitopes targeted by monoclonal antibodies (12).

Other technologies are also available to improve the recognition of an epitope by its cognate antibody that consists in the synthesis of the peptides surrounded by other peptides/molecules with the aim to create a microenvironment that allows a better presentation of the epitope, making it more accessible and diminishing possible steric problems (13).

After optimizing the selected peptides, they could be translated to more affordable formats such as an ELISA or a Lateral flow assay (LFA). Depending of the final format decided for the diagnostic test, the peptide will require additional modifications. If the proof of concept is designed so that the biomarker will directly bind to the solid surface of the format (i.e. polystyrene in ELISA microplates, nitrocellulose in LFAs), then it is a common practice to add a tag molecule, frequently a protein carrier, either at the C- or N-term in order to optimize the binding of the peptides to the desired solid-phase surface; however, unmodified simple peptides can be also used as final bioprobes in diagnostic tests.

When no tags are added to the peptides, their binding to the solid surface will depend on the material and the amino acid sequence of the peptide. Thus, the passive immobilization of the peptide to the desired solid surface could be improved if the pH of the buffer used to coat the biomolecule is below the isoelectric point of the peptide, because the exposure of hydrophobic regions will be optimized. Therefore, acidic, neutral or basic buffers will be used to coat the peptide on the solid surface (14–16).

On the other hand, given the small size of the peptides they are frequently conjugated to molecules of higher size in order to improve their immobilization to the solid surface. The most common molecules for this purpose are biotin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and ovalbumin (OVA) (17,18). Unfortunately, as far as we know, no literature is available on the comparison of the use of these tags to improve the binding of small peptides to solid surfaces. The larger size of KLH and its higher cost in comparison with BSA and OVA, make it not the most preferred tag; however, it has the advantage of rendering lower cross-reactions in the immunoassay. Among the four most used conjugate molecules before mentioned, biotin offers the best option because biotinylated peptides can be subsequently attached to a solid surface coated with avidin or streptavidin. Then, the efficient immobilization of the peptide to the solid surface is optimized thus rendering higher sensitivity. However, a major disadvantage is the high cost that limits its application in the development of affordable tests.

Future development and validation process of the test

A final goal in our research group that goes beyond the objectives set for this study, is to develop a very sensitive and specific test that can be deployed to low-resource settings where they are most needed.

To accomplished this, the selected peptides can be translated to a more affordable assay format, ideally for its implementation as POCTs, that fulfil the ASSURED (affordable, sensitive, specific, user-friendly, rapid, equipment-free, delivered) criteria.

A critical phase in the development of a new test is the validation process because it will determine the fitness of the assay in order to guarantee that it has been properly developed, optimized and standardized for an intended purpose.

The definition of the intended purpose is a fundamental first step in the development of a test, prior the validation process, because it delineates its appropriate future use. In our case, independent validation processes will have to done for the selected peptides in the case its intended use is to (i) estimate seroprevalence of dengue in the population, (ii) confirm diagnosis of suspected dengue cases, or (iii) determine the serostatus of individuals for vaccination. The development of the test also involves the definition of

the sample type to be used, writing the standardize operating protocols (SOPs), the careful selection of reagents, materials and controls in the enough volume/quantity to complete the process. Besides, for the standardization of the assay, depending of the assay format, labor intensive optimizations will be needed that includes the use of checkerboard titrations with the aim to select appropriate reagents and sample dilutions. Furthermore, buffer formulations, reaction times and temperature ranges, as well as essential operational equipment specification are also part of the standardization process. The proper definition of all these parameters will provide with a solid reliable proof of principle.

Typically, the validation pathway of an assay involves three main stages: (i) determine the analytical characteristics (repeatability, calculation of the analytical sensitivity and specificity), (ii) determine the diagnostic characteristics (calculation of the diagnostic sensitivity and specificity, and the cut-off), and (iii) evaluate the reproducibility of the assay (ability of the test to provide consistent results when evaluate in different laboratories).

An important challenge during the validation process will be the selection of well-defined reference controls and test samples, for which the history of previous arboviral infections is well-documented.

Once the validation process has been successfully achieved, the next step is the implementation for its intended application. This step involves not only the diagnostic suitability of the assay, but also other factors such as its ability to meet operational requirements and its acceptability for the scientific community and laboratory or technical staff. At this stage, if the assay shows to be robust enough for its intended use, the eventual marketing may be of interest to commercial companies who could finally be able to produce and scale the assay for its commercial use.

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Appendix 1: Curriculum vitae

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Academic studies

2018 - 2022	PhD Biomedical Sciences University of Antwerp, Belgium
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Professional experience:

2018 - 2022	Doctoral project: "*Assessing the antibody responses against dengue virus in the context of emerging arboviruses – from basic immunology to new diagnostics"* Virology Unit, Institute of Tropical Medicine Supervisor: Dr. Kevin Arien (KArien@itg.be). Co-promotor: Dr. Michael Talledo (michael.talledo@yahoo.com)
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International mobility

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List of publications

2022 **Falconi-Agapito F,** Kerkhof K, Merino X, et al. Peptide Biomarkers for the Diagnosis of Dengue Infection [Internet]. Front. Immunol. 2022. Available from:

https://www.frontiersin.org/article/10.3389/fimmu.2022.793882

2021 **Falconi-Agapito F**, Kerkhof K, Merino X, Michiels J, Van Esbroeck M, Bartholomeeusen K, Talledo M, Ariën KK. Dynamics of the Magnitude, Breadth and Depth of the Antibody Response at Epitope Level Following Dengue Infection. *Front Immunol* (2021) **12**:2625. doi: 10.3389/fimmu.2021.686691.

2020 **Falconi-Agapito F**, Selhorst P, Merino X, Torres F, Michiels J, Fernandez C, et al. A New Genetic Variant of Dengue Serotype 2 Virus Circulating in the Peruvian Amazon. Int J Infect Dis (2020) 96:136–8.

2020 Kerkhof K*, **Falconi-Agapito F***, Van Esbroeck M, Talledo M, Ariën KK. Reliable Serological Diagnostic Tests for Arboviruses: Feasible or Utopia? Trends Microbiol (2020) 28:276–292. doi:10.1016/j.tim.2019.11.005. (*equally contributed)

2015 **Falconi-Agapito F,** Saravia LE, Flores-Pérez A, Fernández-Díaz M. Naturally Occurring β-Nicotinamide Adenine Dinucleotide–Independent Avibacterium paragallinarum Isolate in Peru. Avian Dis (2015) 59:341–343. doi:10.1637/10969-110314-CaseR

2015 Valdivia-Olarte H, Requena D, Ramirez M, Saravia LE, Izquierdo R, **Falconi-Agapito F,** Zavaleta M, Best I, Fernández-Díaz M, Zimic M. Design of a predicted MHC restricted short peptide immunodiagnostic and vaccine candidate for Fowl adenovirus C in chicken infection. Bioinformation (2015) 11:460–465. doi:10.6026/97320630011460

2014 Chamakh-Ayari R, Bras-Gonçalves R, Bahi-Jaber N, Petitdidier E, Markikou-Ouni W, Aoun K, Moreno J, Carrillo E, Salotra P, Kaushal H, et al. In vitro evaluation of a soluble Leishmania promastigote surface antigen as a potential vaccine candidate against human leishmaniasis. PLoS One (2014) 9:e92708–e92708. doi:10.1371/journal.pone.0092708

Conference abstracts (last 6 years):

2021 "Synthetic peptides as biomarkers for the diagnosis of dengue infection".

Francesca Falconi-Agapito, Karen Kerkhof, Xiomara Merino, Diana Bakokimi, Fiorella Torres, Marjan Van Esbroeck, Michael Talledo & Kevin K. Ariën. 62nd Institute of Tropical Medicine Colloquium. Antwerp, Belgium. December, 2021. Virtual meeting.

Poster presentation

2021 "Epitope biomarkers for the detection of anti-dengue antibodies in human sera". **Francesca Falconi-Agapito**, Karen Kerkhof, Marjan Van Esbroeck, Xiomara Merino, Michael Talledo & Kevin K. Ariën. 69th Annual Meeting of the American Society of Tropical Medicine and Hygiene. November, 2021. Virtual Meeting.

Poster presentation

2020 "Profiling of the Epitope Diversity and Evolution of Dengue Binding Antibodies by Peptide Microarray". **Francesca Falconi-Agapito**, Karen Kerkhof, Xiomara Merino, Marjan Van Esbroeck, Michael Talledo & Kevin K. Ariën, 69th Annual Meeting of the American Society of Tropical Medicine and Hygiene. November, 2020. Virtual Meeting. Oral presentation.

2019 "Co-circulation of Dengue, Zika and Chikungunya in the Peruvian Amazon". **Francesca Falconi-Agapito**, Xiomara Merino, Karen Kerkhof, Kevin K. Ariën, Michael Talledo. 68th Annual Meeting of the American Society of Tropical Medicine and Hygiene. November, 2019. National Harbor, MD, USA. Poster presentation.

2018 "Variability of IgM and IgG antibody levels on PCR dengue positive patients in arbovirus endemic areas in Peru". **Falconi-Agapito, F**; Merino X, Nolasco, O, Kerkhof K, Arien K and Talledo, M. 7th European Congress of Virology. April, 2019. Rotterdam, Netherlands.

2017 "Preliminary results of the population genetic structure of *Aedes aegypti* from Peru"

F. Falconi-Agapito, M. Talledo, MA. Miranda, A. Poma, C. Paredes-Esquivel. 7th International Congress of the Society for Vector Ecology (SOVE). October, 2017. Mallorca, Spain. Poster presentation.

2017 "Presence of defective HTLV-1 provirus in Peruvian asymptomatic carriers". R. Mora, J. Rosado, **F. Falconi-Agapito**, C. Ocampo, E. Gotuzzo, C. Alvarez, M. Talledo M. 10th European Congress on Tropical Medicine and International Health. October 2017. Antwerp, Belgium. Oral presentation.

2016 "Genetic diversity of human T-lymphotropic virus 1 in Peru". J. Rosado, **F. Falconi-Agapito**, C. Ocampo, C. Alvarez, G. López, K. Verdonck, E. Gotuzzo, M. Talledo. MEEGID XIII: 13th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases. May, 2016. Antwerp, Belgium. Oral presentation.

Courses/conferences attendance (last 6 years):

62nd Institute of Tropical Medicine Colloquium. Antwerp, Belgium. December, 2021. Virtual meeting.

2021 *Poster presentation*

ASTMH 70th Annual Meeting. Organized by the American Society of Tropical Medicine and Hygiene. November, 2021. *Virtual Meeting.*

Molecular Data for Infectious Diseases. Organized by Institute of Tropical Medicine Antwerp. Distance learning. November 16, 2020-December 11, 2020. March 29, 2021 – April 16, 2021.

2020

ASTMH 69th Annual Meeting. Organized by the American Society of Tropical Medicine and Hygiene. November, 2020. *Virtual Meeting.*

Poster presentation. Travel award recipient.

ASTMH 68th Annual Meeting. Organized by the American Society of Tropical Medicine and Hygiene. November, 2019. National Harbor, MD, USA

Poster presentation. ACAV scholarship recipient.

2019

II Local EBQ course – Peru. "Epidemiology, Biostatistic & Qualitative Research Methods". Organized by Universidad Nacional de la Amazonía Peruana, Universidad Peruana Cayetano Heredia and The Global Health Institute, University of Antwerp

"Multivariate Data Analysis". Organized by StatUa and the Antwerp Doctoral School.

Workshop: "A multidisciplinary approach to the challenge of arboviral diseases in America" Instituto Nacional de Salud Pública. Cuernavaca, Mexico. *Attendant*

I International Symposium: Molecular Epidemiology applied to Infectious Diseases. Molecular Epidemiology Unit, Institute of Tropical Medicine "Alexander von Humboldt" and School of Sciences and Philosophy "Alberto Cazorla Talleri". Universidad Peruana Cayetano Heredia. Lima, Peru. *Chairman*

2018

XV Course and Workshop of Training in Molecular Biology Techniques Applied to Tropical Infectious Diseases. Molecular Epidemiology Unit, IMTAvH and School of Sciences and Philosophy "Alberto Cazorla Talleri" Universidad Peruana Cayetano Heredia. Lima, Peru. Lecturer and Organizer

Course: Infectología y Medicina Tropical para el siglo XXI. Institute of Tropical Medicine "Alexander von Humboldt", Universidad Peruana Cayetano Heredia. *Attendant*

Workshop: Recognition of *Anopheles darlingi* and its importance in research. Fogarty International Center and Universidad Peruana Cayetano Heredia. *Attendant*

2017

Biomedical research – Basic. Training requirements for Investigators and staff involved primarily in biomedical research with human subjects. CITI Programa de Educación en Ética de la Investigación. On-line course

Workshop of Scientific writing. Fogarty International Center and Universidad Peruana Cayetano Heredia. *Attendant*

Immunology International Course. Pasteur Institute and National Health Institute. Lima, Peru. *Attendant*

Conference: Biotechnology, Human Health and Molecular Epidemiology. "Oligopeptides for the new generation of serologic diagnostic in Leishmaniasis". Biological Sciences School. Universidad Nacional de Trujillo. Lecturer

2016 Course: Responsible behaviour in Research. Andean Center for Research and Training in Computer Science for Global Health. On-line course

XIII Course and Workshop of Training in Molecular Biology Techniques Applied to Tropical Infectious Diseases. Molecular Epidemiology Unit, IMTAvH and School of Sciences and Philosophy "Alberto Cazorla Talleri". Universidad Peruana Cayetano Heredia. Lima, Perú. *Lecturer*

Academic Teaching

2015 - 2016 Lecturer at Laboratory Lessons. Cellular and Molecular Biology Course. Medicine School. Universidad Científica del Sur

2013 Invited lecturer. Teaching lessons in Avian Pathology Veterinary School. Universidad Nacional San Luis Gonzaga, Ica-Peru.

2007 - 2008 Invited lecturer. Teaching some lessons of Micology. School of Biological Sciences, Universidad Nacional Mayor de San Marcos, Lima

2003 Laboratory Assistant. Assistance work of Parasitology II School of Biological Sciences. Universidad Nacional Mayor de San Marcos, Lima

Honours and achievements

2020 ASTMH 2020 Annual Meeting Travel Award, supported in part by the Bill & Melinda Gates Foundation

2019 ACAV Student Travel Award to attend the ASTMH Annual Meeting in Maryland, USA.

2019 VLIR-UOS Scholarship to attend the II Local EBQ course – Peru. "Epidemiology, Biostatistic & Qualitative Research Methods" Organized by Universidad Nacional de la Amazonía Peruana, Universidad Peruana Cayetano Heredia and The Global Health Institute, University of Antwerp.

2018	Travel award granted by the Public Health Institute, Cuernavaca, Mexico to attend the workshop "A multidisciplinary approach to the challenge of arboviral diseases in America."
2017	Awarded with the mobilization granted from CONCYTEC to do an internship at The University of the Balearic Islands in molecular phylogeny of *Aedes aegypti*.
2015	Awarded with the mobilization grant from CONCYTEC to attend the 152nd Annual Meeting of the American Association of Avian Pathologists (AAAP) / American Veterinary Medical Association (AVMA). Boston, MA. USA.
2013	Awarded with the best research work at the AMEVEA Peru, rewarded with a full scholarship to attend the Latin American Congress of Poultry. San Salvador, El Salvador.
2009	"Haya de La Torre" scholarship awarded by the Peruvian Government to outstanding students around the country to conduct postgraduate studies.
2005	José Gregorio Paz Soldan Medal "Honorable mention" given by Universidad Nacional Mayor de San Marcos, for organizing I International Student Congress of Microbiology and Parasitology.
2003	Scholarship given by Santander Hispano Bank for high student performance.