# Chapter 8
# Dengue Virus and Other *Flaviviruses* (Zika): Biology, Pathogenesis, Epidemiology, and Vaccine Development

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## 1 Introduction

The *Flaviviridae* family includes a variety of viruses that are distributed worldwide, some of which are associated with high morbidity and mortality. Because there are neither vaccines nor antivirals for most of the *Flavivirus* infections, study of the viral replicative cycle is relevant.

The *Flaviviridae* family comprises three genera: (i) the *Pestivirus*, which infects mammals, including cows and pigs, such as the bovine diarrhea virus 1; (ii) the *Hepacivirus*, which includes only the hepatitis C virus (HCV), an important cause of hepatitis and hepatocellular carcinoma in humans; and (iii) the *Flavivirus*, which contains more than 80 members. A number of *Flaviviruses* are pathogenic to humans and are transmitted via the bite of an arthropod vector (tick or mosquito) to produce an acute cytolytic infection. Examples of flaviruses affecting humans are yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Zika virus, and tick-borne encephalitis virus (TBEV). Most of them cause severe diseases in humans with complex pathologies that on occasions may have fatal results.

The first epidemic of DENV occurred in 1779–1780 in Asia, Africa, and North America. Initially, sporadic outbreaks of the disease were reported, only occurring in its benign form, known as dengue fever. However, after World War II, the infection with DENV spread to different parts of the world, and more than one serotype was

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detected in the same population. This situation increased the number of cases of dengue fever and resulted in the appearance of the most severe form of the infection, known as dengue hemorrhagic fever or severe dengue. Nowadays, one-third of the world's population lives in areas at risk for infection, and more than 100 countries are endemic for dengue, reporting annually more than 400 million cases.

Dengue is endemic in virtually all Latin America (with the exception of Chile and Uruguay). According to the Pan American Health Organization (PAHO) (www.paho.org), in the year 2016, the region reported nearly 650,000 confirmed cases of dengue fever and more than 12,000 cases of severe dengue, with the circulation of all four serotypes. Given the importance of dengue and other *Flavivirus* diseases in the Americas, including the recent emergence of Zika virus, many researchers in the region have devoted their effort to the study and control of this disease. This chapter is aimed to review some of the most relevant findings and contribution made to the biology, epidemiology, and prevention of dengue and also that of Zika disease in the region.

## 2 Dengue Virus Replicative Cycle

### *2.1 Early Events*

Because DENV is an arbovirus, it can replicate efficiently in mammalian and mosquito hosts. DENV is transmitted to humans by female mosquitoes of the genus *Aedes* during a blood meal. In this process, the virus is inoculated in the human skin where the virus comes in contact with several cell types, including skin-resident dendritic cells and macrophages. These cells are then transported to the regional lymph nodes where the virus infects other macrophages and monocytes, amplifying the infection. Different molecules have been described as DENV receptors in mammalian cells, among them, heparan sulfate [50] proteins associated with CD14 [51]; the lectin DC-SIGN [145, 203]; CLEC5A [49]; the heat shock proteins HSP70, HSP90, and GRP78 [101, 175]; cell receptors such as laminin [213] and mannose receptors; prohibitin [106]; and molecules related to lipid detection such as nLc4, Cer L-3, and TIM and TAM receptors [125]. The variety of cell receptors reported suggest that different receptors are used in different cell types and receptor redundancy use or that DENV uses a receptor complex formed by more than one protein in different steps of viral infection, such as attachment, internalization, and signaling pathway triggering. Later, the virus can be detected in remote lymph nodes to finally induce viremia. Viremia precedes the onset of clinical symptoms, and during this phase, the virus is disseminated to other organs such as the liver, spleen, and kidney.

During the viremia stage, humans can transmit the virus to a healthy mosquito. After the blood meal, DENV can infect epithelial cells of the midgut of the insect. The migration of the virus to the hemocele allows it to reach different organs such as fat bodies, the Malpighian tubules, and finally the salivary glands, where it is

ready to be transmitted to humans. Again, glycoproteins of 40 and 45 kDa and polypeptides of 57, 67, and 80 kDa have been identified as DENV putative receptors in mosquito cells, although the final identity of those proteins has been elusive [106, 129, 130, 185, 186, 215, 228].

DENV uses lipid rafts to enter into the host cell, and most of the receptors described for DENV to date are associated with lipid rafts or are recruited to these sections of the membrane at the time of infection [173]. Recent work highlighted the importance of cholesterol and specifically of lipid rafts in the entry and signaling [48, 49] process of DENV in vertebrate cells. The results suggest that the integrity of lipid rafts is required during the infectious process of DENV in monocytes/macrophages in the absence or presence of facilitator antibodies, as well as in mouse neuroblastoma cells N18 [111, 173, 175]. Consistently, a significant reduction in DENV infection was detected in the liver cell line Huh-7 when cells were pretreated with drugs that prevent the formation of these membrane microdomains [199].

Virus attachment is followed by viral entry and decapsidation. Several studies indicate that viral entry occurs by a clathrin-mediated endocytosis [1–3, 138]. The low pH present in late endosomes induces the fusion between viral and endosomal membrane, inducing capsid release into the cytoplasm [33, 134]. Uncoating after viral entry is one of the least studied steps in the *Flavivirus* life cycle, but, recently, it has been described that the capsid is degraded after viral internalization by the host ubiquitin-proteasome system. However, neither the proteasome activity nor capsid degradation is necessary for viral genome release into the cytoplasm, suggesting that DENV capsid degradation is not responsible for genome uncoating. However, DENV genome release requires a non-degradative ubiquitination step [35].

## *2.2 Viral Replication*

The dengue virus, as other RNA viruses, modifies the membranes of the endoplasmic reticulum to concentrate all the factors necessary for replication of the viral genome (replicative complex) [140, 166, 202]. The first step in DENV replication is translation of the viral genome, given that the positive polarity of the viral genome allows it to function as mRNA. This RNA contains a cap structure in the 5′-end and lacks a poly A tail in the 3′-end [118]. Translation of the viral genome occurs by a cap-dependent mechanism. However, in conditions where cap-dependent translation is inhibited, the DENV genome can still be translated. Because viral RNA is monocystronic, a polyprotein is synthesized that is cleaved by cellular and viral proteases to generate mature viral proteins. Next, the new synthesized nonstructural proteins initiate viral replication. RNA elements located within the 5′- and 3′-untranslated regions (UTR) regulate translation and genome replication [8, 85]. It has been described that within the 5′-UTR of about 100 nucleotides (nt) there are three regulatory elements: the stem-loop A (SLA), which is the viral polymerase

promoter [77, 78, 114]; the stem-loop B (SLB), which contains the 5′ upstream of the AUG region (5′-UAR), complementary to the 3′-UTR (3′-UAR) that mediates viral RNA circularization [11]; and a U-rich spacer which enhances viral replication [114]. The 450-nucleotide-long 3′-UTR contains four domains: domain A1, containing a variable region (VR) [196]; domains A2 and A3, with a dumbbell-like secondary structure functioning as enhancer for viral replication [10, 119]; and domain A4, containing the small hairpin (sHP) and the 3′-stem-loop (3′-SL), all necessary for viral replication [221]. It has been extensively described that long-range RNA–RNA interactions between the 5′-cyclization sequence (5′-CS) with the 3′-CS and 5′-AUR (upstream AUG region) and 3′-UAR are necessary for efficient RNA replication [11]. To initiate viral replication, the viral polymerase NS5 interacts with the 5′-end SLA promoter and then moves to the 3′-end initiation site, located very close because of viral genome cyclization [77]. Recently, specific RNA sequences have been identified in the viral 3′-UTR that are essential for viral replication in mosquito cells but dispensable for replication in mammalian cells [77]. These studies provided direct evidence for host-specific functions of viral RNA elements and raised the question whether viral RNA structures are under specific selective pressures during host adaptation [220].

The size limitations of the viral genome require viruses to depend heavily on host cell factors to complete their replicative cycle successfully. In this regard, a number of viral proteins, including La, PTB, and PABP among others, have been identified to bind specifically to the DENV genome UTRs to effectively modulate the replication process [5, 63, 229, 230].

Other important elements for viral replication are the membranes from the endoplasmic reticulum (ER). During DENV infection, extensive rearrangements of the endoplasmic reticulum occur. Three different substructures have been described: (1) invagination of the ER membrane known as vesicle packets involved in viral replication (VPs), (2) virus “bags” where viral progeny accumulate, and (3) convoluted membranes (CMs) with an unknown function [224]. The nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 are located in the invaginations induced in the endoplasmic reticulum.

In mosquito cells, significant differences were observed in the intracellular lipid profile of DENV-infected cells compared to uninfected cells. These new lipids have the ability to alter the structural and functional characteristics of membranes, confirming the idea that infection promotes important rearrangements in membranes through alteration in lipid metabolism [102, 166].

In addition, it has been observed for different *Flavivirus* that if the amount of cholesterol present in these complexes is reduced, the viral genome replication is affected [211]. Furthermore, it has been shown that if the biosynthetic pathway of cholesterol is inhibited (using statins or siRNA against pathway protein cholesterol synthesis), or if capture of cholesterol from the medium (delipidated) is prevented, viral replication in cell lines A549 (lung carcinoma) and K562 (hematopoietic) decreases considerably, showing that cholesterol has a major role in this process [181, 199]. The dependence of DENV replication on cholesterol and lipid metabolism opens possibilities for antiviral treatments. Indeed, compounds that affect lipid

metabolism such as nordihydroguaiaretic acid (NDGA) are demonstrated to be an effective inhibitor of DENV replication in vitro [198].

### *2.3 Virus Morphogenesis*

The DENV structural proteins C, prM, and E are synthesized in the ER; however, C protein accumulates progressively around cellular organelles named lipid droplets (LDs) during infection [99]. LDs are formed by sphingolipids and cholesterol esters and are located close to the ER. These organelles have been involved in viral assembly. Interestingly, the number of lipid droplets per cell increases after infection, linking lipid droplet metabolism and viral replication. Specific hydrophobic amino acids, located in the center of the capsid protein, have been identified as key elements for lipid droplet association [188]. Surprisingly, the N-terminus of C is necessary for efficient particle formation in mosquito cells, but they are crucial for propagation in human cells, suggesting that this function of C is differentially modulated in different host cells [187].

These findings are consistent with the fact that the pharmacological inhibition of fatty acid synthase (FASN) mediated by C75 promotes a significant inhibition of DENV morphogenesis [170, 188].

Once viral RNA is associated with C, the nascent particle buds into the ER where it acquires the viral membrane containing the C and prM proteins. The immature virions traffic through the trans-Golgi secretory pathway and along this pathway; the prM protein is cleaved by host furin-like proteases to generate mature virions [112, 231]. Along the mature virions, soluble NS1 protein is also secreted to the extracellular milieu. Recently, it was reported that NS1 is secreted also from infected mosquito cells and not only by vertebrate cells, as previously supposed [7].

## 3 Virus–Host Interactions

As was described earlier, DENV infection can induce a mild disease or can cause a more severe form of the infection called severe dengue. Severe dengue is characterized by the rapid onset of capillary leakage and is accompanied by significant thrombocytopenia and mild-to-moderate liver injury. Hemorrhagic manifestations include bleeding in the skin and gastrointestinal tract. Although the pathogenesis of the severe forms of dengue infection has been broadly studied, the process is not yet fully understood [52, 121, 150]. Secondary infections are recognized as one of the most important risk factors for the development of severe dengue by a complex mechanism known as antibody-dependent enhancement (ADE) of viral infection. It has been postulated that during secondary infection, the antibodies generated during primary infection are able to form virus–antibody complexes that infect Fc-bearing cells such as human monocytic and dendritic cells, through the Fc receptor.

This mechanism is responsible for an increase in the proinflammatory cytokine response, which has the capacity to disturb the apical junction complex in vitro and to cause an increase in vascular permeability in vivo [174]. Indeed, the association between aberrant cytokine levels and dengue severity has long been apparent; past and recent work carried out in Brazil and other parts of the region has contributed greatly to understand the cytokine profiles in sera of patients with dengue and its association with disease severity [22, 76, 91].

Another key element of the antiviral response to DENV is type 1 interferon (IFNα/β). IFN type 1 is secreted very early after DENV infection by mammalian cells, and it has an important function in protection against viral infections [84, 169]. It has been reported that DENV triggers but also counterattacks many of the signaling pathways involved in the induction of a robust IFNα/β response [5, 6, 137, 141, 142, 160, 177, 178]. In concordance with the antiviral activity of IFN type 1, IFN-α levels in patient sera are rapidly modulated after fever onset, and a better clinical condition correlates with higher IFN-α levels, supporting the idea that IFN response has a role in the pathogenesis of DENV [62].

Special mention of the work in dengue conducted in Latin America shall be made to the dengue cohort study being carried out in Nicaragua to study the natural history and transmission of dengue in children. This ambitious study, carried out for more than 10 years, has enrolled thousands of children and has worked in collaboration with public authorities. Among other findings, the Nicaraguan cohort study had provided evidence for the role of neutralizing antibodies in protection against dengue and the role of secondary infections as a risk factor to develop severe dengue [103, 222].

## 4 Epidemiology of Dengue in Latin America

Dengue reports in the Americas date back to the nineteenth century. In the first moiety of the twentieth century, in Brazil as in other countries of Latin America, the mosquito *Aedes (Ae.) aegypti* was eradicated after a program of the PAHO to control yellow fever, another *Flavivirus* transmitted by the same vector [40]. Unfortunately, this program was discontinued, which led to reinfestation of *Ae. aegypti*, and dengue became one of the most important infectious diseases in Latin America.

As a consequence of this scenario, several efforts have been made to map the epidemiological situation of dengue in the different countries, improve diagnostic tests, identify the circulating serotypes, and better understand the disease with its different forms. The scientific community also focused on studies concerning virus biology, interaction between host and pathogen, and vaccine development. The contribution of Latin America in dengue research was recently analyzed in a bibliometric study, revealing that Brazil was the highest contributor (31.2%), followed by Puerto Rico (12.9%) and Mexico (10.7%) [219].

Epidemiological inquiries have been performed in different regions in Latin America. In 1963 DENV3 was isolated in Jamaica and disseminated to other

Caribbean countries [27]. Later on, the emergence of DENV2 in Cuba in 1981 represented a mark in the epidemiology of dengue in America, with several reported cases of dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). Investigations revealed that most of the DHF cases presented antibodies against DENV1, which was responsible for the epidemic of 1977 [105]. Also in 1981, DENV4 was introduced in eastern Caribbean islands and spread to the rest of the Caribbean, Mexico, and Central and South America [167]. In 1989 a large dengue epidemic was reported in Venezuela with many cases of DHF [161]. In general, the epidemics of dengue in the Americas occur with recurring peaks of cases at 3- to 5-year intervals. More upsetting is the fact that, over time, peaks become progressively higher, with more cases of reported severe forms of the disease [189]. Nowadays, the four dengue serotypes circulate in several countries, which increases the risk of DHF.

The greatest epidemic in the Americas was in 2013, when almost 2.4 million dengue cases were notified, and approximately 1.6% of them evolved to severe forms of the disease [162]. In that year, Brazil, Mexico, Colombia, and Paraguay had the most important outbreaks, totalizing 83% of the dengue cases in America. Brazil leads the rank of dengue fever cases, with an incidence ranging from 313.8 (in 1998) to 722.4 (in 2013) per 100,000 inhabitants [206].

In Brazil, the first outbreak of dengue with laboratory confirmation was in 1981 in a city in the north of the country, Boa Vista, with simultaneous occurrence of DENV1 and DENV4. This episode was immediately controlled, and the virus did not spread to other regions [156]. Dengue only became a health problem after the epidemic of 1986, when serotype 1 was introduced in the state of Rio de Janeiro and spread to different regions [193]. Since then, dengue has become endemic in Brazil, with explosive epidemics marked by the introduction of DENV2 in 1990, DENV3 in 2000, and DENV4 in 2010 [148, 149, 168, 208]. Epidemiological studies were performed to investigate serotype prevalence and virus distribution, not only by regions but also according to age [29, 30, 95, 104, 204, 207]. These works revealed, for instance, a shift in the age pattern of DHF with more children younger than 15 years being affected [205, 207]. A similar scenario was also observed in Venezuela [210].

More recently, studies have been conducted in many countries in Latin America to map the dengue epidemiological situation in these areas and to access the efficacy of vaccines against it [60]. These studies revealed, for instance, that there is a substantial underreporting of dengue in the epidemiological surveillance systems from Brazil, Colombia, and Mexico [192]. Unapparent infections may also be important in the dengue epidemiology, and scientists have started to investigate this as a possible source for mosquito transmission, which would impact the disease burden [90]. Following the same line, other investigations have been performed to establish the burden of dengue and its economic cost in different countries such as Brazil, Colombia, Nicaragua, Mexico, and even Argentina, where dengue is present but at lower magnitude compared to other regions in America [44, 120, 212, 225, 226].

Other works focused on phylogeny studies, aiming to understand the dynamic of DENV population and the origin of the different virus isolates, by comparing sequences obtained from distinct countries in Latin America and all over the world

[9, 13, 43, 57, 70, 153, 227]. These investigations revealed, for example, that the DENV2 that emerged in Brazil in 1990 continued to circulate until 2003, whereas in 2007 a new DENV2 was isolated that belonged to a different genotype, thus suggesting that this virus did not evolve locally but was rather caused by a new introduction, probably coming from the Caribbean [74]. Similar observation occurred in Peru, also with DENV2 [57].

In parallel with these studies, much effort has been expended to improve the clinical and laboratory dengue diagnostic [4, 16, 34, 56, 59, 116, 157, 171]. Reports have shown, for instance, the incidence of dengue infection by blood transfusion and renal transplant [20, 53]. Recently, it was performed a prospective study with a large cohort of patients from several countries in Latin America and Asia, aiming to differentiate between dengue and other common febrile illness and to identify parameters associated with the progression to severe forms of the disease [100]. These works will certainly help to update guidelines for diagnostics and treatment of dengue all over the world.

Other investigations were performed with postmortem materials of suspected dengue cases to establish/improve diagnostics [19, 46]. Studies with samples from confirmed fatal dengue cases have also been reported [15, 147, 152, 158, 159, 172, 176, 184, 209]. All these works provided important information about the pathogenesis of the disease, especially regarding the severe forms of dengue, and helped to map the target organs/cells for virus replication. It showed, for instance, the commitment of some organs that are not commonly associated with dengue, such as the heart and kidney [159, 172, 209], as well as the reinforcement of the involvement of the central nervous system during the disease [15, 147].

## 5 Sylvatic Dengue Cycle

DENV exists as sylvatic and urban cycles in Africa and Asia [223]. Investigations to identify a sylvatic cycle for DENV in the Americas or to find evidence of infection in neotropical forest mammals have yielded contradictory results. Molecular and serological evidence for DENV infection in opossums and especially in several species of bats has been reported in studies conducted in French Guiana and Mexico [64, 200]. Yet, other studies conducted with bats also collected in Mexico have failed to find evidence that bats can sustain DENV replication [36, 37].

## 6 Vaccines

The development of a vaccine against dengue has been pursued since the studies of Sabin in 1945 [183]. One of the major difficulties in this field is to achieve a vaccine against the four dengue serotypes. Studies all over the world showed that infection with one serotype promotes long-term immunity to this serotype, but protection to

heterologous infection is only transient. In fact, results revealed an increased risk for severe forms of dengue during a second infection with a heterologous serotype [80]. It seems that the immunity to a specific serotype may induce an uncontrolled immune response during a secondary infection, leading to the DHF/DSS.

One hypothesis to explain the role of immune response in the development of severe forms of the dengue disease is the antibody-dependent enhancement (ADE) of virus replication. In this phenomenon, antibodies generated by the first dengue infection, mainly against the E and prM proteins, bind to the heterologous virus serotype in the second infection but without neutralizing its ability. Instead, the antibody–virus complex can bind to Fc receptors present in monocytes/macrophages and dendritic cells, which are the primary target cells in dengue infection, facilitating, therefore, virus entrance and replication [93]. Another hypothesis, not necessarily exclusionary, is based on the cellular immune response, named original antigenic sin. In this case, cross-reactive memory T cells generated by the first infection are preferentially activated during the second dengue infection. However, these low-affinity T cells are unable to clear infection and can cause an uncontrolled cytokine production (the cytokine storm), which finally results in the plasma leakage that is characteristic of the DHF/DSS [124].

Consequently, it is a consensus that an effective dengue vaccine has to be tetravalent; otherwise, immunization against one serotype could increase the risk for more severe forms of the disease in individuals infected with other serotypes. Different vaccines are now being tested in clinical trials in Latin America. One of these vaccines, produced by Sanofi Pasteur, is based on the recombinant life-attenuated yellow fever (YF)/dengue virus, in which the E and prM genes from YF are substituted by the genes from each DENV serotype. This tetravalent vaccine, CYD, was tested in Asia and Latin America. In Latin America, it was tested in Brazil, Colombia, Peru, Honduras, Mexico, and Puerto Rico [61, 68, 92, 107]. Unfortunately, the efficacy of this vaccine was below expectations, as protection against DENV2 was not achieved in several children. In general, the efficacy of the vaccine was significantly low in children 2 to 5 years of age, especially in individuals with no previous DENV infection. Also, the vaccination regimen involves three doses given with a 6-month interval, which can have logistical difficulties for its administration. This may be a problem in dengue endemic areas because it can leave the population more susceptible to the development of severe disease until the immunological protection is complete. In fact, a statistically significant increase of hospitalization among vaccinated children (2–5 years old) was observed [92]. Because of this, the Sanofi Pasteur vaccine against dengue was recently licensed for commercial use in Brazil and Mexico only in individuals aged from more than 9 years to 45 years, which does not include an important segment of the population at risk for the development of severe dengue [96]. Although results of the clinical trials with this vaccine were quite disappointing, these studies were the most robust performed to date, and they revealed, for instance, that only neutralizing antibodies seem to be not correlated to protection. In fact, vaccinated children presented high levels of neutralizing antibodies to DENV2, but they were not protected against this virus serotype [182].

Another tetravalent vaccine, developed by the National Institute of Health (NIH), is also being tested in Latin America. This vaccine is composed of a mixture of attenuated recombinant virus, obtained by the deletion of 30 nucleotides in the 3′-UTR of DENV1, DENV3, and DENV4 and by the substitution of prM and E genes from DENV4 to those genes from DENV2 [72–74]. This vaccine was tested first in clinical trials in the USA in *Flavivirus*-naïve healthy adults. It was well tolerated, although production of neutralizing antibodies seemed to be associated with the occurrence of rashes. Results suggested that this vaccine could be administered in one single dose, because antibody levels did not increase after the second dose [73]. This vaccine has recently entered in phase 3 clinical tests in some countries in Latin America, especially Brazil in a partnership with Butantan Institute, but results are not yet available.

The Takeda tetravalent dengue vaccine (TDV), in its turn, was constructed by using the life-attenuated DENV2 derived by serial passages of wild-type virus in primary dog kidney cells (PDK). The prM and E genes from this attenuated DENV2 virus, D2 PDK-53, were substituted by those genes from the other virus serotypes [98]. The tetravalent vaccine is composed of the attenuated DENV2 and the three chimeric DENV1, 3, and 4. This vaccine was tested in Puerto Rico and Colombia, and it was well tolerated and immunogenic for all serotypes in volunteers from 1.5 to 45 years of age [197]. In addition, one inactivated vaccine against dengue will soon be tested in Brazil in studies performed by the Institute of Technology in Immunobiologicals (BioManguinhos), from the Oswaldo Cruz Foundation (Fiocruz). This vaccine was developed by GSK, and it will be administered with adjuvants.

Besides the clinical trials of vaccines against dengue, several preclinical investigations have been performed all over the world, including in Latin America. Studies using life-attenuated recombinant virus were also conducted in Brazil, by constructing several chimeric YF/DENV [45, 81, 123]. This strategy was similar to that of the dengue vaccine developed by Sanofi Pasteur, although analyses were only focused on mice and nonhuman primates.

Other strategies used DNA vaccines encoding structural as well as nonstructural dengue proteins. Usually, the vaccines focused on the E protein together with the prM, which works as a chaperonin for the correct folding of the E protein [65, 113, 167]. However, one study from Brazil showed that a DNA vaccine encoding the ectodomain (domains I, II, and III) of the DENV2 E protein, fused to a strong signal peptide, was able to induce high levels of protection in mice challenged with a lethal virus dose [24]. Furthermore, the authors showed that the combination of this DNA vaccine with a chimeric YF/DENV2, constructed in Brazil [45], generated 100% protection in mice with induction of a synergetic neutralizing antibody response [23]. This study also pointed that the cellular immune response elicited by the DNA vaccine was significantly higher when compared to immunization with the chimeric attenuated virus, which may be important for protection and can explain, in part, the low efficiency of the Sanofi Pasteur vaccine. Other investigations focused on DNA vaccines encoding the DENV NS1 or NS3 proteins [54, 55], which also elicited

protection. Interestingly, one of these studies showed that the cooperation between CD4+ T cells and antibodies, more than CD8+ T lymphocytes, was crucial for protection induced by a DNA vaccine containing the NS1 gene [89]. Reports of DNA vaccines using the entire E and NS1 gene together or only the domain III of the E protein have also been published [126, 127, 139].

Studies with subunit vaccines have been conducted in different countries in Latin America. Some work has been described in Cuba using the recombinant C protein expressed in *Escherichia coli*, which was tested in mice [86, 109, 115]. The importance of CD4+ and CD8+ T-cell response in the protection elicited by this vaccine was also reported [87]. Other research was based on tetravalent formulations, combining the domain III of the envelope protein to the capsid protein, which similarly induced protection in mice and nonhuman primates [201]. Further, heterologous prime-boost protocols were tested using purified proteins and infective virus [214]. In Mexico, researchers tested the ability of different domains of the envelope protein to induce protection, alone or in combination with the NS1 [83]. Following the same line, studies in Brazil have shown that purified recombinant NS1 protein induced protection in mice in combination with detoxified heat-labile toxin from enterotoxigenic *E. coli* as adjuvant [12]. In Argentina, the E protein was expressed in a plant system to use it as a subunit vaccine or in a diagnostic kit [122]. Another report from Mexico explored the approach of expressing one peptide from the NS3 protein on the surface of *Salmonella* and showed its ability to induce a strong cytotoxic T-cell response [117].

Another difficulty in the development of an effective vaccine against dengue is the lack of an ideal experimental animal model that mimics the disease in all its forms, as we observed in humans. Several studies have been performed with immunodeficient mice, which can develop clinical signs similar to those observed in humans [232, 233]. However, although these animal models are extremely valuable for studying the disease, their use for vaccine tests is controversial because the elicited immune response can differ significantly from that observed in immunocompetent individuals. The most traditional immunocompetent mouse model for testing vaccines against *Flavivirus*, including dengue, is the use of brain-adapted virus inoculated by the intracerebral (i.c.) route. Albeit this is not the natural route of infection in humans; studies with dengue patients have revealed more and more the commitment of the central nervous system during the disease [15, 147]. Also, this model provides a straightforward readout parameter for vaccine testing because virus inoculation is usually lethal. Moreover, recently a study in Brazil was published showing the systemic effect of the virus infection by the i.c. route. Authors detected viremia in these animals, especially in late stages of infection, induction of T-cell responses, and tissue damages in peripheral organs, such as the liver [154]. In another work, researchers investigated the use of immunocompetent mice inoculated with macrophages infected in vitro with a DENV isolate, not laboratory adapted. They observed some aspects of the virus tropism described in humans, with detection of the DENV genome in the same organs [26].

## 7 Studies with Zika Virus

Apart from the studies with dengue, much research has begun with the Zika virus (ZIKV) in some countries in Latin America, especially in Brazil. The interest toward ZIKV by the scientific community in Latin America is a consequence of the huge health problem we have been experiencing since the beginning of 2015.

This virus also belongs to the *Flaviviridae* family, genus *Flavivirus*. It has a typical *Flavivirus* organization: an enveloped virus with a single-stranded positive-sense RNA genome. The RNA is translated into a polyprotein that is cleaved, generating three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The serological diagnosis for ZIKV is not fully conclusive because a significant proportion of tested samples showed cross-reactivity to other viruses, especially DENV. Because of this, laboratory testing generally includes polymerase chain reaction (PCR) assays. Transmission of the ZIKV occurs predominantly via the bites of *Aedes* mosquitoes, mainly *Aedes aegypti*. Moreover, transmission from the mother to the fetus via the placenta was recently established [32, 133]. Sexual transmission has also been reported [69, 97]. More recently, Baca-Carrasco and Velasco Hernández [25] performed a mathematical study to analyze the effects of sexual transmission and migration in the spread of the ZIKV. They concluded that transmission through sexual contact was insufficient to influence the spread of the disease as we observed in Latin America, although it may have affected the magnitude and duration of outbreaks. On the other hand, migration was decisive for the rapid spread of this virus. In addition, the viral RNA and infective particles have been detected in the saliva and urine of ZIKV-infected patients [28, 143] and are now being used as diagnostic tools.

The ZIKV was first isolated in 1947 in a sentinel monkey for monitoring yellow fever in a forest in Uganda named Zika. After the first isolation of the ZIKV, symptomatic cases of infection with this virus were reported in some African and Southeast Asiatic countries. However, little attention was paid to these cases because infection with ZIKV was considered to lead to only mild symptoms. The situation changed after the first recognizable outbreak of ZIKV in Micronesia in 2007, characterized as a DENV-like disease [71]. The second ZIKV outbreak occurred in French Polynesia, affecting approximately 28,000 individuals, in which an increase in the number of cases of Guillain–Barré syndrome (an immunomediated neuropathy that can cause paralysis) was reported [42]. More dramatic was the outbreak of this virus in 2015 in Brazil, after which several cases of microcephaly (occipital frontal circumference below the mean for age and gender, related to serious development problems in the children) was reported in babies whose mothers were infected during pregnancy [94, 132]. In adults, infection usually leads to symptoms as low-grade fever, arthralgia, rash, headache, and myalgia, although most infections are asymptomatic [31, 206].

The autochthonous transmission of ZIKV in Brazil was first reported in the northeast of the country in March 2015, almost simultaneously by two research groups [41, 222]. The first suspicion was that this virus was introduced in Brazil

during the World Cup soccer competition in 2014; however, no countries participating in this competition were endemic for ZIKV. Another possibility is that the ZIKV had entered Brazil during the Va'a World Spring Championship canoe in August 2014, when four Pacific countries participated in this event, including French Polynesia [144]. Phylogenetic studies with samples of isolated virus from patients in Brazil also support this hypothesis. In fact, the ZIKV that is circulating in Brazil belongs to the Asian clade and shares 97–100% identity with the virus lineages isolated during the outbreak of 2013 in French Polynesia [38, 88].

After introduction of ZIKV in Brazil, it soon spread throughout Latin America. Colombia was the second country to report circulation of ZIKV in 2015 [21, 39, 146, 191], followed by Mexico, Panama, Haiti, and Puerto Rico [18, 66, 67, 110]. In 2016 many other regions confirmed autochthonous cases of this virus, totalizing 48 countries and territories in the Americas [163].

In Brazil, after the emergence of the ZIKV, it was observed that there was a 20-fold annual increase in the number of microcephaly cases [155, 163, 194]. According to the Brazilian Ministry of Health, between October 2015 and May 2016, a total of 7534 suspected cases of microcephaly and other congenital malformation of the central nervous system (CNS) have been reported [163]. Detection of the virus in pregnant women showing infection symptoms, as well as in amniotic fluid, placenta, and the brains of newborns, intensely reinforced the correlation between infection with ZIKV and malformations of the CNS in newborns, including microcephaly [38, 133, 155]. Mlakar and collaborators [133] published the first data that indicated a strong relationship between ZIKV and microcephaly, describing the case of a Slovenian woman who lived temporarily in the Northeast of Brazil and presented symptoms of the virus infection (febrile illness with rash) at the end of the first trimester of pregnancy. Ultrasonography performed at 29 weeks of gestation revealed microcephaly with calcifications in the fetal brain. The ZIKV RNA was detected in the fetal brain tissue, thus confirming virus transmission from the mother to the fetus. One study with a Brazilian cohort of 88 pregnant women reported that 72 were positive for ZIKV (82%), most of them showing fetal abnormalities [32]. Further, this investigation demonstrated that the fetal abnormalities can happen even when infection with ZIKV occurs after the first trimester of pregnancy. Several other studies also reported the association of ZIKV infection and microcephaly [14, 47, 136, 217]. In one report, the transplacental transmission of ZIKV was evidenced not only by detection of the viral protein and RNA in placental tissues but also by its effects leading to placentitis [151]. The neurotropism of ZIKV was observed by the detection of viral proteins in glial cells and observation of scattered foci of microcalcifications in the fetal brain tissues.

More recently, the term congenital Zika syndrome (CZS) has been preferably used, because it was observed that microcephaly is only one of the clinical signs of this congenital malformation disorder. The clinical features of the CZS are a consequence of direct neurological damages and severe intracranial volume loss. Although the cognitive, sensory, and motor disability components of this syndrome can be shared by other congenital infections, some features seemed to be characteristic: (1) severe microcephaly with partially collapsed skull, (2) thin cerebral cortices with

subcortical calcifications, (3) macular scarring and focal pigmentary retinal mottling, (4) congenital contractures, and (5) marked early hypertonia with symptoms of extrapyramidal involvement [135]. Neurological examination of affected infants has shown hypertonia and spasticity, irritability manifested by excessive crying, dysphagia, and, less frequently, hypotonia [194]. A detailed study described the prenatal evolution and perinatal outcomes of 11 neonates who showed developmental abnormalities and neurological damage associated with ZIKV infection in Brazil [128]. The ZIKV was detected in the amniotic fluid, placenta, and cord blood for all patients, as well as from some neonatal tissues collected post mortem. Most of the infants presented with microcephaly, although the authors also found newborns presenting with severe brain lesions with a normal cephalic perimeter. They observed variable injuries as the consequence of brain lesions related with the virus infection, with a common pattern of brain atrophy and changes associated with disturbances in neuronal migration. Some patients showed mild brain atrophy and calcifications, whereas others presented severe malformations, including the absence of the thalamus and lissencephaly [128]. Histopathological and immunohistochemical analysis of tissues from two postmortem babies revealed multiple small foci of calcification and degenerate nerve cells in the brainstem, histiocyte and microglial proliferation, and gliosis, as well as neuronal and axonal degeneration. Ocular abnormalities were also observed, mainly paresis of the oculomotor and abducens muscles with convergent strabismus and loss of photomotor and consensual reflexes. In fact, different studies have been showing that the ZIKA can cause severe injury in the retina [216–218]. The retinal damages include mild to severe macular pigmentary changes and chorioretinal atrophy [216, 217]. Miranda and collaborators [131] described for the first time vascular changes and hemorrhagic retinopathy probably associated with the intrauterine infection with ZIKV. On the other hand, Ventura and collaborators [218] have evaluated the eyes of eight infants whose mothers were infected with ZIKV during pregnancy. Optical coherence tomography technology showed severe involvement of the neurosensory retina, including the internal and external layers and the choroid in most eyes, indicating severe visual impairment in these newborns.

Besides the CZS, infection with the ZIKV is also associated with neurological disorders in adults. Several cases of Guillain–Barré syndrome (GBS) have been reported after infection with ZIKV in Brazil [17, 31, 79, 164, 179]. One study with two cases from Salvador, Bahia, reported the development of ascending paresis after an acute exanthematous illness, evolving later to tetraparesis and cranial nerve palsy, which resolved after intravenous administration of human immunoglobulin [180]. The studies in Brazil supported the association of GBS and ZIKV infection. Furthermore, they served as an alert to other countries in Latin America, where the virus spread recently, of the potential risk not only for CZS in babies but also for neurological commitments in adults and the need for timely detection, diagnosis, and treatment to prevent mortality and long-term sequelae. In fact, from April 2015 to March 2016, a total of 164,237 confirmed or suspected cases of ZIKV disease and 1,474 cases of GBS were reported in Bahia-Brazil, Colombia, the Dominican

Republic, El Salvador, Honduras, Suriname, and Venezuela [190]. Unfortunately, part of these cases progressed to death, as was reported in one study in Colombia, in which 4% of patients with GBS died after respiratory failure and sepsis [165]. Moreover, although epidemiological studies revealed that females had a 75% higher incidence rate of ZIKV disease than males, the greater apparent risk for developing GBS is in males (28% more incidence of GBS among males than among females) [190]. The development of GBS after ZIKV infection probably involves an autoimmune process as described for other viral infections. However, some reports have showed that the development of GBZ after ZIKV infection may follow the pattern of a para-infectious disorder rather than the classic post-infection profile. Actually, a study in Colombia with 66 patients with GBZ revealed that 48% of these individuals had a rapid onset of neurological symptoms without an asymptomatic period after ZIKV infection symptoms (para-infectious) [165]. There are different hypotheses to explain such a scenario. One of them is that the immune molecular mimicry process against the nervous system may initiate before clinical symptoms of the ZIKV infection appear [165]. Additionally, it has been speculated that simultaneous epidemics of DENV and ZIKV may predispose the development of GBS as a result of sequential virus infection and stimulation of the immune system, triggering to an immunopathogenic process [180]. If this is the case, one must pay attention in the developing of vaccines against both *Flavivirus* because exanthematous immunization against one virus may impact in the development of disease caused by another virus.

In an attempt to understand the mechanism by which the ZIKV leads to microcephaly and other malformations in the fetal brain, researchers started to investigate the effect of the virus infection in human neural stem cells, growing as neurospheres and brain organoids [82]. They showed that ZIKV targeted these cells, reducing their viability and growth, which suggests that the virus abrogates the neurogenesis during brain development. However, there are several gaps in this field, and many studies will probably be performed in the future to answer these questions.

In addition, efforts have been made to establish experimental animal models to study the effect of ZIKV infection in several aspects, including intrauterine infections. Most of these studies are based on immunodeficient mice, in particular those lacking type I and II interferon receptors [195]. Similar to the studies with DENV, such a model is useful for investigations concerning the pathogenesis of the Zika, but its use for vaccine tests is controversial. Another investigation was performed with newborn Swiss mice infected with a ZIKV isolated in Brazil [75]. Inoculation of these mice with ZIKV by the intracerebral route led to severe cerebral lesions, with neuronal death, presence of apoptotic bodies, and degeneration of white matter. When the animals were infected by the subcutaneous route, the authors observed moderate cerebral lesions, morphologically similar to those was found in the previous group and additional myelopathy, with architectural loss, marked by neuronal death and apoptotic bodies. In another study, Cugola and collaborators [58] have shown that the association of birth defects, also using a ZIKV isolated in Brazil, depends on the mouse strain. These authors demonstrated that the offspring of immunocompetent pregnant C57BL/6 mice injected intravenously with ZIKV were

not infected, indicating that the virus did not cross the placenta barrier. On the other hand, pups from SJL pregnant females infected with ZIKV presented severe intrauterine growth restriction, resembling that which we observed in humans, including signs of microcephaly.

The establishment of animal models for ZIKV infection has a direct impact in the development of vaccines against this virus. After the dramatic outbreak of such virus in Latin America, much effort has been done toward the development of a vaccine against ZIKV. Several investigations have been conducted by different groups all over the world. One study was performed by Brazilian researchers in collaboration with groups in the U.S., using DNA vaccines or purified inactivated virus [108]. The DNA vaccine based on the full-length prM/E proteins conferred protection against ZIKV in the murine model of SJL mice previously described [58]. However, protection was only measured by absence of viremia and production of antibodies against the E protein in this animal. Other report of DNA vaccines against ZIKV encoding the prM/E proteins was also published [69]. The immunogenicity of such vaccines was tested in mice and nonhuman primates, and protection was evaluated by the lack of viremia in these animals. Clinical trials of some of these DNA vaccines, as well as a ZIKV purified inactivated vaccine, are already ongoing or are about to start in the U.S. [234]. The speed of which these researches have been conducted is a very positive point. However, many studies are still necessary to obtain an efficient and safe vaccine against ZIKV.

## 8 Conclusions and Future Challenges

Dengue continues to be a major public health problem in the Americas despite efforts and control actions carried out by public health authorities. Research and work carried out in the region have contributed significantly to the understanding of the virus biology, the pathogenesis, the epidemiology, and the diagnosis of this important and burdensome disease. Moreover, researchers in the region have helped in the development of the current licensed dengue vaccine and are also participating in the development of future vaccines and antiviral therapies. Challenges for the future include further understanding of the virus replicative cycle, the pathogenesis of severe dengue, and the immune response to infection to pave the way for effective patient intervention strategies and improved vaccines. Also, a better understanding of the virus–mosquito relationship is needed to implement effective and sustainable mosquito control measurements. Finally, the emergence of Zika virus in the continent, with its severe complications for adults but especially for infected newborns, poses formidable challenges for the region that require urgent attention. In particular, the extensive cross-reactivity between dengue and the Zika virus makes it important to investigate possible effects that vaccination against one *Flavivirus* may have upon the other, in terms of protection and/or pathogenesis, especially in a region where both viruses circulate concomitantly.

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