

# Zika virus challenges for neuropsychiatry

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**Abstract:** Before 2007, Zika virus (ZIKV) was generally considered as an arbovirus of low clinical relevance, causing a mild self-limiting febrile illness in tropical Africa and Southeast Asia. Currently, a large, ongoing outbreak of ZIKV that started in Brazil in 2015 is spreading across the Americas. Virus infection during pregnancy has been potentially linked to congenital malformations, including microcephaly. In addition to congenital malformations, a temporal association between ZIKV infection and an increase in cases of Guillain-Barré syndrome is currently being observed in several countries. The mechanisms underlying these neurological complications are still unknown. Emerging evidence, mainly from in vitro studies, suggests that ZIKV may have direct effects on neuronal cells. The aim of this study was to critically review the literature available regarding the neurobiology of ZIKV and its potential neuropsychiatric manifestations.

**Keywords:** Zika virus, microcephaly, Guillain-Barré syndrome, neurodevelopmental disorders

## Introduction

Zika virus (ZIKV) is an arthropod-borne virus (arbovirus) transmitted by *Aedes* spp. In recent years, outbreaks of ZIKV infection have occurred, and the infection has been linked to severe neurological complications, including microcephaly and Guillain-Barré syndrome (GBS).

In this article, we first briefly review the biology and epidemiology of ZIKV and then critically address the literature available regarding the neurobiology of ZIKV and its potential neuropsychiatric manifestations.

## Virology of ZIKV

ZIKV is a positive single-strand enveloped RNA virus that belongs to the family *Flaviviridae* and genus *Flavivirus*. ZIKV genome includes two noncoding regions and one region encoding a polyprotein that is cleaved into capsid, membrane precursor, and envelope, and also seven nonstructural proteins.<sup>1,2</sup> Based on the phylogenetic tree reported by Weaver et al,<sup>3</sup> ZIKV differs from other *Flavivirus* by 20% nucleotide sequence divergence.

The prototype ZIKV MR 766 was first identified in Africa.<sup>4-7</sup> In Asia, Lanciotti et al<sup>8</sup> described a coding region of ZIKV 2007 EC and the nonstructural protein 5 (NS5) gene, corresponding to a *Flavivirus* sequence, showing a high homology of the Asian strain with the virus originally identified in Africa. Accordingly, it has been proposed that the Asian cluster was phylogenetically derived from the African ZIKV strain.<sup>2,8,9</sup> The first cases of ZIKV infection reported in Brazil were from a strain genetically related to the Asian lineage.<sup>10-12</sup> The introduction of ZIKV in Brazil probably occurred in 2014 due to the arrival of foreign tourists and athletes during the Soccer World

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Cup and/or other sport events.<sup>11,12</sup> Giovanetti et al<sup>13</sup> described the complete genome of ZIKV isolated in Brazil, confirming that this cluster is similar to the virus isolated in French Polynesia.<sup>13,14</sup>

## Early studies and epidemiology of ZIKV

The denomination Zika came from a forested area in Uganda, where ZIKV was first identified in rhesus monkeys during a research about yellow fever cycle and other arboviruses.<sup>5</sup> The virus was also identified in mosquitoes.<sup>4,6,7</sup> Later, Boorman and Poeterfield<sup>15</sup> proved the transmission from laboratory-infected *Aedes aegypti* mosquitoes to rhesus monkeys. Simpson<sup>16</sup> reported the first proved case of vector transmission and human disease.

Dick<sup>6</sup> inoculated ZIKV in mice and detected virus replication in the brain. Some mice exhibited motor weakness and paralysis, and susceptibility to infection appeared to decrease with age. Therefore, ZIKV was considered neurotropic since its original description.

The first evidence of human infection based on the identification of antibodies against ZIKV came from patients in Uganda and nearby areas.<sup>6</sup> Later, Macnamara<sup>17</sup> reported the isolation of ZIKV in one patient and the rise of antibody serum titres in two patients during an investigation of jaundice in Nigeria.

Sporadic cases of ZIKV infection had been reported in Africa and Asia until 2007, when an outbreak occurred in Yap State, Federated States of Micronesia.<sup>8,18</sup> Lanciotti et al<sup>8</sup> identified several cases of ZIKV infection by using serological and molecular analysis during this outbreak. Duffy et al<sup>18</sup> investigated suspected cases of ZIKV presenting macular or papular rash, arthritis or arthralgia, and non-purulent conjunctivitis. From 185 suspected cases, 49 (26%) cases were confirmed by positive reverse transcriptase polymerase chain reaction (RT-PCR) for ZIKV. In addition, 414 (74%) of 557 investigated subjects had IgM anti-ZIKV in blood samples, but only 156 of those reported clinical symptomatic illness.<sup>18</sup>

More recently, in October 2013, a “mild dengue-like illness” was described in French Polynesia.<sup>19,20</sup> Approximately 19,000 suspected cases were estimated, but ZIKV infection was confirmed in only 294 (49.5%) of 584 patients submitted to serological tests.<sup>19</sup> Subsequently, the virus spread to other countries, and several cases were described throughout the world, including Japan, Australia, Europe, and Latin America.<sup>12,21</sup>

The first autochthonous cases in Brazil were published in June 2015 that referred to patients who acquired the

infection by March 2015.<sup>10</sup> These patients exhibited fever, rash, conjunctivitis, and arthralgia with negative molecular and serological examinations for dengue and chikungunya viruses. ZIKV infection was confirmed by RT-PCR.<sup>10</sup> A case of ZIKV infection acquired in March 2015 by an Italian traveler returning from Brazil was also reported in June 2015.<sup>22</sup> In the first semester of 2015, health services in two states of the northeast of Brazil (Pernambuco and Bahia) reported a significant rise in the frequency of patients with mild fever, maculopapular rashes, headaches, non-purulent conjunctivitis, arthralgia, and myalgia. Serological tests for dengue and chikungunya viruses were negative, and ZIKV was detected by RT-PCR.<sup>11,23</sup> Another outbreak was described in the southeast region, in Rio de Janeiro, with the identification of 364 suspected cases.<sup>24</sup> Among 262 cases tested for ZIKV infection, 119 (71.9%) had ZIKV RNA in serum collected during the acute phase of the disease.<sup>24</sup> Subsequently, the Brazilian Ministry of Health reported that autochthonous transmission of ZIKV occurred in 18 states of the country from April to November 2015.<sup>25</sup>

In April 2016, a total of 91,387 cases of ZIKV infection had been reported in all Brazilian states, with an incidence of 44.7/100,000 inhabitants.<sup>26</sup> Simultaneous infections of ZIKV and dengue virus were also described.<sup>27</sup>

From Brazil, ZIKV spread to other Latin American countries due to the large availability of the vector *A. aegypti*. Even in temperate regions with limited presence of the vector, there is great concern with possible autochthonous cases, especially through sexual transmission.<sup>28,29</sup>

## Infection during pregnancy and vertical transmission of ZIKV

The first evidence of perinatal transmission of ZIKV was confirmed in two cases during the largest outbreak in French Polynesia.<sup>30</sup> Both mothers presented cutaneous rash after delivery. One of the newborns presented a transient skin rash occurring 3 hours after phototherapy. Both mothers and newborns were serum positive by RT-PCR, and vertical transmission possibly occurred by transplacental route or during delivery.<sup>30</sup>

In October 2015, the Brazilian Ministry of Health issued an official communicate stating that 26 cases of neonatal microcephaly were notified from public and private services in Pernambuco state.<sup>31</sup>

Microcephaly is a neurological syndrome that can be associated with other malformations and is closely related to the development of the central nervous system (CNS).<sup>32</sup> Its definition and clinical relevance vary among authors.<sup>33–35</sup> For

instance, cutoff points of head circumference measures  $\leq 2$  or 3 standard deviation (SD) (from the mean expected values for age and sex) have been adopted. Children with head circumference  $\leq 3$  SD are considered as having severe microcephaly, and they are more likely to have other structural abnormalities, more severe developmental impairment, and cognitive and behavioral problems than those with milder microcephaly.<sup>34,35</sup>

Until November 17, 2015, 399 suspected cases of microcephaly were identified in seven states of the northeast of Brazil. The majority of cases were concentrated in Pernambuco. From that moment onward, the Brazilian Ministry of Health recommended that all cases of microcephaly should be immediately reported.<sup>36</sup> This event was reported to the World Health Organization (WHO) and classified as a potential public health emergency.<sup>37</sup>

It is worth mentioning that, until December 2015, the official parameter adopted for microcephaly definition in Brazil considered the cephalic perimeter  $< 33$  cm for term newborns.<sup>38</sup> From that point until March 2016, the parameter adopted for microcephaly notification was changed to a cephalic perimeter  $< 32$  cm for term newborns of both sexes.<sup>38</sup> This criterion change was proposed to increase the specificity of the test.<sup>39</sup>

In parallel to those official reports, confirmed cases of ZIKV infection in fetus and newborns have been published.<sup>40–42</sup> The first reports identified virus genome in the amniotic fluid of two pregnant women whose fetuses had microcephaly,<sup>41,42</sup> and in samples collected from a deceased neonate with 19 cm head circumference and severe hydrops who died just after birth.<sup>40</sup> Calvet et al<sup>41</sup> and Oliveira Melo et al<sup>42</sup> described two cases in which amniotic fluid was collected from two pregnant Brazilian women who developed symptoms compatible with ZIKV infection at 10 and 18 weeks of gestation and whose fetuses had microcephaly. Ultrasound (US)-guided transabdominal amniocentesis was performed at 28 weeks of gestation, and ZIKV genome was identified in the amniotic fluid of both pregnant women, but not in their urine or serum. Fetal US at 25–27 weeks of gestation revealed severe CNS abnormalities, including microcephaly, asymmetric hemispheres, ventriculomegaly, hypoplastic cerebellum, and hypoplasia/absence of the cerebellar vermis. Calcification areas were seen in later examinations. One infant was born at 40 weeks of gestation with a head circumference of 30 cm, while the other newborn presented severe ventriculomegaly, microphthalmia, cataract, and severe arthrogryposis in legs and arms.<sup>41,42</sup>

Due to the temporal coincidence of these events, a possible association of microcephaly and ZIKV infection was hypothesized by the Brazilian Ministry of Health.<sup>40,43</sup> Since then, a special task force was established to investigate the association between microcephaly and ZIKV infection during pregnancy. Notification of all cases of microcephaly became mandatory and in any case of pregnant women with suspected ZIKV infection pregnancy outcome must be monitored.

On December 1, 2015, given the increase in congenital abnormalities, GBS, and other neurological manifestations possibly related to ZIKV, the WHO released an epidemiologic alert with recommendations, including surveillance, case definition, diagnosis, vector control, and prevention measures for public health proceedings.<sup>37</sup> On January 22, 2016, a report including the participation of the Brazilian Medical Genetics Society–Zika Embryopathy Task Force was posted in the Centers for Disease Control and Prevention (CDC) Morbidity and Mortality Weekly Report.<sup>44</sup> In this report, Schuler-Faccini et al<sup>44</sup> described 35 infants with microcephaly that had been born from August to October 2015 in eight among 26 states of Brazil. Four (11%) infants had low birth weight, while arthrogryposis, talipes, microphthalmia abnormalities in fundoscopic examination were also reported. All mothers had lived in and/or visited ZIKV-affected areas during pregnancy, and 74% presented a skin rash during the first ( $n=21$ ) or second ( $n=5$ ) trimesters of pregnancy. Tests for other congenital infections were negative. Results of cerebrospinal fluid analysis were not available. Computed tomography (CT) scans and transfontanelar cranial USs identified brain calcifications (periventricular, parenchymal, thalamic, and in the basal ganglia areas), lissencephaly, pachygyria, and ventricular enlargement.<sup>44</sup>

Other studies reported ZIKV infection during pregnancy with confirmation of the virus by RT-PCR in both mother and fetus samples. On February 10, 2016, in Slovenia, Mlakar et al<sup>45</sup> described a 25-year-old woman who previously lived in the northeast of Brazil and exhibited, during the 13th week of gestation, a generalized maculopapular rash, high fever, severe musculoskeletal, and retro-ocular pain and itching, parallel to ZIKV epidemic in that community. The ultrasonography, at 29 weeks of gestation, identified intrauterine growth retardation (fetal weight at third percentile), microcephaly, moderate ventriculomegaly, and numerous calcifications in the placenta. Pregnancy was terminated at 32 weeks and fetal autopsy confirmed microcephaly, almost complete agyria, hydrocephalus, and multifocal dystrophic calcifications in the cortex and in the subcortical white matter.<sup>45</sup> Simultaneously, another two cases

of fetal losses and two cases of early neonatal deaths were described by Martines et al.<sup>46</sup> All four mothers presented rash during the first trimester of pregnancy, but they were not tested for ZIKV. The two newborns died within 20 hours of life, and the histopathologic evaluation of brain showed parenchymal calcification, microglial nodules, gliosis, cell degeneration, and necrosis. Brain tissues were positive for ZIKV in RT-PCR. Tissues of the two miscarriages were also positive for ZIKV in RT-PCR. Immunohistochemistry also detected ZIKV antigen in tissues of two among four cases. Investigation for all other infectious agents related to vertical transmission was negative.<sup>46</sup> On February 25, 2016, Sarno et al<sup>47</sup> reported a case of fetal death at 32 weeks of pregnancy, without a clear previous history of ZIKV infection. Obstetric ultrasonography showed microcephaly, hydranencephaly, intracranial calcifications, and destructive lesions of posterior fossa. Pregnancy interruption occurred at the 32nd week, and RT-PCR was positive for ZIKV in cerebral, cortex, and medulla, and in amniotic fluid.<sup>47</sup> In March 2016, Driggers et al<sup>48</sup> published a case of a 33-year-old North American woman who had been in Mexico, Guatemala, and Belize with her husband on holidays. This woman developed fever, myalgia, ocular pain, and a skin rash in her eleventh week of gestation. Serologic analyses were positive for IgG antibodies and negative for IgM antibodies against dengue virus, and positive for both IgG and IgM antibodies against ZIKV, and the virus was also detected by RT-PCR assay at 16 and 21 weeks of gestation. Diffuse atrophy of the cerebral mantle, especially in the frontal and parietal lobes, and of “corpus callosum” was identified at the 20th week by magnetic resonance imaging (MRI), and fetal head circumference decreased in sequential US images from the 47th to 24th percentile between 16 and 20 weeks of gestation. On post-mortem analysis, the highest viral loads were found in fetal brain, but ZIKV was also identified in placenta, umbilical cord, and other fetal tissues, including muscle, liver, lung, and spleen.<sup>48</sup> In March 2016, the CDC also published a retrospective analysis of cases from the period of August 2015 to February 2016.<sup>49</sup> A total of 257 women were evaluated and 97% tested negative for recent ZIKV infection. Among the nine cases with confirmed ZIKV infection (RT-PCR, immunohistochemistry, and/or serologic tests in amniotic fluid, placenta, or serum), six pregnant women reported symptoms during the first trimester: two early pregnancy losses occurred, two pregnancies were electively terminated, one infant with microcephaly was born alive, and one pregnancy was still continuing when the article was published. In three women whose symptoms occurred during the second

and third trimesters, no abnormalities were identified in the newborns.<sup>49</sup>

A cohort study from Rio de Janeiro/Brazil enrolled 88 pregnant women (from 5 to 38 weeks of gestation) who had started with cutaneous rash within the 5 days prior the study recruitment.<sup>46</sup> ZIKV infection was identified by real-time RT-PCR in 72 (82%) of them in blood and/or urine samples. US was performed in 42 women with positive ZIKV infection, and fetal abnormalities (in utero growth restriction with or without microcephaly, ventricular calcifications, abnormal amniotic fluid volume, or altered cerebral or umbilical artery flow) were detected in 29% of them. Two fetal deaths occurred at 36 and 38 weeks of gestation.<sup>50</sup>

In April 2016, three studies described image changes in Brazilian cases of microcephaly.<sup>51–53</sup> A study from Recife, the capital of the state of Pernambuco/Brazil, evaluated 23 infants with congenital microcephaly and epidemiology suggestive of congenital ZIKV infection.<sup>51</sup> ZIKV IgM antibodies were detected in the cerebrospinal fluid of seven infants. Other infections were excluded by serological analysis. Brain CT showed intracranial calcifications in all cases, mainly in frontal and parietal lobes and at the corticomedullary junction, basal ganglia, and thalamus. Ventriculomegaly (severe in 53%) and global hypogyration of the cerebral cortex were also found in all infants. Cerebellar hypoplasia and abnormal hypodensity of the white matter were also described.<sup>51</sup> In another study from Pernambuco, neuroradiological examinations of children with a diagnosis of microcephaly, who were born during the ZIKV outbreak in 2015/2016, were retrospectively evaluated.<sup>52</sup> After the exclusion of other known causes of microcephaly, the final sample with suspect ZIKV infection comprised 23 infants. In six cases, cerebrospinal fluid was analyzed and IgM antibodies against ZIKV were positive. In the remaining 17 infants, the diagnosis was suspected, but without laboratory confirmation. Descriptions of the radiological abnormalities identified in CTs included decreased brain volume, ventriculomegaly, and cerebral calcifications (in cortical and subcortical junctions, basal ganglia, and periventricular areas). Simplified gyral pattern, hypogenesis/hypoplasia of corpus callosum, and delayed myelination were also described in MRI examinations.<sup>52</sup> Cavalheiro et al<sup>53</sup> evaluated 13 term newborns with microcephaly, whose mothers presented cutaneous rash within the first 4 months of pregnancy. Other infectious agents were excluded. In all of them, decreased cerebral mantle, increased subarachnoid space and ventriculomegaly, lissencephaly, and hypoplasia of the corpus callosum were observed in



brain CT and/or MRI. Only one case exhibited periventricular calcifications, whereas, in others, calcifications were mostly localized in the subcortical/cortical transition area and in the basal ganglia.<sup>53</sup> In May 2016, another case report from Rio de Janeiro described a 27-year-old woman, within the 12th week of gestation exhibiting symptoms of fever, maculopapular rash, and arthralgia.<sup>54</sup> Serology testing for congenital infections and dengue and chikungunya virus was negative. Normal fetal anatomy was seen in US examinations at 12 and 21 weeks of gestation, but microcephaly was suspected at the 32-week scan. Microcephaly was confirmed at the 37-week scan along with diffuse brain calcifications. Fetal MRI additionally revealed reduced gyration and asymmetric colpocephaly. Neonatal head circumference was 29.0 cm, and transfontanelar US examination identified subcortical and periventricular calcifications with ventricular dilatation. Postnatal CT also revealed brain calcifications, cortical atrophy, small anterior fontanel, and premature closure of coronal and metopic sutures.<sup>54</sup> Up to May 7, 2016, the Brazilian Ministry of Health reported 7,438 suspected cases of microcephaly, among which 2,679 cases were excluded and 3,433 were still under investigation. A total of 1,326 cases of microcephaly were confirmed, but only 205 of them had laboratorial confirmation of ZIKV infection.<sup>55</sup>

Studies also retrospectively evaluated the frequency of fetal abnormalities possibly due to ZIKV outbreak in French Polynesia that affected ~66% of the population.<sup>56,57</sup> In the 23-month period included in the study, eight cases of microcephaly were identified, of which five resulted in termination of pregnancy by medical abortion.<sup>56</sup> This study estimated the risk of microcephaly after ZIKV infection in pregnancy in 0.95%, and the first trimester seems to be the period at highest risk.<sup>56</sup> It is worth mentioning that, based on the analysis of Brazilian data, Johansson et al<sup>57</sup> proposed the risk of microcephaly ranging from 0.88% to 13.2% of ZIKV infection occurring in the first trimester of pregnancy, depending on the overall infection rate estimates. The lower end of this range was similar to the ~1% risk estimated for French Polynesia. Still there, a retrospective evaluation of 19 cases of congenital cerebral malformations, including five cases of microcephaly, described positive identification of ZIKV RNA by RT-PCR in four of them.<sup>58</sup> Another study reviewed the occurrence of fetal abnormalities in 4,285 births in the Polynesia Hospital Center.<sup>59</sup> Twelve cases of malformations were identified, and among them three cases of fetuses with severe CNS abnormalities and positive PCR for ZIKV in the amniotic fluid. Calcifications and ventriculomegaly were observed in all cases in fetal US.

Microcephaly, low cerebellar biometry, occipital subependymal pseudocysts, polymicrogyria with laminar necrosis and opercular dysplasia, absent/or hypoplastic corpus callosum, and hypoplastic brainstem were detected through MRI.<sup>59</sup>

The studies describing radiological abnormalities of the CNS in fetuses and neonates with presumed congenital ZIKV infection are summarized in Table 1.

Ocular abnormalities in neonates possibly associated with ZIKV infection have also been reported. In a sample of 29 infants, from Salvador, Bahia, Brazil, with microcephaly and a presumed diagnosis of congenital ZIKV infection, ocular abnormalities were found in ten children (34.5%), and, in seven, the alterations were bilateral. The authors described focal pigment mottling of the retina and chorioretinal atrophy, optic nerve abnormalities, bilateral iris coloboma, and lens subluxation. From this sample, 23 mothers reported symptoms compatible with ZIKV infection during pregnancy, of which 18 occurred in the first trimester, four in the second trimester, and one in the third trimester.<sup>60</sup> A study from Recife, Pernambuco, Brazil, showed ten infants with similar ocular abnormalities, including gross pigment mottling and/or chorioretinal atrophy and optic nerve abnormalities. Seven mothers (70%) referred symptoms during pregnancy, six of them in the first trimester.<sup>61</sup> Unfortunately, the diagnosis of ZIKV infection was based on the clinical symptoms of the mother during pregnancy, and also the exclusion of other infections such as syphilis, HIV, toxoplasmosis, cytomegalovirus, herpes simplex virus, and rubella.

## Controversies on causal association between ZIKV infection and microcephaly

The absence of a unified definition of microcephaly has had important epidemiological consequences. Since March 2016, the official criteria used to diagnose microcephaly in Brazil have been based on the WHO recommendations:<sup>61,62</sup> 1) for term babies, international parameters of the WHO should be used and 2) for preterm newborns, the intergrowth parameters should be used. Initially, the definition of cases of microcephaly in the surveillance system included all full-term infants with a head circumference  $\leq 33$  cm, and later the inclusion criteria were modified to  $\leq 32$  cm.<sup>63</sup> However, the National Live Birth Information System adopted a definition of microcephaly when head circumference measure was  $\leq 3$  SD from the mean expected for age and sex.<sup>64,65</sup> Therefore, variations in the number of suspected cases may have occurred according to the definitions adopted and may have resulted in lower diagnostic specificity, leading to possible overestimation

**Table 1** Main radiological abnormalities of the CNS described in fetuses and neonates with presumed congenital ZIKV infection

References	Country, region/states	Subjects	Other infectious agents evaluated	ZIKV identification method	Clinical features	Radiological findings
Aragao et al <sup>52</sup>	Brazil, Pernambuco <sup>a</sup>	23 children with microcephaly	Paired serology (IgM and IgG) of infants and mothers for CMV, toxoplasmosis, rubella, HIV syphilis	IgM antibody-capture ELISA performed in six CSF samples: all positive. Others not tested	Birth weight categorized as small for gestational age (39%). Premature closure of anterior fontanel (87%). Microcephaly	CT (22 infants): decreased brain volume (95%), ventriculomegaly (86%), calcifications in cortical and subcortical junctions (100%), basal ganglia (59%), periventricular (45%), hypoplasia of cerebellum (27%), and brainstem (45%). MRI (eight infants): decreased brain volume and ventriculomegaly (100%), simplified gyral pattern (75%), hypogenesis/hypoplasia of corpus callosum (75%), and delayed myelination (88%).
Brasil et al <sup>50</sup>	Brazil, Rio de Janeiro	72 pregnant women with positive ZIKV infection and 16 noninfected, followed prospectively	Immunes to rubella and CMV; negative for syphilis; positive dengue-IgG antibodies in 88%	Real-time RT-PCR assays for ZIKV on blood and urine samples of pregnant women <sup>b</sup>	Two fetal deaths at 36 and 38 weeks of gestation in ZIKV-positive women. Six newborns: macular lesions (33%), small for age (33%), EEG abnormalities	Fetal US (12 ZIKV-positive women): abnormal amniotic fluid volume (25%), growth restriction (42%), microcephaly (33%), and brain calcifications (33%). Newborn CT (one case): cerebral calcifications and global cerebral atrophy
Cavalleiro et al <sup>53</sup>	Brazil, Pernambuco/ Maranhão/ Rio Grande do Norte	13 infants with congenital microcephaly; radiologic examinations retrospectively reviewed	Negative serological tests for toxoplasmosis, rubella, CMV, herpes virus, and syphilis	Mothers presented with rash during pregnancy (100%). ZIKV infection was not laboratory confirmed in infants or mothers	Infants were born after the 37th gestational week by cesarean section; no other clinical features were described	CT/MRI: decreased cortical and white matter (100%), hypoplastic corpus callosum (100%), secondary ventriculomegaly (100%), lissencephaly (100%), calcifications in subcortical/cortical transition regions and basal ganglia (92%)
Calvet et al <sup>41</sup>	Brazil, Paraíba	Two pregnant women with symptoms whose fetuses had microcephaly	Toxoplasmosis, HIV, syphilis, measles, rubella, CMV, herpes simplex, parvovirus B19 screens were negative. RT-qPCR for dengue and chikungunya virus were negative in urine and serum	Amniocentesis at gestational week 28: RT-qPCRs for ZIKV were positive in the amniotic fluids and negative in urine and serum samples in both patients	Symptoms (rash, arthralgia, fever) at 10 and 18 weeks of gestation. Neonate 1: head circumference of 30 cm at 40 weeks. Neonate 2: microphthalmia, cataract, arthrogryposis in legs and arms	Patient 1: fetal US at 16 weeks: normal; at 21 weeks: microcephaly, moderate ventriculomegaly, partial agenesis of the cerebellar vermis; at 27 weeks: relevant dilation of ventricles, asymmetry of hemispheres, hypoplastic cerebellum with complete absence of the cerebellar vermis; at 40 weeks: microcephaly, calcification areas. Patient 2: fetal US at 22 weeks: mild cerebellar vermis hypoplasia; at 25 weeks: hypoplasia of the cerebellar vermis, enlargement of the posterior fossa, microcephaly

Driggers et al <sup>48</sup>	Washington, DC, USA (travels to Mexico, Guatemala, and Belize)	Case report: 33 year-old pregnant woman	Dengue virus: positive IgG antibodies and negative IgM. Serologic test for chikungunya virus was negative	Positive IgG and IgM antibodies. Positive RT-PCR against ZIKV in maternal serum, fetal brain, placenta, and other fetal tissues	At eleventh gestational week: ocular pain, myalgia, mild fever, and skin rash. Pregnancy was terminated at 21st week	Fetal US 13, 16, and 17 weeks of gestation: no evidence of microcephaly or intracranial calcifications. At 19 weeks: reduced cerebral mantle, enlarged frontal horns (intraventricular hemorrhage questioned), dilation of the third ventricle, dilation of the frontal horns of the lateral ventricles Fetal MRI at 20 weeks: diffuse atrophy of the cerebral mantle (frontal and parietal lobes) with the absence of normal lamination pattern, hypoplastic corpus callosum Fetal US: calcifications and ventriculomegaly (100%). Fetal MRI: microcephaly (100%), polymicrogyria with laminar necrosis and opercular dysplasia (100%), absent/or hypoplastic corpus callosum (67%), low cerebellar biometry (67%) CT: ventriculomegaly and global hypomyelination (100%). Calcifications (100%): frontal and parietal lobes, basal ganglia and thalamus; cerebellar hypoplasia (74%), white matter hypodensity (100%) Patient 1: Fetal US at 20 weeks of gestation: absence of the corpus callosum, ventriculomegaly, and brain atrophy. Fetal MRI: severe brain atrophy
Guillemette-Artur et al <sup>49</sup>	French Polynesia	Retrospectively analyzed MRI findings of three fetuses	PCR for CMV and lymphocytic choriomeningitis virus was negative	PCR for ZIKV was positive	Two mothers had ZIKV-like symptoms during the first trimester of pregnancy. In all cases, pregnancies were electively terminated	
Hazin et al <sup>51</sup>	Brazil, Pernambuco <sup>c</sup>	23 infants with congenital microcephaly	Toxoplasmosis, syphilis, varicella, parvovirus, HIV, rubella, CMV, and herpes simplex were excluded by serological tests	ZIKV IgM antibodies were detected in the cerebrospinal fluid in seven of 23 infants	No clinical description	
Meaney-Delman et al <sup>49</sup>	The USA (travels to American Samoa, Brazil, El Salvador, Guatemala, Haiti, Honduras, Mexico, Puerto Rico, and Samoa)	Nine pregnant women with positive ZIKV		RT-PCR, IHC, and serologic testing	All women had rash. Four cases were described: one had radiological examinations	
Mrakar et al <sup>45</sup>	Ljubljana, Slovenia (lived in northeast of Brazil)	Case report: 25-year-old pregnant woman	PCR assays were negative for other flaviviruses, CMV, rubella virus, varicella-zoster virus, herpes simplex virus, parvovirus B19, enteroviruses, and <i>Toxoplasma gondii</i>	Positive results for ZIKV were obtained by RT-PCR in fetal brain	At 13th week of gestation, there were symptoms of generalized rash, fever, musculoskeletal, and retroocular pain. Pregnancy was terminated at 32 weeks.	Fetal US at 14 and 20 weeks: normal fetal growth and anatomy. At 29 weeks of gestation: intrauterine growth retardation, microcephaly, moderate ventriculomegaly; placenta calcifications

(Continued)

Table 1 (Continued)

References	Country, region/states	Subjects	Other infectious agents evaluated	ZIKV identification method	Clinical features	Radiological findings
Schuler-Faccini et al <sup>44</sup>	Brazil, eight states, not specified	35 infants with congenital microcephaly	Negative serological tests for syphilis, toxoplasmosis, rubella, CMV, and herpes simplex virus	Reported maternal rash during pregnancy (71%). ZIKV infection was not laboratory confirmed in infants or mothers	Fetal autopsy: microcephaly, agyria, hydrocephalus, and multifocal dystrophic calcifications in the cortex and in the subcortical white matter Low birth weight (26%), arthrogryposis (11%), hypertonia/spasticity (37%), hyperreflexia (20%), irritability (20%), tremors (11%), and seizure Symptoms 12th week of gestation. Male infant born at 38 weeks, with microcephaly. Cephalic circumference of 32 cm at 1-month-old. No other clinical features were described	CT and TUS: lissencephaly, pachygyria (33%), cortical/subcortical atrophy, nonhypertensive ventricular enlargement (44%), periventricular, parenchymal, thalamic, basal ganglia brain calcifications (74%)
Werner et al <sup>54</sup>	Rio de Janeiro, Brazil	Case report: 27-year-old pregnant woman	Serology testing for toxoplasmosis, rubella, CMV, and herpes simplex, dengue and chikungunya virus was negative			Fetal US: microcephaly and diffuse brain calcifications were identified at 37 weeks. Fetal MRI: reduced gyration and asymmetric colpocephaly. Transfontanellar US: subcortical and periventricular calcifications, ventricular dilatation. Postnatal CT: brain calcifications, cortical atrophy, small anterior fontanel and premature closure of coronal and metopic sutures. Postnatal MRI: pachygyria, corpus callosum dysgenesis, brain calcifications

**Notes:** Association for AACD. <sup>a</sup>QuantiTect Probe RT-PCR Kit (Qiagen NV, Venlo, the Netherlands); blood and urine samples. <sup>b</sup>Professor Fernando Figuera Integral Medicine Institute (MIP).

**Abbreviations:** AACD, Assistance of Disabled Children; CMV, cytomegalovirus; CNS, central nervous system; CSF, cerebrospinal fluid; CT, computed tomography; EEG, electroencephalogram; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; IHC, immunohistochemical; IgM, immunoglobulin M; IgG, immunoglobulin G; MRI, magnetic resonance imaging; PCR, polymerase chain reaction; qPCR, quantitative PCR; RT, reverse transcriptase; TUS, transfontanellar ultrasound; US, ultrasound; ZIKV, Zika virus.



of affected infants.<sup>39,65</sup> Furthermore, the real incidence of microcephaly in Brazil, prior to the occurrence of ZIKV infectious, was possibly underestimated, especially in the northeast region, and the obligatory notification of cases may falsely inflate the real incidence.<sup>39,66,67</sup> In a communicate, the Latin American Collaborative Study of Congenital Malformations (ECLAMC 2015) questioned the number of reported cases, stating that they were much higher than the expected considering the incidence of ZIKV infection in the Brazilian population.<sup>68</sup>

Other possible etiologies of microcephaly including environmental, alcohol-related, infectious or genetic, and/or associated factors (malnutrition, coinfection with other agents) also must be more carefully investigated.<sup>66</sup> Congenital microcephaly is clearly associated with genetic and acquired conditions (for review, refer Nunes et al<sup>69</sup>). Exposure to drugs, including alcohol, maternal infection, and nutritional deficiency is the major cause of acquired congenital microcephaly. Regardless of the cause, the etiopathogenic factor must influence the proliferation phase of the CNS development, around third and fourth months of pregnancy, to determine microcephaly. Accordingly, ZIKV infection could cause microcephaly only when occurring in the first months of pregnancy. In this regard, convincing epidemiological data are missing.<sup>70</sup> Although this relationship still needs further clarification, some of the evidence described so far suggests the possibility of ZIKV-related teratogenic effects.<sup>44,71,72</sup> In terms of public health safety measures, while prospective studies are not finalized and/or more robust evidence is available, precautions to control vector multiplication and to reduce individual exposure to the virus, especially in fertile and pregnant women, are recommended throughout the world.<sup>31,37,73</sup> The continuous surveillance of suspected cases is also maintained, and a recent report from the WHO<sup>74</sup> enlists eight countries that have reported cases of microcephaly and/or CNS malformations that were potentially associated with ZIKV infection: Brazil, Cape Verde, Colombia, French Polynesia, Martinique, Panama, Slovenia, and the USA. In the last two countries, the probable place of infection was located in South/Central America.

Table 2 depicts arguments for and against a causal association between ZIKV infection and microcephaly.

## Neuronal damage by ZIKV: evidence from experimental models and unanswered questions

ZIKV infection has been linked to severe neurological complications, that is, microcephaly and GBS. While the

first condition involves the CNS and the second is related to peripheral nerves lesion. The mechanisms by which ZIKV can determine these two distinct syndromes are still uncertain. The virus could directly affect CNS and peripheral neurons. Alternatively, neuronal lesion could result from ZIKV-elicited processes, including immune response and loss of glial support. So far, studies have focused on the first hypothesis implicating a direct effect of ZIKV on neurons.

Although suggestive, the detection of ZIKV in the brain of microcephalic human fetus<sup>45</sup> does not prove either causality or pathogenic mechanism. To overcome the limitations of human studies, *in vitro* and *in vivo* experimental approaches have been pursued.

Two independent studies showed that ZIKV can infect neural stem cells *in vitro*, inducing caspase-mediated cell death.<sup>75,76</sup> The most compelling evidence for a direct role played by ZIKV in microcephaly came from an elegant study using induced pluripotent stem cells cultured as neurospheres and brain organoids, in *in vitro* models of embryonic brain development. Garcez et al<sup>76</sup> showed that ZIKV reduced the viability and growth of neural stem cells in these models, disrupting the formation of both structures. To control for unspecific effects of viral infection on neurogenesis, the same experiments were performed with dengue virus 2, a flavivirus with genetic and serological similarities to ZIKV. Neurospheres and brain organoids exposed to dengue virus 2 did not have their growth and development altered, suggesting that neurogenesis impairment is not a general feature of the flavivirus family.<sup>76</sup>

Until recently, only a very limited number of studies had been performed in rodents, indicating that ZIKV can replicate and damage CNS cells.<sup>77,78</sup> Among the concerns regarding these older studies, there is the use of the prototype MR 766 strain of ZIKV that had undergone multiple passages in suckling mouse brains, possibly changing its biological properties, including virulence. Alterations in molecular components of ZIKV overtime could also be responsible for its increased neuropathogenicity lately. To overcome these limitations, Lazear et al<sup>79</sup> assessed four ZIKV strains (three strains isolated from mosquitoes in Africa and one contemporary clinical isolate) in wild-type (WT) C57BL/6 mice and immune-deficient transgenic mice. Four- to six-week-old WT mice inoculated with ZIKV by subcutaneous route did not develop any clinical sign of disease, while different strains of mice lacking interferon  $\alpha/\beta$  (or type 1) signaling developed neurological signs (muscle weakness) and succumbed to the infection. *Ifnar1*<sup>-/-</sup> mice, which cannot respond to interferon  $\alpha/\beta$ , were highly vulnerable to the infection

**Table 2** Supporting and controversial evidence of the association between ZIKV infection during pregnancy and fetal abnormalities

Supporting evidence	Controversial evidence
Fetal involvement with maternal infection occurring during first trimester or early second trimester	
Presence of ZIKV antibodies in maternal serum <sup>45,48,49</sup>	ZIKV infection inferred only by epidemiological/clinical criteria <sup>44,49,53,54,56</sup>
Isolation of ZIKV during pregnancy (maternal serum/urine, amniotic fluid) <sup>3,41,42,49,50,58</sup>	Risk of infection after first trimester could not be excluded
Isolation of ZIKV antibodies in samples from fetus/neonates (fetal necropsy, cerebrospinal fluid) <sup>45–48,51,52</sup>	
Common phenotype in cases with isolation of ZIKV in fetus/neonates	
Microcephaly <sup>41,42,47,48,51,52,58</sup>	
Fetal death <sup>40,47,49,50</sup>	
Intracranial calcifications <sup>47,51–53</sup>	Despite the fact that infection seems to cause more severe disease in fetus during the first trimester, data regarding exposure and disease cannot be totally explained
Cerebral atrophy <sup>45,48,52</sup>	
Destructive lesions <sup>47</sup>	
Anomalies of cortical development/gyration <sup>51–53</sup>	Mild phenotypes could also be associated with ZIKV infection, since only severe cases have been reported
Common phenotype in cases with presumed infection	
Ocular lesions <sup>60,61</sup>	
Reports of fetuses and infants with microcephaly who are born to women who travel to countries with active ZIKV transmission	
In Slovenia, a pregnant woman who was living in the northeast of Brazil during ZIKV outbreak. <sup>45</sup> A pregnant woman who had been to Mexico, Guatemala, and Belize on holiday <sup>48</sup>	
Six pregnant women who had traveled to areas with active transmission of ZIKV <sup>49</sup>	
Virus presence in fetal and neonatal samples, and similar findings are presented in other congenital infections	The evaluation of placental tissues, fetal death and neonatal death are necessary to determine the effect of gestational age during maternal illness on fetal outcomes.
The findings of microcephaly, premature fetal death, intracranial calcifications, and CNS abnormalities are similar to the effects of other known congenital infectious agents and environmental toxins <sup>69</sup>	Experimental evidence of infection of neural stem cells in vitro <sup>75,76</sup> and animal models <sup>76,77</sup>
Strong temporal and geographical association of ZIKV and neurological involvement	Due to changes in microcephaly definition, obligatory notification, and absence of confirmation of etiology in several cases, an overestimation of ZIKV-affected infants should be considered in Brazil. <sup>39,65–67</sup> Other etiologies of microcephaly should also be ruled out. <sup>69</sup>
Brazil	Ideally, consistent findings should be performed by two or more high-quality epidemiologic studies, with the control of confounding factors, sufficient numbers, exclusion of positive and negative bias factors, prospective studies if possible, and relative risk higher than 6.0. However, literature until now includes only surveys and retrospective or short-term cohort studies. Therefore, until a large epidemiological study is published, it is not possible to confirm the association between maternal exposure and fetal involvement
Brazilian Ministry of Health <sup>36,55</sup>	
Northeast <sup>51,52</sup>	
Southeast <sup>54</sup>	
French Polynesian	
Retrospective studies <sup>56,58,59</sup>	

**Abbreviations:** CNS, central nervous system; ZIKV, Zika virus.

and exhibited high levels of ZIKV in all tissues evaluated, including blood, spleen, testes, brain, and spinal cord. Interestingly, ZIKV could cause disease in non-weaned WT mice, that is, 1-week-old, when the innate immune response is not yet mature, indicating an age-dependent vulnerability to disease.<sup>79</sup> Besides proposing a model for therapeutic and pathogenesis investigation, this study confirmed a pivotal role played by interferon  $\alpha/\beta$  in restricting ZIKV infection, as already described for dengue virus infection.

Rossi et al<sup>80</sup> also suggested a significant role played by type I interferon in the resistance against ZIKV infection.

Using a Cambodian isolate inoculated by intraperitoneal route, they found that 3-week-old 129sv mice lacking the interferon  $\alpha/\beta$  receptor (A129) succumbed to the infection, while immunocompetent mice survived. An age-dependent vulnerability to disease was also demonstrated in this model with 3-week-old mice exhibiting more severe signs of infection than 5- and 11-week-old mice. ZIKV was detected in the brain on day 3 postinfection and caused neurological signs (lethargy, tremor) by day 5 postinfection, especially in 3-week-old A129.<sup>80</sup> Mice lacking both interferon  $\alpha/\beta$  and  $\gamma$  (or type II) receptors (AG129) infected by intraperitoneal

or intradermal routes also developed a severe disease, dying on day 6 postinfection. Given the antiviral activity of interferons, these results were not surprising, being also corroborated by an *in vitro* study showing the potential of interferons  $\alpha/\beta$  and  $\gamma$  in controlling ZIKV replication.<sup>81</sup> The major limitation of the models proposed so far pertains to the fact that susceptible animals lack a key component of the innate immune response against viral infection. This certainly influences how the immune system deals with the ZIKV infection, affecting the evaluation of the real role played by immune mechanisms on disease pathogenesis. In fact, mechanistic insights are still missing in *in vivo* studies, particularly those related to neuronal damage. In addition, it is uncertain whether ZIKV promotes widespread or localized brain damage, and careful neuropathological analysis coupled with behavioral assessment is warranted in these and future animal models of ZIKV. The age-dependent vulnerability to disease must be further explored, including intrauterine infection at different stages of fetus development. This latter issue is of paramount importance taking into consideration the controversies regarding the association between prenatal ZIKV infection and microcephaly. More importantly, it is uncertain the consequences of ZIKV infection after this critical period on CNS development. Theoretically, neuronal migration, organization, and myelination could be impaired by CNS infection, leading to different conditions, including major (eg, lissencephaly and schizencephaly) and minor (eg, focal dysplasias) brain malformations, and “functional” neuropsychiatric syndromes including intellectual impairment and autism. Longitudinal cohorts of newborns from epidemic areas, involving systematic assessment of neurodevelopmental milestones, can help answering this issue.

## GBS and other autoimmune-mediated disorders

The first link between ZIKV infection and a neurological complication occurred in the ZIKV outbreak in French Polynesia from October 2013 to March 2014.<sup>82</sup> Cao-Lormeau et al<sup>83</sup> reported 42 patients with GBS during this ZIKV outbreak, corresponding approximately to a 20-fold increase in the expected number of cases of GBS for that region. GBS is an acute autoimmune peripheral polyneuropathy characterized by the development of ascending motor weakness associated with variable degrees of cranial nerves palsy, sensory changes, and dysautonomia.<sup>84</sup> The disease is self-limiting, reaching its nadir after 2–4 weeks of onset. Depending on its severity, it can affect respiratory muscles, requiring artificial ventilation support.

In the reported cases, most patients (88%) had a previous history of a short-lasting viral syndrome characterized by fever, rash, arthralgia, and/or conjunctivitis, and all were positive for ZIKV serological tests. The GBS in these patients differed from the typical syndrome as it was marked by a fast progression to a nadir (<1 week) and a short plateau phase. Facial palsy was a common manifestation (64%), and one-third of patients required respiratory assistance. Besides the combination of fast onset and fast recovery, patients lacked the typical demyelinating pattern of the disease, exhibiting the acute motor axonal neuropathy (AMAN) type.<sup>83</sup> In contrast with non-ZIKV-related AMAN cases, the patients did not show the characteristic anti-ganglioside antibodies. The atypical presentation along the lack of AMAN-related antibodies cast doubt on the anti-glycolipid antibody role on disease pathogenesis.<sup>83,85</sup> In this scenario, a direct effect of ZIKV can be implicated again. All patients in Cao-Lormeau et al's<sup>83</sup> series were treated with intravenous immunoglobulin, one of the standard treatments for GBS along with plasmapheresis.<sup>84</sup> It remains to be defined whether the favorable clinical outcome observed in this study could be achieved with plasmapheresis and/or lack of specific treatment. The latter may happen in regions with limited economic and health resources.

As with obstetric complications, including microcephaly, there are several challenges ahead. First, the epidemiological data must be confirmed. Preliminary reports from different Latin American countries seem to corroborate the association between ZIKV and GBS.<sup>86</sup> This also undermines an eventual role played by ethnic-related factors, such as Human Leukocyte Antigen alleles, in post-ZIKV GBS.<sup>78</sup> However, direct and/or indirect pathogenic roles for ZIKV must be proved. In this regard, animal models may be helpful, but so far no study has evaluated peripheral nerves lesion or dysfunction following ZIKV. It is also tempting to speculate whether ZIKV can be implicated in other autoimmune neurological diseases, as occurred with dengue virus.<sup>87</sup> Mécharles et al<sup>88</sup> reported a case of acute myelitis possibly due to ZIKV infection. In 2016, at the American Academy of Neurology Meeting, Doctor Ferreira<sup>89</sup> reported two Brazilian patients with acute disseminated encephalomyelitis and ZIKV infection, calling great attention to this issue. To prevent unnecessary alarm, sporadic reports must be confirmed by careful epidemiological analysis, especially taking into consideration the concurrent circulation of other arboviruses, such as dengue. To date, there is no evidence of an increased rate of acute disseminated encephalomyelitis or related autoimmune conditions in regions facing ZIKV outbreak.

**Table 3** Unanswered questions regarding ZIKV infection and neuropsychiatric complications

What are the mechanisms underlying ZIKV-induced neuronal damage?  
 What is the role of the immune response against ZIKV in the pathogenesis of neurological complications?  
 What are the risk and protective factors related to the development of neurological complications after ZIKV infection?  
 Are there differences in ZIKV infection during the first trimester of pregnancy according to maternal immune response and/or viral load?  
 Is there any neurological and/or psychiatric consequence for the fetus of ZIKV infection in the second half of pregnancy?  
 Is the ZIKV infection associated with other immune-mediated neurological diseases?

**Abbreviation:** ZIKV, Zika virus.

## Conclusion

There is a clear temporal association between the increased reporting of cases of microcephaly and of GBS and the ZIKV outbreak. However, there are several unanswered questions that must be investigated (Table 3). For instance, studies addressing the mechanisms underlying ZIKV-induced neuronal damage are warranted. Reliable diagnostic criteria for the identification of cases of microcephaly are still required. In addition, ongoing longitudinal cohort studies can show the long-term neurological and psychiatric consequences of congenital and after-birth ZIKV infection. From a personal perspective, as neither pharmacological treatment nor vaccines are available, prevention of ZIKV infection relies on protective measures against mosquito bites.

Further studies on ZIKV infection are urgently needed and may help the development of therapeutic strategies against ZIKV and other arboviruses, such as dengue and chikungunya.

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## Disclosure

The authors report no conflicts of interest in this work.

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