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Immuno-virological, clinical and imaging determinants of congenital Zika virus infection

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Immuno-virological, Clinical and Imaging Determinants of Congenital Zika Virus Infection

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Abstract

Zika virus is an emergent pathogen that has been associated with severe neurological anomalies in newborns during the 2015-16 pandemic. In a prospective cohort study in French Guiana, we aim to estimate the rates of maternal-fetal transmission and subsequent fetal, neonatal and infantile adverse outcomes; as well as placental and maternal risk factors for congenital Zika infections.

Among 291 fetuses from Zika-infected mothers enrolled in French Guiana, 26% (76/291) had a confirmed congenital infection at birth (positive IgM or RT-PCR in placenta or fetal samples). Infected fetuses presented a higher risk of severe adverse outcomes (fetal loss or severe neurological anomalies) when compared to those who tested negative at birth: 14% (11/76) and 21% (16/76) versus 0.5% (1/215) and 5% (10/215), respectively: RR 6.9 [95%CI 3.6-13.3]. Placentomegaly was observed more frequently and earlier in case of congenital infection (39.5%, 30/76) compared to placentas from non-infected fetuses (17.2%, 37/215) or controls (7.2%, 24/334): aHR 2.02 [95%CI 1.22-3.36] and aHR 3.23 [95%CI 1.86-5.61], respectively. Adverse perinatal outcomes and congenital infections were more common in fetuses from mothers with a prolonged Zika viremia (>30 days post-infection), compared to those from infected mothers without prolonged viremia or controls: 40% and 60% versus 5.3% and 26.3% versus 6.6% and 0%, aRR 7.2 [95%CI 0.9-57.6] and aRR 6.7 [95%CI 3.0-15.1]) for adverse outcomes, and aRR 2.3 [95%CI 0.9-5.5] for congenital infections. Infected neonates presented higher risks of infantile adverse outcomes (neurologic impairments, neurosensory alterations and / or delay in motor acquisitions) and suspected delay in neurodevelopment: 40% (6/15) vs 5% (5/96) aRR 7.5 [95%CI 2.6-21.4]; and 64% (7/11) vs 14% (7/51), aRR 4.3 [1.8-9.9], respectively.

Le virus Zika est un pathogène émergent qui a été associé à de graves anomalies neurologiques chez les nouveau-nés au cours de la pandémie de 2015-16. Dans une étude de cohorte prospective en Guyane française, nous avons cherché à estimer le taux de transmission materno-fœtale et les conséquences néfastes durant les périodes fœtale, néonatale et la petite enfance; ainsi que les facteurs de risque placentaire et maternel de ces infections congénitales à virus Zika.

En Guyane, parmi les 291 fœtus de mères infectées recrutées, 26% (76/291) avaient une infection congénitale confirmée à la naissance (IgM ou RT-PCR positive dans des échantillons placentaires ou foetaux). Les fœtus infectés présentaient un risque plus élevé d'issues défavorables (perte fœtale ou anomalies neurologiques graves) comparé aux non-infectés: 14 % (11/76) et 21 % (16/76) contre 0,5 % (1/215) et 5 % (10/215), respectivement : RR 6,9 [95%CI 3,6-13,3]. Des placentomégalies ont été observées plus fréquemment et plus tôt en cas d'infection congénitale (39,5 %, 30/76) comparé aux placentas de fœtus non-infectés (17,2 %, 37/215) ou aux témoins (7,2 %, 24/334) : aHR 2,02 [95 % IC 1,22-3,36] et aHR 3,23 [95 % IC 1,86-5,61], respectivement. Les issues fœtales défavorables et les infections congénitales étaient plus fréquentes chez les fœtus de mères présentant une virémie Zika prolongée (>30 jours), par rapport à ceux de mères infectées sans virémie prolongée ou aux témoins: 40% et 60% contre 5,3% et 26,3% contre 6,6% et 0%, aRR 7,2 [95%CI 0,9-57,6] et aRR 6,7 [95%CI 3,0-15,1]) pour les issues défavorables, et aRR 2,3 [95%CI 0,9-5,5] pour les infections congénitales. Les nouveau-nés infectés présentaient des risques plus élevés d'issue infantile défavorable (troubles neurologiques, altérations neurosensorielles et/ou retard des acquisitions motrices) et de retard neuro-développemental : 40 % (6/15) contre 5 % (5/96), aRR 7,5 [95 % IC 2,6-21,4] ; et 64% (7/11) contre 14% (7/51), aRR 4,3 [1,8-9,9], respectivement.

I. Introduction

1. Characteristics and epidemiology of Zika virus

Zika virus (ZIKV) is a single-stranded RNA virus belonging to the *Flavivirus* genus in the *Flaviviridae* family[1], similarly to dengue (DENV), West Nile and yellow fever viruses. This large family is part of the arboviruses group, (i.e. viruses transmitted through arthropods). ZIKV comprises a single polyprotein cleaved into three structural proteins (capsid, precursor membrane and envelope) and seven non-structural proteins (NS1, N2A, N2B, N3, N4A, N4B and N5), non-structural proteins playing a role in viral replication and modulation of the cell antiviral response[2,3]. ZIKV was isolated for the first time in 1947 in Africa, and has circulated for several decades in sub-Saharan Africa and South East Asia with only sporadic transmission to humans. Its emergence in the Pacific, in 2007, was associated with the first outbreak that occurred in Yap Islands, which was followed by the second large epidemic in French Polynesia in October 2013. From there, it spread through the Pacific, and the Americas[4,5], where it caused the massive 2015/2016 epidemic, during which the severe neurological neonatal complications were brought to light[6]. ZIKV circulation declined from late 2016 but it is still circulating in late 2018, as exemplified by the first outbreaks reported in India[7-10].

ZIKV isolates have been clustered into the ancestral African lineage and the emerging Asian lineage[11]. The strains that emerged in the Pacific and subsequently spread in the Americas are from the Asian lineage. The increase in virulence and epidemicity observed since ZIKV emerged in French Polynesia in 2013, is potentially associated with viral mutations. Indeed, a first mutation on NS1 (A118V) has been observed from 2007 in Micronesia, and could be related to an increase of its epidemicity. The S139N mutation affecting the prM protein has been observed in the strains sequenced in French Polynesia and in the Americas, and could contribute to its neurologic virulence[12] (Figure 1).

This does not mean, however, that strains without these mutations cannot cause severe complications [12-14]. *In vitro* and animal experiments showed that the ZIKV African lineage could infect human and mouse neuronal stem cells and is at least as efficient as the Asian strains implicated in the recent epidemics [15-17].

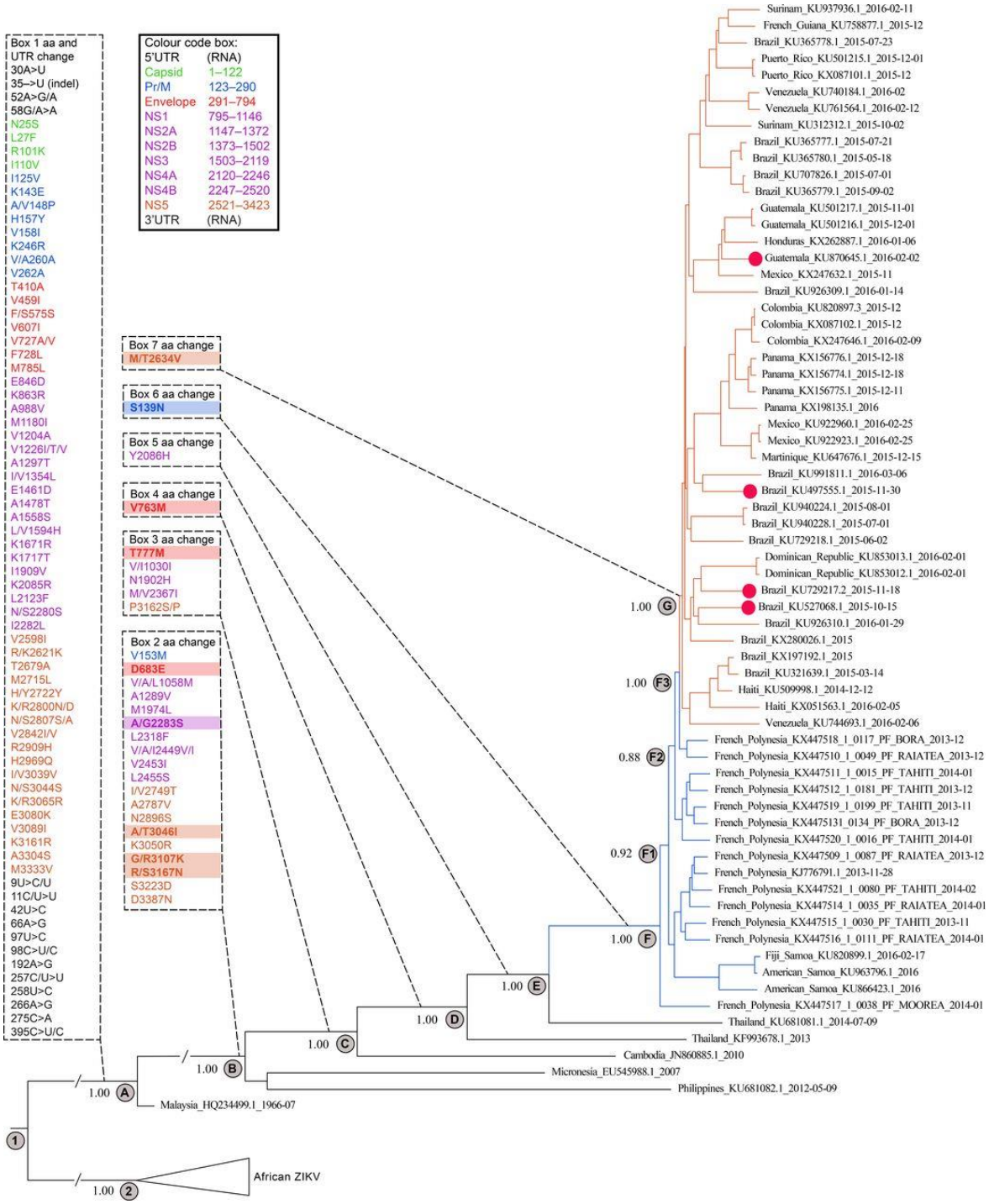

Figure1: ZIKV Phylogeny of African, Asian, Pacific and American isolates.
From "How Did Zika Virus Emerge in the Pacific Islands and Latin America?" by Pettersson et al., mBio, 2016[18,19]

Economic growth in tropical developing countries was a major driver for unprecedented and unplanned urban growth, which provided the ideal ecological conditions to increase the *Aedes* mosquito population, the mosquito vector of both ZIKV and other arboviruses such as DENV or chikungunya virus[6]. This, combined with ineffective mosquito control and increased travel exchanges of humans and goods, provided the ideal mechanism for the emergence and spread of vector-borne transmitted diseases[20]. Moreover, previous immunity against another *Flavivirus* may be a co-factor for clinically more severe ZIKV infections through a phenomenon called Antibody Dependent Enhancement (ADE), but this has not been demonstrated[21].

The risk of a new epidemic exists in all areas where *Aedes* competent mosquitoes are endemic and where the population is non-immune (although it is currently unclear whether immunity is protective or might induce ADE)[22]. ZIKV was first limited to enzootic circulation between non-human primates and sylvatic Aedes mosquitoes, before it gained the capacity to be transmitted by human adapted Aedes spp mosquitoes[23]. Since ZIKV has proven to adapt rapidly to new hosts and vectors, however, it is possible that ZIKV may emerge or reemerge in other settings than those mentioned above.

2. Transmission

Like other flaviviruses, ZIKV is mainly transmitted by infected mosquitoes. The main vectors of ZIKV are *Aedes* spp. mosquitoes, principally *Aedes aegypti*, which is highly prevalent in tropical and subtropical areas, particularly in an urban setting. Other mosquitoes have proven to be competent vectors for ZIKV, in particular *Aedes albopictus*, but to date there are no reports of ZIKV outbreaks driven by *Ae. albopictus*. Sexual transmission of ZIKV has been described, which is unique amongst flaviviruses[24]. Transmission of ZIKV via blood transfusion is also

possible. In endemic areas, the proportion of infection due to each route of infection is, understandably, not possible to evaluate due to continuous exposure to mosquito bites.

The first reported perinatal transmission, likely occurring during delivery, was described in French Polynesia by Besnard *et al*.[25]. Subsequently, Oliveira Melo *et al*. isolated ZIKV in the amniotic fluid of two fetuses with significant cerebral malformations, confirming transplacental transmission[26]. The link between ZIKV and congenital abnormalities has subsequently been confirmed [27-29]. Transplacental transmission is also described for other arboviruses, but ZIKV and Venezuelan equine encephalitis virus remain the only arboviruses associated with congenital CNS malformations[30].

ZIKV has been isolated in human milk, but milk-borne transmission has not been confirmed[31].

3. ZIKV infection in pregnant women

Prevalence. The cumulative risk of ZIKV infection for pregnant women living in epidemic areas was reported to be 21% to 44% in cohorts from Colombia, Puerto Rico and French Guiana[28,32,33], but depended mainly on local incidence of ZIKV, which ranged between 1% in Brazil after the first epidemic wave and 75% in Yap Island during the outbreak in 2007[34,35]. Risk of infection for travelers was estimated to be 1.3% during the worldwide epidemic[36], depending on the areas visited, home conditions and use of mosquito repellants, but has significantly dropped since the decline in circulation of ZIKV.

Symptoms and complications. Pregnant women were symptomatic in 17-56% of cases of ZIKV infection[21,28,37,38]. Symptom onset may appear from the second day after infection and may last up to two weeks. Symptomatic infections are characterized by a maculopapular rash, mild fever, asthenia, pruritus, arthralgia, retro-orbital cephaleas, myalgia, conjunctivitis or conjunctival hyperemia, and/or edema of the extremities[39-41]. Rare severe neurological

complications might occur, particularly Guillain-Barré syndrome which may be a life-threatening condition in pregnant women (prevalence of 0.02 to 1.23% in general population)[42-44]. However, pregnancy is not associated with more frequent or more severe maternal complications[37]. The presence and the severity of maternal symptoms are not associated with a higher risk of birth defects or fetal loss[45,46]. Although a persistent maternal viremia was initially suspected to be associated with a higher risk of birth defects, there is still a lack of evidence to confirm this hypothesis[28,46,47].

Diagnosis. Since most ZIKV-infected pregnant women are asymptomatic and symptoms are non-specific[37], a biological confirmation of the infection is required. According to the United States Centers for Disease Control and Prevention (CDC) guidelines, ZIKV testing is recommended for every symptomatic pregnant women with possible ZIKV exposure, for asymptomatic pregnant women with ongoing possible ZIKV exposure and for ZIKV-exposed pregnant women whose fetus presents with prenatal US findings consistent with congenital ZIKV infection. ZIKV testing may also be considered for asymptomatic pregnant women with recent possible but not ongoing exposure to ZIKV (i.e. travelers)[48]. However, since birth defects were described in asymptomatic ZIKV-infected pregnant woman returning from endemic areas[49], ZIKV testing should be offered to all pregnant women possibly exposed to ZIKV. If symptoms compatible with ZIKV infection are identified, nucleic acid test (NAT) or ZIKV RNA amplification by reverse transcription polymerase chain reaction (RT-PCR) should be performed in serum/blood and urine as soon as possible and up to 12 weeks after symptom onset, according to CDC guidelines. ZIKV can be detected in blood most often within the first weeks after symptoms onset. In urine, the window of detection is increased up to 2-3 weeks after infection. A positive NAT or RT-PCR in any body fluid confirms the diagnosis. Nevertheless, as false positive results have been described, the CDC recommends positive

NAT, to be confirmed by a second set of testing, an approach that may not always be possible during an active epidemic, due to limited laboratory capabilities. A negative result does not exclude ZIKV infection, due to the transient presence of the virus in infected patients. CDC recommends performing NAT three times during pregnancy for asymptomatic pregnant women with ongoing exposure to ZIKV. However, due to differences in serological and virological assays available, particularly in developing and low-income countries, testing guidelines may differ from country to country and ZIKV-serology may also be considered for women living in an area of active ZIKV transmission[48].

Serologic evaluation for ZIKV infection includes an initial screening by enzyme-linked immunosorbent assay (ELISA) to detect specific class M immunoglobulins (IgM) against ZIKV followed by confirmation testing by plaque reduction neutralization test (PRNT) due to cross-reactivity with other flaviviruses[50]. Indirect immunofluorescence and ELISA are both adequate approaches for detecting ZIKV-IgG, but must be considered jointly with other laboratory results, particularly PRNT, due to low specificity of IgG. The sensitivity and negative predictive value of the serological results are controversial, since some patients remain serologically negative despite proven infection (RNA amplification), and false positive results due to cross reaction are possible even using PRNT[51]. For women with ongoing exposure, serological assays are not appropriate to the determination of timing of the acute infection in relation to the beginning of the pregnancy. Nevertheless, serological diagnosis based on IgM remains a reliable tool, particularly in women without co/previous exposure to arboviruses[52].

A recent update in the WHO classification for ZIKV cases defines a suspected case as a person presenting with a rash and/or fever and at least one of arthralgia, arthritis or conjunctivitis. Probable cases are defined as presence of specific IgM antibodies against ZIKV with an epidemiological link. Confirmed cases are defined as detection of ZIKV RNA or antigen in any

body fluid or presence of IgM antibodies against ZIKV plus PRNT for ZIKV with a titer ≥20 and titer ratio ≥ 4 compared to other flaviviruses[53].

4. Congenital ZIKV infection

The concern on congenital ZIKV infections started after the temporal association between an increase in the rash reported by pregnant women in Brazil and an epidemic of neonatal microcephaly 8-9 months later[54,55]. Then, it has been shown that ZIKV can infect the human placenta, leading to chronic placentitis with villous inflammation (histiocytic-predominant villitis)[56]. Hyperplasia in villous Hofbauer cells, stromal lymphocytic cells and histiocytes in the intervillous spaces are reported[56,57]. After having infected the placenta, ZIKV might be using the Hoffbauer cells and their migratory ability to reach the fetal vessels and contaminate the fetus[56,58] (Figure 2). However, type III interferons produced by the trophoblast could confer protection against congenital ZIKV infection[59].

After a maternal-fetal transmission, several reports demonstrated that ZIKV envelope protein and ZIKV RNA were present in brain tissues from newborns with microcephaly[60-62]. However, the presence of the viral envelope protein was not observed in other organs, confirming that the brain was the main target organ for viral replication in the fetus[56,62]. Tang *et al*. highlighted that ZIKV could infect human cortical neural progenitor cells (hNPCs), producing infectious ZIKV particles and dysregulating the cell cycle and transcription of hNPCs[63].

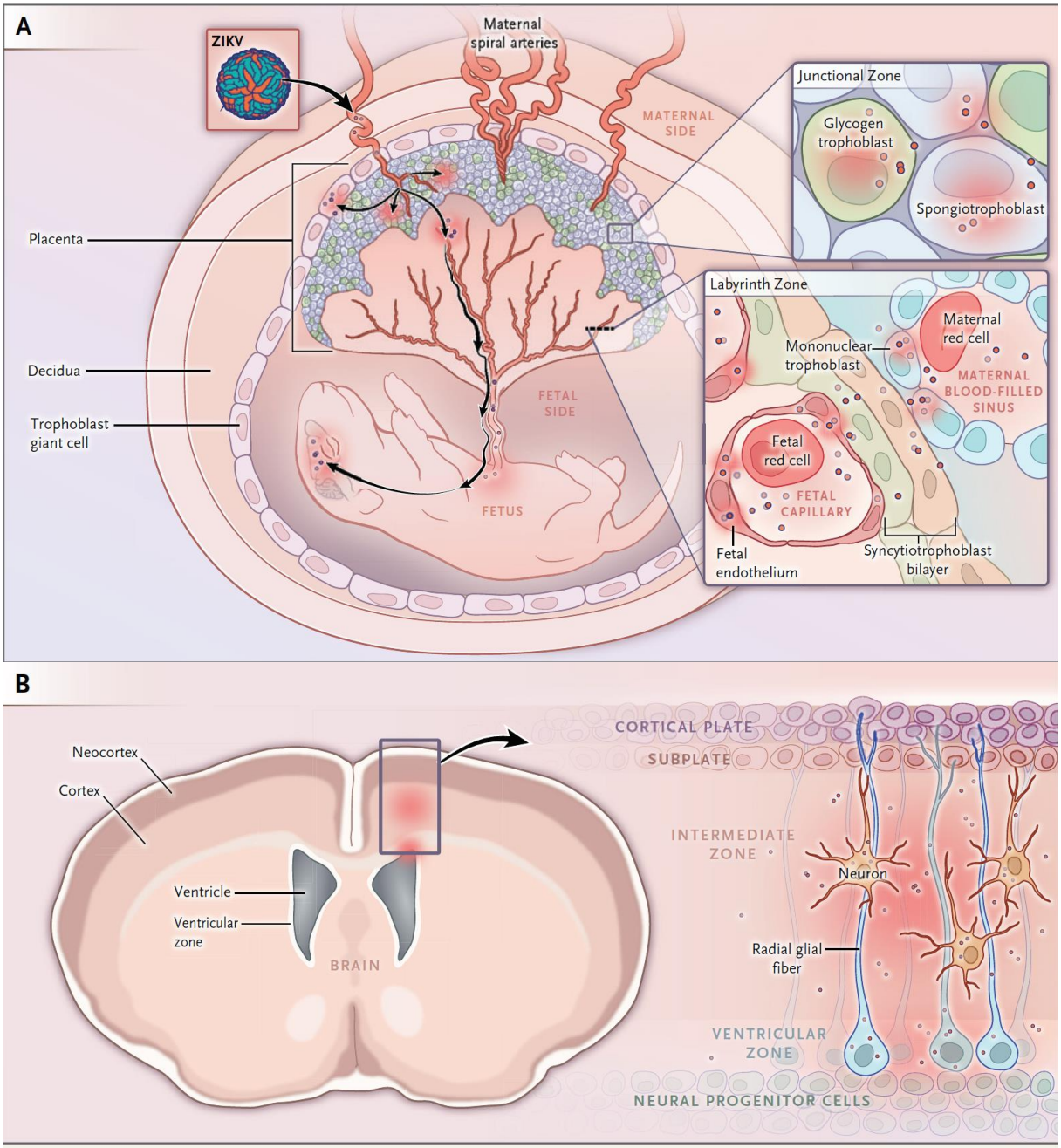

Figure 1. Cellular Sites of Zika Virus (ZIKV) Infection in Pregnancy.
The mouse placenta comprises both maternal- and fetal-derived components, including the junctional and labyrinth zones (Panel A). ZIKV can infect placental trophoblasts including the trophoblast giant cells, glycogen trophoblasts, and spongiotrophoblasts in the junctional zone and the mononuclear trophoblasts, maternal sinusoids, and fetal endothelial cells that line fetal capillaries in the labyrinth zone (insets). ZIKV infects the mouse fetal brain with tropism for cells of the cerebral cortex and ventricular zone, including cortical neural progenitor cells and radial glial cells (Panel B). ZIKV infection leads to reduced cortical layers and ventricular cavity owing to increased cell death in cortical neural progenitors and their processes, especially in the intermediate and ventricular zones (inset).

Figure 2: Cellular sites and mechanisms for congenital ZIKV infections in a mouse model.
From "Modeing Zika Virus Infection in Pregnancy" by Mysorekar et al., NEJM, 2016[64]

In the malformative pattern of congenital ZIKV infections, microcephaly seems to be just the "tip of the iceberg" [26,61]. Histologically, microcephaly and ventriculomegaly are associated with

a diffuse severe brain damage with extensive destruction and calcifications mainly at the gray and white matter junction, besides perivascular cuffs of lymphocytes, microglial nodules, and reactive gliosis. Cerebellar hypoplasia and meningeal and parenchymal inflammation varied in intensity and distribution, with predilection for a periventricular and perivascular distribution [65]. In neonates presenting arthrogryposis at birth, various degrees of destructive, calcification, hypoplasia and migration disturbances were described[66].

The spectrum of lesions can be summarized as follow[67]:

- Disturbances of migration in cerebral, cerebellar hemispheres, and brainstem represented by abnormal clusters or bands of germinal matrix towards the cortex, meningeal glioneuronal heterotopias, polymicrogyria, and cortical dysplasia, in those who were infected earlier in pregnancy.
- Destructive lesions with nerve cell degeneration; apoptosis; coarse and filamentous calcifications in the hemispheres, basal ganglia, thalami, brainstem, and spinal cord; and spinal motor nerve cell loss.
- Hypoplastic lesions defined by lack of descending fibers leading to small basis pontis, pyramids, and spinal corticospinal tracts.
- Inflammation is mild with predominance of CD8+ T lymphocytes and no active necrotizing lesions.

The histology of ZIKV-infected brains is concordant with radiological and ultrasonographic findings in congenital ZIKV infections[68,69]. Although still controversial, the CDC defines Congenital Zika Syndrome (CZS) as a proven *in utero* ZIKV infection associated with: severe microcephaly in which the skull has partially collapsed, decreased brain tissue with a specific pattern of brain damage (including subcortical calcifications), damage to the back of the eye

(including macular scarring and focal pigmentary retinal mottling), congenital contractures (clubfoot or arthrogryposis), hypertonia or restricted body movement soon after birth[70,71].

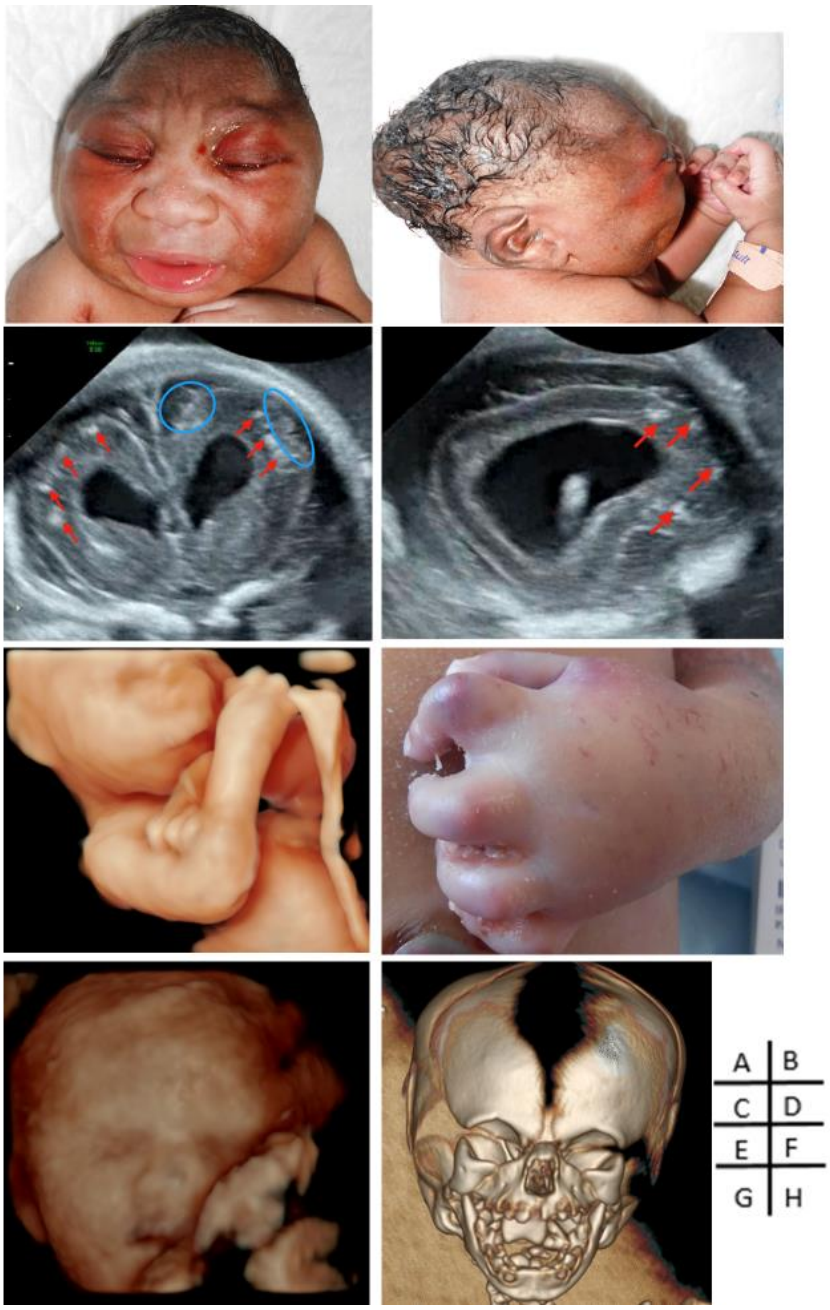

Figure 3: Clinical and imaging features of CZS
Microcephaly at birth, with an occipital horn and an excess of skin between the occiput and the neck (a and b); multiple ponctiform calcifications and focal polymicrogyria (c, prenatal US); ventriculomegaly with irregular ventricle border, septa and calcifications (d, prenatal US); arthrogryposis (e, prenatal US; d, at birth); severe bilateral microphtalmy (e, prenatal US; f, prenatal CT).
Adapted from "The Zika virus epidemic in French Guiana" by Lambert, Pomar and Malinger, Ultrasound Obstet Gynecol, 2017[72]

A recent *in vivo* study on non-immune pregnant primates infected by ZIKV demonstrated an abnormal oxygen transport within the placenta, which could explain other adverse perinatal outcomes in congenital ZIKV infections (intra-uterine growth restriction and fetal losses)[60,62,73].

In an early report on the ZIKV epidemic in French Guiana, we reported a higher rate of neurological anomalies observed on prenatal US in ZIKV-infected pregnant women compared to controls (9% vs 4%), and first results of ZIKV serology performed on umbilical cord blood documented a congenital ZIKV-infection in 11% of fetuses/newborns form ZIKV-infected pregnant women [28]. However, the burden of the disease in fetuses and newborns, as well as the rates of maternal-fetal transmission, congenital Zika syndrome and other adverse outcomes remain unclear. Moreover, maternal, fetal and placental determinants for trans-placental contamination and subsequent adverse effects are not yet described.

II. Objectives

Globally, this research thesis had three main objectives:

1. Define congenital ZIKV infection and estimate the rate of maternal-fetal infection by searching ZIKV RNA or specific ZIKV IGM in placenta and fetal samples. To do so, we performed a literature review to evaluate the different guidelines defining congenital ZIKV infections at birth, and to identify clinical, imaging and biological features associated with CZS (Paper 1). We also followed a cohort of infected pregnant women in French Guiana and performed a systematic testing of their fetuses/newborns by RT-PCR and/or ELISA in placenta, urine, blood, amniotic fluid and/or cerebrospinal fluid, to define the rates of maternal-fetal transmission and subsequent adverse perinatal outcomes (Paper 2).

2. Define specific determinants for congenital ZIKV infection, congenital Zika syndrome and other adverse outcomes, by highlighting placental and maternal risk factors (Papers 3 & 4).

3. Define the consequences during infancy and childhood of congenital ZIKV infections. In addition to the cohort of infected pregnant women, we enrolled their neonates in a pediatric cohort (Paper 5).

III. Summary of the results

1. Congenital Zika infection and adverse perinatal outcomes

Paper 1:

Zika virus during pregnancy: From maternal exposure to congenital Zika virus syndrome

Prenatal Diagnosis, 2019, https://doi.org/10.1002/pd.5446

Léo Pomar , Didier Musso, Gustavo Malinger, Manon Vouga, Alice Panchaud, David Baud

In this review, we described the risks and complications of maternal and subsequent fetal infection by ZIKV, and we provided an extended definition of clinical, imaging and biological features associated with Congenital Zika Syndrome (CZS). Among infected pregnant women, the risk of any adverse fetal/neonatal outcome was estimated at 5% to 42%, with 1% to 4% of fetal loss and 4% to 9% of CZS. Findings associated with CZS were microcephaly (33%-64%), ventriculomegaly (63%-92%), calcifications (71%-92%), malformations of cortical development (79%-82%), anomalies of the corpus callosum (71%-100%) and of the posterior fossa (21%-82%), arthrogryposis (10%-25%), eye abnormalities (25%), and extra-neurologic signs such as intra uterine growth restriction (14%), placentomegaly, transient hepatitis, mild anemia. Infants who presented with CZS at birth suffered from motor abnormalities (77%-100%), epilepsy (9%-54%), hearing loss, and neurologic impairments.

We also present a review of the guidelines for the follow-up of infected pregnant women and their fetuses / neonates.

Author's contribution: LP performed all the literature research, as well as the manuscript drafting. All authors performed a critical review of the manuscript's draft and improved this review in their domain of expertise.

Paper 2:

Maternal-fetal transmission and adverse perinatal outcomes in pregnant women infected with Zika virus: prospective cohort study in French Guiana

BMJ, 2018; doi: https://doi.org/10.1136/bmj.k4431

Léo Pomar, Manon Vouga, Véronique Lambert, Céline Pomar, Najeh Hcini, Anne Jolivet, Guillaume Benoist, Dominique Rousset, Séverine Matheus, Gustavo Malinger, Alice Panchaud, Gabriel Carles, David Baud.

The main objective of this original research was to estimate the rates of maternal-fetal transmission of ZIKV, adverse fetal/neonatal outcomes, and subsequent rates of asymptomatic/symptomatic congenital ZIKV infections. To do so, we recruited a cohort of pregnant women at any stage of pregnancy with a laboratory confirmed symptomatic or asymptomatic ZIKV infection during the epidemic period in western French Guiana. The cohort enrolled 300 participants and prospectively followed their 305 fetuses/newborns.

Maternal-fetal transmission was documented in 26% (76/291) of fetuses/newborns with complete data. Among the ZIKV positive fetuses/newborns, 45% (34/76) presented with no signs/complications at birth, 20% (15/76) with moderate signs potentially related to CZS, 21% (16/76) with severe complications compatible with CZS (major sign or multiple minor signs including brain anomalies), and 14% (11/76) with fetal loss. Compared with the ZIKV positive fetuses/neonates, those that were identified as negative for ZIKV (215/291) were less likely to present with severe complications (5%; 10/215) or fetal loss (0.5%; 1/215; Relative Risk (RR) 6.9, [95%CI 3.6-13.3). Association between congenital ZIKV infection and any adverse fetal/neonatal outcome was also significant (RR 4.4 [95%CI 2.9-6.6]).

When considering a more restrictive definition of congenital ZIKV infection, excluding placental samples from the analysis, maternal-fetal transmission was documented in 18% (52/282) of fetuses / newborns with other samples tested, and the results were similar to those

of the main analysis for “any adverse outcomes” (RR 4.2 [95%CI 2.7-6.0]) and “severe adverse outcomes (RR 5.4 [95%CI 2.8-10.2]).

Author’s contribution: LP, VL, CP, AJ, GC, MV, AP and DB conceived and designed the study. LP, VL, CP, GC, and NH provided care to mothers and prospectively collected the clinical data and samples. LP and CP collected data on neonatal outcomes. DR and SM did all the viral investigations. AP, LP, MV, and DB interpreted the results. LP, MV, and DB wrote the first version of the report, and all authors critically reviewed and approved the final version.

2. Maternal and placental determinants of congenital ZIKV infection

Paper 3:

Placental infection by Zika virus in French Guiana

Ultrasound in Obstetrics and Gynecology, 2019; doi: https://doi.org/10.1002/uog.21936

Léo Pomar, Véronique Lambert, Yoann Madec, Manon Vouga, Céline Pomar, Séverine Matheus, Arnaud Fontanet, Alice Panchaud, Gabriel Carles, David Baud

The main objective of this original research was to correlate placental thickness during pregnancy and histopathological results with trans-placental ZIKV infection.

Among 291 fetuses/placentas from proven infected mothers, trans-placental infection was confirmed in 76 cases, of which 16 resulted in Congenital Zika Syndrome (CZS) and 11 in fetal loss. The 215 remaining placentas without evidence of ZIKV infection represented the exposed group. A total of 334 placentas from ZIKV-negative pregnant women represented the non-exposed group.

Placentomegaly (thickness>40 mm) was observed more frequently in infected placentas (30/76, 39.5%) compared to exposed placentas (37/215, 17.2%) or controls (24/334, 7.2%), even when considering gestational age at diagnosis and co-morbidities (adjusted Hazard Ratio [aHR] 2.02 [95%CI 1.22-3.36] and aHR 3.23 [95%CI 1.86-5.61], respectively), and appeared earlier in infected placentas. Placentomegaly was observed even more frequently in case of CZS (62.5%) or fetal loss (45.5%) compared to asymptomatic congenital infection (30.6%) (aHR 5.43 [95%CI 2.17-13.56] and aHR 4.95 [95%CI 1.65-14.83], respectively). Umbilical artery Doppler anomaly was observed more frequently in case of trans-placental infection resulting in fetal loss (30.0% vs 6.1%; adjusted Relative Risk [aRR] 4.83 [95%CI 1.09-20.64]). Infected placentas also exhibited a higher risk of any pathological anomalies than exposed placentas (aRR 2.60 [1.40-4.83]).

Author's contribution: LP, YM, AP and DB conceived and designed the study. LP and VL performed the ultrasound assessments and the postnatal / postmortem examination of the placentas. SM did the viral investigations. AP, LP, MV, AF and DB interpreted the results. LP and YM performed the statistical analysis and wrote the first version of the report, and all authors critically reviewed and approved the final version.

Paper 4:

Prolonged maternal Zika viremia as a marker of adverse perinatal outcomes

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In this prospective cohort study, we enrolled ZIKV-infected pregnant women with a positive polymerase chain-reaction at inclusion, and non-infected pregnant women tested by serology in each trimester and at delivery from January to July 2016. Prolonged viremia was defined as ongoing virus detection at least 30 days post infection.

Adverse outcomes (fetal loss or neurologic anomalies) were more common in fetuses and neonates from mothers with prolonged viremia (6/15; 40.0%) compared to those from infected mothers without prolonged viremia (1/19; 5.3%, RR 7.6 [95%CI 1.0-56.5], RR adjusted for maternal comorbidities, trimester of maternal infection and considering twin pregnancies in the variance (aRR) 7.2 [95%CI 0.9-57.6]) or those from non-infected mothers (20/332; 6.6%, RR 6.6 [95%CI 3.1-14.0], aRR 6.7 [95%CI 3.0-15.1]), respectively. Congenital infections were confirmed more often in fetuses and neonates from mothers with prolonged viremia compared to others (60% vs 26.3% vs 0.0%): RR 2.3 [95%CI 1.0-5.4], aRR 2.3 [95%CI 0.9-5.5]. A sensitivity analysis found similar results when considering the evolution of qPCR values between the inclusion and the first follow-up instead of prolonged viremia as a binary variable.

Author's contribution: LP, VL, AP, and DB conceived and designed the study. LP, VL, CP, GC, and NH provided care to mothers and prospectively collected the clinical data and samples. DR and SM did all the viral investigations. LP, AP, MV, and DB interpreted the results, did the literature review, and provided critical inputs to the paper. LP, AP and DB wrote the first version of the report, and all authors critically reviewed and approved the final version.

3. Consequences during infancy and childhood of congenital ZIKV infections

Paper 5:

Association between laboratory confirmed congenital Zika infection at birth and outcomes up to three years of life

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In a cohort of 129 neonates from ZIKV-infected mothers, we compared neonatal, 2-year infantile and 3-year neurodevelopment outcomes among children diagnosed with a congenital Zika infection at birth with those of children tested negative.

Among these neonates, 18 (14%) had laboratory evidences of a congenital ZIKV infection at birth. Infected neonates had a higher risk of adverse neonatal outcomes (death, structural brain anomalies or neurologic symptoms) than those who tested negative at birth: 8/18 (44%) vs 4/111 (4%), aRR 10.1 [3.5-59.0]. In infants still followed at the pediatric clinic of the CHOG at two years of life (n=111), neurologic impairments, neurosensory alterations and / or delay in motor acquisitions were more common in those diagnosed with a congenital ZIKV infection at birth, even when considering only those without structural brain anomalies: 6/15 (40.0%) vs 5/96 (5.2%), aRR 7.5 [95%CI 2.6-21.4]; and 2/10 (20.0%) vs 3/93 (3.2%), aRR 6.0 [1.0-30.9], respectively. Finally, infected children had also a higher risk to be referred for a suspected delay in neurodevelopment (<-2SD) at three years of life, even when considering only those without structural brain anomalies: 7/11 (64%) vs 7/51 (14%), aRR 4.3 [1.8-9.9]; and 3/7 (42.9%) vs 7/49 (14.3%), aRR 2.9 [1.0-8.8], respectively.

Author's contribution: NH and LP conceived and designed the study. NH, YK, ZH, VL, MM, GC and LP provided care to the mothers and their infants, and collected the data. AP, DB and LP interpreted the results. NH and LP wrote the first version of the report and did the literature review. All authors critically reviewed and approved the final version.

IV. Discussion

1. Main results

In this cohort, maternal-fetal transmission seemed to occur in approximately a quarter of exposed fetuses and was associated with early adverse fetal/neonatal outcomes (fetal loss or severe neurological anomalies compatible with CZS) in a third of infected fetuses. Infected fetuses and neonates presented a 7-fold increased risk of adverse outcomes compared to non-infected fetuses. Concerning the placental risk factors for congenital ZIKV infection, early placentomegaly was more frequent in case of vertical transmission, notably in case of CZS or fetal loss. Umbilical Doppler anomaly could also be associated to fetal loss in case of maternal-fetal transmission. Concerning the maternal parameters, we found that a prolonged maternal viremia (>30 days post infection) could be associated with a 7-fold increased risk of fetal/neonatal adverse outcomes, and with a 2-fold increased risk of confirmed congenital infection when compared to pregnancies without prolonged viremia.

When considering consequences of congenital ZIKV infections during infancy and childhood, infected neonates had a 7-fold increased risk to present infantile adverse outcomes (neurologic impairments, neurosensory alterations and / or delay in motor acquisitions) and a 4-fold increased risk to be referred for a suspicion of neurodevelopmental delay at three years of life.

2. Strengths and limitations

The prenatal cohort enrolled all the patients followed at the prenatal diagnosis unit of the CHOG and willing to participate in the study. The CHOG being the only referral center for prenatal diagnosis in western French Guiana and the support of the French Ministry of Health to offer an enhanced prenatal follow-up to ZIKV-exposed pregnant women during the epidemic permitted to limit as much as possible selection biases in this cohort. Apart the geographical

distance, these patients were not restricted in their access to prenatal care, and the few rate of lost to follow-up indicated a large and non-selective inclusion of the population of exposed pregnant women living in western French Guiana in this cohort. Furthermore, after inclusion, a monthly follow-up was planned with possibilities to recall patients who missed their appointment, and organization of delocalized ultrasound monitoring in primary care centers by one of the investigators for patients living in isolated areas, in order to homogenize their follow-up. Prenatal monitoring was carried out by only two experienced sonographers working with a multidisciplinary prenatal diagnosis centre, which guarantees high quality prenatal imaging.

Another strength of this cohort is the standardized testing and follow-up of exposed fetuses and newborns, limiting the risk of misclassification at birth. 95% (291/305) and 82% (334/405) of the placentas / fetuses / newborns from infected and non-infected pregnant women were tested at birth for congenital ZIKV infection, and all of them underwent clinical examination and imaging, according to national and international guidelines.

In the context of French Guiana, infants present an important risk to be lost to follow-up after post-partum discharge, as some of them live in isolated area and are followed in primary care centers. These infants were not included in the cohort to avoid misclassification bias. Thus, the postnatal cohort enrolled only neonates planned at the pediatric clinic of the CHOG for their follow-up. In infants enrolled in the cohort, in case of a missed appointment, the parents were recalled to schedule another evaluation. We did not enroll infants referred to the CHOG for advanced cares who were not included initially in the cohort, to avoid selection biases.

Nonetheless, the studies on the cohort from French Guiana presented several limitations.

First of all, the sensitivity and specificity of IgM to diagnose maternal ZIKV infections remain debatable since cross-reactivity with other arboviruses are described. Serological testing (including DENV and CHIKV) was performed at the French Guiana National Reference Centre

for arboviruses. For Zika virus, we used an in-house MAC-ELISA assay, with a sensitivity, when correlated with PCR results, varying between 87% for serum samples collected between five and 20 days from symptom onset to 98% for those collected after seven days[37]. Specificity varies depending on the presence of co-infections with other arboviruses, reaching 80% in negative patients, but dropping in the case of co-infections. In such cases (n=18), we obtained confirmation with a micro-neutralising assay. Serological cross reactions with other Flaviviridae were expected to be minimal, as circulation of DENV has been low in French Guiana since 2014 and no significant circulation of other Flaviviridae has been seen during the ZIKV epidemic[74].

Although ZIKV testing was based on the previous guidelines relevant during the 2015-2016 epidemic with adaptation to local capacities, it does not follow the more recent CDC guidelines[75] (i.e. patients considered as non-infected underwent serological assessment in each trimester rather than nucleic acid tests, as is currently recommended). Patients included as non-infected, however, remained negative for IgM and IgG in each trimester and at delivery, limiting the risk of exposure misclassification. In the study investigating the impact of maternal prolonged viremia, women with only positive IgM testing (i.e. without RT-PCR) were excluded from this analysis as some may have had undetected prolonged viremia, which would have led to an exposure misclassification as well resulting in an underestimation of the consequences of prolonged viremia.

Information regarding the sensitivity and specificity of placenta, fetal and neonatal testing are limited. In particular, several studies have demonstrated the progressive disappearance of ZIKV RNA in the maternal-fetal compartments[76,77]. In contrast to CMV, which may be detected for several months in urine of congenitally infected newborns, ZIKV RNA was rarely detected in urine samples (7/76; 9.2%). Though the sensitivity of amniocentesis seems to be limited in cases of congenital ZIKV infection[76], it may help to diagnose early foetal infections but was

only performed in 12 cases when prenatal US was suggestive of congenital infection. In that context, we cannot exclude false negative results. Indeed, viral RNA is very susceptible to degradation, which occurs through hydrolysis and ribonucleases activity. Clinical samples are particularly vulnerable to RNA degradation by the action of host nucleases[78]. In the case of diagnosis of vertical transmission of viruses, RNA is much less stable than DNA in placenta and fetal samples and require more steps for detection at the laboratory level. A critical challenge for RNA preservation and detection in these samples is to prevent degradation by the nuclease during the sampling and purification processes[79]. The storage and transportation of clinical samples are also at risk of RNA hydrolysis, which represents a limitation for healthcare settings with a decentralized laboratory[80-82]. Therefore, the absence of detection of an RNA virus does not necessarily mean that the infection of the given tissue is absent. Of note, severe complications potentially compatible with CZS were observed in some newborns without laboratory evidence of ZIKV infection, as well as placentomegalies and anatomo-pathologic anomalies were observed in negative placentas; either we were not able to detect ZIKV in these cases, or other etiologies may have induced similar complications.

Furthermore, we considered placental and umbilical cord samples in the diagnosis of congenital ZIKV infection, which may be questionable due to the risk of maternal contamination of these samples[70]. Nevertheless, the risk of false positive results due to maternal contamination seems low in this cohort. First, ZIKV-status based on umbilical cord blood samples was confirmed at day three of life in all but 4 neonates. Secondly, we detected ZIKV RNA and specific IgM in placental and fetal umbilical cord samples in 7/8 cases with a laboratory confirmed congenital ZIKV infection even when maternal blood and urine were RT-PCR negative. When excluding placental samples from our analysis, maternal-fetal transmission was documented in 18.4% (52/282), of which 32.7% (17/52) suffered from severe complications at birth. Association

between a laboratory confirmation of congenital ZIKV infection and outcomes did not change in our sensitivity analysis.

Our postnatal radiological analysis was based on transfontanellar ultrasound, for which the sensitivity for CNS anomalies is lower compared to MRI or CT-scan for calcifications and skull anomalies. The closest MRI was located 300 km and therefore used only for symptomatic cases or those with abnormal prenatal or postnatal ultrasound. Similarly, CT-scan was not routinely available due the limited resources of our radiological unit. Specialized evaluations by neurologist and infectious diseases specialist specialised in pediatrics are not routinely available in French Guiana, possibly resulting in an underestimation of the proportion of symptomatic congenital infections at birth. We therefore developed a definition of complications compatible with CZS based on specific and non-specific characteristics for congenital ZIKV and TORCH infections observable up to the first week of life[83], adapted to the local medical capacities of our hospital. This classification might be more applicable in low resource setting hospitals, often present in tropical regions, at risk of emergence and re-emergence of ZIKV.

Thirdly, conclusions regarding the impact of the timing of infection on maternal-fetal transmission and subsequent outcomes was difficult to establish since the timing of diagnosis of maternal infection reported here may occur much later than the timing of maternal infection. Thus, association between trimester of infection and outcomes could not be assessed in our studies, and we preferred to present the trimester of maternal infection diagnosis in the exposure characteristics. In the study investigating the impact of maternal prolonged viremia, molecular testing has been proposed at different stages of pregnancy depending on the presence of maternal symptoms or fetal signs. Thus, we cannot exclude that some of these patients were in fact infected earlier in pregnancy and had an undetected prolonged viremia resulting in an exposure misclassification. This bias would result in an underestimation of the consequences of prolonged viremia as the case that presented with neurological anomalies (CZS) in the

reference group from infected mothers without prolonged viremia could in fact be related to prolonged viremia. Similarly, we cannot exclude a potential selection bias, as some patients were tested by RT-PCR because their fetus presented with anomalies at inclusion. As this proportion did not differ between the groups (3/14 vs 4/19), even if we consider only anomalies suggestive of fetal infection at inclusion (2/14 vs 2/19), we did not expect that relative risks may be significantly affected. However, this potential selection bias could overestimate absolute frequencies of fetal anomalies in RT-PCR positive patients, and this bias seem to be inherent in contemporary cohorts as inclusion after the observation of fetal anomalies potentially related to Zika were widely common.

The recruitment of infected pregnant women occurred at the time of their first US performed at the prenatal diagnosis unit, and was therefore not conducive to evaluation of early fetal consequences of maternal ZIKV infection before 12 weeks gestation. The rate of early miscarriages, some of them occurring in none-recognized pregnancies or at home without hospital consultation, was thus difficult to determine. Furthermore, since we excluded pregnant patients for whom the diagnosis of ZIKV infection was done at delivery, because of the lack of specific follow-up during pregnancy and early postnatal life, our results were not able to provide information on the consequences of a late infection in pregnancy.

The postnatal cohort might suffer from similar biases, but is mainly impacted by the important proportion of loss to follow-up decreasing the sample size from 129 to 111 after 2 years and to 62 after 3 years and introducing a potential selection bias. This proportion was the same between the two groups (≈15% after 2 years and 40% after 3 years) which suggest that a potential selection bias on the outcome has been undifferentiated between the two groups. Yet, it is difficult to know if the loss to follow up has selected the more severe cases or not. One would argue that the lack of clinical concern by parents, particularly in asymptomatic cases,

might have driven the loss to follow-up. This would have overestimated the absolute risks of infantile adverse outcomes and the suspicion of neurodevelopment delay in the cohort. Thus, absolute risk in this study should be considered carefully.

Another source of potential selection bias is linked to practical limitations to perform a follow-up at the CHOG for newborns from mothers living along the Maroni river or in isolated areas in Suriname. Thus, among the newborns born at CHOG, only those followed at the pediatric clinic of the CHOG were enrolled resulting in the exclusion of 50% of the newborns born at CHOG from ZIKV-infected mothers and leading to the initial inclusion of only 129 infants. This might have selected infants stemming from family with a higher socio-economic status. This would have led to a possible underestimation of the absolute risk but again it is not expected that this selection bias has been differentiated between both groups.

The last limitation was that a control group of children born from uninfected mothers who underwent neurodevelopmental testing using the CDAS was not available. In the general population, a normal distribution of neurodevelopmental scores would be expected when using a standardized tool such as the CDAS, but this score has never been used in French Guiana and cognitive scales in particular may include items that could be influenced by the cultural context. In a cross-sectional study evaluating the neurodevelopment of Polynesian infants born during the ZIKV outbreak versus a control group of Canadian infants, Subissi and colleagues described that confounding factors such as socioeconomic status and cultural factors may play an important role in infantile neurodevelopment[84].

3. Interpretation

Maternal-fetal transmission of ZIKV and adverse outcomes: Our study provided the first estimation of the rate of maternal-fetal transmission of ZIKV. These results are congruent with a study performed in Brazil on 54 pregnant women with RT-PCR confirmed ZIKV infections,

where vertical transmission was documented in 18/51 (35.3%) newborns tested, while 15 newborns (27.8%) exhibited mild/moderate signs[85]. These included isolated ultrasound anomalies such as lenticulostriate vasculopathy or subependymal cysts, abnormal otoacoustic emissions, chorioretinitis and intra-uterine growth restriction; severe anomalies were not described. The broader number of patients included in our cohort may have enabled the detection of more uncommon severe anomalies and provided a better estimation of maternal-fetal transmission. Shortly after, the cohort from New-York City reported positive IgM in 7% of the neonates from mothers with a probable or confirmed ZIKV infection[86]. A recent Brazilian cohort suspected congenital infections in 65% of the infants followed until their first year of life[87]. In this cohort, infants were tested up to their first year of life whereas testing was only completed at birth in the other cohorts. 28% of them were tested after their 3 months of life, and positive results were considered as congenital infections whereas these infants could have been infected in the post-natal period. Another concern is the possibility of a selection bias: 12.4% of the infants tested had structural brain anomalies and 6.1% had microcephaly, whereas none of the untested infants had these outcomes. The number of samples, the diversity of assays used and, most importantly the age at testing seem to have greatly contributed to the differences observed between these studies and ours.

The rates of severe fetal/neonatal anomalies (8.9%) and pregnancy losses (4.1%) in ZIKV-infected pregnant women in French Guiana were similar to those reported in non-endemic countries; the US Zika Pregnancy and Infant registry described rates of 5% for severe anomalies and 3% for pregnancy loss in exposed pregnancies[88]. Our results were concordant with the Zika-DFA study performed in a similar population, in which neurologic defects and fetal losses were reported in 7% and 1.1% of 555 exposed fetuses, respectively[40]. This highlights that the

majority of exposed fetuses (>90%) seem to remain asymptomatic or pauci-symptomatic at birth, even in the case of a confirmed fetal infection.

Placental infection with ZIKV: Placentomegaly identified via US within few weeks after infection may be the consequence of placental inflammation due to recent maternal and trans-placental infection[56]. The higher rate of low birthweight/placental weight ratio we observed in case of congenital infection was likely related to the additional impact associated with placentomegaly, which reflected the persistent enlargement of placentas. Placental enlargement could result from fibrinoid deposition and small vascularized villi that form to compensate for *in utero* hypoxia, recently described in congenital ZIKV infections[73] and other congenital infections[89]. The relatively low rate of a low birthweight/placental weight ratio compared to the rate of placentomegaly could also indicate that some of the instances of placentomegaly observed are transient and do not affect placental volume at birth. These transient placentomegalies may be associated with other co-morbidities and natural growth of the placenta, which may be why placentomegaly was identified in the third trimester in the exposed and control groups, and is thus unrelated to ZIKV. Abnormal umbilical Doppler measurements tended to occur more frequently in infected placentas but less so than placentomegaly. These results may indicate that placental infection resulting in placentomegaly does not always lead to placental dysfunction. However, an abnormal umbilical Doppler associated with placentomegaly in infected placentas may predict an acute risk of fetal loss (even if the fetus does not present with growth restriction at diagnosis)[58,90].

The sensitivity of placentomegaly to predict congenital ZIKV infection (39.5%) is debatable but is similar for other congenital infections [89]. The sensitivity, however is increased when predicting CZS or fetal loss. The NPV >90% for congenital infection and >98% for adverse outcomes of placenta thickness <40 mm may help to reassure pregnant patients in poor resource

areas where molecular and serological testing are not available during an epidemic peak. Moreover, placental thickness measurement by ultrasound is easy to perform and does not require advanced expertise in comparison to neurosonograms.

Placental inflammation consists of diffuse vascular inflammation, chorioamnionitis lesions, villitis and calcifications, such as described in cCMV infection[91]. ZIKV showed its capacity to specifically infect human placental macrophages and trophoblasts[92], but placental inflammation was inconsistently described[93-95] and could only occur in early stages of congenital infections [57]. Our study highlighted a higher rate of ischemic necrosis with fibrin deposits (INFD) in ZIKV-infected placentas, which could increase placental growth, such as in Syphilis congenital infections[96]. Subchorionic thrombosis and congestive capillaries observed more often in infected placentas have been previously described in congenital ZIKV infections, and could contribute to fetal hypoxia[66]. We showed Hofbauer cell hyperplasia and leukocytic infiltration in ZIKV-infected placentas, as described by others as a specific finding in ZIKV-infected placentas[58,94,97]. We cannot exclude that ZIKV infection may be restricted to the placenta with no further involvement of the fetus, as described in some CMV infections[98]. However, placentomegaly may represent an early sign of congenital infection, before congenital Zika virus syndrome or other adverse outcomes become apparent[99].

Prolonged maternal viremia: Driggers *et al.* were the first to highlight a possible association between prolonged maternal viremia and congenital infection with CZS[47]. In their cohort study, Rodo *et al*. described nine cases of prolonged maternal viremia, among which two resulted in congenital ZIKV infection and one of these exhibited severe neurological anomalies[100]. The rates of congenital infection and fetal/neonatal adverse outcomes in women with prolonged viremia seem to be higher in our study (9/15 for congenital infections, and 4/15 for CZS). This difference may be explained by the exclusive use of amniocentesis for diagnosis in the Rodo *et*

al. cohort, whereas multiple fetal/neonatal samples were tested in our study. Our results are congruent with Meaney *et al.* who identified prolonged ZIKV RNA detection in four symptomatic pregnant women in the U.S. Zika Pregnancy Registry, of which one pregnancy (25%) resulted in congenital Zika syndrome[101,102]. In their report, Suy *et al*. described a case of CZS with prolonged maternal viremia where the viral load in the maternal serum remained stable for 14 weeks and then became negative, instead of decreasing progressively, as would be expected[103]. They suggested that the prolonged viremia that was detected in the mother could be the result of viral replication in the fetus or placenta, which thus "acts as a reservoir". However, it still lacks a consensual threshold to define "prolonged viremia". In our study, we define prolonged viremia as ongoing viral detection at least 30 days after symptom onset or after initial detection of viremia for a question of feasibility. Indeed, many of our patients were living around the Maroni river, in isolated areas, and came monthly to the CHOG for their clinical follow-up. In this context of geographical distance, we decided that monthly RT-PCR in case of initial detection of viremia was the most appropriate. In the context of a smaller area with local facilities, it would have been interesting to test patients every two weeks to fulfill the threshold used in other studies [100,102].

Negative and positive predictive values of prolonged maternal viremia for congenital infections and adverse outcomes related to ZIKV seem to be moderate as fetal/neonatal adverse outcomes and congenital infections also occur in pregnant women without identified prolonged viremia. One explanation could be that prolonged viremia might reflect viral replication in the placenta without further involvement for the fetus[104]. In addition, some of our cases with prolonged maternal viremia (6/15) did not exhibit congenital infections, suggesting that prolonged maternal viremia might also reflect persistent viral replication in other reservoirs than the fetus or the placenta. The study of Rodo *et al*. and the CDC report also described fetuses without congenital infection or adverse outcomes from mothers with prolonged viremia[100,102].

Consequences of congenital ZIKV infections during infancy and childhood: Brain structural malformations and ocular anomalies associated with congenital Zika infection have been well described worldwide, particularly in congenital Zika Syndrome[40,71,105-109]. Infancy outcomes and developmental outcomes of infants exposed to ZIKV in utero has been studied less extensively and often with no stratification by infant status towards ZIKV infection at birth. The study by Nielsen-Saines and colleagues found similar results to our findings with abnormal neurodevelopment and/or abnormal eye or hearing assessments in 31.5% of children evaluated between 7 and 32 months of age[110]. In this study, the cognitive and language domain was also the most affected (35% of 146 children). When comparing neuroimaging findings to neurodevelopmental performance in ZIKV-exposed infants, Lopes Moreira et al. noted a significant association between normal results on brain imaging and higher Bayley-III scores[111]. However, they failed to predict severe developmental delay in 2% of children and normal development in 16%. Similarly, in our cohort overall (i.e. regardless of the status towards ZIKV infection at birth), around 20% (10/56) of the children without structural brain anomalies had a suspicion of neurodevelopment delay in at least one domain at three years of life, whereas one third (2/6) with brain anomalies were not detected as at risk for abnormal development. The results of the Colombian cohort reported by Mulkey *et al.*, indicate that neurodevelopmental delay in a child that is healthy at birth could worsen with age[112].

Brasil and colleagues performed a study stratifying infants by their status towards ZIKV infection at birth. They described neurodevelopment outcomes of 130 children born from ZIKV-infected mothers, of whom 84 (65%) were tested positive for ZIKV between birth and 1 year of age[87]. They could only observe trends towards an association between laboratory confirmed infection and specific abnormalities (structural brain anomalies, vision and hearing deficits, abnormal neurologic exam, developmental delay). The difference between their results

and ours may be explained by the difference in testing for congenital infections, as a positive result after post-partum discharge do not differentiate congenital infection from post-natal acquired infection. They might have experienced a high proportion of exposure misclassification biasing their estimated toward a null association.

Overall, our results and others seem to indicate that normal antenatal and neonatal evaluation cannot provide complete reassurance in infants exposed to ZIKV in utero, and close infantile follow-up remains crucial, particularly in those with a confirmed congenital infection at birth.

4. Perspectives

It seems important to test the validity of our results in another population than pregnant women living in endemic areas. During the ZIKV pandemic, pregnant women who travelled in endemic zones were subject to special attention, with recommendations that are still drastic today. Thus, through an international registry for ZIKV during pregnancy[113], we aim to compare the risks of maternal infection, maternal-fetal transmission and symptomatic congenital infections among travellers versus pregnant women living in endemic areas. A first analysis comparing the data of Swiss, Spanish, French and American travellers versus those of pregnant women living in French Guiana is ongoing.

In congenital CMV infections, as much as 13.5% of newborns who presented no signs/complications at birth will subsequently develop permanent sequelae, such as motor, cognitive or vision impairment and hearing loss[114]. This proportion could be similar in case of exposure to ZIKV in utero, as reported by our first results and other studies [110,111,112]. However, the other studies presenting the follow-up of exposed infants until 2-3 years did not correlate their development with their testing for congenital infection at birth[115]. Thus, we aim to include our data in an individual patient data meta-analysis conducted by the WHO, to investigate fetal,

neonatal and infantile consequences of ZIKV exposure in utero in a worldwide study[116]. Participation to the working groups of this meta-analysis would help to correlate long-term sequelae with testing at birth and to identify variables of interest that require a large number of patients to be investigated (maternal prolonged viremia, antibodies titers, ADE). To pool our results with other cohorts has already permit to identify other prognostic factors in case of congenital ZIKV infection: infection in the first trimester of pregnancy and male infants seem to present an increased risk of microcephaly[117].

We will apply the methodology of these studies to other emergent pathogens in order to present their prevalence during pregnancy, as well as their maternal, placental, fetal and neonatal consequences. As a first step, we have adapted the Zika registry to the SARS-COV-2 pandemic, in order to provide a worldwide multicentric cohort investigating the consequences of SARS-COV-2 infections during pregnancy[113,118]. We have also reported one of the first case of fetal loss related to placental infection with SARS-COV-2[119].

V. Conclusion

Maternal-fetal transmission of ZIKV seem to occur in a quarter of exposed fetuses and is associated with early adverse fetal/neonatal outcomes (fetal loss or severe neurological anomalies) in a third of infected fetuses. Early placentomegaly and maternal prolonged viremia could be associated with increased risks of congenital infections and subsequent adverse fetal / neonatal outcomes. Infants diagnosed with a congenital ZIKV infection at birth seem to present higher risks of neurologic impairments, neurosensory alterations and neurodevelopmental delay, even in cases without structural brain anomalies.

Like other congenital infections, asymptomatic ZIKV infections at birth could result in late-onset alterations, and their consequences during childhood remain to be investigated.

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Paper 1

Zika virus during pregnancy: From maternal exposure to congenital Zika virus syndrome

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REVIEW

WILEY PRENATAL DIAGNOSIS

Zika virus during pregnancy: From maternal exposure to congenital Zika virus syndrome

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Abstract

Zika virus (ZIKV), a vector-borne virus similar to dengue virus, was responsible for a global epidemic between 2013 and 2017 and has emerged as a new agent responsible for severe fetopathies. We present a review to describe the risks and complications of maternal and subsequent fetal infection by ZIKV. The risk of ZIKV infection during pregnancy depends on the incidence of the disease, which is highly variable in different affected geographic areas (less than 1% to 75%). Among infected pregnant women, the risk of any adverse fetal/neonatal outcome was estimated at 5% to 42%, with 1% to 4% of fetal loss and 4% to 9% of suspected congenital Zika syndrome (CZS). The estimated rate of maternal-fetal transmission ranges between 7% and 26%, depending on the methodology of the study.

Findings associated with CZS are microcephaly (33%-64%), ventriculomegaly (63%-92%), calcifications (71%-92%), malformations of cortical development (79%-82%), anomalies of the corpus callosum (71%-100%) and of the posterior fossa (21%-82%), arthrogryposis (10%-25%), eye abnormalities (25%), and extra-neurologic signs such as intra uterine growth restriction (14%), placentomegaly, transient hepatitis, mild anemia. Infants who present with CZS at birth suffer from motor abnormalities (77%-100%), epilepsy (9%-54%), hearing loss, and neurologic impairments.

Prenatal ultrasound with advanced neurosonography and appropriate virological follow-up represent the state-of-the art approach to adequately monitor at-risk pregnancies, in order to diagnose early signs of CZS and to inform parents about the neonatal prognosis.

1 | INTRODUCTION

1.1 | Characteristics and epidemiology

Zika virus (ZIKV) is a single-stranded RNA virus belonging to the *Flavivirus* genus in the *Flaviviridae* family,[1] similarly to dengue (DENV), West Nile, and yellow fever viruses. This large family is part of the arboviruses group (ie, viruses transmitted through arthropods). ZIKV was isolated for the first time in 1947 in Africa and has circulated for several decades in sub-Saharan Africa and South East Asia with only sporadic transmission to humans. Its emergence in the Pacific, in 2007, was associated with the first outbreak that occurred in Yap Islands, which was followed by the second large epidemic in French Polynesia in October 2013. From there, it spread through the Pacific and the Americas,[2,3] where it caused the massive 2015/2016 epidemic, during which the severe neurological neonatal complications

Correction added on 16 April 2019, after first online publication: An additional affiliation has been incorporated in this version.

were brought to light. ZIKV circulation declined from late 2016, but it is still circulating in late 2018, as exemplified by the first outbreaks reported in India.[4-7]

ZIKV isolates have been clustered into the ancestral African lineage and the emerging Asian lineage.[8] The strains that emerged in the Pacific and subsequently spread in the Americas are from the Asian lineage. The increase in virulence and epidemicity observed since ZIKV emerged in French Polynesia in 2013 is potentially associated with viral mutations. This does not mean, however, that strains without these mutations cannot cause severe complications.[9-11] *In vitro* and animal experiments showed that the ZIKV African lineage could infect human and mouse neuronal stem cells and is at least as efficient as the Asian strains implicated in the recent epidemics.[12-14]

Economic growth in tropical developing countries was a major driver for unprecedented and unplanned urban growth, which provided the ideal ecological conditions to increase the *Aedes* mosquito population, the mosquito vector of both ZIKV and other arboviruses such as DENV or chikungunya virus. This, combined with ineffective mosquito control and increased travel exchanges of humans and goods, provided the ideal mechanism for the emergence and spread of vector-borne transmitted diseases.[15] Moreover, previous immunity against another *Flavivirus* may be a cofactor for clinically more severe ZIKV infections through a phenomenon called Antibody Dependent Enhancement (ADE), but this has not been demonstrated.[16]

The risk of a new epidemic exists in all areas where *Aedes* competent mosquitoes are endemic and where the population is nonimmune (although it is currently unclear whether immunity is protective or might induce ADE).[17] ZIKV was first limited to enzootic circulation between nonhuman primates and sylvatic Aedes mosquitoes, before it gained the capacity to be transmitted by human adapted Aedes spp mosquitoes.[18] Since ZIKV has proven to adapt rapidly to new hosts and vectors, however, it is possible that ZIKV may emerge or reemerge in other settings than those mentioned above.

1.2 | Transmission

Like other flaviviruses, ZIKV is mainly transmitted by infected mosquitoes. The main vectors of ZIKV are *Aedes* spp. mosquitoes, principally *Aedes aegypti*, which is highly prevalent in tropical and subtropical areas, particularly in an urban setting. Other mosquitoes have proven to be competent vectors for ZIKV, in particular *Aedes albopictus*, but to date, there are no reports of ZIKV outbreaks driven by *Ae. albopictus*. Sexual transmission of ZIKV has been described, which is unique among flaviviruses.[19] Transmission of ZIKV via blood transfusion is also possible. In endemic areas, the proportion of infection due to each route of infection is, understandably, not possible to evaluate because of continuous exposure to mosquito bites.

The first reported perinatal transmission, likely occurring during delivery, was described in French Polynesia by Besnard et al.[20] Subsequently, Oliveira Melo et al isolated ZIKV in the amniotic fluid of two fetuses with significant cerebral malformations, confirming transplacental transmission.[21] The link between ZIKV and congenital abnormalities has subsequently been confirmed.[22-24] Transplacental transmission is also described for other arboviruses, but ZIKV and Venezuelan equine encephalitis virus remain the only arboviruses associated with congenital CNS malformations.[25]

ZIKV has been isolated in human milk, but milk-borne transmission has not been confirmed.[26]

What is already known about the topic?

The Zika virus (ZIKV) emergence and its consequences during pregnancy led to an increasing number of data about the maternal and fetal consequences of ZIKV infections.

What does this study add?

This review evaluates risks of maternal ZIKV infection, maternal-fetal transmission, congenital Zika syndrome (CZS), other adverse perinatal outcomes, and long-term sequelae among pregnancies exposed to ZIKV. This review also provides contemporary criteria and guidelines for the follow-up of exposed fetuses, based on prenatal and postnatal features of CZS.

2 | METHODS

A PubMed search was carried out using the terms "Zika pregnancy," "congenital Zika," and "Zika infants" and identified 1307 papers between June 2009 and November 2018. Alternative spellings were used for search terms with multiple accepted spellings (for example "CZS"). We reviewed all titles and abstracts when available and limited the search to articles reporting rates of maternal infection, maternal-fetal transmission, congenital ZIKV syndrome (CZS), adverse perinatal outcomes, and long-term sequelae among pregnancies exposed to ZIKV. We also selected papers reporting description of symptomatic maternal and congenital ZIKV infections. Guidelines providing recommendations for the diagnosis and the follow-up of ZIKV-exposed pregnancies, fetuses, and infants were also included. At least two reviewers evaluated the articles and extracted data. Searches were limited to English language. The process of article selection and the number of articles are described in a Figure S1.

3 | MATERNAL INFECTION

3.1 | Prevalence

The cumulative risk of ZIKV infection for pregnant women living in epidemic areas was reported to be 21% to 44% in cohorts from Colombia, Puerto Rico, and French Guiana,[23,27,28] but depended mainly on local incidence of ZIKV, which ranged between 1% in Brazil

after the first epidemic wave and 75% in Yap Island during the outbreak in 2007.[29,30] Risk of infection for travelers was estimated to be 1.3% during the worldwide epidemic,[31] depending on the areas visited, home conditions, and use of mosquito repellants, but has significantly dropped since the decline in circulation of ZIKV.

3.2 | Symptoms and complications

Pregnant women were symptomatic in 17% to 56% of cases of ZIKV infection.[16,23,32,33] Symptom onset may appear from the second day after infection and may last up to 2 weeks. Symptomatic infections are characterized by a maculopapular rash, mild fever, asthenia, pruritus, arthralgia, retro-orbital cephaleas, myalgia, conjunctivitis or conjunctival hyperemia, and/or edema of the extremities.[34-36] Rare severe neurological complications might occur, particularly Guillain-Barré syndrome, which may be a life-threatening condition in pregnant women (prevalence of 1.23% (95% CI, 1.17%-1.29%) in general infected-population).[37,38] However, pregnancy is not associated with more frequent or more severe maternal complications.[32] The presence and the severity of maternal symptoms are not associated with a higher risk of birth defects or fetal loss.[39,40] Although a persistent viremia was initially associated with a higher risk of birth defects, no recent studies have been able to determine if viral load or prolonged viremia represented risk factors for adverse fetal or neonatal outcomes.[23,40,41]

3.3 | Diagnosis

Since most ZIKV-infected pregnant women are asymptomatic and symptoms are nonspecific,[32] a biological confirmation of the infection is required. According to the United States Centers for Disease Control and Prevention (CDC) guidelines, ZIKV testing is currently recommended for every symptomatic pregnant women with possible ZIKV exposure, for asymptomatic pregnant women with ongoing possible ZIKV exposure and for ZIKV-exposed pregnant women whose fetus presents with prenatal US findings consistent with congenital ZIKV infection. ZIKV testing may also be considered for asymptomatic pregnant women with recent possible but not ongoing exposure to ZIKV (ie, travelers)[42] (https://www.cdc.gov/pregnancy/zika/testing-follow-up/documents/testing-algorithm-asymptomatic.pdf).[43] However, since birth defects were described in asymptomatic ZIKV-infected pregnant women returning from endemic areas,[44] ZIKV testing should be offered to all pregnant women possibly exposed to ZIKV. If symptoms compatible with ZIKV infection are identified, nucleic acid test (NAT) or ZIKV RNA amplification by reverse transcription polymerase chain reaction (RT-PCR) should be performed in serum/blood and urine as soon as possible and up to 12 weeks after symptom onset, according to CDC guidelines. ZIKV can be detected in blood most often within the first weeks after symptoms onset. In urine, the window of detection is increased up to 2 to 3 weeks after infection. A positive NAT or RT-PCR in any body fluid confirms the diagnosis. Nevertheless, as false positive results have been described, the CDC recommends positive NAT, to be confirmed by a second set of testing, an approach that may not always be possible during an active epidemic, because of limited laboratory capabilities. A negative result does not exclude ZIKV infection, because of the transient presence of the virus in infected patients. CDC recommends to perform NAT three times during pregnancy for asymptomatic pregnant women with ongoing exposure to ZIKV. However, because of differences in serological and virological assays available, particularly in developing and low-income countries, testing guidelines may differ from country to country, and ZIKV serology may also be considered for women living in an area of active ZIKV transmission.[43]

Serologic evaluation for ZIKV infection includes an initial screening by enzyme-linked immunosorbent assay (ELISA) to detect specific class M immunoglobulins (IgM) against ZIKV followed by confirmation testing by plaque reduction neutralization test (PRNT) due to cross-reactivity with other flaviviruses.[45] Indirect immunofluorescence and ELISA are both adequate approaches for detecting ZIKV-IgG, but must be considered jointly with other laboratory results, particularly PRNT, due to low specificity of IgG. The sensitivity and negative predictive value of the serological results are controversial, since some patients remain serologically negative despite proven infection (RNA amplification), and false positive results due to cross reaction are possible even using PRNT.[46] For women with ongoing exposure, serological assays are not appropriate to the determination of timing of the acute infection in relation to the beginning of the pregnancy. Nevertheless, serological diagnosis based on IgM remains a reliable tool, particularly in women without co/previous exposure to arboviruses.

A recent update in the World Health Organization (WHO) classification for ZIKV cases defines a suspected case as a person presenting with a rash and/or fever and at least one of arthralgia, arthritis, or conjunctivitis. Probable cases are defined as presence of specific IgM antibodies against ZIKV with an epidemiological link. Confirmed cases are defined as detection of ZIKV RNA or antigen in any body fluid or presence of IgM antibodies against ZIKV plus PRNT for ZIKV with a titer greater than or equal to 20 and titer ratio greater than or equal to 4 compared with other flaviviruses.[47]

4 | CONGENITAL ZIKV SYNDROME

4.1 | Definition of Congenital ZIKV syndrome

Although still controversial, the CDC defines CZS as a proven *in utero* ZIKV infection associated with severe microcephaly in which the skull has partially collapsed, decreased brain tissue with a specific pattern of brain damage (including subcortical calcifications), damage to the back of the eye (including macular scarring and focal pigmentary retinal mottling), congenital contractures (clubfoot or arthrogryposis), hypertonia, or restricted body movement soon after birth.[48]

A more restrictive definition of CZS was published by Moore et al, based on advanced clinical and neurological features, postnatal imaging (magnetic resonance imaging [MRI]), and fundoscopy.[49]

In the cohort from French Guiana, we proposed a definition based on early neonatal clinical, biological, and imaging features,

differentiating neonates with signs potentially associated with CZS and those with complications compatible with CZS.[50]

In future epidemics, other more subtle signs might be associated with CZS, and some infected infants may only develop anomalies in childhood.[51,52]

Semiology and characteristics of CZS are described in Table 1.

4.2 | Prenatal features of CZS

Although the first reports showed an association between microcephaly and ZIKV materno-fetal infection,[21,53] many case series and cohorts have shown that microcephaly is not consistently present in CZS, and that ultrasound (US) examination must pay particular attention to the brain anatomy.[23,24,54,55]

4.2.1 | Microcephaly

Most current guidelines define microcephaly as a head circumference (HC) below the third percentile (less than 2 standard deviations [SD]), and the term "severe microcephaly" is used for a HC of less than 3 SD on reference charts (Intergrowth 21st references can be used[56]). For different reasons, however, caution is required on prenatal US, since HC measurements are not always accurate, most fetuses with an HC between −2 and −3 SD will develop normally, and the estimation of brain growth may be distorted by a normal head size with large peri-

TABLE 1 Characteristics of congenital Zika virus syndrome

		Major Signs	Minor Signs
Prenatal and postnatal imaging/Birth defects (Ultrasonography, MRI, CT-scan, and autopsy)	Cerebral	Fetal brain disruption sequence[a]	
		Severe microcephaly < −3 SD	Mild microcephaly < −2 SD
		Ventriculomegaly >12 mm	Mild ventriculomegaly >10 mm
		Cisterna magna >10 mm	subependymal cysts
		Multiple linear or punctiform calcifications	Isolated intracerebral calcification
		Dys/agenesis of corpus callosum	Hypoplasia of corpus callosum
		Vermian dysgenesis	Vermian hypoplasia
		Brainstem dysgenesis	Lenticulostriate vessels vasculopathy
		Porencehaply	Choroid plexus cysts
		Periventricular cystic lesions	Irregular periventricular halo
		MCD[b]	Intraventricular adhesions
	Extra-cerebral	Fetal hydrops	Oligo/polyhydramnios
		Arthrogryposis	IUGR
		Ocular anomalies	Hyperechogenic bowel
			Ascite, subcutaneous edema
			Placentomegaly >40 mm
			Hepato/splenomegaly
Clinical signs		Hypertonia	Hypotonia
		Swallowing disorder	SGA
		HC < −3 SD	HC < −2 SD
		Arthrogryposis	Partial immobilism
		Epilepsy	Hepato/splenomegaly
			Jaundice
			Tremors and extrapyramidal symptoms
			Cognitive disabilities
			Hearing impairment
			Hyperexcitability, impatient crying
			Sleeping disorders
Ocular anomalies		Microphtalmia	Cataract
		Coloboma	Posterior anomalies
			Chorioretinal atrophy
			Focal pigmentary mottling
			Optic nerve hypoplasia/atrophy
Biological parameters			Hb < 140 g/L
			AST > 100 U/L
			ALT > 100 U/L

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hb, hemoglobin; HC; head circumference; IUGR, intra-uterine growth restriction; SD, standard deviation; SGA, small for gestational age; Tc, Thrombocytes. Adapted from Pomar et al.[50]

[a]Fetal brain dysruption sequence: Severe microcephaly, premature closure of fontanels, collapsed skull, overlapping sutures, redundant scalp skin

[b]Malformations of cortical development (MCD): lissencephaly, agyria, pachygyria, polymicrogyria, and heterotopia

cerebral spaces, masking a "micro-encephaly." Microcephaly related to CZS is generally associated with an atypical skull shape and an occipital excess of skin, suggesting a fetal brain disruption sequence. In our and others experience, microcephaly is always associated with other findings and seems to be the consequence of viral brain injury. Prevalence of microcephaly in CZS is 33.3% to 64 %, but brain volume loss seems to be much more frequent (92% of cases).[57-59]

4.2.2 | Ventriculomegaly

Ventriculomegaly induced by ZIKV is often asymmetrical or unilateral, suggesting focal injury more than a compression model found in other infections (stenosis of the Sylvius aqueduct and obstruction by a necrotic process).[57] Ventriculomegaly associated with ZIKV is probably related to the atrophic brain, peri-ventricular, and germinative necrosis, as suggested by the thin cortical mantle frequently observed. Prevalence of ventriculomegaly in CZS is 63.1% to 92%.[57-59]

4.2.3 | Calcifications

Calcifications caused by focal necrosis are common in CZS. These calcifications are often weakly echogenic without any posterior shadowing on prenatal US[60-63] and can intensify in echogenicity and size during pregnancy, which may lead to multiple punctiform macrocalcifications.[57,60] Prevalence of calcifications in CZS is 71% to 92%.[57,58]

The calcifications can be localized to any part of the brain with particular predilection for the cortical-subcortical junction and the periventricular zone; calcifications have also been identified in the midbrain, nucleus caudate, brainstem, cerebellum, basal ganglia, and spinal cord as described in other TORCH (toxoplasmosis, others, rubella, cytomegalovirus, herpes virus) infections.[57,64]

4.2.4 | Malformations of cortical development

Abnormal neuronal migration and disorders of cortical development are visualized by reduction in gyration (pachy/agyria), polymicrogyria, heterotopias, and subsequent microcephaly.[57,65] Malformations of cortical development were described in 79% to 82% of CZS cases.[57,58]

In addition, destructive cystic diseases such as porencephaly, schizencephaly, or hydranencephaly are frequently observed because of necrosis of neural progenitors. Similar destructive lesions are frequently observed in other congenital infections.[66-68] Ventricular hemorrhages, which reflect bleeding in the highly vascularized germinal matrix have not been associated with CZS, whereas lesions of the germinal matrix, including subependymal pseudocysts,[60] have been observed in several reports.[23,69] Subependymal pseudocysts are thought to precede germinal matrix hemorrhages and are frequently observed in premature newborns.[70] Although most of these pseudocysts are benign, they can be associated with dismal prognosis due to early destruction of the germinal matrix when they are located in the occipital or temporal horn.[71] Intraventricular synechiae and periventricular cystic degeneration may also develop, as in congenital cytomegalovirus (CMV), and was reported in 58% of CZS cases in the Colombian cohort.[57,72]

4.2.5 | Corpus callosum dysgenesis

Dysgenesis of the corpus callosum is frequently described including partial or complete agenesis and callosal calcifications.[57,64,69] Prevalence of malformations of the corpus callosum range between 71% and 100%.[57,58]

4.2.6 | Posterior fossa anomalies

Mega cisterna magna is also described, which may represent a Dandy-Walker malformation or vermian hypoplasia/dysgenesis, especially when an enlarged cisterna magna is found early in pregnancy.[23,57,65] Vermian hypoplasia and global cerebellar hypoplasia were reported in 42% and 21% to 82% of CZS cases, respectively.[57,73] The brainstem can also be hypoplastic or dysplastic and is associated with extra-cerebral abnormalities such as swallowing disorders and hydramnios (25%) as well as partial immobilization or arthrogryposes (10%-25%).[57,60,64,74,75]

4.2.7 | Eye abnormalities

The eye disorders observed on prenatal US or MRI can range from the more common unilateral microphthalmia to anophthalmia.[21,23,57] The spectrum of optical abnormalities found postnatally is larger, including signs that can be found in prenatal imaging: optic chiasm hypoplasia, coloboma of the retina, and cataract.[23,76] Prevalence of eye abnormalities in CZS is approximately 25%.[57,77]

4.2.8 | Extra-cerebral anomalies

Signs of placental inflammation such as increased thickness (placentomegaly) and calcifications have been observed in some cases.[61,78] Placental dysfunction induced by ZIKV infection may contribute to the development of fetal damage or intrauterine growth restriction (IUGR), particularly in cases of early infection when placental circulation is not yet established.[79] Overall, IUGR was observed in 14% of CZS cases and could be the result of both fetal infection and placental insufficiency.[34,79]

4.3 | Clinical features of CZS at birth

Mild anemia, cholestasis, and transient hepatitis have been found in fetuses and newborns infected by ZIKV.[80,81]

Neurologic impairments such as swallowing dysfunction, movement abnormalities, and epilepsy have been described in infants suffering from CZS as well as in infants asymptomatic at birth.[82]

The predominant neurologic findings in young infants with suspected congenital ZIKV infection are extreme irritability, hyperreflexia, and hypertonia with spasticity, tremors, and extrapyramidal symptoms, hypotonia, or a combination of hyper- and

hypotonia.[36,63,83,84] Motor abnormalities affected 77.3% to 100% of infants with CZS at birth.[82,85] Epilepsy is associated with 9% to 95.5% of congenital ZIKV infection.[59,82-87]

Another study reported feeding challenges, sleeping difficulties, severe motor impairment, vision and hearing abnormalities, and/or seizures disorders in all 18- to 24-month old infants born with CZS.[88] Long-term sequelae, particularly regarding neuro-development and cognitive function of these infants, however, remain insufficiently investigated.[75]

5 | MANAGEMENT OF EXPOSED PREGNANCIES AND RECOMMANDATIONS

5.1 | Maternal-fetal transmission of ZIKV

In a cohort evaluated in French Guiana, we first estimated the maternal-fetal transmission rate at 10.9%, based on amniocentesis and serology results of the newborns.[23] After extensive investigation on fetal/neonatal (amniotic fluid, fetal and neonatal blood, cerebro-spinal fluid, urine) and placental samples of 291 fetuses/newborns, the vertical transmission rate was estimated at 26.1%. When positive placenta samples were removed from the analysis because of the theoretical risk of placental contamination by maternal viremia, the vertical transmission rate was estimated at 18%.[50] The New-York City cohort of ZIKV-exposed pregnant women reported proof of a congenital infection in 7% of confirmed or probable maternal ZIKV infections.[89] Available data do not seem to indicate an increased rate of maternal-fetal transmission with ongoing gestation, as for example in congenital toxoplasmosis. However, more studies are required to conclude on the evolution of transplacental infection according to the timing of maternal infection.

Overall, maternal-fetal transmission of ZIKV remains difficult to estimate because of the debatable sensitivity and specificity of ELISA, that ZIKV RNA is only transiently present in blood, urine, and amniotic fluid[81] and depends on the fetal samples investigated.[50]

5.2 | Prevalence of adverse outcomes

At the beginning of the ZIKV epidemic, first reports announced fetal abnormalities linked to maternal ZIKV infection in more than 40% of cases.[36] These reports included "any abnormalities," such as an isolated Doppler anomaly or "small HC < −1 SD" and led to a global overestimation of the consequences of ZIKV infections during pregnancy.

Recent reports, with more extensive investigations of fetuses and newborns, reported CZS in 4% to 9% of pregnancies exposed to ZIKV, when exposure is defined by proven maternal infection.[23,33,35,57,61,87]

In a recent study investigating the rate of adverse outcomes in proven infected fetuses/newborns, 45% presented with no signs/complications, 20% had mild/moderate signs potentially correlated to congenital Zika virus infection (cZIKV) infection, 21% had severe complications compatible with CZS, and 14% resulted in fetal loss.[50]

Maternal infection during the first trimester of pregnancy is associated with a higher risk of miscarriage, fetal loss or CZS; whereas an infection in late pregnancy seems to have less fetal and early neonatal consequences, with unspecific signs, as for others TORCH infections.[23,35,60,90,91]

A recent report of the US Zika Pregnancy and Infant Registry noted a risk of neurodevelopmental abnormality in 9% of infants (1 year of age) born from infected mothers.[87] Overall, the risk of postnatal neurological sequelae in congenitally infected infants without prenatal findings indicative of CZS remains unknown. Ongoing investigation of several cohorts might increase the knowledge on later sequelae associated with congenital ZIKV infection.

Risks associated with maternal and fetal ZIKV infection are described in Figure 1.

5.3 | Follow-up of exposed pregnant women

An enhanced US follow-up schedule for all exposed pregnant women regardless of their status, with detailed fetal neurosonography for those who presented with positive laboratory testing, aims to increase the sensitivity of the detection of CZS. The "International Society of Ultrasound in Obstetrics and Gynecology" (ISUOG) and several national organizations recommend detailed US examination on a monthly basis.[23,92,93] When ZIKV maternal infection is suspected or confirmed, neurosonographic examinations of the fetus should be performed in a referral center, as is done for other congenital infections during pregnancy.

When time of infection is known, a delay[23,57,63] of 3 to 15 weeks[63] was observed before identification of early fetal abnormalities.

In addition to US, MRI represents a complementary tool when neurosonography is limited or not available to evaluate congenital ZIKV infections, particulary after 30 weeks gestation, to facilitate an appropriate examination of the gyration and to highlight increased cerebro-spinal fluid, migration disorders, or cortical development anomalies.[21,57,73]

Recommendations for prenatal and postnatal follow-up of exposed fetuses are available in Figure 2.

5.4 | Prenatal diagnosis and management of CZS

The prenatal diagnosis of CZS depends on the attribution of the observed lesions to ZIKV.

When fetal status is unknown in an infected mother, any US abnormality can predict a CZS in only 41.3% of cases in an epidemic area.[94] Even if a severe CNS malformation is present, other etiologies must be disproved.[95]

If one or more of the abnormalities described above are encountered, an amniocentesis should be proposed in order to perform ZIKV RT-PCR, other TORCH PCRs and karyotype (and/or CGH array) to confirm the diagnosis of CZS and to exclude other possible causes.

By analogy to other congenital infections, the virus is probably only shed in the amniotic fluid once a sufficient time has elapsed for the

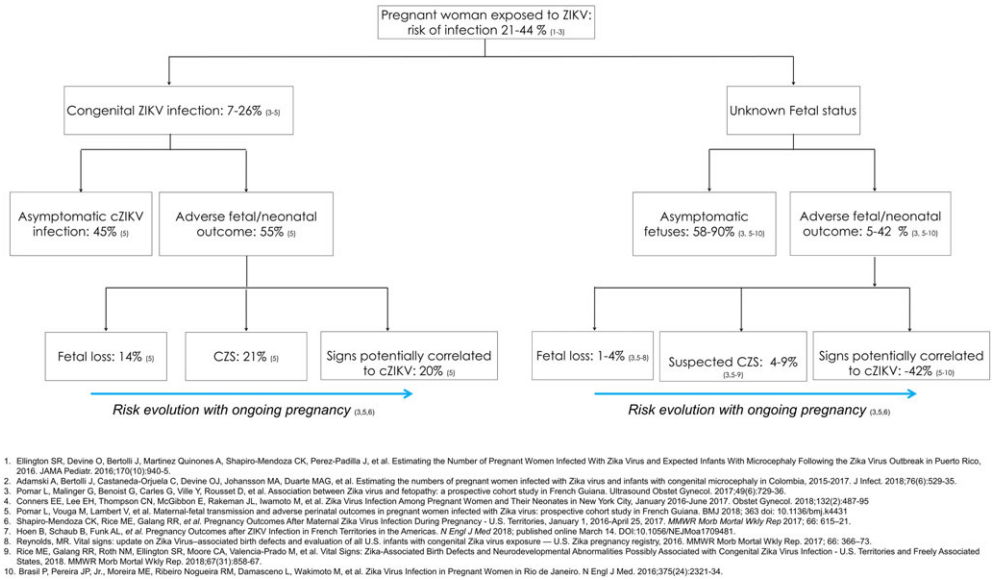

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FIGURE 1 Risks of maternal infection, fetal infection and adverse outcomes. Abbreviations: cZIKV, congenital zika virus infection; CZS, congenital zika virus syndrome; ZIKV, zika virus (Adapted from Pomar et al[50]) [Colour figure can be viewed at wileyonlinelibrary.com]

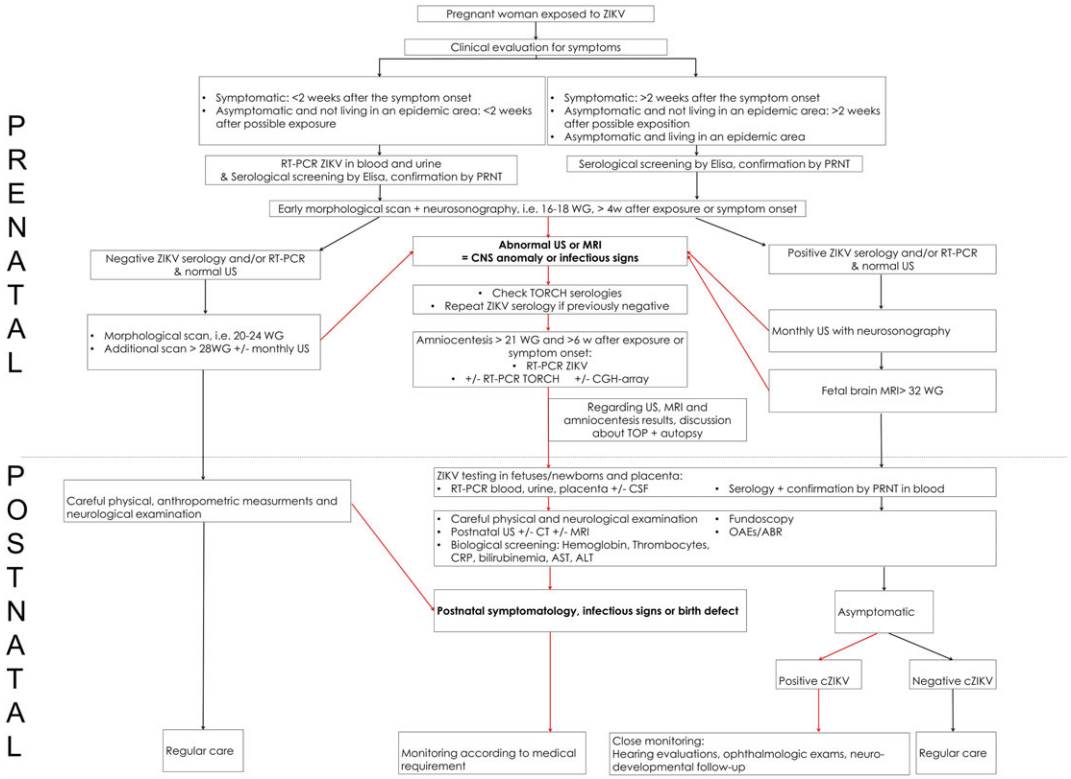

FIGURE 2 Clinical follow-up of exposed pregnant women, fetuses, and newborns. Abbreviations: ABR, auditory brainstem response; CNS, central nervous system; CRP, C-reactive protein; CSF, cerebro-spinal fluid; cZIKV, congenital zika virus infection; MRI, magnetic resonance imaging; OAE, oto-acoustic emission; PRNT, plaque reduction neutralization test; RT-PCR, reverse transcriptase polymerase chain reaction; TOP, termination of pregnancy; US, ultrasound; WG, weeks' gestation; ZIKV, Zika virus (Adapted from Vouga et al[97]) [Colour figure can be viewed at wileyonlinelibrary.com]

virus to breach the placental barrier (6-8 weeks after infection) and once the fetal kidneys produce sufficient urine.[96,97] The sensitivity of amniocentesis remains unknown because of the lack of knowledge about the evolution of viremia. Schaub et al showed that ZIKV RNA amplification could be transient in the amniotic fluid, fetal blood, and placenta.[81] Thus, ZIKV may no longer be detectable after a prolonged

time from the initial infection, since ZIKV secretion by the fetal kidney is probably transient, and RNA is less stable than DNA (ie, CMV). Current recommendations, based on these highlights, suggest that amniocentesis should be offered only in the presence of fetal signs, 6 to 8 weeks after suspected maternal exposure and after 21 weeks' gestation,[97] taking into account that a negative result does not rule out ZIKV congenital infection. Also, a positive ZIKV result does not exclude other fetal chromosomal or infectious pathologies.[95]

Schaub et al described the biological responses to ZIKV infection in fetal blood, such as transient anemia or increased liver enzyme levels.[81] It is yet unknown whether these markers of infection or viral loads in fetal blood are prognostic factors, such as in congenital CMV infection.[98]

Unrelated to the serological findings, the presence of a CNS malformation related to intrauterine infection is associated with a poor prognosis in more than 90% of cases, and in these cases, termination of pregnancy should be considered in accordance with the country's laws.

5.5 | Postnatal diagnosis of CZS and follow-up

The confirmation of an *in-utero* infection can be made from a positive RT-PCR on cord blood, neonatal blood, urine, placenta, or cerebrospinal fluid, as well as the presence of specific IgM.

Positive cord blood should be confirmed on neonatal blood in order to exclude potential maternal contamination.

Postnatal recommendations include clinical, biological, and imaging follow-up, adapted to the ZIKV-status of the newborn and to the presence of signs or symptoms (described in Figure 2).

6 | TREATMENT AND PREVENTION

6.1 | Prevention

Pregnant women and couples planning to start a pregnancy should avoid travel in epidemic areas. When there is a need to travel to or for those living in endemic areas, avoiding mosquito bites using long clothing and repellant is proposed.

Because of the prolonged persistence of ZIKV RNA in semen, the first recommendations issued by the European Centers for Disease Control and Prevention, the WHO, and different health ministries suggested postponement of any pregnancy attempts for at least 6 months for men and 2 months for women after the last possible exposure to the virus.[47] Because of the decline of ZIKV and exceptional persistence of infective viral particles in semen,[99] this delay has been reduced to 3 months for men and remains 2 months for women.[100]

6.2 | Potential therapeutic options

To our knowledge, there is currently no drug licensed against any arboviruses. We review the ongoing research and clinical trials for Zika below.

6.2.1 | Vaccines

Promising DNA, mRNA, and purified inactivated virus vaccines that could be used to prevent ZIKV are currently in phase I[101] or phase II clinical trials.[102,103]

6.2.2 | Antiretroviral

To limit the development of CZS, two strategies might be evaluated: to treat the infected mother in order to reduce maternal viral replication and subsequent transplacental transmission and to focus on the reduction of symptoms in infected fetuses, as it has been tried in cCMV infections with Valacyclovir.[104] Recent *in vitro* and *in vivo* models show good efficacy of Sobosbuvir to reduce the viral burden and vertical transmission in animals.[105]

7 | CONCLUSION

This review presents an estimation of the risk of infection by ZIKV for pregnant women and their fetus/newborn and subsequent risks of complications. Prenatal US with advanced neurosonography and appropriate virological follow-up represent a gold standard to adequately monitor at-risk pregnancies, in order to diagnose early signs of CZS and to inform parents about the neonatal prognosis. Long-term sequelae are still not well described, and long-term cohorts are needed to accurately define the burden of ZIKV in childhood.

DISCLOSURES

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare no support from any organization for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years, no other relationships or activities that could appear to have influenced the submitted work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Paper 2

Maternal-fetal transmission and adverse perinatal outcomes in pregnant women infected with Zika virus: prospective cohort study in French Guiana

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Maternal-fetal transmission and adverse perinatal outcomes in pregnant women infected with Zika virus: prospective cohort study in French Guiana

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ABSTRACT

OBJECTIVES

To estimate the rates of maternal-fetal transmission of Zika virus, adverse fetal/neonatal outcomes, and subsequent rates of asymptomatic/symptomatic congenital Zika virus infections up to the first week of life.

DESIGN

Cohort study with prospective data collection and subsequent review of fetal/neonatal outcomes.

SETTINGS

Referral centre for prenatal diagnosis of the French Guiana Western Hospital.

PARTICIPANTS

Pregnant women at any stage of pregnancy with a laboratory confirmed symptomatic or asymptomatic Zika virus infection during the epidemic period in western French Guiana. The cohort enrolled 300 participants and prospectively followed their 305 fetuses/newborns.

MAIN OUTCOME MEASURES

Rate of maternal-fetal transmission of Zika virus (amniotic fluid, fetal and neonatal blood, urine, cerebrospinal fluid, and placentas); clinical, biological, and radiological outcomes (blindly reviewed); and adverse outcomes defined as moderate signs potentially related to congenital Zika syndrome (CZS), severe complications compatible with CZS, or fetal loss. Associations between a laboratory confirmed congenital Zika virus infection and adverse fetal/neonatal outcomes were evaluated.

RESULTS

Maternal-fetal transmission was documented in 26% (76/291) of fetuses/newborns with complete data. Among the Zika virus positive fetuses/newborns, 45% (34/76) presented with no signs/complications at birth, 20% (15/76) with moderate signs potentially related to CZS, 21% (16/76) with severe complications compatible with CZS, and 14% (11/76) with fetal loss. Compared with the Zika virus positive fetuses/neonates, those that were identified as negative for Zika virus (215/291) were less likely to present with severe complications (5%; 10/215) or fetal loss (0.5%; 1/215; relative risk 6.9, 95% confidence interval 3.6 to 13.3). Association between a positive Zika virus test and any adverse fetal/neonatal outcome was also significant (relative risk 4.4, 2.9 to 6.6). The population attributable fraction estimates that a confirmed congenital Zika virus infection contributes to 47% of adverse outcomes and 61% of severe adverse outcomes observed.

CONCLUSION

In cases of a known maternal Zika virus infection, approximately a quarter of fetuses will become congenitally infected, of which a third will have severe complications at birth or fetal loss. The burden of CZS might be lower than initially described in South America and may not differ from other congenital infections.

WHAT IS ALREADY KNOWN ON THIS TOPIC

Many reports have described the spectrum of congenital Zika virus syndrome in severely affected fetuses/newborns during the recent epidemics

Early reports suggested a risk of fetal anomalies up to 40%, whereas more recent reports agree on a rate of Zika virus related birth defects of 4-8% in cases of confirmed maternal infection

The absolute risk of maternal-fetal infection remains difficult to establish owing to the lack of fetal/neonatal testing, especially in apparently healthy newborns, and is therefore rarely reported

WHAT THIS STUDY ADDS

This paper presents the results of fetal/neonatal testing and early clinical outcomes of 291 fetuses/newborns from Zika virus infected pregnant women during the recent epidemic in French Guiana

Maternal-fetal transmission seems to occur in approximately a quarter of exposed fetuses and is associated with early adverse fetal/neonatal outcomes in a third of infected fetuses

Introduction

The recent epidemics in French Polynesia and the Americas have confirmed vertical trans-placental transmission of Zika virus and its association with congenital anomalies, particularly severe central nervous system lesions.[1-3] Nevertheless, the exact burden of disease remains unclear, especially in endemic countries. Similarly to congenital cytomegalovirus and toxoplasmosis infections, vertical transmission is not systematic and does not always lead to fetuses/infants with apparent signs of infection.[4] The risk of congenital Zika virus syndrome (CZS) was estimated, at first, to be higher than 40% in a cohort of women who developed symptomatic Zika virus infection during pregnancy in Brazil,[5] whereas more recent data from the US Zika pregnancy registry suggest an overall risk of 5% and up to 8% in cases of maternal infection in the first trimester.[6] The lack of fetal/neonatal testing in previous studies has impaired

accurate estimations of maternal-fetal transmission and risk of symptomatic congenital infection.

We conducted a cohort study among pregnant women in western French Guiana during the recent Zika virus epidemic and evaluated the results of comprehensive fetal/neonatal testing for Zika virus. Our primary objective was to estimate the absolute risk of maternal-fetal infection. The secondary objectives were to estimate the percentage of fetuses/newborns with overt signs of infection or related complications within the first week of life, by reviewing fetal/neonatal outcomes blinded to Zika virus status; to estimate the relative risk of adverse perinatal outcomes in infected fetuses; and to estimate the population attributable fraction of a confirmed congenital Zika virus infection for any adverse outcome and for severe adverse outcomes.

Methods

Study population

The study was conducted at the French Guiana Western Hospital Center (Centre Hospitalier de l'Ouest Guyanais; CHOG) during the Zika virus epidemic. French Guiana is a French department located in South America, and in 2015 it had an estimated total population of 252 338 and 6800 births.[7] The Zika virus epidemic in French Guiana lasted nine months from January to September 2016, with a total of 9790 suspected cases, affecting mostly the coast and western part of French Guiana.[8 9] All pregnancies in the territory were offered monitoring by real-time polymerase chain reaction (RT-PCR) and/or detection of Zika virus antibodies as the consequence of an awareness policy adopted in the French Departments of America.[10] During this period, a total of 1105 pregnant women presented with a positive Zika virus test and were monitored in three referral centres—the CHOG, the Centre Hospitalier de Cayenne, and the Centre Medico-Chirurgical de Kourou.[8 11]

The CHOG is located in the western part of French Guiana, in Saint Laurent du Maroni. With a total of 284 beds, it is the second largest hospital in French Guiana and includes a maternity unit providing neonatal intensive care. The catchment population of the CHOG is quite similar of that of all French Guiana, but some particularities come from the fact that a part of the western population live along the Maroni river and are more exposed to poverty, difficult living conditions, and subsequent medical comorbidities (higher rates of pregnancy among adolescents and higher risks of prematurity, vascular diseases, lead poisoning, and anaemia). We identified patients for inclusion in the study either through routine serological testing of all pregnant women admitted to the prenatal unit of the CHOG (irrespective of the trimester of pregnancy or presence of symptoms) or through serological and molecular testing of pregnant women with Zika virus symptoms admitted in our department (fig 1). We included patients with a confirmed Zika virus infection during pregnancy from 1 January to 15 July 2016.[8 11] The enrolment period thus occurred in the early stages of the Zika virus epidemic in French Guiana. We excluded patients not monitored in our prenatal diagnosis unit after the diagnosis of Zika virus infection owing to the lack of prenatal follow-up, patients with fetal losses before 14 weeks' gestation, and patients for whom the diagnosis of Zika virus infection was based on serology performed at delivery. We excluded the last group because of the lack of prospective follow-up during pregnancy and comprehensive fetal/neonatal assessment during the first week of life, as Zika virus status was available only after discharge from hospital (at the peak of the epidemic, results were delayed by as much as two weeks owing to limited technical and human resources).

Patients included in the study provided written informed consent after discussing the objectives of the study. We collected data on demographic characteristics, medical parameters, and possible risk factors for congenital diseases.[11] Pregnancies were monitored as clinically indicated, except for the addition of prenatal ultrasound scans performed monthly during the Zika virus epidemic as recommended by the French authorities. In France, prenatal screening for aneuploidy, HIV, toxoplasmosis, rubella, and syphilis are offered to all pregnant patients during the first trimester. Screening for cytomegalovirus, herpes simplex viruses, and parvovirus B19 (TORCH screening) is proposed in cases of suspected maternal/fetal infection. Gestational age was based on the crown-rump length on an ultrasound scan performed between 11 and 13^{+6} weeks' gestation. Prenatal care as outlined above was supported by the French maternity insurance available to all pregnant women, regardless of their socioeconomic conditions.

Laboratory confirmation of Zika virus infection

We defined pregnant patients as positive for Zika virus either by a positive RT-PCR result performed with the Realstar Zika Kit (Altona Diagnostics GmbH, Hamburg, Germany) in blood and/or urine samples or by the presence of Zika virus specific IgM after detection of anti-Zika virus antibodies in the blood. Serological testing (including Dengue and Chikungunya) was performed at the French Guiana National Reference Centre for arboviruses. For Zika virus, we used an in-house MAC-ELISA assay, with a sensitivity, when correlated with PCR results, varying between 87% for serum samples collected between five and 20 days from symptom onset to 98% for those collected after seven days.[10] Specificity varies depending on the presence of co-infections with other arboviruses, reaching 80% in negative patients, but dropping in the case of co-infections.[10] In such cases (n=18), we obtained confirmation with a micro-neutralising assay. Serological cross reactions with other *Flaviviridae* were expected to be minimal, as circulation of Dengue virus has been low in French Guiana since 2014 and no significant circulation of other *Flaviviridae* has been seen.[12]

We defined a confirmed congenital Zika virus infection either by Zika virus RNA amplification by RT-PCR from at least one fetal/neonatal sample (placenta, amniotic fluid, cerebrospinal fluid, urine, or blood) or identification of Zika virus specific IgM in

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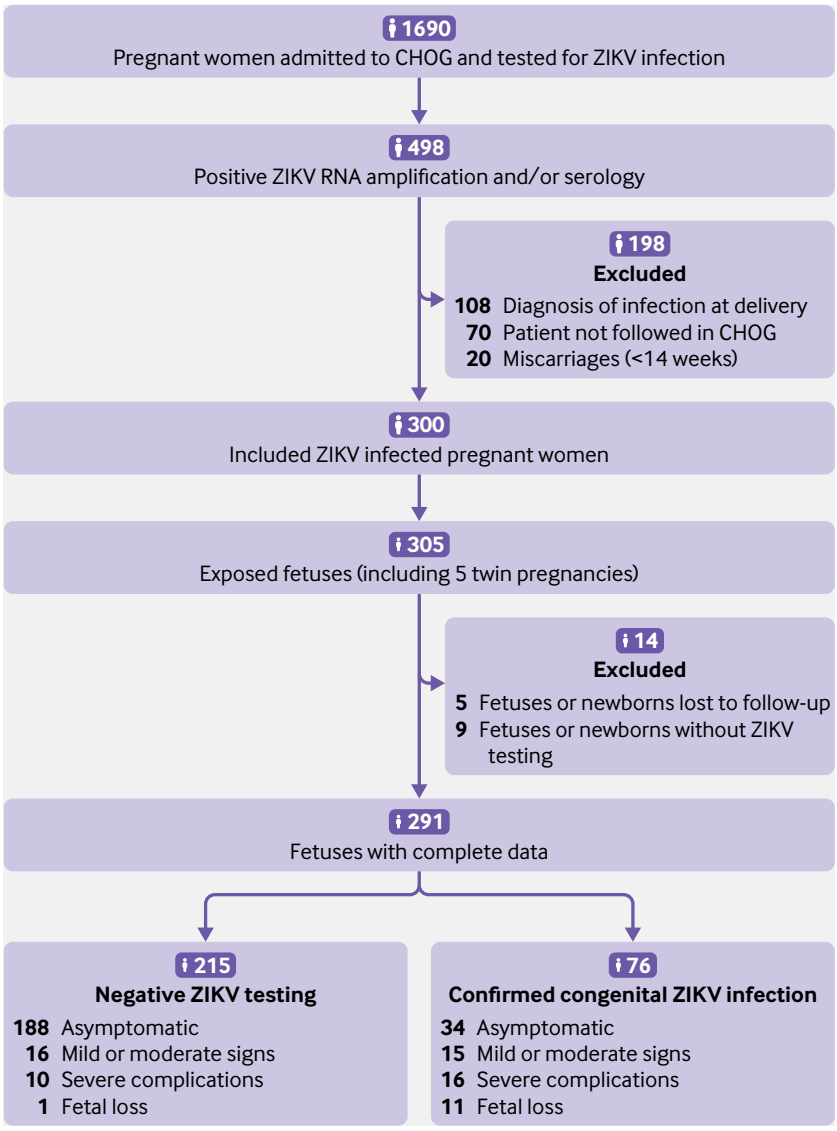

Fig 1 | Prospective maternal cohort and neonatal/fetal outcomes. Pregnant women admitted to French Guiana Western Hospital Center (Centre Hospitalier de l'Ouest Guyanais; CHOG) were routinely tested for Zika virus specific IgM and/or Zika virus RNA. Patients with a positive test were offered participation in the study. ZIKV=Zika virus

the umbilical cord/neonatal blood or in cerebrospinal fluid. Zika virus status was confirmed at day three of life by IgM serology to exclude maternal contamination of umbilical cord blood in all liveborn neonates (except in four neonates whose parents declined). In cases of fetal loss, blood status was defined only by umbilical cord samples.

Fetal/neonatal outcomes

We followed fetuses/newborns from mothers positive for Zika virus up to their first week of life and collected data as well as results of neonatal/fetal testing.

Laboratory tests

When a fetal malformation was suspected, invasive testing was offered to complete TORCH PCRs, karyotype, and comparative genomic hybridisation array if necessary, after discussion with an expert fetal multidisciplinary centre (Caen University Hospital, France), according to French legislation. All fetuses underwent haematological and biochemical screening at birth, performed on cord and/or neonatal blood before the third day of life.

Fetal/neonatal imaging

Pregnant women had monthly ultrasound examinations from the time of diagnosis of Zika virus infection until delivery, with standardised biometric measurements and anatomical evaluation, paying special attention to the brain anatomy, as recommend by national and international medical societies.[13 14] All fetal ultrasound examinations were performed by two experienced sonographers (VL, LP) using E8 and E10 Voluson ultrasounds with abdominal (RM6C) and transvaginal (RIC5-9-D) transducers (General Electric Medical System, Milwaukee, USA).

A transfontanellar ultrasound scan was offered for all neonates during the first week of life, using Phillips EPIQ 7g ultrasound with a neonatal cephalic (C8-5) transducer (Phillips Medical Systems, Cleveland, USA). Computed tomography scanning was not offered routinely owing to the limited capacity of the local radiology unit and was done only if calcifications or skull abnormality were suspected (on prenatal or postnatal ultrasound scans or clinical assessment for skull abnormalities). The radiology unit did not offer magnetic resonance imaging, and the nearest scanner was located 300 km away. Because of these limitations, computed tomography and magnetic resonance imaging examinations were performed after the first week of life and data are not reported in this study.

Neonatal clinical assessments

All neonates underwent clinical examination at birth by a midwife and at day three of life by a senior neonatologist. A complete physical examination was performed with special attention to anthropometric measurements, neurological status, and signs of infection, as recommended by international medical societies.[15] Anthropometric measurements were assessed according to the Intergrowth charts available at https://intergrowth21.tghn.org/standards-tools/.

Fetal/neonatal outcome definitions

On the basis of previously published criteria to define congenital Zika virus and cytomegalovirus infections,[16-19] we used minor and major signs to define four categories (appendix 1). (1) Asymptomatic was defined as no major signs and less than two minor signs. (2) Mild/moderate signs potentially associated with CZS were defined as no major signs and at least two minor signs. (3) Severe complications compatible with CZS were defined as one major sign or three minor signs including at least one cerebral anomaly identified on prenatal or postnatal ultrasound. (4) Fetal loss was defined as the spontaneous demise of the fetus after 14 weeks' gestation. Fetal loss includes late miscarriages (14-24 weeks)[20] and stillbirths (fetal demise >24 weeks) but not intrapartum nor early postpartum

Table 1 | Characteristics of pregnant women admitted to French Guiana Western Hospital Center (Centre Hospitalier de l'Ouest Guyanais; CHOG) between 1 January and 15 July 2016. Values are numbers (percentages) unless stated otherwise

Characteristics	Laboratory confirmed cZIKV infection (n=76)	Negative fetal/neonatal ZIKV testing (n=215)
Median (interquartile range) maternal age, years	26.7 (23.0-32.4)	27.5 (22.3-33.1)
Maternal age >35 years	12 (16)	40 (19)
Any maternal comorbidities*	21 (28)	42 (20)
Diabetes (previous or gestational)	2 (3)	10 (5)
Vascular pathologies	6 (8)	14 (7)
Thrombophilia	2 (3)	2 (1)
Anaemia	4 (5)	4 (2)
Co-infections	3 (4)	7 (3)
Lead poisoning	2 (3)	5 (2)
Alcohol consumption	1 (1)	1 (0.5)
Others	3† (4)	3‡ (1)
Risk of fetal aneuploidy:		
High risk (≥1/250)	2 (3)	3 (1)
Low risk (<1/250)	47 (62)	117 (54)
Late follow-up§	27 (36)	95 (44)
Trimester of suspected maternal infection:		
First	16 (21)	52 (24)
Second	44 (58)	111 (52)
Third	16 (21)	52 (24)

cZIKV=congenital Zika virus.
*Including patients with multiple comorbidities.
†Anti-Lea alloimmunisation; denutrition; vitamin K deficiency.
‡Increased human chorionic gonadotropin concentrations; history of mucopolysaccharidosis.
§Clinical follow-up started after first trimester.

deaths.[21] We defined "any adverse outcomes" as mild/moderate signs potentially associated with CZS or severe complications compatible with CZS or fetal loss (2+3+4) and "severe adverse outcomes" as severe complications compatible with CZS or fetal loss (3+4).

Three independent reviewers (LP, MV, DB) blinded to Zika virus status reviewed all fetal/neonatal outcomes and classified them into the four categories described above on the basis of prenatal/transfontanellar ultrasound findings, symptoms at birth, and haematological and biochemical blood analyses (appendix 1). Discrepant cases were discussed between reviewers to determine the most appropriate classification.

Statistical analyses

We compared the demographic and clinical variables of Zika virus positive and negative fetuses/newborns. We used the binomial Wilson score to calculate confidence intervals of single proportions and the Pearson exact method to calculate confidence intervals of risk ratios and medians. We present denominators where data for the secondary outcome are missing. We defined the population attributable fraction as (Re−Run)/Re=(RR−1)/RR, calculated using Stata. To test the robustness of our findings, we did a sensitivity analysis. As the placenta might be contaminated by maternal blood, we redefined the criteria for a laboratory confirmed congenital Zika virus infection to exclude placentas and removed them from the analysis. We used Stata 14 for data analyses.

Patient and public involvement

This research was done without patient involvement. Patients were not invited to comment on the study design and were not consulted to develop patient relevant outcomes or interpret the results. Patients were not invited to contribute to the writing or editing of this document for readability or accuracy. We have invited the public to help us to develop our dissemination strategy.

Results

From 1 January to 15 July 2016, 300 pregnant women with a positive Zika virus test, of whom 52 (17.3%) presented with compatible symptoms, were monitored in the prenatal diagnosis unit of the CHOG and included in the study, representing a total of 305 exposed fetuses (including five twin pregnancies). Zika virus testing was available for 291 fetuses/newborns and clinical outcomes for 300 fetuses/newborns (fig 1).

Laboratory confirmation of maternal-fetal transmission

Maternal-fetal transmission was documented in 76/291 (26%, 95% confidence interval 21% to 32%) of fetuses/newborns with complete data. Positive Zika virus results were obtained from 48/280 (17%) umbilical/neonatal cord blood samples (confirmed at day three of life for liveborn neonates), 51/232 (22%) placentas, 7/247 (3%) urine samples, 5/12 (42%) amniotic fluid samples, and 4/7 (57%) cerebrospinal fluid samples. When we excluded placental samples from the analysis, maternal-fetal transmission was documented in 52/282 (18%, 14% to 23%) fetuses/newborns with other samples tested.

Among fetuses/newborns with negative testing, 4/215 (2%) had four different samples tested, 167/215 (78%) had three different samples tested, 36/215 (17%) had two different samples tested, and 8/215 (4%) had only one sample tested. Among fetuses/newborns with a laboratory confirmed Zika virus

infection, 1/76 (1%) had five different samples tested, 3/76 (4%) had four different samples tested, 38/76 (50%) had three different samples tested, 15/76 (20%) had two different samples tested, and 19/76 (25%) had only one sample tested (appendix 2).

As shown in table 1, no significant differences in baseline maternal characteristics existed between the two groups. We also observed similar baseline maternal characteristics between patients included in and excluded from the cohort, as well as in all patients delivered at CHOG during 2016 (appendix 3). Cases of maternal co-infection among fetuses/neonates with a laboratory confirmed Zika virus infection included two active hepatitis B infections. In fetuses with a negative Zika virus test, two HIV, two primary toxoplasmosis, one human T-lymphotropic virus, one Coxsackie virus, one primary varicella zoster virus, and one leptospirosis were recorded. The timing of diagnosis of maternal infection was similar between fetuses/newborns with a confirmed Zika virus infection and those with no laboratory evidence of a Zika virus infection.

Fetal/neonatal outcomes

Among exposed fetuses (n=291), 210 (72%, 67% to 77%) presented with no signs/complications at birth, 31 (11%, 8% to 15%) presented with mild/moderate signs potentially related to CZS, 26 (9%, 6% to 13%) presented with severe complications compatible with CZS (including three medical termination of pregnancy), and 12 (4%, 2% to 7%) fetal losses were recorded (fig 2).

Among the 76 fetuses/neonates with a documented congenital Zika virus infection, 34 (45%, 34% to 56%) presented with no signs/complications, 15 (20%, 12% to 30%) had mild/moderate signs, 16 (21%, 13% to 33%) had severe complications, and 11 (14%, 8% to 24%) resulted in fetal loss (table 2). In contrast, among the 215 fetuses/neonates that tested negative for Zika virus, 188 (87%, 82% to 91%) presented with no signs/complications, 16 (7%, 5% to 12%) had mild/moderate signs, 10 (5%, 3% to 8%) had severe complications, and 1 (0.5%, 0.1% to 3%) resulted in fetal loss (table 2). A full description of each fetus/newborn with an adverse outcome is available in appendix 4.

Association between Zika virus exposure and fetal/neonatal outcomes

Fetuses/newborns with a laboratory confirmed congenital Zika virus infection had a higher risk of "any adverse outcome" (that is, mild/moderate signs or severe complications or fetal loss) (42/76; 55% (44% to 66%) versus 27/215; 13% (9% to 18%)) than did those who were considered Zika virus negative by laboratory testing (relative risk 4.4, 95% confidence interval 2.9 to 6.6). Similarly, the risk of "severe adverse outcomes" (defined as severe complications or fetal loss) was higher in cases of confirmed congenital Zika virus infection (relative risk 6.9, 3.6 to 13.3). The population attributable fraction of a confirmed congenital Zika virus infection was 47% for any adverse outcome and 61% for severe adverse outcomes.

When we did our analysis using a more restrictive definition for a confirmed congenital Zika virus infection (that is, sensitivity analysis by excluding placental Zika virus samples owing to potential maternal contamination), the results were similar to those of the main analysis for "any adverse outcomes" (relative risk 4.2, 2.7 to 6.0) and "severe adverse outcome" (5.4, 2.8 to 10.2).

On examination of individual symptoms (table 3), fetuses/newborns with a laboratory confirmed congenital Zika virus infection presented with more frequent jaundice (19/76 (25%) *v* 20/215 (9%)), hypotonia (10/76 (13%) *v* 11/215 (5%)), hypertonia (5/76 (7%) *v* 3/215 (1%)), and swallowing dysfunction (4/76 (5%) *v* 1/215 (0.5%)) than did those that tested negative for Zika virus. In newborns with measurements available, head circumference below 2 standard deviations (that is, microcephaly) was observed in 10% (27/273) of newborns and four newborns presented with a head circumference below 3 standard deviations. The rate of microcephaly was similar between fetuses/neonates with a laboratory confirmed congenital Zika virus infection and those who tested negative (8/61 (13%) *v* 19/212 (9%) for head circumference <2 SD). Biological parameters measured in newborns with a laboratory confirmed congenital Zika virus infection (table 3) identified an increased incidence of anaemia (17/57 (30%) *v* 8/192 (4%) for haemoglobin <140 g/L) and elevated liver enzymes (32/52 (62%) *v* 40/155 (26%) for aspartate aminotransferase >50 U/L). Other biological parameters, such as thrombocytopenia often associated with other congenital infections, did not differ between the groups.

Discussion

In this paper, we have presented the results of fetal/neonatal testing and early neonatal outcomes for 291 fetuses/newborns of mothers infected with Zika virus. All fetal/neonatal outcomes were reviewed independently and blindly for Zika virus status. Maternal-fetal transmission was documented in 26% of fetuses/newborns and was significantly associated with "severe adverse outcomes."

Comparison with other studies

In our cohort, only 13% (approximately one in eight) of all fetuses/newborns born to mothers positive for Zika virus presented with "severe adverse outcomes." This is comparable to rates for other congenital infections, such as congenital cytomegalovirus. Maternal-fetal transmission rates of congenital cytomegalovirus are estimated to be 30-35% in cases of maternal primary infection. Among fetuses infected with cytomegalovirus, only 10-15% are estimated to present without signs/complications at birth.[22] This rate can be up to 30% when considering all observed anomalies and related terminations of pregnancy.[23] Our large cohort study with complete and comprehensive analysis of early neonatal outcomes including neonatal test results facilitates a well informed estimate of the burden of disease in countries with active Zika

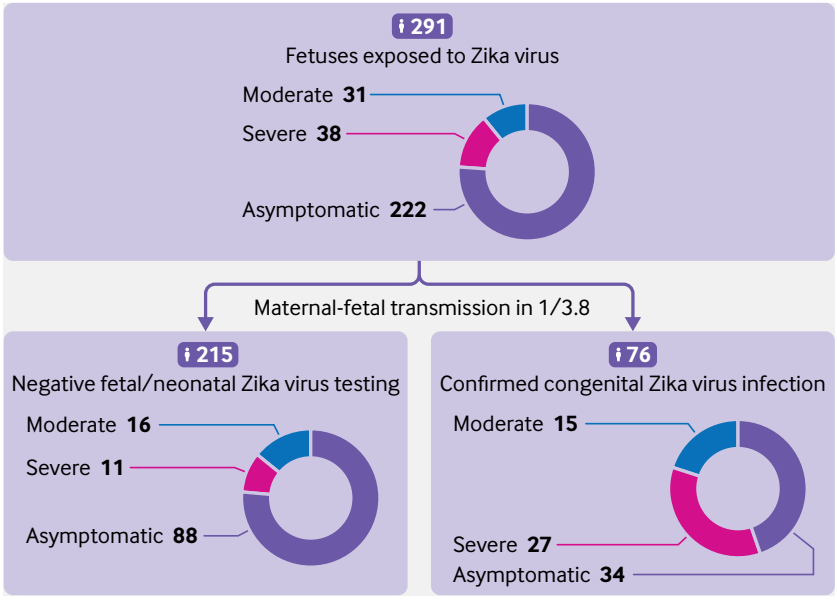

Fig 2 | Maternal-fetal transmission rate and primary fetal/neonatal outcomes. Outcomes and results of fetal/neonatal testing were available for 291 fetuses/newborns. The rate of maternal-fetal transmission was evaluated on the basis of fetal/neonatal testing. A confirmed congenital Zika virus infection was considered when either Zika virus RNA was amplified by real-time polymerase chain reaction from at least one fetal/neonatal sample (placenta, amniotic fluid, cerebrospinal fluid, urine, or blood) or when Zika virus specific IgM was identified in the umbilical cord/neonatal blood or in cerebrospinal fluid. Each case was reviewed by three independent reviewers, blinded to Zika virus status, and classified into four categories based on prenatal ultrasound findings, symptoms at birth, biological parameters, and postnatal transfontanellar ultrasound (see appendix 1). Discordant classifications were discussed between the three reviewers

virus circulation. Although large cohort studies have described neonatal outcomes,[5] including the recent report from the US Zika pregnancy and infants registry encompassing 2464 infants and the Zika-DFA study including 555 fetuses, the results of laboratory testing were not described.[6 24] Our results are congruent with another recent study performed in Brazil among 54 pregnant women with RT-PCR confirmed Zika virus infections, in which vertical transmission was documented in 18/51 (35%) newborns tested, whereas 15 (28%) newborns had mild/moderate signs. These included isolated ultrasound anomalies such as lenticulostriate vasculopathy or subependymal cysts, abnormal otoacoustic emissions, chorioretinitis, and intrauterine growth restriction; severe anomalies were not described.[25] The broader number of patients included in our cohort may have enabled the detection of more uncommon severe anomalies and provided a better estimation of maternal-fetal transmission. The rates of severe anomalies (9%) and pregnancy losses (4%) in our whole cohort of exposed pregnancies are similar to those reported in non-endemic countries; the US Zika pregnancy and infant registry describes rates of 5% for severe anomalies and 3% for pregnancy loss in exposed pregnancies.[6] Our results are concordant with the recent Zika-DFA study performed in a similar population, in which neurological defects and fetal losses were reported in 7% and 1% of 555 exposed fetuses, respectively (compared with 9% and 4% in our study).[24] Our study also considered clinical and biological aspects up to the first week of life, which may have increased the rate of severe outcomes.

In our study, the most common clinical symptom reported was jaundice, and neonates with a laboratory confirmed Zika virus infection had a moderate elevation of aspartate aminotransferase. Although it was initially believed that Zika virus is not associated with systemic manifestations, mild anaemia, cholestasis, and a moderate elevation of aspartate aminotransferase have been previously described in infected fetuses.[26] Furthermore, a transient hepatitis, with spontaneous resolution at 4 months of age, has been described in a peripartum infected newborn in French Polynesia.[27] This suggests that transient liver damage might be part of a moderate CZS, similarly to what is known for congenital cytomegalovirus.[17] We observed more frequent neurological impairment (hypotonia, hypertonia, and swallowing dysfunction) among fetuses/newborns with a laboratory confirmed congenital Zika virus infection than in those that tested negative. Brainstem dysfunction, manifested by absence of sucking and swallowing, have also been described by others,[28] even in newborns without microcephaly or severe cerebral radiological anomalies.[29] Thus, newborns from mothers exposed to Zika virus during their pregnancy should be systematically screened for dysphagia and other subtle neurological impairments, even in the absence of neuroimaging findings.

Strengths and limitations of study

Our study has several limitations. First of all, information about the sensitivity and specificity of neonatal testing is limited.[1] In particular, several

Table 2 | Fetal/neonatal outcomes. Values are numbers (percentages, 95% confidence intervals)

Outcomes	Laboratory confirmed cZIKV infection (n=76)	Negative fetal/neonatal ZIKV testing (n=215)
Asymptomatic	34 (45, 34 to 56)	188 (87, 82 to 91)
Any adverse outcomes	42 (55, 44 to 66)	27 (13, 9 to 18)
Mild/moderate signs	15 (20, 12 to 30)	16 (7, 5 to 12)
Severe adverse outcomes	27 (36, 26 to 47)	11 (5, 3 to 9)
Severe complications	16 (21, 13 to 33)	10 (5, 3 to 8)
Fetal loss	11 (14, 8.3 to 24)	1 (0.5, 0.1 to 3)

Outcomes and results of fetal/neonatal testing were available for 291 fetuses/newborns. Prenatal and postnatal imaging, postnatal examination, and sample collection were realised in the Centre Hospitalier de l'Ouest Guyanais (prenatal diagnosis, maternity and paediatric units). real-time polymerase chain reactions and serologies were performed in the national reference centre of arboviruses, Pasteur Cayenne. Each case was reviewed and classified by three independent reviewers blinded to Zika virus status (Materno-fetal and Obstetrics Research Unit, Centre Hospitalier Universitaire Vaudois). Discordant classifications were discussed between the three reviewers.
cZIKV=congenital Zika virus.

Table 3 | Secondary fetal/neonatal outcomes. Values are numbers (percentages) unless stated otherwise

	Laboratory confirmed cZIKV infection (n=76)		Negative fetal/neonatal ZIKV testing (n=215)	
Details of clinical outcomes	**No (%) or median (IQR)**	**95% CI**	**No (%) or median (IQR)**	**95% CI**
Median (IQR) gestational age at delivery, weeks	38.1 (35.3-39.4)	37.6 to 39.0	38.4 (37.6-39.3)	38.2 to 38.6
Gestational age <37 weeks at delivery*	8/62 (13)	6.7 to 23.4	24 (11)	7.6 to 16.1
Biometry				
Median (IQR) birth weight*, g	2970 (2630-3330)	2865 to 3120	3035 (2780-3432)	3010 to 3129
Birth weight <P3*	1/59 (2)	0.3 to 9.0	5/196 (3)	1.1 to 5.8
Birth weight <P10*	7/59 (12)	5.9 to 22.5	19/196 (10)	6.3 to 14.6
Head circumference <2 SD*	8/61 (13)	6.8 to 23.8	19/212 (9)	5.8 to 13.6
Head circumference <3 SD*	2/61 (3)	0.9 to 11.2	2/212 (1)	0.3 to 3.4
Clinical examination				
Jaundice	19 (25)	16.6 to 35.8	20 (9)	6.1 to 13.9
Hepatomegaly	5 (7)	2.8 to 14.5	5 (2)	1.0 to 5.3
Hypotonia	10 (13)	7.3 to 22.5	11 (5)	2.9 to 8.9
Hypertonia	5 (7)	2.8 to 14.5	3 (1)	0.4 to 4.0
Swallowing dysfunction	4 (5)	2.1 to 12.8	1 (0.5)	0.1 to 2.6
Biological parameters—No/No tested (%)				
C reactive protein >10 mg/L	6/53 (11)	5.3 to 22.6	13/191 (7)	4.0 to 11.3
Haemoglobin <140 g/L	17/57 (30)	19.5 to 42.7	8/192 (4)	2.1 to 8.0
Thrombocytes <150 g/L	7/57 (12)	6.1 to 23.2	9/194 (5)	2.5 to 8.6
Thrombocytes <100 g/L	0/57 (0)	0.0 to 6.3	4/194 (2)	0.8 to 5.2
Median (IQR) total bilirubin, mmol/L	180 (134-230)	162 to 209	172 (145-184)	165 to 178
Severe hyperbilirubinaemia†	4/47 (9)	3.3 to 19.9	9/161 (6)	3.0 to 10.3
Aspartate aminotransferase >50 U/L	32/52 (62)	48.0 to 73.5	40/155 (26)	19.6 to 33.2
Aspartate aminotransferase >100 U/L	6/52 (12)	5.4 to 23.0	8/155 (5)	2.6 to 9.8
Alanine aminotransferase >50 U/L	1/52 (2)	0.3 to 10.1	2/155 (1)	0.3 to 4.6

cZIKV=congenital Zika virus; IQR=interquartile range.
*Live births.
†Defined as plasma bilirubin concentrations requiring treatment: >320 µmol/L in infant >35 weeks' gestation and >2500 g, >200 µmol/L in infant >35 weeks' gestation and <2500 g, or >200 µmol/L in preterm infant <35 weeks' gestation.

studies have shown the progressive disappearance of Zika virus RNA in the maternal-fetal compartments (fetal and maternal blood, amniotic fluid, and neonatal blood and urine).[26 30] In contrast to cytomegalovirus, which may be detected for several months in the urine of congenitally infected newborns, Zika virus RNA was rarely detected in urine samples (7/76; 9%). Although the sensitivity of amniocentesis seems to be limited in cases of congenital Zika virus infection,[26] it may help to diagnose early fetal infections but was only performed in 12 cases when prenatal ultrasound scans were suggestive of congenital infection. In that context, we cannot exclude false negative results. Of note, severe complications compatible with CZS were observed in 10 (5%) newborns without laboratory evidence of Zika virus infection; either we were not able to detect Zika virus in these cases or other aetiologies may have induced similar complications (the rate of brain anomalies in the general population is estimated to be 3%).[31] The high number of fetuses/neonates with negative results that underwent multiple Zika virus neonatal tests (80% (171/215) had at least three different samples tested) ensures a low probability of false negative results.

Furthermore, we considered placental and umbilical cord samples in the diagnosis of congenital Zika virus infection, which may be questionable owing to the risk of maternal contamination of these samples.[15] Nevertheless, the risk of false positive results due to maternal contamination seems to be low in this study. Zika virus status based on umbilical cord blood samples was confirmed at day three of life in all but four neonates. Additionally, we previously detected Zika virus RNA and specific IgM in placental and fetal umbilical cord samples in seven of eight cases with a laboratory confirmed congenital Zika virus infection, even when maternal blood and urine were negative.[26] When we excluded placental samples from our analysis, maternal-fetal transmission was documented in 18% (52/282) cases, of which 33% (17/52) had severe complications at birth. Association between a laboratory confirmation of congenital Zika virus infection and outcomes did not change in our sensitivity analysis.

Secondly, our study focuses on immediate neonatal outcomes. In congenital cytomegalovirus, as many as 13.5% of newborns who present with no signs/complications at birth will subsequently develop permanent sequelae, such as motor, cognitive, or vision impairment and sensorineural hearing loss.[32] Study of developmental milestones and visual and auditory capacity in exposed fetuses will be important. These might be difficult to monitor owing to the lack of follow-up, particularly in newborns with no symptoms. Furthermore, our postnatal radiological analysis was based on transfontanellar ultrasound scans, for which the sensitivity for central nervous system anomalies is lower than for magnetic resonance imaging or computed tomography scanning for calcifications and skull anomalies. The closest magnetic resonance imaging scanner was located 300 km away and was therefore not available for this study. Similarly, computed tomography scanning was not routinely available owing to the limited resources of our radiological unit.

When performed, it was often done after the first week of life and therefore not included here. We cannot exclude the possibility that some mild anomalies were not identified. In this study, nine newborns with severe complications had no anomalies identified on prenatal ultrasound scans. Overall, we recognise that several authors have proposed a broader definition of CZS,[33 34] based on both advanced techniques (magnetic resonance imaging, computed tomography scanning) and specialised evaluation (ophthalmologist, infectious diseases specialist, and neurologist specialised in paediatrics). Such evaluations are not routinely available in French Guiana, explaining why fundoscopy and results of auditory testing are not described in this paper. We therefore developed a definition of complications compatible with CZS based on specific and non-specific characteristics for congenital Zika virus and TORCH infections observable up to the first week of life, adapted to the local medical capacities of our hospital. This classification might be more applicable in hospitals in low resource settings, often present in tropical regions, at risk of emergence and re-emergence of Zika virus.

Thirdly, conclusions about the impact of the timing of infection on maternal-fetal transmission are difficult to establish as the diagnosis of maternal infection reported here may have occurred much later than the actual maternal infection. Thus, we could not assess the association between trimester of infection and outcomes. The recruitment of infected pregnant women occurred at the time of their first ultrasound scan performed at the prenatal diagnosis unit and was therefore not conducive to evaluation of early fetal consequences of maternal Zika virus infection before 12 weeks' gestation. The rate of early miscarriages, some of them occurring in unrecognised pregnancies or at home without hospital consultation, is thus difficult to determine and was not the focus of our research. Furthermore, as we excluded pregnant patients for whom the diagnosis of Zika virus infection was done at delivery, because of the lack of specific follow-up during pregnancy and early postnatal life, our results cannot provide information on the consequences of late infection in pregnancy.

Finally, our study aimed to describe the burden of congenital Zika virus infection in an epidemic population with a high birth rate and limited access to invasive testing. We cannot exclude the possibility that some of the signs observed were unrelated to congenital Zika virus infection; as illustrated by the population attributable fraction, a confirmed congenital Zika virus infection contributes to only 47% of adverse outcomes and 61% of severe adverse outcomes observed here. Some maternal information may have been missed, and invasive testing and complete genetic analyses were not systematically performed (for evident ethical reasons). Nevertheless, potential missing information or additional diagnoses not reported would result in an overestimation of the burden of congenital Zika virus infection observed in this cohort.

Selection bias is expected to be limited, as basic maternal characteristics were similar between patients included in this study and the whole obstetric population delivering at the CHOG in 2016 (see appendix 3). Considering these results when counselling potentially exposed couples living in tropical areas at risk of emergence and re-emergence of Zika virus therefore seems reasonable.[35]

Conclusions

Our study provides a large comprehensive description of maternal-fetal transmission rates of Zika virus, as well as the burden of congenital infection, during the recent Zika virus epidemic in French Guiana. Despite significant maternal-fetal transmission, the burden of disease seems to be lower than initially suspected and might not differ from those of other well know congenital infections. Although caution is needed, our results suggest that in cases of maternal Zika virus infection, approximately one in four fetuses will become congenitally infected, of which one in three will be affected by severe complications at birth or fetal loss. The population attributable fraction estimates that a confirmed congenital Zika virus infection contributes to 47% of adverse outcomes and 61% of severe adverse outcomes observed. This information will help healthcare providers conducting parental counselling.

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Contributors: LP, VL, CP, AJ, GC, MV, and DB conceived and designed the study. LP, VL, CP, GC, and NH provided care to mothers and prospectively collected the clinical data and samples. LP and CP collected data on neonatal outcomes. GM and GB, as fetal central nervous system experts, contributed to the interpretation of sonograms and the management of congenital Zika virus syndrome cases. DR and SM did all the viral investigations. AP, MV, and DB interpreted the results, did the literature review, and provided critical inputs to the paper. LP, MV, and DB wrote the first version of the report, and all authors critically reviewed and approved the final

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Ethical approval: The study protocol was approved by the institutional review board of the Centre Hospitalier de l'Ouest Guyannais (CHOG). Patients included in the study provided written informed consent after discussing the objectives of the study.

Data sharing: Technical appendix and statistical code are available from the corresponding author at david.baud@chuv.ch.

Transparency declaration: The corresponding author affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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Appendix 1-4

Paper 3

Placental infection by Zika virus in French Guiana

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Placental infection by Zika virus in French Guiana

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KEYWORDS: congenital infection; placental pathology; placentomegaly; ultrasound; Zika

CONTRIBUTION

What are the novel findings of this work?
Vertical transmission of Zika virus (ZIKV) is not systematic and does not always lead to placental dysfunction with associated adverse outcome. This original research presents the placental findings on prenatal ultrasound and anatomopathological examination in a large cohort of ZIKV-infected placentae and controls.

What are the clinical implications of this work?
ZIKV-infected placentae exhibit a higher risk of placentomegaly and pathological anomalies than do non-infected placentae, and early placentomegaly may represent the first sign of congenital ZIKV infection. Identification of placentomegaly could be particularly useful in low-income countries in which access to tertiary centers may be restricted.

ABSTRACT

Objectives *To describe placental findings on prenatal ultrasound and anatomopathological examination in women with Zika virus (ZIKV) infection, and to assess their association with congenital ZIKV infection and severe adverse outcome, defined as fetal loss or congenital Zika syndrome (CZS).*

Methods *This was a prospective study of pregnancies undergoing testing for maternal ZIKV infection at a center in French Guiana during the ZIKV epidemic. In ZIKV-positive women, congenital infection was defined as either a positive reverse transcription polymerase chain reaction result or identification of ZIKV-specific immunoglobulin-M in at least one placental, fetal or neonatal sample. Placental ZIKV-infection status was classified as non-exposed (placentae from non-infected women), exposed (placentae from ZIKV-infected women without congenital infection) or infected (placentae from ZIKV-infected women with proven congenital infection). Placentae were assessed by monthly prenatal ultrasound examinations, measuring placental thickness and umbilical artery Doppler parameters, and by anatomopathological examination after live birth or intrauterine death in women with ZIKV infection. The association of placental thickness during pregnancy and anatomopathological findings with the ZIKV status of the placenta was assessed. The association between placental findings and severe adverse outcome (CZS or fetal loss) in the infected group was also assessed.*

Results *Among 291 fetuses/neonates/placentae from women with proven ZIKV infection, congenital infection was confirmed in 76 cases, of which 16 resulted in CZS and 11 resulted in fetal loss. The 215 remaining placentae from ZIKV-positive women without evidence of congenital ZIKV infection represented the exposed group. A total of 334 placentae from ZIKV-negative pregnant women represented the non-exposed control group. Placentomegaly (placental thickness > 40 mm) was observed more frequently in infected placentae (39.5%) than in exposed placentae (17.2%) or controls (7.2%), even when adjusting for gestational age at diagnosis and comorbidities (adjusted hazard ratio (aHR), 2.02 (95% CI, 1.22–3.36) and aHR, 3.23 (95% CI, 1.86–5.61), respectively), and appeared earlier in infected placentae. In the infected group, placentomegaly was observed more frequently in cases of CZS (62.5%) or fetal loss (45.5%) than in those with asymptomatic congenital infection (30.6%) (aHR, 5.43 (95% CI, 2.17–13.56) and aHR, 4.95 (95% CI, 1.65–14.83), respectively). Abnormal umbilical artery Doppler was observed more frequently*

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in cases of congenital infection resulting in fetal loss than in those with asymptomatic congenital infection (30.0% vs 6.1%; adjusted relative risk (aRR), 4.83 (95% CI, 1.09–20.64)). Infected placentae also exhibited a higher risk for any pathological anomaly than did exposed placentae (62.8% vs 21.6%; aRR, 2.60 (95% CI, 1.40–4.83)).

Conclusions *Early placentomegaly may represent the first sign of congenital infection in ZIKV-infected women, and should prompt enhanced follow-up of these pregnancies.*

INTRODUCTION

The recent worldwide Zika virus (ZIKV) epidemic has confirmed vertical transplacental transmission of ZIKV and its association with congenital anomalies, in particular severe central nervous system (CNS) lesions[1–3].

In congenital viral infections, the placenta is the major route of maternal–fetal transmission, through which the virus spreads during maternal viremia[4,5]. Viral replication in the placenta could impair vascular remodeling and cause fibrosis, leading to placental dysfunction and reducing maternal–fetal circulation[6–8]. A recent *in-vivo* study on non-immune pregnant primates infected by ZIKV demonstrated a robust maternal–placental–fetal inflammatory response associated with abnormal oxygen transport within the placenta[9]. Placentitis and placentomegaly have also been described in human placentae infected by ZIKV[10–12]. Studies reporting on the features of congenital ZIKV infection have described both CNS and non-CNS lesions, which could be a consequence of placental infection with or without fetal infection[3,13,14].

Similar to other TORCH (toxoplasmosis, other, rubella, cytomegalovirus, herpes simplex virus) group infections, vertical transmission of ZIKV is not systematic and does not always lead to placental dysfunction with associated adverse outcome. In a previous study investigating the rate of maternal–fetal infection, we demonstrated transplacental infection by ZIKV in 76/291 (26.1%) fetuses/neonates born to infected mothers, of which 27 (35.5%) exhibited severe adverse outcome (11 fetal losses and 16 cases of a severe CNS anomaly suggestive of congenital Zika syndrome (CZS))[15].

The aim of this study was to describe placental findings on prenatal ultrasound and anatomopathological examination in women with ZIKV infection, and to assess their association with congenital ZIKV infection and severe adverse outcome (fetal loss or CZS).

PATIENTS AND METHODS

Study population

Details of the study protocol are described elsewhere[14,15]. Briefly, the study was conducted at the French Guiana Western Hospital Center (Centre Hospitalier de l'Ouest Guyanais (CHOG)) at the beginning of the ZIKV epidemic from 1 January to 15 July 2016. Initial inclusion of ZIKV-infected pregnant women and controls occurred either through routine serological testing (performed in each trimester of pregnancy and at birth) of all pregnant women admitted to the prenatal diagnosis unit of the CHOG or through serological and molecular testing in the presence of maternal symptoms. Molecular and serological testing were performed at the French Guiana National Reference Center for arboviruses (Pasteur Cayenne), using the Realstar Zika Kit (Altona Diagnostics GmbH, Hamburg, Germany) for reverse transcription polymerase chain reaction (RT-PCR), and in-house M antibody-capture enzyme-linked immunosorbent assay and micro-neutralizing assays for serological testing.

Patients were monitored according to the clinical standard of care in France, with the exception of prenatal ultrasound being performed monthly in patients who were positive for ZIKV, and two supplementary ultrasound examinations being performed in patients who were negative for ZIKV (at 26–28 weeks' gestation and 36–38 weeks' gestation), as recommended by the French authorities and others[16–18]. Data regarding demographic, medical and obstetric characteristics and possible risk factors for congenital diseases were collected prospectively. The study protocol was approved by the institutional review board of the CHOG.

Study groups

Pregnant women were defined as ZIKV positive based on either a positive RT-PCR result in blood and/or urine, or by the presence of ZIKV-specific immunoglobulin-M (IgM) after anti-ZIKV antibody detection in the blood, confirmed by a micro-neutralizing assay in cases of suspected coinfection with other arboviruses (expected to be minimal, as circulation of Dengue virus has been low in French Guiana since 2014).

Confirmed congenital ZIKV infection was defined as either ZIKV-RNA amplification by RT-PCR from at least one fetal/neonatal sample (placenta, amniotic fluid, cerebrospinal fluid, urine or blood) or identification of ZIKV-specific IgM in umbilical cord/neonatal blood or in cerebrospinal fluid.

Placental ZIKV-infection status was classified into three categories as follows: (1) non-exposed control placentae from women who tested negative for ZIKV up to delivery; (2) exposed placentae from women with proven ZIKV infection without reported congenital infection in the newborn; (3) infected placentae from women with proven ZIKV infection and proven congenital infection in the newborn.

Infected placentae were then classified into those resulting in CZS, fetal loss or asymptomatic congenital infection at birth.

Placental analysis

Sonographers, clinicians and pathologists were aware of the ZIKV status of the pregnant women, as well as

potential fetal malformations at the time of prenatal and postnatal placental examinations, but were unaware of the fetal virological status for ZIKV, which was defined after postnatal testing.

Prenatal placental examination

All prenatal ultrasound examinations were performed by two experienced sonographers (V.L., L.P.) using E8 and E10 Voluson ultrasound machines with transabdominal (RM6C) and transvaginal (RIC5-9-D) transducers (GE Healthcare, Zipf, Austria). Placental maximum vertical thickness was measured at each prenatal ultrasound examination, by longitudinal (non-oblique) scanning with the ultrasound probe and beam perpendicular to the chorionic plate. The measurements were performed avoiding areas of amniochorionic detachment and periods of uterine contractility. Placentomegaly was defined as a placental thickness greater than 40 mm[19,20]. Umbilical artery Doppler was performed on a free vertical loop of cord at each prenatal ultrasound examination in ZIKV-infected pregnant women. Umbilical artery resistance index (RI) was reported and correlated with gestational age. Abnormal umbilical artery Doppler was defined as a RI greater than the 95th percentile[21].

Postnatal placental examination

Routine examination of the placenta at the time of delivery was completed by a midwife and/or an obstetrician following live birth or intrauterine death (IUD). We intended to offer anatomopathological examination of the placenta to all patients with confirmed ZIKV infection during pregnancy, but during the peak of the epidemic this was limited owing to a lack of storage capacity, fixation material and availability of pathologists. The placentae were fixed in 10% buffered formalin for 48 h. Placental weight was measured and birth weight/placental weight ratio was calculated using the method of Thompson *et al.*[22]. Low and high birth weight/placental weight ratios were defined as a value $< 3^{rd}$ percentile and $> 97^{th}$ percentile, respectively (according to gestational age and sex)[22]. Samples from the umbilical cord, membrane and placental parenchyma, including the decidua and chorionic plates, were collected. Specific immunochemistry examination was offered for cases when CZS was identified prenatally.

Anatomopathological anomalies included abnormal birth weight/placental weight ratio, signs of placental inflammation (chorioamnionitis lesions, villitis and intervillositis, calcifications), infarcts, ischemic necrosis with fibrin deposits (INFD), thrombosis, leukocytic infiltration and Hofbauer cell hyperplasia (Appendix S1).

Covariates

Gestational age was recorded at each prenatal measurement of placental thickness and at the time of delivery (after live birth or IUD). Maternal comorbidities (diabetes, vascular pathologies, thrombophilia, severe anemia, lead poisoning, alcohol consumption, malnutrition, vitamin-K deficiency) or coinfections (TORCH group infections) and risk of aneuploidy > 1/250 were evaluated as effect modifiers for the association of placental thickness, Doppler abnormalities or pathological results with the ZIKV status of the placenta.

Statistical analysis

Baseline maternal and fetal/neonatal characteristics were compared according to the ZIKV status of the placenta. Categorical variables were compared using the chi-square or Fisher's exact test, while the distribution of continuous variables was compared using the Kruskal–Wallis test.

Placental thickness measurements were compared every month between the three groups (infected placentae, exposed placentae and controls) using ANOVA. The weekly incidence of placentomegaly (> 40 mm thickness) was compared between the three groups using Kaplan–Meier analysis, and the cumulative incidence was compared using the logrank test. The weekly incidence of placentomegaly in the infected group was also compared between those with CZS, fetal loss or asymptomatic congenital infection at birth, using the same statistical methods. Factors associated with the incidence of placentomegaly throughout pregnancy were evaluated using the Cox proportional hazards model. Factors associated with abnormal umbilical artery Doppler measurements or anatomopathological findings, including placental ZIKV status, were evaluated using a logistic regression model. Abnormal Doppler measurements and anatomopathological findings in the infected group were also compared between those with CZS, fetal loss or asymptomatic congenital infection at birth, using the same statistical methods. CIs for relative risks (RR) and hazard ratios (HR) were calculated using the exact method. When data are missing, denominators are presented. Statistical analysis was conducted using Stata version 14 (StataCorp., College Station, TX, USA).

RESULTS

From 1 January to 15 July 2016, a total of 1690 pregnant women were tested for ZIKV in the CHOG, of whom 498 had confirmed maternal ZIKV infection (Figure 1). Among 291 fetuses/neonates/placentae with available test results for ZIKV from mothers with proven infection, congenital ZIKV infection was confirmed in 76 (26.1%) cases, representing the infected group, of which 27/76 (35.5%) resulted in fetal loss ($n = 11$) or CZS ($n = 16$). The remaining 215 (73.9%) fetuses/neonates/placentae from infected mothers without evidence of congenital ZIKV infection represented the exposed group. A total of 334 fetuses/neonates/placentae from mothers who tested negative for ZIKV throughout their pregnancy represented the non-exposed control group.

No differences in baseline maternal characteristics and delivery parameters were observed between the three study groups (Table 1). Fetal loss was more prevalent in cases of congenital infection than in exposed placentae and controls (14.5% *vs* 0.5% and 1.2%; $P < 0.001$). The timing of diagnosis of maternal ZIKV infection was similar between infected and exposed placentae.

Association between prenatal ultrasound findings and placental ZIKV status

Placental thickness was measured at least once during pregnancy, with a median number of four, four and three measurements in infected, exposed and control placentae, respectively. Infected placentae tended to be thicker than exposed placentae between 18 and 22 weeks' gestation ($P = 0.05$) and were significantly thicker after 26 weeks ($P < 0.001$). Similarly, infected placentae were significantly thicker than control placentae after 18 weeks ($P < 0.001$) (Figure 2). The kinetics of placental thickness according to gestational age, between control, exposed and infected placentae is shown in Figure S1.

Placentomegaly (> 40 mm thickness) was observed more frequently in infected placentae (39.5%) (Figure S2) than in exposed placentae (17.2%) or controls (7.2%), even when adjusting for gestational age at diagnosis and comorbidities (2.20% *vs* 0.79% and 0.43% per week; adjusted HR (aHR), 2.02 (95% CI, 1.22–3.36); $P = 0.007$, and aHR, 3.23 (95% CI, 1.86–5.61); $P < 0.001$, respectively) (Figure 3). Placentomegaly was no more frequent in exposed placentae than in controls after adjusting for gestational age and comorbidities (aHR, 1.34 (95% CI, 0.78–2.29); $P = 0.288$). Placentomegaly appeared earlier in infected placentae (at a median of 30 weeks and as early as 18 weeks) compared with exposed placentae (at a median of 33 weeks and as early as 23 weeks) and with controls (at a median of 34 weeks and as early as 22 weeks) ($P < 0.001$).

When considering infected placentae, placentomegaly was observed more frequently in those from pregnancies

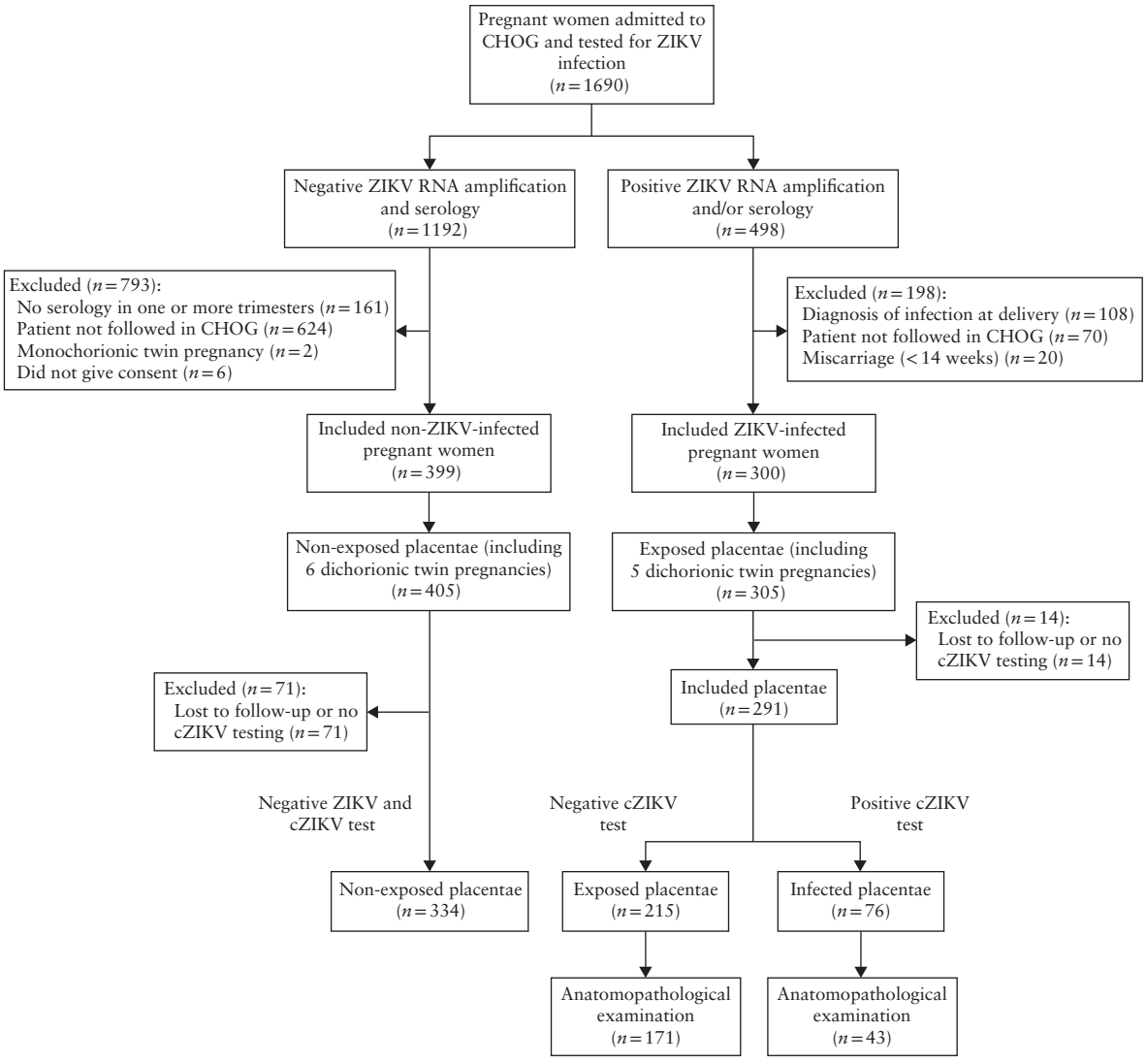

Figure 1 Flowchart summarizing inclusion of pregnancies undergoing testing for Zika virus (ZIKV) infection. CHOG, Centre Hospitalier de l'Ouest Guyanais; cZIKV, congenital ZIKV.

with CZS (10/16 (62.5%)) or fetal loss (5/11 (45.5%)) than in those from pregnancies with asymptomatic congenital ZIKV infection (15/49 (30.6%)), even when adjusting for gestational age at diagnosis and comorbidities (4.09% and 3.14% *vs* 1.48% per week; aHR, 5.43 (95% CI, 2.17–13.56); $P < 0.0001$, and aHR, 4.95 (95% CI, 1.65–14.83); $P = 0.004$, respectively) (Figure 4). Placentomegaly appeared earlier in placentae from pregnancies with CZS (as early as 18 weeks) or fetal loss (as early as 19 weeks) compared with those from pregnancies with asymptomatic congenital infection (as early as 26 weeks) ($P = 0.044$ and $P = 0.048$, respectively).

Table 1 Clinical characteristics of pregnancies that underwent testing for Zika virus (ZIKV) infection, according to maternal and placental ZIKV status

Characteristic	*Infected placenta* (n = *76*)	*Exposed placenta* (n = *215*)	*Controls* (n = *334*)
Maternal age (years)	27 (23–32)	28 (22–33)	28 (23–34)
Maternal age > 35 years	12 (15.8)	40 (18.6)	55 (16.5)
Maternal comorbidity*	21 (27.6)	42 (19.5)	79 (23.7)
Diabetes (chronic or gestational)	2 (2.6)	10 (4.7)	17 (5.1)
Vascular pathology	6 (7.9)	14 (6.5)	19 (5.7)
Thrombophilia	2 (2.6)	2 (0.9)	3 (0.9)
Anemia	4 (5.3)	4 (1.9)	11 (3.3)
Co-infection†	3 (3.9)	7 (3.3)	9 (2.7)
Lead poisoning	2 (2.6)	5 (2.3)	8 (2.4)
Alcohol consumption	1 (1.3)	1 (0.5)	7 (2.1)
Other‡	3 (3.9)	3 (1.4)	8 (2.4)
Dichorionic twins	1 (1.3)	3 (1.4)	6 (1.8)
Risk of fetal aneuploidy			
> 1/250	2 (2.6)	3 (1.4)	14 (4.2)
< 1/250	47 (61.8)	117 (54.4)	264 (79.0)
Late follow-up (>14 w)	27 (35.5)	95 (44.2)	56 (16.8)
Trimester of maternal infection diagnosis			
First	16 (21.1)	52 (24.2)	—
Second	44 (57.9)	111 (51.6)	—
Third	16 (21.1)	52 (24.2)	—
Number of prenatal US	4 [1–7]	4 [1–7]	3 [1–6]
Fetal loss	11 (14.5)	1 (0.5)	4 (1.2)
GA at fetal loss (weeks)	25 (18–32)	33	33 (28–35)
GA at delivery (weeks)	38 (35–39)	38 (37–39)	37 (35–39)
GA at delivery < 37 w	12/62 (19.4)	24/214 (11.2)	35 (10.5)
Birth weight < 3rd p	1/59 (1.7)	5/196 (2.6)	7 (2.1)
Fetal/neonatal findings suggestive of CZS	16 (21.1)	—	—

Data are given as median (interquartile range), *n* (%), median [range] or *n*/*N* (%). Infected placentae were from pregnancies with congenital ZIKV infection, exposed placentae were from those with maternal ZIKV infection without congenital ZIKV infection and control placentae were from those with no maternal or congenital ZIKV infection. *Some patients had multiple comorbidities. †Infected group: two active hepatitis-B and one HIV infection; exposed group: two primary toxoplasmosis, one human T-lymphotropic virus, one Coxsackie virus, two primary varicella-zoster virus (VZV) and one leptospirosis infection; controls: two primary toxoplasmosis, one primary cytomegalovirus, three HIV and three primary VZV infections. ‡Anti-Lea alloimmunization, malnutrition, vitamin-K deficiency, increased human chorionic gonadotropin levels, history of mucopolysaccharidosis. CZS, congenital Zika syndrome; GA, gestational age; p, percentile; US, ultrasound scans; w, weeks.

When considering placentomegaly > 40 mm as a predictor of congenital ZIKV infection, sensitivity was 39.5%, specificity was 88.8%, positive predictive value (PPV) was 32.3% and negative predictive value (NPV) was 91.3%. As a predictor for CZS, sensitivity was 62.5%, specificity was 86.7%, PPV was 11.0% and NPV was 98.9%. As a predictor of fetal loss related to congenital ZIKV infection, sensitivity was 45.5%, specificity was 86.0%, PPV was 5.5% and NPV was 98.9%.

Umbilical artery Doppler measurements were available for 75/76 infected placentae (not performed in a case of IUD at 18 weeks' gestation) and for all exposed placentae (Figure S3). Umbilical artery RI > 95th percentile tended to be more frequent in those with infected placentae than in those with exposed placentae, although not statistically significantly so (12.0% *vs* 6.0%, adjusted for maternal comorbidities: adjusted RR (aRR), 1.95 (95% CI, 0.85–4.19); $P = 0.0945$). When considering infected placentae, umbilical artery RI > 95th percentile was more frequent in those from pregnancies with fetal loss (3/10 (30.0%)) compared

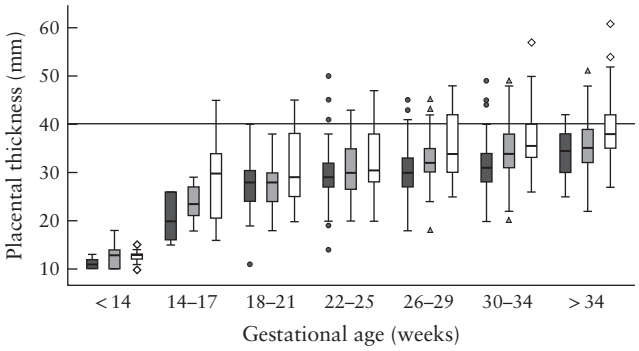

Figure 2 Box-and-whiskers plot showing prenatal thickness of placentae from pregnancies with congenital Zika virus (ZIKV) infection (□), placentae from pregnancies with maternal ZIKV infection without congenital ZIKV infection (▩) and control placentae (■), according to gestational age. Horizontal line indicates cut-off for placentomegaly (thickness > 40 mm). Boxes represent median and interquartile range (IQR), whiskers represent range excluding outliers more than 1.5 × IQR from upper or lower quartile, and circles, triangles and diamonds represent outliers.

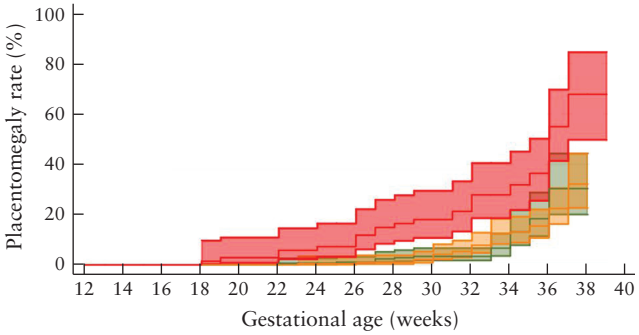

Figure 3 Kaplan–Meier plot showing probability of placentomegaly in placentae from pregnancies with congenital Zika virus (ZIKV) infection (——), placentae from pregnancies with maternal ZIKV infection without congenital ZIKV infection (——) and control placentae (——), according to gestational age. Shaded areas are 95% CI.

with those from pregnancies with asymptomatic congenital infections (3/49 (6.1%); aRR, 4.83 (95% CI, 1.09–20.64); $P = 0.0292$), but not compared with cases of CZS (3/16 (18.8%); aRR, 3.0 (95% CI, 0.60–13.09); $P = 0.13$).

Association between histopathological findings and placental ZIKV status

Pathological examinations were available for 43/76 (56.6%) infected placentae and 171/215 (79.5%) exposed placentae (Figure 1). No differences in baseline maternal characteristics or pregnancy outcome were observed between these two groups, apart from more frequent fetal loss in the infected group ($P < 0.001$) (Table S1). No differences in baseline maternal characteristics or pregnancy outcome were observed according to whether pathological examination was available (data not shown).

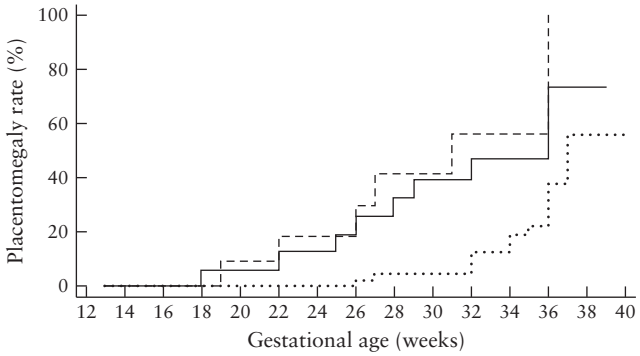

Figure 4 Kaplan–Meier plot showing probability of placentomegaly in placentae from pregnancies with congenital Zika virus infection that resulted in congenital Zika syndrome (——) or fetal loss (– – –) and in those with asymptomatic congenital infection (·····), according to gestational age.

Among infected placentae, 27/43 (62.8% (95% CI, 48.3–77.2%)) demonstrated pathological anomalies: five chorioamnionitis lesions, nine infarcts, 14 cases with INFD, eight cases with chronic villitis or intervillositis, two cases with subchorionic thrombosis and two calcifications. Leukocytic infiltration and Hofbauer cell hyperplasia were demonstrated by immunohistochemistry in two placentae of fetuses with CZS (following termination of pregnancy at 27 weeks and stillbirth at 33 weeks, respectively).

Among exposed placentae, 37/171 (21.6% (95% CI, 15.5–27.8%)) demonstrated anomalies on pathological examination: four chorioamnionitis lesions, 10 infarcts, five cases with INFD, five cases with chronic villitis or intervillositis, five cases with subchorionic thrombosis and eight calcifications.

Infected placentae exhibited a higher risk of any pathological anomaly than did exposed placentae (RR, 2.90 (95% CI, 2.01–4.19); $P < 0.0001$), even when adjusting for gestational age at delivery and comorbidities (aRR, 2.60 (95% CI, 1.40–4.83); $P = 0.002$). Frequencies of anatomopathological findings are presented in Table 2. Low birth weight/placental weight ratio was also more frequent in infected placentae than in exposed placentae (aRR, 2.78 (95% CI, 1.12–8.78); $P = 0.035$).

When considering infected placentae, pathological anomalies were no more frequent in those from pregnancies with CZS (8/12 (66.7%)) or fetal loss (8/10 (80.0%)) than in those from pregnancies with asymptomatic congenital infection (11/21 (52.4%)) after adjusting for gestational age at birth/IUD and comorbidities (aRR, 1.22 (95% CI, 0.66–2.17); $P = 0.42$ and aRR, 1.49 (95% CI, 0.82–2.48); $P = 0.14$, respectively).

Among infected placentae, positive RT-PCR of the placenta was found in 51/58 (87.9% (95% CI, 76.7–95.0%))

Table 2 Placental findings in pregnancies with maternal Zika virus (ZIKV) infection, according to placental ZIKV status

	Infected placenta (n = *76*)		*Exposed placenta* (n = *215*)	
Variable	n/N	*% (95% CI)*	n/N	*% (95% CI)*
Placentomegaly (thickness > 40 mm)	30/76	39.5 (28.5–50.5)	37/215	17.2 (12.2–22.3)
Umbilical artery RI > 95th percentile	9/75	12.0 (4.6–50.5)	13/215	6.0 (2.9–9.2)
Birth weight/placental weight ratio				
< 3rd percentile	7/43	16.3 (5.3–27.3)	10/171	5.8 (2.3–9.4)
> 97th percentile	4/43	9.3 (0.6–18.0)	14/171	8.2 (4.1–12.3)
Pathology findings	27/43	62.8 (48.3–77.2)	37/171	21.6 (15.5–27.8)
Chorioamnionitis lesions	5/43	11.6 (2.1–21.2)	4/171	2.3 (0.1–4.6)
Infarcts	9/43	20.9 (8.8–33.1)	10/171	5.8 (2.3–9.4)
Ischemic necrosis with fibrin deposits	14/43	32.6 (18.6–46.6)	5/171	2.9 (0.4–5.4)
Villitis and/or intervillositis	8/43	18.6 (7.0–30.2)	5/171	2.9 (0.4–5.4)
Chronic villitis with congestive capillaries	6/43	14.0 (3.6–24.3)	4/171	2.3 (0.1–4.6)
Subchorionic thrombosis	2/43	4.7 (1.6–10.9)	5/171	2.9 (0.4–5.4)
Calcifications	5/43	11.6 (2.1–21.2)	8/171	4.7 (1.5–7.9)
Leukocytic infiltration	2/43	4.7 (1.6–10.9)	0/171	—
Hofbauer cell hyperplasia	2/43	4.7 (1.6–10.9)	0/171	—
Positive RT-PCR of placenta	51/58	87.9 (76.7–95.0)	0/174	—
With placentomegaly	26/51	51.0 (37.3–64.7)	—	—
With umbilical artery RI > 95th percentile	6/51	11.8 (2.9–20.6)	—	—
With pathological findings	21/35	60.0 (43.8–76.2)	—	—

Infected placentae were from pregnancies with congenital ZIKV infection and exposed placentae were from those with maternal ZIKV infection without congenital ZIKV infection. RI, resistance index; RT-PCR, reverse transcription polymerase chain reaction.

placentae tested and was not associated with a higher risk for any placental pathology ($P = 0.80$) or low birth weight/placental weight ratio ($P = 0.69$).

DISCUSSION

Principal findings

In this study, ZIKV-infected placentae were thicker after 26 weeks' gestation, and placentomegaly was more prevalent and presented earlier in gestation, particularly in cases of fetal/neonatal adverse outcome (CZS and/or fetal loss), than in non-infected placentae. Infected placentae also exhibited a higher risk of anatomopathological anomalies than did non-infected placentae.

Limitations

While the accuracy of molecular and serological testing for maternal and congenital ZIKV infection is still under debate, we assessed ZIKV status at multiple time points and in numerous samples[15], thus reducing the risk of false-negative results. Non-infected pregnant women remained negative on ZIKV testing during all trimesters of pregnancy and at delivery[14]. The ZIKV status of infants born to ZIKV-positive and -negative pregnant women was assessed in multiple samples, including umbilical-cord and neonatal blood, placenta, urine, amniotic fluid and/or cerebrospinal fluid for symptomatic cases, limiting the risk of misclassification[15]. Misclassification would result in potential underestimation of the observed differences between the three groups. The risk of a false-positive ZIKV result was limited because pregnant women were tested in the early stage of the epidemic, without significant circulation of other *Flaviviridae* during this period, and possible cross-reactions were minimized using a micro-neutralizing assay[15].

Interpretation

Placentomegaly was observed in 39.5% of infected placentae and was associated significantly with congenital ZIKV infection. Low birth weight/placental weight ratio was also associated with congenital ZIKV infection. Placentomegaly identified on ultrasound within a few weeks after infection may be the consequence of placental inflammation due to recent maternal and transplacental infection[10]. The higher risk of low birth weight/placental weight ratio in the infected group is probably related to the increase in placental weight associated with placentomegaly, which could result from fibrinoid deposition and small vascularized villi that form to compensate for *in-utero* hypoxia, described recently in congenital ZIKV infection and other congenital infections[9,23]. Placentomegaly appeared earlier in the infected group than in the exposed and control groups, and may be an early sign of transplacental infection. Placentomegaly was observed in cases of both asymptomatic and symptomatic congenital infection. In fetuses with CZS, placentomegaly appeared after the fetal anomalies in one case, at the same time in two cases and before the appearance of CZS in seven cases (from 2 to 13 weeks before).

The relatively low rate of low birth weight/placental weight ratio compared with the rate of placentomegaly could also indicate that some of the instances of placentomegaly observed were transient and did not affect placental volume at birth. These transient cases of placentomegaly may be associated with other comorbidities and natural growth of the placenta, which may be why placentomegaly was identified in the third trimester in the exposed and control groups, and was thus unrelated to ZIKV. Abnormal umbilical artery Doppler measurements tended to occur more frequently in infected than exposed placentae, but to a lesser extent than placentomegaly. These results may indicate that placental infection resulting in placentomegaly does not always lead to placental dysfunction. However, abnormal umbilical artery Doppler associated with placentomegaly in infected placentae may predict an acute risk of fetal loss, even if the fetus does not present with growth restriction at diagnosis[24,25].

The sensitivity of placentomegaly in the prediction of congenital ZIKV infection is debatable, but is similar to that for other congenital infections[23]. The sensitivity is, however, higher when predicting CZS or fetal loss. The NPV of $> 90\%$ for congenital infection and of $> 98\%$ for adverse outcome of placental thickness < 40 mm may help to reassure pregnant patients in low-resource areas in which molecular and serological testing are not available during an epidemic peak. Moreover, placental thickness measurement by ultrasound is easy to perform and does not require advanced expertise, as compared with neurosonograms.

It is important to keep in mind that increased placental thickness is not specific to congenital ZIKV infection and is associated with other congenital infections, chromosomal and fetal abnormalities and maternal pathologies[26–29].

Compared to exposed placentae, infected placentae exhibited a 2.6-fold increased risk of any histopathological anomaly. Placental inflammation consisted of diffuse vascular inflammation, chorioamnionitis lesions, villitis and calcifications, as described in congenital cytomegalovirus (cCMV) infection[30]. It has been shown that ZIKV is able to specifically infect human placental macrophages and trophoblasts[31], but placental inflammation has been described inconsistently[11,32,33] and reported to occur only in the early stages of congenital infection[34]. Our study highlights a higher rate of INFD in ZIKV-infected placentae, which could increase placental growth, as in congenital syphilis infection[35]. Only excessive INFD was considered and was found in all cases of fetal loss; however, this finding does not seem to be specific to ZIKV infection, as it is also often found in fetal loss of other origin[36]. Subchorionic thrombosis and congestive capillaries, which have been described previously in congenital ZIKV infection, were observed more often in infected placentae, and could contribute to fetal

hypoxia[37]. We observed Hofbauer cell hyperplasia and leukocytic infiltration in ZIKV-infected placentae, which has been described by others as a specific finding in ZIKV-infected placentae[11,24,38].

Histopathological examination of the placenta highlights the potential long-term consequences of placental disorders during pregnancy, and the anomalies found may be the result of different pathologies impacting on the placenta[27]. We cannot exclude the possibility that some of the placental signs observed may be partially caused by other comorbidities; however, the similarities seen in maternal comorbidities and delivery characteristics between the exposed and infected groups point to a reduced influence of other factors. The main difference between these groups is the rate of fetal loss, but this may be due to the fact that fetal loss is a known consequence of fetal and placental infection by ZIKV[5,15].

Overall, ZIKV infection of the placenta precedes viral transmission to the fetus. We cannot exclude the possibility that ZIKV infection may be restricted to the placenta with no further involvement of the fetus, as described in some cases of CMV infection[39]. Placentomegaly may represent an early sign of congenital infection, before CZS or other adverse outcomes become apparent[40].

Conclusions

Our study provides a comprehensive description of the placental consequences of ZIKV infection. Infected placentae exhibited a higher risk for placentomegaly, low birth weight/placental weight ratio and histopathological anomalies. Although placentomegaly is non-specific for ZIKV infection, early placentomegaly may represent the first sign of congenital ZIKV infection and should lead to enhanced prenatal follow-up of ZIKV-exposed pregnancies.

DISCLOSURE STATEMENT

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Appendix S1 Definitions of placental anomalies

Figure S1 Non-parametric Lowess curves showing kinetics of placental thickness according to ZIKV-infection status.

Figure S2 Ultrasound image showing placentomegaly related to congenital Zika virus infection at 22 weeks' gestation.

Figure S3 Fetal growth, umbilical artery RI and kinetics of placental thickness, according to gestational age and ZIKV status of placenta. Estimated fetal weight was calculated using Hadlock 3 formula ($Log_{10}EFW = 1.326 - (0.00326 \times AC \times FL) + (0.0107 \times HC) + (0.0438 \times AC) + (0.158 \times FL)$) and 3rd percentile curve refers to INTERGROWTH-21st charts. Placentae associated with small-for-gestational-age (SGA) fetus (confirmed by birth weight) were no more frequent in cases of ZIKV infection (1/59 (1.7%)) compared with ZIKV-exposed cases (5/196 (2.6%)) or controls (7/334 (2.1%)). When considering thick placentae (> 40 mm), SGA fetuses were no more frequent in infected placentae (1/29 (3.4%)) compared with exposed placentae (2/37 (5.4%)) or controls (2/24 (8.3%)).

Table S1 Characteristics of placentae with anatomopathological examination available, according to placental ZIKV status

Paper 4

Prolonged maternal Zika viremia as a marker of adverse perinatal outcomes

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Prolonged Maternal Zika Viremia as a Marker of Adverse Perinatal Outcomes

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Whether prolonged maternal viremia after Zika virus infection represents a risk factor for maternal–fetal transmission and subsequent adverse outcomes remains unclear. In this prospective cohort study in French Guiana, we enrolled Zika virus–infected pregnant women with a positive PCR result at inclusion and noninfected pregnant women; both groups underwent serologic testing in each trimester and at delivery during January–July 2016. Prolonged viremia was defined as ongoing virus detection ≥30 days postinfection. Adverse outcomes (fetal loss or neurologic anomalies) were more common in fetuses and neonates from mothers with prolonged viremia (40.0%) compared with those from infected mothers without prolonged viremia (5.3%, adjusted relative risk [aRR] 7.2 [95% CI 0.9–57.6]) or those from noninfected mothers (6.6%, aRR 6.7 [95% CI 3.0–15.1]). Congenital infections were confirmed more often in fetuses and neonates from mothers with prolonged viremia compared with the other 2 groups (60.0% vs. 26.3% vs. 0.0%, aRR 2.3 [95% CI 0.9–5.5]).

The recent worldwide epidemic confirmed maternal–fetal transmission of Zika virus (ZIKV) and its association with adverse perinatal outcomes, particularly severe central nervous system lesions and fetal losses (*1*–*3*). Whether prolonged viremia after ZIKV infection in pregnant women represents a risk factor for maternal–fetal transmission, congenital Zika syndrome (CZS), or other adverse outcomes is on ongoing controversy (*4*).

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ZIKV is detectable in maternal blood by reverse transcription PCR (RT-PCR) during the acute phase of infection. ZIKV viremia usually lasts from 2 days before to 16 days after symptom onset; median time of ZIKV RNA clearance is 5 days (*5*). Driggers et al. (*6*) detected ZIKV RNA in maternal serum samples 8 weeks after onset of clinical symptoms; they suggested that prolonged viremia might occur as a consequence of viral replication in the fetus or placenta and might be correlated with CZS. In other reports, however, prolonged maternal viremia has been described in pregnant women with both normal and adverse fetal outcomes (*6*–*10*).

In a cohort of pregnant women exposed to ZIKV in French Guiana, we investigated the impact of prolonged viremia on fetal and neonatal adverse outcomes (fetal loss or neurologic anomalies) compared with infected pregnant women without prolonged viremia and noninfected pregnant women. We also compared the rates of congenital infections between these groups.

Methods

Study Population, Recruitment, and Follow-up

The study was conducted at the Centre Hospitalier de l'Ouest Guyanais (CHOG; Saint-Laurent-du-Maroni, in French Guiana) during January 1–July 15, 2016, at the beginning of the ZIKV epidemic. Persons for inclusion were initially identified either through routine serologic testing of all pregnant women admitted to the prenatal diagnosis unit of CHOG (performed in each trimester of pregnancy and at birth), or through serologic and molecular testing in cases of maternal symptoms, acute exposure in the previous 2 weeks (patients who arrived in French Guiana from a nonendemic country or patients who arrived from an

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endemic country [e.g., Brazil in January 2016] before the epidemic began in French Guiana), fetal or neonatal central nervous system anomalies, or in cases of amniocentesis performed for suspected CZS or other indications (i.e., aneuploidy diagnosis). All pregnant women with ZIKV testing available with ongoing follow-up at CHOG were invited to participate in the study. Written consent was obtained from all participants. The study received ethics approval from the CHOG institutional review board (*11*). Data regarding demographic, medical, and obstetrical characteristics and possible risk factors for congenital diseases were collected prospectively at inclusion.

Patients were monitored in accordance with the clinical standard of care in France, with the exception that prenatal ultrasound was performed monthly for patients who tested positive for ZIKV. Two supplementary ultrasounds were provided to patients who tested negative for ZIKV (at 26–28 and 36–38 weeks' gestation), as recommended by health authorities in France and other organizations (*12*–*15*). In cases of fetal loss (>14 weeks' gestation) or termination of pregnancy, a postmortem examination was offered, including macroscopic imaging and anatomic–pathologic examination.

In cases of live birth, a clinical examination (with particular attention to neurologic and systemic symptoms such as hypertonia, swallowing disorders, hypotonia, hepatomegaly, and jaundice) and testing for congenital ZIKV infection were performed for all neonates. Biologic, ophthalmologic, and imaging follow-up was offered for neonates from ZIKV-infected pregnant women (*16*).

Pregnant women not followed at the CHOG prenatal diagnosis unit after ZIKV testing, as well as patients who only delivered at CHOG without appropriate prenatal follow-up, were excluded from this study. Fetuses or neonates who did not undergo testing for ZIKV at birth or an appropriate postnatal or postmortem examination also were excluded.

Definition of Maternal ZIKV Infection

Molecular and serologic testing was performed at the French Guiana National Reference Center for arboviruses (Institut Pasteur of French Guiana, Cayenne, French Guiana) using the Realstar Zika Kit (Altona Diagnostics GmbH, https://altona-diagnostics.com) for RT-PCR, in-house IgM and IgG antibody-capture ELISA, and microneutralization assays for serologic testing. The limit of detection for serum samples tested using the Realstar Zika Kit was 0.61 (95% CI 0.39–1.27) copies/μL (*17*). A cycle threshold (C_t) value ≤37 was considered positive.

When ZIKV RNA was initially detected, molecular diagnosis was performed monthly and at delivery on maternal serum samples. Prolonged viremia was defined as ongoing viral detection ≥30 days after symptom onset or after initial detection of viremia in asymptomatic patients. Absence of prolonged viremia in infected patients was defined as a subsequent negative molecular test ≤30 days after symptom onset or after initial detection of viremia in asymptomatic patients.

In all cases, ZIKV serologic tests were performed in each trimester of pregnancy and at delivery. Patients with only positive IgM without RT-PCR testing or with a negative RT-PCR result were excluded from the analysis.

Maternal symptoms potentially related to ZIKV were recorded at each prenatal visit and at birth. These symptoms included rash, fever, asthenia, pruritus, arthralgia, retro-orbital headache, myalgia, conjunctival hyperemia, edema of the extremities, and neurologic complications (*18*). Asymptomatic pregnant women who remained ZIKV-negative on serologic tests during their pregnancy and at delivery were considered noninfected.

Definition of Fetal and Neonatal Adverse Outcomes and Congenital Infection

Fetal and neonatal outcomes were reviewed by 3 independent reviewers, including 2 maternal–fetal medicine specialists who had not been in contact with these patients previously. Cerebral anomalies were defined as ≥1 major cerebral sign, based on an extended definition of CZS (*14*,*19*,*20*) (Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/27/2/20-0684-App1.pdf). Fetal loss was defined as a spontaneous fetal demise at >14 weeks' gestation, including late miscarriages (14–24 weeks' gestation) and stillbirths (fetal demise >24 weeks' gestation up to intrapartum); intrapartum and early postpartum deaths were excluded. For the analysis, fetuses and neonates who had major cerebral anomalies, fetal losses, or both were categorized as having adverse outcomes. Termination of pregnancy for reasons other than major cerebral abnormalities were not considered as adverse outcomes in this analysis.

All fetuses and neonates underwent ZIKV testing at birth or after fetal loss. Prenatal testing by amniocentesis was offered in those with fetal anomalies, if an amniocentesis was indicated for other indications (i.e., aneuploidy diagnosis), or both. A confirmed congenital ZIKV infection was defined either by ZIKV RNA amplification by RT-PCR from ≥1 fetal or neonatal sample (e.g., placenta, amniotic fluid, cerebrospinal fluid, urine, or blood) or identification of ZIKV-specific

IgM in the umbilical cord or neonatal blood or in cerebrospinal fluid. Details of congenital ZIKV testing are discussed elsewhere (*19*).

Statistical Analyses

Standardized differences were calculated to compare baseline characteristics of patients with prolonged viremia to those of the reference groups (i.e., pregnant women with positive RT-PCR results without prolonged viremia and noninfected pregnant women). These characteristics were considered unbalanced when the standardized difference was >0.15.

Relative risks (RRs) and 95% CIs were calculated for fetal and neonatal adverse outcomes by using generalized linear regression. Adjusted RRs (aRRs) were calculated for variables reflecting unbalanced baseline characteristics that could represent confounding factors (maternal age and maternal underlying conditions). When fetuses from mothers with prolonged viremia were compared with those from infected mothers without prolonged viremia, RRs were also adjusted for the trimester of maternal infection diagnosis. A robust SE option was used for twins in order to not affect the variance in considering twins as separate cases.

We conducted a sensitivity analysis to test the robustness of our findings, using different criteria for the diagnosis of maternal prolonged viremia. We compared fetuses and neonates from patients with stable or increasing quantitative PCR (qPCR) values between the inclusion and the first follow-up with those from mothers with decreasing qPCR values.

The missing data were considered to be random, and thus we performed a complete case analysis. All statistical analyses were conducted by using Stata 15 (StataCorp, https://www.stata.com).

Results

Recruitment and Maternal ZIKV Diagnosis

During January 1–July 15, 2016, a total of 1,690 pregnant women were admitted to CHOG and tested for ZIKV infection (Figure 1). Among 498 women with a positive test, 198 were not prospectively followed in the CHOG prenatal diagnosis unit (including 20 patients with early miscarriages, 70 who were followed elsewhere after initial diagnosis of ZIKV infection, and 108 with a diagnosis at delivery without appropriate prenatal follow-up). A total of 300 pregnant women (including 5 with dichorionic twin pregnancies) with a positive ZIKV test were monitored in the unit. Full fetal and neonatal testing and follow-up was available for 287 of them (including 4 with twin pregnancies). Among these 287 ZIKV-positive patients, 254 (including 3 with twin pregnancies) had ZIKV infection diagnosed only by positive IgM, without molecular testing or with negative molecular testing, preventing calculation of the start of viremia. These patients were excluded from the analysis. Positive molecular testing was found in 33 patients (including 1 with a twin pregnancy); 30 were positive by RT-PCR in maternal blood, 9 were positive by RT-PCR in urine samples. Among these ZIKV RNA–positive pregnant women, 14 (including 1 with a twin pregnancy) exhibited a prolonged viremia, whereas the other 19 became subsequently negative within 30 days. Details of positive molecular testing are presented in Appendix 1 Table 2 and the evolution of qPCR values for each positive patient in Appendix 2 Figure (https://wwwnc.cdc.gov/EID/article/27/2/20-0684-App2.xlsx).

During the same period, 399 pregnant women (including 6 with dichorionic twin pregnancies) with negative serologic test results for ZIKV were followed for routine scans at CHOG. Full maternal, placental, and neonatal testing was available for 326 of them (including 6 with twin pregnancies). These patients remained negative for ZIKV during the entire pregnancy and at delivery, and constituted the noninfected group. The recruitment process is summarized in Figure 1.

Baseline Characteristics of Participants

As shown in Appendix 1 Table 3, baseline characteristics were similar between pregnant women with prolonged viremia and the reference groups, except for a history of congenital abnormalities or intrauterine fetal demise, maternal underlying conditions, rate of dichorionic twins, and high risk for fetal aneuploidy (>1/250), for which standardized differences >0.15 were observed. Maternal ZIKV infections were diagnosed earlier in pregnancies with prolonged viremia than in those without prolonged viremia.

Fetal and Neonatal Adverse Outcomes and Congenital Infections

ZIKV testing and outcomes were available for 15 fetuses from 14 infected pregnant women with prolonged viremia (including 1 with a dichorionic twin pregnancy) (Appendix 1 Tables 4, 5). Two pregnancies (2/15 [13.3%]) were terminated for severe neurologic anomalies, and 2 fetal losses (2/15 [13.3%]) were recorded. Among fetuses with imaging studies and examinations available, 4 (4/14 [28.6%]) exhibited neurologic anomalies and 4 (4/11 [36.4%]) ocular anomalies (Appendix 1 Table 4). Congenital ZIKV infections were confirmed in 9 (9/15 [60.0%]) of these fetuses or newborns, of which 6 (6/9 [66.7%]) cases resulted in adverse outcomes (4 suspected CZS and 2

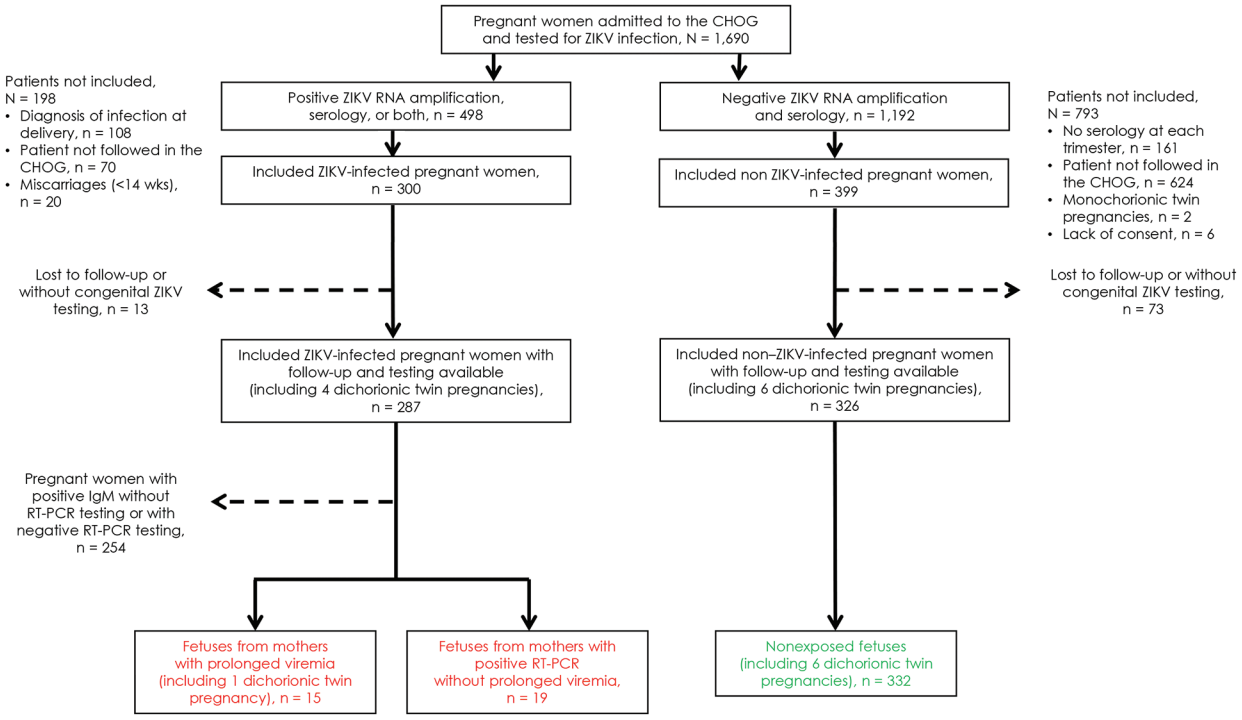

Figure 1. Flowchart of pregnant women admitted to CHOG, French Guiana, January 1–July 15, 2016. All women were routinely tested for ZIKV-specific IgM and IgG in each trimester of pregnancy and at delivery. In cases of maternal symptoms, acute exposure in the previous 2 weeks, fetal anomalies, or if an amniocentesis was indicated, pregnant women were also tested for ZIKV RNA by RT-PCR in blood and urine. Patients with a positive RT-PCR result were offered to participate in the study and underwent monthly RT-PCR testing up to clearance or delivery. Prolonged viremia was defined as ongoing viral detection ≥30 days after symptom onset or after initial detection of viremia. Asymptomatic patients who remained negative for ZIKV IgM during the whole pregnancy were recruited and considered as non–ZIKV-infected. Patients with only positive IgM without or with a negative RT-PCR test result were excluded of this analysis because of the inability to accurately date the onset and clearance of viremia. Patients without appropriate monthly follow-up were also excluded from this study (e.g., those who had early miscarriages, late diagnosis of infection at delivery, or were not followed in our center after the diagnosis). After expulsion, fetal losses were tested by RT-PCR (as well as by IgM, if available). Fetuses with anomalies were tested by RT-PCR on amniotic fluid. Neonates were tested for ZIKV at birth (RT-PCR on placenta, urine, blood and IgM on blood [as well as on cerebrospinal fluid, if symptomatic]). Fetuses and neonates without appropriate testing and examination after fetal loss or birth were excluded from this analysis. Overall, 15 fetuses from 14 infected pregnant women with prolonged viremia (including 1 with a twin pregnancy), 19 fetuses from 19 infected pregnant women without prolonged viremia, and 332 fetuses from 326 noninfected pregnant women (including 6 with twin pregnancies) were included. CHOG, Centre Hospitalier de l'Ouest Guyanais (Saint-Laurent-du-Maroni, French Guiana); RT-PCR, reverse transcription PCR; ZIKV, Zika virus.

fetal losses). All pregnancy outcomes for women with prolonged viremia are detailed in Figure 2. Figure 3 presents an example of CZS related to maternal prolonged viremia.

Among 19 fetuses from mothers with an initially positive RT-PCR result without prolonged viremia, no fetal loss or termination of pregnancy was recorded. Neurologic anomalies were found in 1 (1/19 [5.3%]) of these fetuses, who also had ocular anomalies (confirmed at birth). Congenital ZIKV infections were confirmed in 5 (5/19 [26.3%]) of these newborns, of which 1 (1/5 [20%]) resulted in adverse outcomes (suspected CZS).

ZIKV testing and outcomes were available for 332 fetuses or newborns from noninfected pregnant women (including 6 with dichorionic twin pregnancies). Four pregnancies (4/332 [1.2%]) resulted in termination of pregnancy (1 for severe neurologic anomalies and 3 for extraneurologic anomalies [congenital heart disease, skeletal dysplasia, and Down syndrome]). Four (4/332 [1.2%]) fetal losses were recorded. Among the fetuses with imaging studies and examination available, 17 (17/331 [5.1%]) had neurologic anomalies. None of these fetuses or neonates were found to be ZIKV-positive at birth.

Associations between Prolonged Viremia, Fetal and Neonatal Adverse Outcomes, and Congenital Infections

Overall, fetuses or neonates from mothers with a prolonged viremia during pregnancy exhibited a higher

risk for adverse outcomes (6/15 [40%] with fetal loss, neurologic anomalies, or both) compared with those from infected mothers without prolonged viremia (1/19 [5.3%], RR 7.6 [95% CI 1.0–56.5]) and noninfected mothers (20/332 [6.0%], RR 6.6 [95% CI 3.1–14.1]). Similar results were observed for fetal losses and neurologic anomalies when analyzed independently (Appendix 1 Table 4). After adjustment for maternal underlying conditions and considering the twin pregnancies in the variance, these associations and trends persisted (aRR 7.2 [95% CI 0.9–57.6] when compared with fetuses from infected mothers without prolonged viremia and aRR 6.7 [95% CI 3.0–15.1] when compared with fetuses from noninfected mothers). In the comparison with fetuses from infected mothers without prolonged viremia, the analysis was also adjusted for the trimester of maternal infection diagnosis (Appendix 1 Tables 4, 6).

Congenital infections were confirmed more frequently in fetuses from mothers with prolonged viremia (9/15 [60.0%]) when compared with those from infected mothers without prolonged viremia (5/19 [26.3%], RR 2.3 [95% CI 1.0–5.4]) and noninfected mothers (0/332 [0.0%]). After adjustment for the trimester of maternal infection diagnosis and consideration of twin pregnancies in the variance, this trend persists (aRR 2.3 [95% CI 0.9–5.5]) (Appendix 1 Tables 4, 6).

Our sensitivity analysis found similar results when considering the evolution of qPCR values between the inclusion and the first follow-up instead of prolonged viremia as a binary variable (Appendix 1 Table 7). Neurologic or systemic symptoms at birth were also more frequent in newborns from mothers with prolonged viremia compared with those from infected mothers without prolonged viremia or noninfected mothers (Appendix 1 Table 5).

Discussion

The main findings of this cohort study are 2-fold. First, maternal ZIKV infection with prolonged viremia is associated with a 7-fold increased risk for fetal or neonatal adverse outcomes compared with pregnancies without prolonged viremia. Second, maternal prolonged

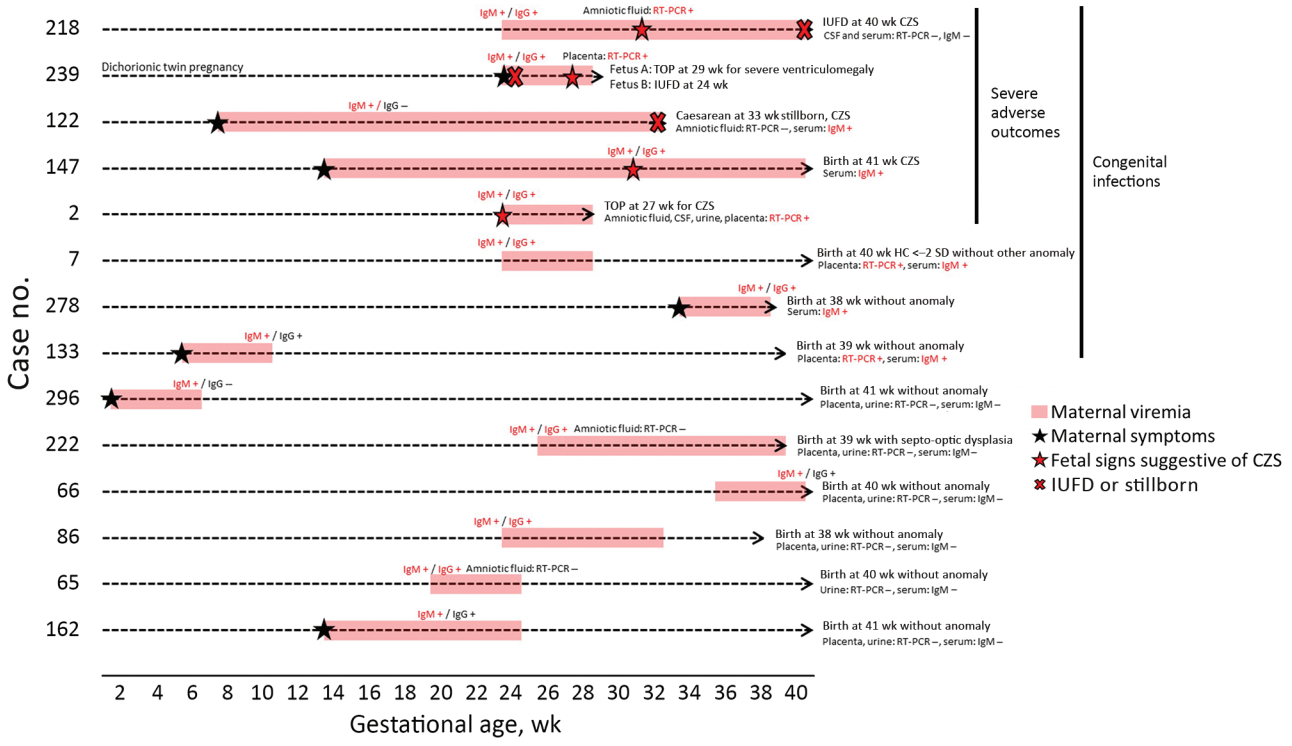

Figure 2. Pregnancy outcomes of patients with prolonged viremia in a cohort study of pregnant women admitted to Centre Hospitalier de l'Ouest Guyanais, French Guiana, January 1–July 15, 2016. Description and outcomes of 14 pregnancies with prolonged maternal ZIKV viremia (including 1 with dichorionic twin pregnancy). Congenital ZIKV infection was confirmed in 9/15 (60%) fetuses, and 6/15 (40%) fetuses had adverse outcomes. Four of them had multiple abnormalities consistent with CZS (1 live birth, 1 TOP, 1 IUFD, and 1 stillbirth). The 2 fetuses from the dichorionic twin pregnancy also had adverse outcomes, with an IUFD at 24 wk and a TOP for severe ventriculomegaly in the other fetus at 29 wk. CSF, cerebrospinal fluid; CZS, congenital Zika syndrome; HC, head circumference; IUFD, intrauterine fetal demise; RT-PCR, reverse transcription PCR; TOP, termination of pregnancy; ZIKV, Zika virus.

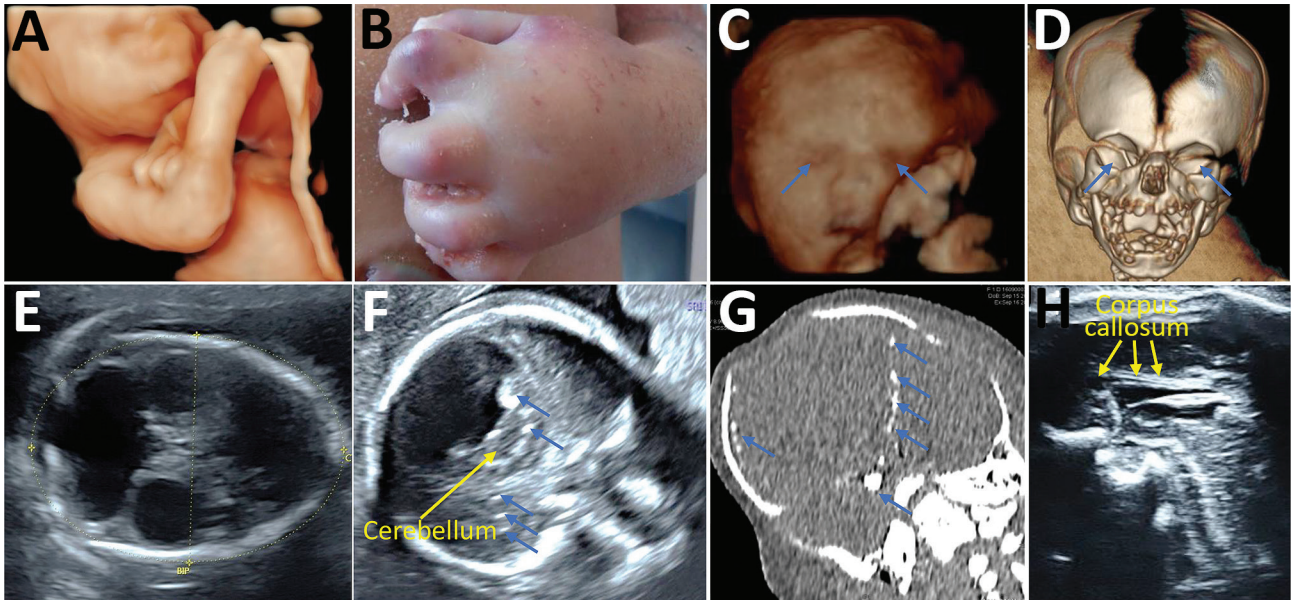

Figure 3. Prenatal ultrasound, computed tomography, and postmortem aspect of a fetus with congenital Zika syndrome related to maternal prolonged viremia in patient (case no. 122) in a cohort study of pregnant women admitted to Centre Hospitalier de l'Ouest Guyanais, French Guiana, January 1–July 15, 2016. The mother had symptomatic acute Zika virus infection at 8 weeks' gestation (and had ongoing viremia until birth of her stillborn child with signs of congenital Zika syndrome. Severe microcephaly, ventriculomegaly, and calcifications were detected by ultrasound at 13 weeks' gestation. Overall, this fetus had arthrogryposis detected on 3-D ultrasound (A) and postmortem (B); severe bilateral microphthalmia detected on 3-D ultrasound (C) and fetal computed tomography (D); microcephaly with atrophic cortex detected on ultrasound (E) showing a head circumference of 160 mm at 25 weeks' gestation (−5 SDs); ventriculomegaly detected on ultrasound (E); brain calcifications detected on ultrasound (F) and computed tomography (G); pontocerebellar hypoplasia detected on ultrasound (F); and corpus callosum dysgenesis detected on ultrasound (H). In panels F and G, blue arrows indicate intracerebral calcifications.

viremia is associated with a 2-fold increased risk for confirmed congenital infection compared with infected mothers without prolonged viremia.

Our study was limited by the sample size of the infected group because only patients with a positive ZIKV RT-PCR result at enrollment were included in the analysis and followed up with monthly RT-PCR testing until clearance or delivery. Because the measured effect size was high, the sample size was sufficient to identify an association. The limited number of cases with prolonged viremia, however, forced us to group all adverse outcomes together to conduct an analysis with sufficient power and prevented the evaluation of its association with individual signs or symptoms or new characteristics (*21*,*22*).

Although ZIKV testing was based on the previous guidelines relevant during the 2015–2016 epidemic with adaptation to local capacities, testing does not follow the more recent CDC guidelines (*23*) (i.e., patients considered as noninfected underwent serologic testing in each trimester rather than nucleic acid testing, as is currently recommended). Patients included as noninfected, however, remained negative for IgM and IgG in each trimester and at delivery, limiting the risk for exposure misclassification. Similarly, women with only positive IgM testing were excluded from the study because some of them might have had undetected prolonged viremia, which would have led to an exposure misclassification and an underestimation of the consequences of prolonged viremia.

Information about the sensitivity and specificity of neonatal testing remains limited, and several studies have shown the progressive disappearance of ZIKV RNA in the maternal–fetal compartments (*24*). Although the identification of IgM in fetal or neonatal blood was used to avoid congenital ZIKV false negatives, we cannot exclude an outcome misclassification because some neonates with negative results could have been infected by ZIKV without viremia and immunity against ZIKV detectable at birth. This risk is likely low given that >80% of fetuses or neonates from infected pregnant women underwent testing in ≥3 different samples (including blood, urine, placenta, cerebrospinal, and amniotic fluid) (*19*), and all neonates from noninfected pregnant women underwent serologic testing at birth. Undetected congenital infections in the 2 reference groups might result in overestimation of the effect of prolonged viremia overall.

Neonates from noninfected mothers underwent postnatal transfontanellar ultrasound (as well as by computed tomography and magnetic resonance imaging, if available) only in the case of an abnormal prenatal ultrasound or symptoms at birth, in contrast to those from infected mothers who underwent routine postnatal imaging. However, when they are asymptomatic, some neonates from noninfected mothers might have undetected cerebral anomalies at birth, resulting in an overestimation of the consequences of prolonged Zika viremia. We cannot totally exclude this bias; however, all neonates from noninfected mothers underwent multiple prenatal ultrasound assessments (enhanced by 2 supplementary examinations with neurosonograms during the epidemic), reducing the risk for undetected cerebral anomalies.

Molecular testing has been proposed for use at different stages of pregnancy depending on the presence of maternal symptoms. Thus, symptomatic patients might have had ZIKV infection diagnosed earlier than asymptomatic patients for whom a molecular diagnosis was proposed in cases of fetal anomalies, amniocentesis (for fetal signs or other indications [i.e., aneuploidy diagnosis]), or both, occurring later in pregnancy. Among infected pregnant women without prolonged viremia, 7 were asymptomatic with a continuous exposure and did not have molecular testing before amniocentesis, preventing accurate identification of the time of infection during pregnancy. Thus, we cannot exclude that some of these patients were in fact infected earlier in pregnancy and had an undetected prolonged viremia resulting in an exposure misclassification. This bias would result in an underestimation of the consequences of prolonged viremia because a case that included neurologic anomalies (CZS) in the reference group from infected mothers without prolonged viremia could in fact be related to prolonged viremia. Similarly, we cannot exclude a potential selection bias given that some patients were tested by RT-PCR because their fetus had anomalies at inclusion. Because this proportion did not differ between the groups (3/14 vs. 4/19), even if we consider only anomalies suggestive of fetal infection at inclusion (2/14 vs. 2/19), we would expect relative risks to be significantly affected. However, this potential selection bias could overestimate absolute frequencies of fetal anomalies in RT-PCR–positive patients, and this bias seems to be inherent in contemporary cohorts because inclusion after the observation of fetal anomalies potentially related to Zika were common.

Driggers et al. (*6*) were the first to highlight a possible association between prolonged maternal viremia and congenital infection with CZS. In their cohort study, Rodo et al. (*25*) described 9 cases of prolonged maternal viremia, among which 2 resulted in congenital ZIKV infection, with 1 of those 2 infections resulting in severe neurologic anomalies. The rates of congenital infection and fetal or neonatal adverse outcomes in women with prolonged viremia seem to be higher in our study (9/15 for congenital infections and 4/15 for CZS). This difference might be explained by the exclusive use of amniocentesis for diagnosis in the Rodo et al. cohort, whereas multiple fetal or neonatal samples were tested in our study (>80% of the fetuses or newborns had ≥3 different samples tested). Our results are congruent with Meaney et al. (*10*), who identified prolonged ZIKV RNA detection in 4 symptomatic pregnant women in the US Zika Pregnancy Registry, of which 1 pregnancy (25%) resulted in congenital Zika syndrome. Suy et al. (*8*) described a case of CZS with prolonged maternal viremia where the viral load in the maternal serum sample remained stable for 14 weeks and then became negative, instead of decreasing progressively, as would be expected. Suy et al. suggested that the prolonged viremia that was detected in the mother could be the result of viral replication in the fetus or placenta, which thus might act as a reservoir. However, their study still lacks a consensual threshold to define prolonged viremia. In our study, we defined prolonged viremia as ongoing viral detection ≥30 days after symptom onset or after initial detection of viremia for a question of feasibility. Indeed, many of our patients were living around the Maroni River, in isolated areas, and came monthly to CHOG for their clinical follow-up. In light of this geographic distance, we decided that monthly RT-PCR testing in case of initial detection of viremia was the most appropriate. In the context of a smaller area with local facilities, testing patients every 2 weeks to fulfill the threshold used in other studies might have been useful (*10*,*25*).

Negative and positive predictive values of prolonged maternal viremia for congenital infections and adverse outcomes related to ZIKV seem to be moderate because fetal and neonatal adverse outcomes and congenital infections also occur in pregnant women without identified prolonged viremia. One explanation could be that prolonged viremia might reflect viral replication in the placenta without further involvement for the fetus (*27*). In addition, some of our cases with prolonged maternal viremia (6/15) did not exhibit congenital infections, suggesting that prolonged maternal viremia might also reflect persistent viral replication in other reservoirs than the fetus or

the placenta. The study of Rodo et al. (*10*) and the CDC report (*25*) also described fetuses without congenital infection or adverse outcomes from mothers with prolonged viremia (*10*,*25*).

Our results also indicate that noninfected women exhibited a 5.1% risk for fetal neurologic anomalies and 1.2% risk for fetal losses (higher than the estimation of 3% risk for neurologic anomalies and 0.5%–1.0% risk for fetal losses in developed countries), reflecting that other etiologies for adverse perinatal outcomes remain present even in the context of a ZIKV epidemic (*28*), particularly in French Guiana, where pregnant women are exposed to lead poisoning, poverty, and higher risk for underlying conditions (*29*). To reduce the impact of these cofounding factors on our assessment of adverse neonatal outcomes related to prolonged ZIKV viremia, we chose to adjust the RR estimates for unbalanced maternal underlying conditions that might have an effect on the exposure and on adverse perinatal outcomes.

In conclusion, prolonged maternal ZIKV viremia could be a marker for an increased risk for maternal–fetal transmission and subsequent adverse perinatal outcomes. Even if prolonged maternal viremia is not consistently present in cases of congenital infection, it might reflect active viral replication in the fetal–placental compartment and should lead to an enhanced prenatal and neonatal follow-up.

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L.P., V.L., A.P., and D.B. conceived and designed the study. L.P., V.L., C.P., G.C., and N.H. provided care to mothers and prospectively collected the clinical data and samples. D.R. and S.M. did all the viral investigations. L.P., A.P., M.V., and D.B. interpreted the results, did the literature review, and provided critical inputs to the paper. L.P., A.P., and D.B. wrote the first version of the report, and all authors critically reviewed and approved the final version. The corresponding author attests that all listed authors meet authorship criteria, that no others meeting the criteria have been omitted, that this manuscript is an honest, accurate, and transparent account of the study being reported, and that no important aspects of the study have been omitted.

Individual participant deidentified data that underlie the results will be shared with researchers who provide a methodologically sound proposal for multicentric study, particularly individual participant data metaanalysis. Proposals should be directed to L.P. (leo.pomar@chuv.ch).

About the Author

Mr. Pomar was a midwife with a master degree in ultrasound and fetal medicine and a specialization in fetal brain imaging when he enrolled and followed these patients. He is now conducting this research to obtain a PhD degree at Lausanne University Hospital, Lausanne, Switzerland.

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Paper 5

Association between laboratory confirmed congenital Zika infection at birth and outcomes up to three years of life

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ASSOCIATION BETWEEN CONFIRMED CONGENITAL ZIKA INFECTION AT BIRTH AND OUTCOMES UP TO THREE YEARS OF LIFE

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ABSTRACT

Little is known about the long-term development of children diagnosed with congenital Zika infection at birth. In this cohort study, we correlate the outcomes up to three years of life of children exposed to Zika virus *in utero* with their laboratory results for congenital Zika infection testing at birth.

Among 129 newborns tested at birth, 18 (14%) had a laboratory confirmed congenital Zika infection. Infected neonates had a higher risk of adverse neonatal outcomes (death, structural brain anomalies or neurologic symptoms) than those who tested negative: 8/18 (44%) vs 4/111 (4%), aRR 10.1 [3.5-59.0]. During infancy, neurologic impairments, neurosensory alterations or delay in motor acquisitions were more common in infants with a congenital Zika infection at birth: 6/15 (40%) vs 5/96 (5%), aRR 7.5 [2.6-21.4]. Finally, infected children had also a higher risk to be referred for a suspected delay in neurodevelopment at three years of life: 7/11 (64%) vs 7/51 (14%), aRR 4.3 [1.8-9.9]. Infected infants without structural brain anomalies seem also to present increased risks of neurological anomalies but to a lesser extent. It seems paramount to offer a systematic testing for congenital ZIKV infection in case of *in-utero* exposure, and to adapt counseling on these results.

Key words: Zika virus, congenital Zika infection, prenatal diagnosis, neonatal outcomes, neurodevelopment.

INTRODUCTION

In the last decade, ZIKV has spread through the Pacific Islands[1] and the Americas[2], leading to a worldwide epidemic. It is now well demonstrated that ZIKV is associated with multiple congenital abnormalities, particularly affecting the central nervous system[3-5]. Long-term disabilities, including cerebral palsy, epilepsy and neuro-sensory alterations have been described in infants from the American and Brazilian cohorts[6-9]. Infants included in these cohorts were mostly affected by congenital Zika Syndrome (CZS) with cerebral anomalies or neurological impairments at birth. The evolution of those with a laboratory confirmed congenital infection but who were asymptomatic and without cerebral anomalies at birth remains poorly described. Overall, the correlation between laboratory testing for congenital ZIKV infection at birth and long-term disability including sensory and cognitive deficits is still lacking and may have major clinical and public health implications[10].

The French Guiana Western Hospital Center (CHOG, Centre Hospitalier “Franck Joly”, referral center for the western French Guiana), was confronted with the ZIKV epidemic from January to September 2016. In a previous study of the ZIKV-infected pregnant women cohort followed at the CHOG, we estimated that 279 infants were liveborn to these mothers[11].

In this paper, we aim to compare neonatal, 2-year infantile and 3-year neurodevelopment outcomes among children with a laboratory confirmed congenital Zika infection at birth with those of children tested negative.

RESULTS

Study population

Between January and September 2016, 132 newborns from 128 ZIKV-infected mothers (4 dichorionic twins) were enrolled for prospective follow-up at the pediatric clinic of the CHOG. Three of them were not tested for ZIKV before post-partum discharge and were excluded. Among 129 tested for ZIKV at birth, 18 (14.0%) had a laboratory confirmed congenital infection, and 111 (86.0%) tested negative (details of fetal and neonatal testing are presented in Appendix1, Figure 1 describes the enrollment).

Baseline characteristics

Median maternal age at delivery was 25 and 26 years old in the group of congenital infections and in the negative group, respectively. Infection in the 1st trimester of pregnancy was more frequent in mothers of infected newborns (38.9% vs 24.3%). Cesarean-sections occurred more often in these mothers (27.8% vs 12.6%). Twins were also more frequent in infected newborns (11.1% vs 5.4%). Alcohol consumption was more frequent in mothers of newborns tested negative for ZIKV at birth (10.8% vs 5.6%). Other environmental or recreative exposures and co-morbidities during pregnancy, low socio-economic status, infant's gender, rates of prematurity and neonatal adaptation were not different between these groups (Table 1). Three mothers presented a TORCH co-infection during pregnancy, but newborns were tested negative during pregnancy and at birth (one primary toxoplasmosis and two primary cytomegalovirus infections).

Neonatal outcomes

Among 18 neonates with a confirmed congenital ZIKV infection at birth, 8 (44.4%) presented an adverse outcome during the neonatal period: 6 (33.3%) had severe structural brain

anomalies, of whom one died in his first day of life (congenital Zika syndrome with arthrogryposis and severe brainstem dysfunction), and 2 others suffered of severe neurological symptoms. The risk of neonatal adverse outcomes was higher in infected newborns compared to those tested negative at birth (4/111, 3.6%), even after adjustment for maternal infection in the first trimester of pregnancy: aRR 10.0 [95%CI 3.5-29.0]. When considering only neonates without structural brain anomalies, the risk of severe neurological symptoms was also higher in those diagnosed with a congenital ZIKV infection (2/12, 16.7% vs 1/108, 0.9%): aRR 20.5 [95%CI 2.0-207.7] (Table 2). At two months of life, microcephaly, abnormal OAE and ocular anomalies were confirmed in 2/17 (11.8%), 2/12 (16.7%) and 3/10 (30%) infected newborns; and in 1/111 (0.9%), 1/43 (2.3%) and 2/34 (5.9%) newborns tested negative at birth, respectively (Table 3).

Infantile outcomes

At 2 years of life, 15 infants with a confirmed congenital ZIKV infection at birth were still followed at the CHOG pediatric clinic. Among them, 5 (33.3%) presented neurologic impairments: 2 cerebral palsy and 3 severe dystonia, with seizures in 3 of them. Two of the infants with neurologic impairments presented a delay in motor acquisitions, a partial or complete blindness, and one hearing deficits. Hearing deficit was also diagnosed in another infected children, without neurologic impairments (Table 4). Overall, the risk of infantile adverse outcomes was higher in infected infants (6/15, 40.0%) compared to infants tested negative at birth (5/96, 5.2%), even when considering only children without brain structural anomalies (2/10, 20.0% vs 3/93, 3.2%): aRR 7.5 [95%CI 2.6-21.4] and aRR 6.0 [1.0-30.9], respectively (Table 2).

Neurodevelopment at three years of life

Eleven children of the infected group and 51 children of the group tested negative at birth came for neurodevelopment screening in August and September 2019. The median age at evaluation was 35 months in the infected group and 36 months in the group of children tested negative at birth. Developmental score below -2SD ("Referral" zone) in at least one domain was observed in 7/11 (63.6%) infected children, the cognitive and language domain being the most affected (6/11, 54.5%). Details of the CDAS are presented in Table 4 and Figure 2. Children with a confirmed congenital ZIKV infection at birth had a higher risk of CDAS in the "referral zone" (<-2SD) compared to children tested negative (7/51, 13.7%), even when considering only those without structural brain anomalies: aRR 4.3 [1.8-9.9] and aRR 2.9 [1.0-8.8], respectively (Table 2).

Children with CDAS in the "referral zone" were the same who had an infantile adverse outcome among the infected. In addition, one infected infant without structural brain anomalies and who remained asymptomatic until 2 years of age was screened as at risk for developmental delay at 3 years of life.

DISCUSSION

Main results

In this study we assessed the development of children with laboratory confirmed congenital ZKV infection up to 3 years of age. Our results indicate that infected neonates present a higher risk of neurological symptoms at birth (27.8%), even when no structural brain anomalies are observed (16.7%), when compared to neonates tested negative at birth (0.9%). At 2 years of age, infection at birth was still associated with a higher risk of neurologic impairments or neurosensory alterations (40.0% vs 5.2%). At three years of life, suspicion of neurodevelopment delay (<-2SD) was more common in children tested positive at birth (63.6% vs 13.7%). All the children tested positive at birth with structural brain anomalies had a suspicion of neurodevelopment delay (<-2SD) (4/4), while for those without structural brain anomalies the proportion was less than half (3/7).

Interpretation

Brain structural malformations and ocular anomalies associated with congenital Zika infection have been well described worldwide, particularly in congenital Zika Syndrome[5,7,12-16]. Infancy outcomes and developmental outcomes of infants exposed to ZIKV in utero have been studied less extensively and often with no stratification by infant status towards ZIKV infection at birth. The study by Nielsen-Saines and colleagues found similar results to our findings with abnormal neurodevelopment and/or abnormal eye or hearing assessments in 31.5% of children evaluated between 7 and 32 months of age[17]. In this study, the cognitive and language domain was also the most affected (35% of 146 children). When comparing neuroimaging findings to neurodevelopmental performance in ZIKV-exposed infants, Lopes Moreira et al. noted a significant association between normal results on brain imaging and higher Bayley-III scores[6]. However, they failed to predict severe developmental delay in 2% of children and normal

development in 16%. Similarly, in our cohort overall (i.e. regardless of the status towards ZIKV infection at birth), around 20% (10/56) of the children without structural brain anomalies had a suspicion of neurodevelopment delay in at least one domain at three years of life, whereas one third (2/6) with brain anomalies were not detected as at risk for abnormal development. The results of the Colombian cohort reported by Mulkey *et al.*, indicate that neurodevelopmental delay in a child that is healthy at birth could worsen with age[18].

Brasil and colleagues performed a study stratifying infants by their status towards ZIKV infection at birth. They described neurodevelopment outcomes of 130 children born from ZIKV-infected mothers, of whom 84 (65%) were tested positive for ZIKV between birth and 1 year of age[19]. They could only observe trends towards an association between laboratory confirmed infection and specific abnormalities (structural brain anomalies, vision and hearing deficits, abnormal neurologic exam, developmental delay). The difference between their results and ours may be explained by the difference in testing for congenital infections, as a positive result after post-partum discharge do not differentiate congenital infection from post-natal acquired infection. They might have experienced a high proportion of exposure misclassification biasing their estimated toward a null association.

Overall, our results and others seem to indicate that normal antenatal and neonatal evaluation cannot provide complete reassurance in infants exposed to ZIKV in utero, and close infantile follow-up remains crucial, particularly in those with a confirmed congenital infection at birth.

Study limitations

The first limitation of this study is the important proportion of loss to follow-up decreasing the sample size from 129 to 111 after 2 years and to 62 after 3 years and introducing a potential selection bias. Loss to follow-up is very important in determining a study's validity because patients lost to follow-up might have a different outcome than those who complete the study.

In our study, the proportion of loss to follow up were however the same between the two groups (≈15% after 2 years and 40% after 3 years) which suggest that a potential selection bias on the outcome has been undifferentiated between the two groups. Yet, it is difficult to know if the loss to follow up has selected the more severe cases or not. One would argue that the lack of clinical concern by parents, particularly in asymptomatic cases, might have driven the loss to follow-up. This would have overestimated the absolute risks of infantile adverse outcomes and the suspicion of neurodevelopment delay in the cohort. Thus, absolute risk in this study should be considered carefully.

Another source of potential selection bias is linked to practical limitations to perform a follow-up at the CHOG for newborns from mothers living along the Maroni river or in isolated areas in Suriname. Thus, among the newborns born at CHOG, only those followed at the pediatric clinic of the CHOG were enrolled resulting in the exclusion of 35% of the newborns born at CHOG from ZIKV-infected mothers and leading to the initial inclusion of only 129 infants. This might have selected infants stemming from family with a higher socio-economic status. This would have led to a possible underestimation of the absolute risk but again it is not expected that this selection bias has been differentiated between both groups.

The second limitation of this study is the testing performance to confirm congenital infections. In fetuses and neonates it has been demonstrated that viremia is transient in blood, amniotic fluid and urine[20]. Thus, the window to detect congenital infections using RT-PCR may be shorter than for other congenital infections (i.e. CMV). In infants, congenital ZIKV infections are difficult to confirm retrospectively, due to serological test cross-reaction and the possibility of an infection after birth in the context of a continuous exposure. To avoid false negative or false positive results, neonatal serology was performed before postpartum discharge. As no other *flavividae* was significantly circulating during this period, we considered the risk of cross-reactions low, and positive neonatal IgM without positive RT-PCR were considered as a

laboratory confirmed congenital infection, although we should have considered them probable if we had used the CDC definitions[21]. We tried to reduce as much as possible misclassification biases, in performing neonatal testing on different samples (Appendix 1), but we can't exclude that some newborns classified as uninfected had undetectable viremia and immune response at birth.

The follow-up of infants and children was based on the French recommendations[22] and adapted to local capacities, but we cannot exclude that routine MRI, auditory brainstem response testing and consultation with a pediatric neurologist, as recommended by the CDC, would have diagnosed more subtle and specific signs of congenital ZIKV infection.

The third limitation is linked to a problem of language skills. Some children or mothers have difficulties when using French language. These difficulties could have wrongly led to a lower score when using the French version of the CDAS to evaluate the cognitive & language domain resulting in a misclassification of the outcome. The two practitioners who evaluated these children, however, were also able to speak the local language and translate questions limiting the poor understanding of the CDAS assessment. This would have led to an overestimation of the difficulties in the cognitive and language domain, but the problem would have been present in both groups, with a less impact in the comparison of these groups.

The last limitation was that a control group of children born from uninfected mothers who underwent neurodevelopmental testing using the CDAS was not available. In the general population, a normal distribution of neurodevelopmental scores would be expected when using a standardized tool such as the CDAS, but this score has never been used in French Guiana and cognitive scales in particular may include items that could be influenced by the cultural context. In a cross-sectional study evaluating the neurodevelopment of Polynesian infants born during the ZIKV outbreak versus a control group of Canadian infants, Subissi and colleagues described

that confounding factors such as socioeconomic status and cultural factors may play an important role in infantile neurodevelopment[23].

CONCLUSION

A laboratory confirmed congenital ZIKV infection at birth could be associated with a 10-fold increased risk of neonatal adverse outcomes (death, structural brain anomalies or neurological symptoms), a 7-fold increased risk of infantile adverse outcomes (neurologic impairments, neurosensory or motor alterations), and a 4-fold risk of suspected neuro-developmental delay at 3 years of age, in comparison to infants tested negative at birth. Infected neonates without structural brain anomalies at birth seem also to present increased risks of neurological complications and late-onset anomalies but to a lesser extent. Therefore, it seems paramount to offer a systematic testing for congenital ZIKV infection at birth in case of in-utero exposure, and to adapt counseling according to these results.

METHODS

Study settings and participants

This prospective cohort study included newborns from mothers infected with ZIKV during pregnancy and followed at the CHOG after the 2016 ZIKV-epidemic. The CHOG offers the only maternity and neonatal intensive care units of the western part of French Guiana. During the ZIKV-epidemic (January to September 2016), all pregnant women in the territory underwent laboratory screening by ZIKV serology in each trimester and at delivery, as well as reverse transcription - polymerase chain reaction (RT-PCR) in urine and plasma samples in case of symptoms.

All infected women were followed in the fetal medicine unit of the CHOG. Fetal ultrasound (US) examinations were performed every 3-4 weeks using E8 and E10 Voluson scanners with abdominal (RM6C) and transvaginal (RIC5-9-D) transducers (General Electric Healthcare, Zipf, Austria). Additional investigations (MRI, computed tomography (CT), amniocentesis) were performed based on ultrasound results and after a discussion with a multi-disciplinary team. All neonates from ZIKV-infected mothers living in the area (supplementary figure) were offered ongoing follow-up at the CHOG until the third year of life and participation in this study. Asymptomatic neonates from mothers living along the Maroni river, outside of the Saint-Laurent du Maroni area, were discharged with their mother after day 3-5 post-partum and were followed in the nearest primary healthcare center, and only came back to the CHOG in case of emergency or need for advanced care. Thus, these infants were not included in this cohort.

The study received ethics approval from the institutional review board of the CHOG and a written consent of the mother was obtained.

Laboratory testing for congenital ZIKV infection and Exposure definition

During pregnancy, RT-PCR in amniotic fluid was offered for cases with fetal anomalies or if an amniocentesis was performed for another indication (i.e. aneuploidy diagnosis). After birth, all newborns underwent ZIKV serology for detection of specific IgM before day three of life. RT-PCRs were performed in cord blood, neonatal urine and placenta. Additional testing on cerebrospinal fluid was proposed in cases with neurological symptoms or demise.

We defined a **laboratory confirmed congenital ZIKV infection** either by positive RT-PCR from at least one fetal/neonatal sample (amniotic fluid, cerebrospinal fluid, urine, blood) or identification of specific IgM in neonatal blood or in cerebrospinal fluid. Details of maternal, fetal and neonatal testing are available in our previous studies[4,11].

Neonates from ZIKV-infected mother without a confirmed congenital ZIKV infection were classified as controls.

Outcome definition and time of measurement

Newborns underwent cerebral imaging and neurosensory testing, and were followed by a pediatrician up to their three years of life. The last evaluation included a neurodevelopmental screening using the Child Development Assessment Scale (CDAS)[24].

Neonatal outcome

All ZIKV-exposed neonates, regardless of their testing result at birth, underwent clinical examination with special attention to anthropometric measurements, neurological status, and signs of infection. The head circumference (HC) measurements were confirmed 24 hours after birth to avoid the effects of delivery sequelae. In addition to clinical examination, they were assessed by transfontanellar ultrasound, hearing evaluation by evoked otoacoustic emission (OAE) testing and fundoscopy (1 to 2 months after birth). Every abnormal examination was

reconfirmed, and more explorations (MRI, CT) were requested depending on the clinical orientation.

Neonatal adverse outcomes were defined as neonatal death (between birth and 2months of life, intra-partum demise not included), structural brain anomalies, or severe neurological symptoms (according to Pomar *et al.*, BMJ, 2018; Prenat diagnosis, 2019)[11,25].

Infantile follow-up and outcome

All neonates enrolled were scheduled for medical consultation at the pediatric clinic of the CHOG at 2, 6, 9, 12, 18 and 24 months of life. These pediatric examinations included parental questioning on infant development, anthropometric measurements, a control of motor acquisitions, a neurological examination and an auditory and visual assessment, following the French high council of public health recommendations (HCSP)[22].

Infantile adverse outcomes were defined as the observation of neurologic impairments (cerebral palsy, severe dystonia, tremors or seizures) or delay in motor acquisitions (sitting position > 9 months or walking > 18 months of age) or neurosensory alterations (impaired response to visual or auditory stimuli) until the age of 24 months.

Neurodevelopmental outcome

In August and September 2019, at three years of life, the children were screened for neurocognitive development by the French version of the CDAS[24]. Adapted to children 0–5 years of age, the results allow the user to evaluate the child's cognitive, language, motor, and social-emotional development using a validated and standardized scale. Results in the "comfort zone" (blue, >-1SD) indicate normal development. Results in the "to be monitored zone" (grey, [-2SD - -1SD]) suggest that interventions with the child should be adapted according to identified difficulties, and that the child should be reassessed at a later date. Finally, results in

the “referral zone” (red, <-2SD) indicate that the child should be referred for an exhaustive developmental assessment. To avoid any bias of administration, all children were evaluated by two medical doctors trained to perform the CDAS test and blinded for the results of congenital Zika infection testing.

A suspected delay in neurodevelopment was defined as a CDAS below -2SD (“referral zone”) in at least one domain at three years of life.

Mitigation of bias

In the context of French Guiana, infants present an important risk to be lost to follow-up after post-partum discharge, as some of them live in isolated area and are followed in primary care centers. These infants were not included in the cohort to avoid misclassification bias. In infants enrolled in the cohort, in case of a missed appointment, the parents were recalled to schedule another evaluation. We did not enroll infants referred to the CHOG for advanced cares who were not included initially in the cohort, to avoid selection biases.

OAE testing was implemented in May 2016 and infants born before were not systematically tested at birth. After the epidemic peak, we encountered human and technical limitations to perform fundoscopy in all children born from ZIKV-positive mothers. To avoid selection and classification biases, we did not consider abnormal fundoscopies or OAE in primary outcomes. We preferred to consider abnormal response to auditory or visual stimuli in infancy, as all the infants were tested for these outcomes.

Statistical analysis

Baseline characteristics of mothers and newborns were obtained at enrollment, and presented as absolute and relative frequencies for those diagnosed with a laboratory confirmed congenital Zika infection at birth and those that tested negative. Timing of maternal infection was

estimated based on symptoms onset, or on laboratory results in case of asymptomatic infection; and grouped into 1st or 2nd and 3rd trimesters for the analysis. Gestational age at birth was considered as a binary variable for the analysis ("prematurity < 37 wg").

The Relative Risks (RR) associated with laboratory confirmed congenital ZIKV infection were assessed using generalized linear models, and were adjusted (aRR) for confounding factors (trimester of maternal ZIKV infection), and controlled for potential interactions with exposures during pregnancy, maternal age, co-morbidities and socio-economic status, infant gender, twins, prematurity and the mode of delivery. Structural brain anomalies were also tested as effect-modifiers for severe neurological symptoms, infantile adverse outcomes and suspicion of neurodevelopmental delay <-2SD. In case of interaction, the analysis was stratified for effect-modifiers.

We performed a complete case analysis, thus using different denominators for the neonatal, infantile and neurodevelopmental outcomes.

Data was collected using Excel software and analyzed using Stata15 (Stata Corporation, College Station, TX, USA).

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Authors contributions

Najeh Hcini conceived and designed the study, provided care to mothers and children, collected the data and draft the initial manuscript.

Yaovi Kugbe and Zo Hasina Linah Rafalimanana provided care to children, collected data, and reviewed and revised the manuscript.

Véronique Lambert, Meredith Mathieu and Gabriel Carles participated to the study design, provided care to mothers, collected data, and reviewed and revised the manuscript.

David Baud and Alice Panchaud analyzed and interpreted the data, and reviewed and revised the manuscript.

Léo Pomar conceived and designed the study, provided care to mothers, analyzed and interpreted the data, and draft the initial manuscript.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Data sharing

Individual participant de-identified data that underlie the results will be shared with researchers who provide a methodologically sound proposal for multi-centric study, particularly individual participant data meta-analysis. Proposals should be directed to leo.pomar@chuv.ch.

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Table 1: Baseline characteristics of ZIKV-exposed pregnancies and newborns

Age, weight and term variables are presented as a median with extreme values. For qualitative variables, absolute frequencies and relative frequencies (percentages) are presented.

Characteristics	Confirmed congenital infection at birth N= 18	Negative testing at birth N= 111	Study cohort N= 129
Maternal age at birth (years) - median (min-max)	25 (18-38)	26 (18-43)	26 (18-43)
Maternal socio-economic status – no. (%)			
Low	6 (33.3%)	38 (34.2%)	44 (34.1%)
Moderate	8 (44.4%)	46 (41.4%)	54 (41.9%)
High	1 (5.6%)	8 (7.2%)	9 (7.0%)
Unknown	3 (16.7%)	19 (17.1%)	22 (17.0%)
Maternal exposure during pregnancy – no. (%)			
Alcohol consumption	1 (5.6%)	12 (10.8%)	13 (10.1%)
Drug use	0 (0.0%)	1 (0.9%)	1 (0.8%)
Current smoker	1 (5.6%)	5 (4.5%)	6 (4.6%)
Lead poisoning	1 (5.6%)	6 (5.4%)	7 (5.4%)
Any maternal comorbidities[π] – no (%)	3 (16.7%)	15 (13.5%)	18 (14.0%)
Diabetes (previous or gestational)	1 (5.6%)	7 (6.3%)	8 (6.2%)
Vascular pathologies	1 (5.6%)	5 (4.5%)	6 (4.7%)
Severe anemia	1 (5.6%)	5 (4.5%)	6 (4.7%)
Co-infections*	1 (5.6%)	2 (1.8%)	3 (2.3%)
Maternal Zika infection – no (%)			
Symptomatic	4 (22.2%)	22 (19.8%)	26 (20.2%)
Asymptomatic	14 (77.8%)	89 (80.2%)	103 (79.8%)
Trimester of Maternal Zika infection – no. (%)			
T1 infection	7 (38.9%)	27 (24.3%)	34 (26.4%)
T2 infection	7 (38.9%)	37 (33.3%)	44 (34.1%)
T3 infection	2 (11.1%)	25 (22.5%)	27 (20.9%)
Unknown	2 (11.1%)	22 (19.8%)	24 (18.6%)
Dichorionic twins	2 (11.1%)	6 (5.4%)	8 (6.2%)
Fetus gender – no. (%)			
Female	10 (55.6%)	63 (56.8%)	73 (56.6%)
Male	8 (44.4%)	48 (43.2%)	56 (43.4%)
Term at birth (weeks' gestation) - median (min-max)	39 (32-41)	39 (36-41)	39 (32-41)
Premature birth <37wg	1 (5.6%)	6 (5.4%)	7 (5.4%)
Mode of delivery – no. (%)			
Normal	13 (72.2%)	97 (87.4%)	110 (85.3%)
C-section	5 (27.8%)	14 (12.6%)	19 (14.7%)
Neonatal adaptation			
Abnormal Apgar score (<6 at 5 min) – no (%)	2 (11.1%)	13 (11.7%)	15 (11.6%)
Abnormal Lactate level (> 4.5) – no (%)	2 (11.1%)	16 (14.4%)	18 (13.9%)

[π] including multiple maternal co-morbidities

* one primary cytomegalovirus infection in the infected group; one primary CMV and one primary toxoplasmosis infections in the control group

Alcool consumption was defined as ongoing consumption during the pregnancy after its diagnosis

Current smoking was defined as ongoing smoking during pregnancy after its diagnosis

Trimester of maternal Zika infection was estimated based on symptoms onset, or on laboratory results (asymptomatic)

Table 2: Main outcomes and associations with congenital ZIKV infection at birth

Main outcomes	Confirmed congenital infections	Negative neonatal testing	RR [95%CI]	p	aRR* [95%CI]	p
Neonatal adverse outcomes[1]	**8/18 (44.4%)**	**4/111 (3.6%)**	**12.3 [4.1-36.8]**	**<0.001**	**10.1 [3.5-29.0]**	**<0.001**
Neonatal demise	1/18 (5.6%)	0/111 (0.0%)		0.14		
Structural brain anomalies	6/18 (33.3%)	3/111 (2.7%)	12.3 [3.4-45.0]	<0.001	9.2 [1.7-31.1]	<0.001
Severe neurologic symptoms	5/18 (27.8%)	1/100 (0.9%)	30.8 [3.8-248.9]	0.001	26 [3.2-208.3]	0.002
- in neonates with structual brain anomalies	3/6 (50.0%)	0/3 (0.0%)		0.464		
- in neonates without structural brain anomalies	2/12 (16.7%)	1/108 (0.9%)	18 [1.8-184.1]	0.015	20.5 [2.0-207.7]	0.011
Infantile adverse outcomes at 2 years of life[2]	**6/15 (40.0%)**	**5/96 (5.2%)**	**7.7 [2.7-22.1]**	**<0.001**	**7.5 [2.6-21.4]**	**<0.001**
- in infants with structural brain anomalies	4/5 (80.0%)	2/3 (66.7%)	1.2 [0.45-3.0]	0.673	1.1 [0.4-2.8]	0.712
- in infants without structural brain anomalies	2/10 (20.0%)	3/93 (3.2%)	6.2 [1.2-32.8]	0.019	6.0 [1.0-30.9]	0.047
Neurologic impairments	5/15 (53.3%)	4/96 (4.2%)	8.0 [2.4-26.5]	<0.001	7.9 [2.4-26.3]	0.001
Delay in motor acquisitions	2/15 (13.3%)	1/96 (1.0%)	12.9 [1.2-134.0]	0.006	11.4 [1.1-113.8]	0.038
Neurosensory alterations	3/15 (20.0%)	1/96 (1.0%)	19.4 [2.2-174.5]	<0.001	17.8 [2.0-159.8]	0.010
Referral for suspicion of neurodevelopment <-2SD in at least one domain at 3 years of life[3]	**7/11 (63.6%)**	**7/51 (13.7%)**	**4.6 [2.0-10.5]**	**<0.001**	**4.3 [1.8-9.9]**	**0.001**
- in children with structural brain anomalies	4/4 (100.0%)	0/2 (0.0%)		0.194		
- in children without structural brain anomalies	3/7 (42.9%)	7/49 (14.3%)	3.0 [1.0-9.0]	0.05	2.9 [1.0-8.8]	0.054
Motor	2/11 (18.2%)	1/51 (2.0%)	9.3 [0.9-93.4]	0.059	8.6 [0.9-82-2]	0.060
Cognitive and language	6/11 (54.5%)	3/51 (5.9%)	9.3 [2.7-31.5]	<0.001	7.8 [2.3-27.2]	0.001
Socio-affective	4/11 (36.4%)	6/51 (11.8%)	4.2 [1.3-13.5]	0.015	3.8 [1.2-11.9]	0.024

[1] 129 neonates evaluated from birth to 2 months of life

[2] 111 infants evaluated up to 2 years of life

[3] 62 infants evaluated at 3 years of life using the Child Development Assessment Scale

* Adjusted on the trimester of maternal Zika infection. Exposures during pregnancy, maternal age, socio-economic status and co-morbidities, infant gender, twins, prematurity and the mode of delivery were tested as effect-modifiers. Structural brain anomalies were also tested as effect-modifiers for severe neurological symptoms, infantile adverse outcomes and suspicion of neurodevelopment <-2SD. In case of interaction, the analysis is stratified on effect-modifiers.

Table 3: Neonatal outcomes up to two months of life

Weight and head circumference (HC) variables are presented as medians with extreme values. For qualitative variables, absolute frequencies and relative frequencies (percentages) are presented.

Neonatal outcomes - From birth to 2 months of life	Confirmed congenital infection at birth N= 18	Negative testing at birth N= 111	Study cohort N= 129
Status at two months of live - no (%)			
Alive	17 (94.4%)	111 (100.0%)	128 (99.2%)
Neonatal demise	1 (5.6%)	0 (0.0%)	1 (0.8%)
Head circumference			
HC at birth (cm) - median (min-max)	32.8 (27.5-35.5)	33 (29-38)	33 (27.5-38)
Microcephaly < -3SD* at birth - no (%)	2 (11.1%)	1 (0.9%)	3 (2.3%)
HC at 1 month[1] (cm) - median (min-max)	37 (33-39)	37 (36.5-38)	37 (33-39)
HC at 2 months[1] (cm) - median (min-max)	38 (33-40)	38.5 (37-42)	38 (33-42)
Microcephaly < -3 SD** at 2m - no (%)	2/17 (11.8%)	1 (0.9%)	3/128 (2.3%)
Weight			
Birth weight (grams) – median (min-max)	2910 (1910-3310)	3185 (1930-4620)	3108 (1910-4620)
Birth weight < -2 SD*	2 (11.1%)	12 (10.8%)	14 (10.9%)
Weight at 1 month[1] (kg) - median (min-max)	3.8 (3.6 - 4.1)	4.3 (2.4-4.8)	4.3 (2.4-4.8)
Weight at 2 months[1] (kg) - median (min-max)	4.8 (4.0-6.2)	5.2 (3.5-6.5)	5.0 (3.5-6.5)
Weight < - 2 SD at 2m** - no (%)	2/17 (11.8%)	8/111 (7.2%)	10/128 (7.8%)
Structural brain anomalies - no (%)	6 (33.3%)	3 (2.7%)	9 (7.0)
Cortical development anomaly	4 (22.2%)	0 (0.0%)	4 (3.1%)
Corpus callosum anomaly	4 (22.2%)	2 (1.8%)	6 (4.7%)
Calcifications or cystic lesions	5 (27.8%)	1 (0.9%)	6 (4.7%)
Posterior fossa anomaly	4 (22.2%)	0 (0.0%)	4 (3.1%)
Ventriculomegaly	4 (22.2%)	1 (0.9%)	4 (3.1%)
Ocular anomalies - no (%)			
Microphtalmy	1 (5.6%)	0 (0.0%)	1 (0.8%)
Fundoscopy anomalies	3/10 (30.0%)	2/34 (5.9%)	5/44 (11.4%)
Subretinal hemorrhage	2/10 (20.0%)	1/34 (2.9%)	3/44 (6.8%)
Chorioretinal lacunae	2/10 (20.0%)	1/34 (2.9%)	3/44 (6.8%)
Macula atrophy	1/10 (10.0%)	1/34 (2.9%)	2/44 (4.5%)
Abnormal otoacoustic emission - no (%)	2/12 (16.7%)	1/43 (2.3%)	3/55 (5.5%)
Severe neurologic symptoms - no (%)	5 (27.8)	1 (0.9%)	6 (4.7 %)
Arthrogryposis	1 (5.6%)	0 (0.0%)	1 (0.8%)
Hypertonia	3 (16.7%)	0 (0.0%)	3 (2.3%)
Dysphagia / swallowing disorders	2 (11.8%)	0 (0.0%)	2 (1.6%)
Seizures	1 (5.6%)	1 (0.9%)	2 (1.6%)
NICU Admission – no (%)	3 (16.7%)	14 (12.6%)	17 (13.2%)

[1] 128 alive neonates evaluated at 1 and 2 months of life

* According to Intergrowth21 charts

**Aaccording to WHO Child Growth Standards

Table 4: Infantile and children outcomes up to three years of life

Weight and head circumference (HC) variables are presented as medians with extreme values. For qualitative variables, absolute frequencies and relative frequencies (percentages) are presented.

Infantile and children outcomes - up to 3 years of life	Confirmed congenital infection at birth N= 15	Negative testing at birth N= 97	Study cohort N= 112
HC (cm) at 1 year - median (min-max)	44 (37-47)	46 (43-49)	46 (37-49)
at 2 years[1]	46 (38-50)	48 (44-53)	48 (40-53)
at 3 years[2]	50 (39-51)	50 (39-54)	50 (39-54)
HC < 3rd percentile*	2/11 (9.1%)	1/51 (2.0%)	3/62 (4.8%)
Weight (kg) at 1 year	10 .0 (6.0-11.3)	10.5 (6.4-13.0)	10.4 (6.0-13.0)
at 2 years[1]	11.6 (9.6-14.0)	12.0 (10.3-15.0)	12.0 (9.6-15.0)
at 3 years[2]	14.5 (11.4-20)	14.8 (12.0-19.4)	14.7 (11.4-20.0)
Weight < 5th percentile*	1/11 (9.1%)	3/51 (5.9%)	4/62 (6.5%)
Neurologic impairments at 2y[1] – no (%)	5 (33.3%)	4/96 (4.2%)	9/111 (8.1%)
Cerebral palsy	2 (13.3%)	0/96 (0.0%)	2/111 (1.8%)
Severe dystonia or tremors	3 (20.0%)	3/96 (3.1%)	6/111 (5.4%)
Seizures	3 (20.0%)	2/96 (2.1%)	5/111 (4.5%)
Motor acquisitions			
Age at sitting position (m) - median (min-max)	6 (3-24)	6 (4-11)	6 (4-24)
Delay for sitting position (>9m) – no (%)	2 (13.3%)	1 (1.0%)	3 (2.7%)
Age at walking (m) - median (min-max	11 (8-24)	11 (7-17)	11 (7-24)
Delay for walking[1] (>18m) – no (%)	1 (6.7%)	0/96 (0.0%)	1/111 (0.9%)
Vision and hearing evaluation			
Impaired response to visual stimuli – no (%)	2 (13.3%)	1 (1.0%)	3 (2.7%)
Impaired response to auditory stimuli – no (%)	2 (13.3%)	1 (1.0%)	3 (2.7%)
Age at CDAS evaluation[2] (m) - median (min-max)	35 (33-39)	36 (34-40)	36 (34-40)
Global assessment – no (%)			
"Comfort" zone (>-1SD)	3/11 (27.3%)	30/51 (58.8%)	33 (53.2%)
"To be monitored" zone ([-2SD;-1SD])	1/11 (9.1%)	14/51 (27.5%)	15 (24.2%)
"Referral" zone (<-2SD)	7/11 (63.4%)	7/51 (13.7%)	14 (22.6%)
Motor domain – no (%)			
"Comfort" zone (>-1SD)	7/11 (63.4%)	47/51 (92.2%)	54 (87.1%)
"To be monitored" zone ([-2SD;-1SD])	2/11 (18.2%)	3/51 (5.8%)	5 (8.1%)
"Referral" zone (<-2SD)	2/11 (18.2%)	1/51 (2.0%)	3 (4.8%)
Socio-emotional domain – no (%)			
"Comfort" zone (>-1SD)	7/11 (63.4%)	41/51 (80.4%)	48 (77.4%)
"To be monitored" zone ([-2SD;-1SD])	0/11 (0.0%)	4/51 (7.8%)	4 (6.5%)
"Referral" zone (<-2SD)	4/11 (36.4%)	6/51 (11.8%)	10 (16.1%)
Cognitive and language domain – no (%)			
"Comfort" zone (>-1SD)	3/11 (27.3%)	32/51 (62.7%)	35 (56.5%)
"To be monitored" zone ([-2SD;-1SD])	2/11 (18.2%)	16/51 (31.4%)	18 (29.0%)
"Referral" zone (<-2SD)	6/11 (54.5%)	3/51 (5.9%)	9 (14.5%)

[1] 111 infants evaluated at 2years of life / Zika= 15

[2] 62 children evaluated at 3 years of life using the CDAS / Zika= 11

* according to WHO Child Growth Standards <2 years and CDC growth charts > 2 years of life

Supplementary Table: Baseline characteristics of infants followed up to 2years of life and children followed up to 3 years of life

Age, weight and term variables are presented as a median with extreme values. For qualitative variables, absolute frequencies and relative frequencies (percentages) are presented.

	Characteristics of infants followed up to 2years of life (n=111)		Characteristics of children followed up to 3years of life (n=62)	
	Confirmed congenital infection at birth N= 15	Negative testing at birth N= 96	Confirmed congenital infection at birth N= 11	Negative testing at birth N= 51
Maternal age at birth (years) - median (min-max)	25 (18-33)	26 (18-43)	25 (18-33)	26 (18-43)
Maternal socio-economic status – no. (%)				
Low	4 (26.7%)	34 (35.4%)	3 (27.3%)	19 (37.3%)
Moderate	8 (43.3%)	40 (41.7%)	7 (63.6%)	21 (41.2%)
High	1 (6.7%)	7 (7.3%)	1 (9.1%)	4 (7.8%)
Unknown	2 (13.3%)	15 (15.6%)	0 (0.0%)	7 (13.7%)
Maternal exposure during pregnancy – no. (%)				
Alcohol consumption	1 (6.7%)	10 (10.4%)	1 (9.1%)	10 (19.6%)
Drug use	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Current smoker	1 (6.7%)	4 (4.2%)	0 (0.0%)	3 (5.9%)
Lead poisoning	1 (6.7%)	5 (5.2%)	1 (9.1%)	3 (5.9%)
Any maternal comorbidities[π] – no (%)	2 (13.3%)	13 (13.5%)	1 (9.1%)	7 (13.7%)
Diabetes (previous or gestational)	0 (0.0%)	6 (6.3%)	0 (0.0%)	3 (5.9%)
Vascular pathologies	1 (6.7%)	5 (5.2%)	1 (9.1%)	4 (7.8%)
Severe anemia	0 (0.0%)	3 (3.2%)	0 (0.0%)	1 (2.0%)
Co-infections*	1 (6.7%)	2 (2.1%)	0 (0.0%)	1 (2.0%)
Maternal Zika infection – no (%)				
Symptomatic	3 (20.0%)	18 (18.8%)	2 (18.2%)	10 (19.6%)
Asymptomatic	12 (80.0%)	78 (82.2%)	9 (81.8%)	41 (80.4%)
Trimester of Maternal Zika infection – no. (%)				
T1 infection	6 (40.0%)	26 (27.1%)	4 (36.4%)	13 (25.5%)
T2 infection	6 (40.0%)	36 (37.5%)	4 (36.4%)	16 (31.4%)
T3 infection	2 (13.3%)	21 (21.9%)	2 (18.2%)	13 (25.5%)
Unknown	1 (6.7%)	13 (13.5%)	1 (9.1%)	9 (17.6%)
Dichorionic twins	2 (13.3%)	6 (6.3%)	2 (11.1%)	4 (7.8%)
Fetus gender – no. (%)				
Female	8 (53.3%)	55 (57.3%)	6 (54.6%)	28 (54.9%)
Male	7 (46.7%)	41 (42.7%)	5 (45.4%)	23 (45.1%)
Term at birth (weeks' gestation) - median (min-max)	39 (32-40)	39 (36-41)	39 (37-40)	39 (36-41)
Premature birth <37wg	1 (6.7%)	6 (6.3%)	0 (0.0%)	3 (5.9%)
Mode of delivery – no. (%)				
Normal	12 (80.0%)	85 (88.5%)	10 (90.9%)	44 (86.3%)
C-section	3 (20.0%)	11 (11.5%)	1 (9.1%)	7 (13.7%)
Neonatal adaptation				
Abnormal Apgar score (<6 at 5 min) – no (%)	1 (6.7%)	10 (10.4%)	1 (9.1%)	6 (11.8%)
Abnormal Lactate level (> 4.5) – no (%)	2 (13.3%)	13 (13.5%)	2 (18.2%)	9 (17.6%)
Structural brain anomalies	5 (33.3%)	3 (3.1%)	4 (36.4%)	2 (3.9%)
Neurological symptoms at birth	4 (26.7%)	1 (1.0%)	4 (36.4%)	1 (2.0%)
Neurologic impairments during infancy	NA	NA	4 (36.4%)	3 (5.9%)
Neurosensory alterations	NA	NA	4 (36.4%)	1 (2.0%)

[π] including multiple maternal co-morbidities

* one primary cytomegalovirus infection in the infected group; one primary CMV and one primary

Alcool consumption was defined as ongoing consumption during the pregnancy after its diagnosis

Current smoking was defined as ongoing smoking during pregnancy after its diagnosis

Trimester of maternal Zika infection was estimated based on symptoms onset, or on laboratory results (asymptomatic)

Appendix: Details of laboratory testing for congenital Zika infection among 132 newborns from Zika-infected mothers

	Positive	Negative	Not tested
Total	18	111	3
RT-PCR			
cord blood	1	100	31
urine	1	111	20
amniotic fluid	2	2	128
cerebrospinal fluid	1	2	129
placenta	9	103	20
IgM			
neonatal serum	12	117	3
cerebrospinal fluid	1	0	131

Figure1: Flowchart of newborns exposed to Zika virus in utero

All newborns from Zika-infected mothers, living in western French Guiana and followed at the pediatric clinic of the CHOG, were enrolled in this cohort following an informed consent process. At birth, they underwent clinical examination (including anthropometric measurements and a special focus on the neurological status), transfontanellar ultrasound (US) and testing for congenital Zika infection (PCR on urine, blood and placenta; serology; and testing in cerebro-spinal fluid if symptomatic). After postpartum discharge, they were recalled at 2, 6, 9, 12, 18 and 24 months of life for a pediatric examination. At 3 years of life (33-42 months), they were recalled for an evaluation of their development using the Child Development Assessment Scale (CDAS).

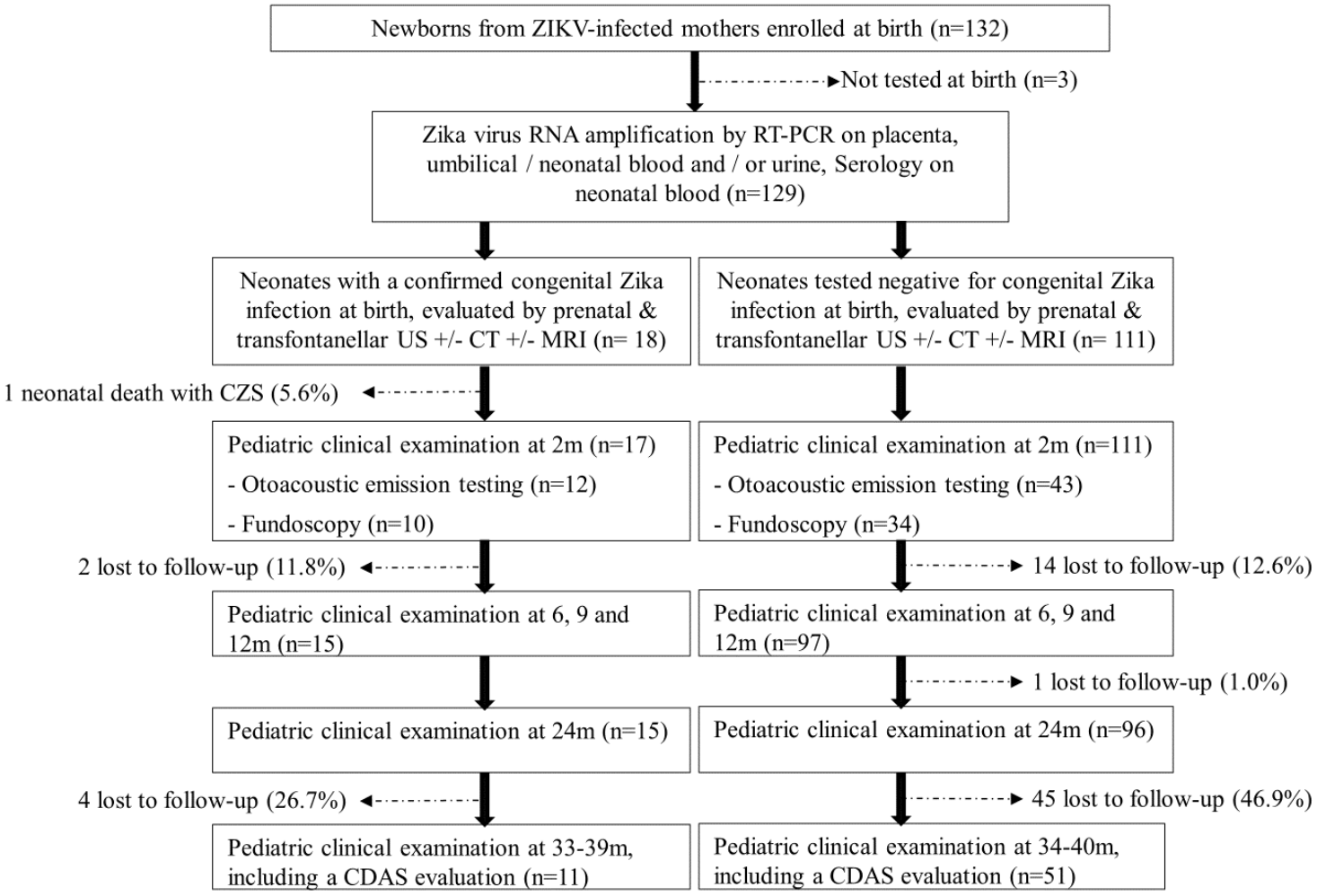
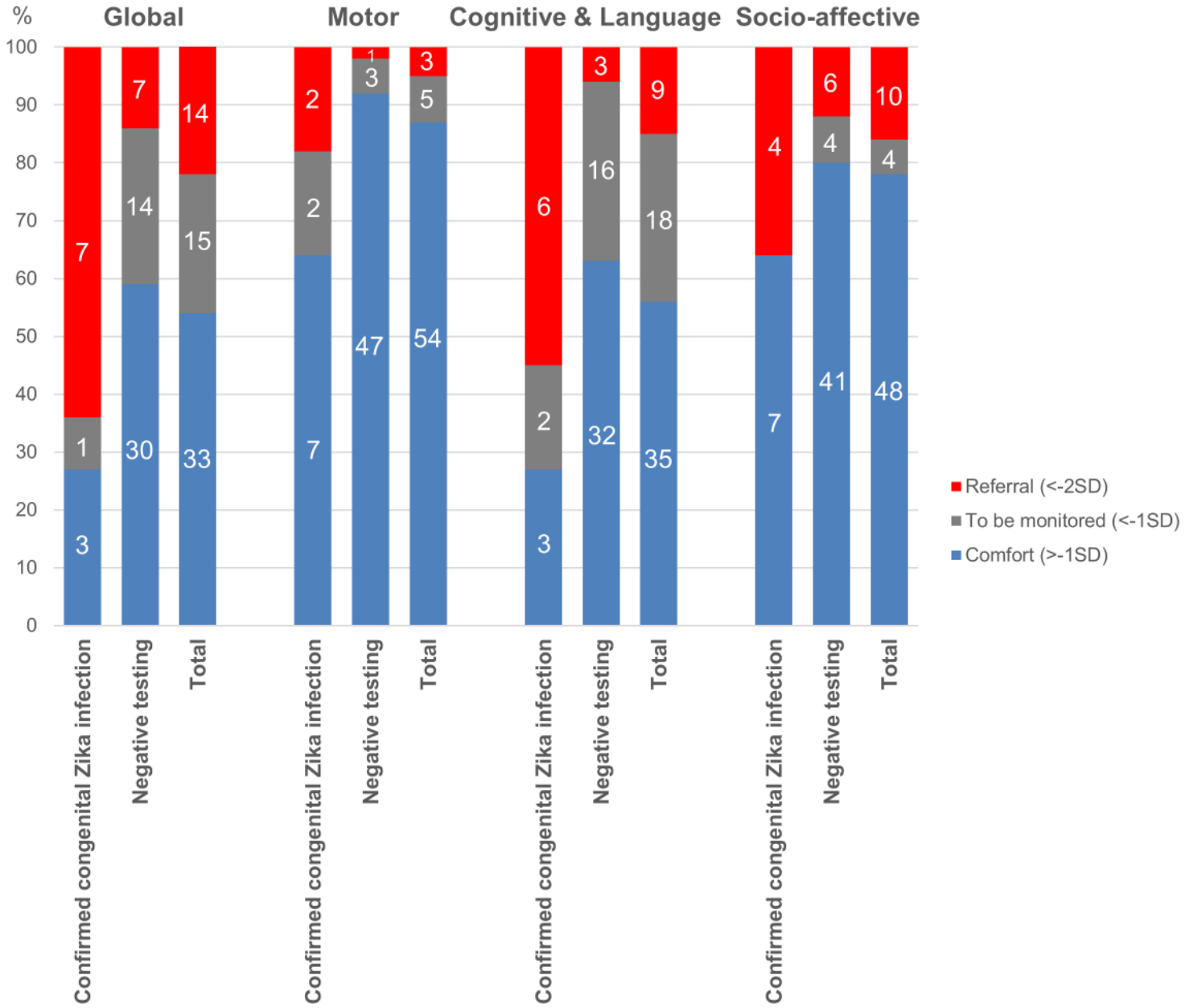


Figure2: Childhood development at three years of life

All children followed for in utero ZIKV exposure were recalled for a developmental evaluation using the Child Development Assessment Scale at three years of life (33 to 42 months, n=62). Normal results are classified in the “comfort” or “blue” zone (>-1SD). Intermediate results are classified in the “to be monitored” or “grey” zone ([-2SD;-1SD]). Suspicion of delays are classified in the “referral” or “red” zone (<-2SD). The motor, socio-emotional and cognitive & language domains were evaluated using this scale. Results of these domains were synthetized in a global evaluation.



Supplementary Figure: Healthcare services in western French Guiana