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ZIKA virus infection and congenital Zika syndrome: A systematic review

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Abstract

Zika virus (ZIKV), studied since 1950s and known for occasionally causing a mild, febrile illness in humans, became a public health emergency and a global threat to health, with its most recent major outbreak, in Brazil (2015-2016), declared a Public Health Emergency of International Concern by the WHO. This literature review aims to provide a short summary of the research and current knowledge about ZIKV, its epidemiology and the associated medical conditions, with a particular focus on fetal and neonatal microcephaly.

Purpose of work: This literature review aims to provide a general introduction to the epidemiology of the Zika virus, with a particular emphasis on the recent outbreaks and the microcephaly association.
Materials and methods: Literature search and review.
Keywords: “Zika”, “ZIKV”, “microcephaly”, “congenital Zika syndrome”, “CZS”, “Zika outbreak”, “epidemic”

Introduction

Viruses can be causal agents of a range of neurological disorders (1,2). The Zika Virus (ZIKV) outbreak of 2015-2016 brought the world’s attention to an exotic pathogen and its previously unreported, but serious clinical implications for fetal development. ZIKV, a single stranded RNA virus and a member of the Flaviviridae family was first isolated in 1947, in the Ziika Forest in Uganda, from a rhesus macaque (3–5). It has been known to possess the ability to

infect humans and studied since 1950, with the first recognized case of infection in humans described in 1954, from Nigeria (6–8). In 1956 a ZIKV infection was induced in a human volunteer and resulted in fever and headache (no rash was present) (6,9). The outline of early ZIKV epidemiology as well as its structure, biology, life cycle, circulation and mechanisms of transmission (including sexual or peripartum) have been reviewed in detail by several authors (4–7,9). ZIKV infection is usually a vector-borne disease frequently propagated by the *Aedes aegypti* and *Aedes africanus* mosquitos (and thus also categorized as arboviral) (6,7). ZIKV enters cells by an as yet unknown mechanism and alters the endoplasmic reticulum producing large vacuoles leading to cell death, possibly by paraptosis (5,10). Its primary target cells are those of the epidermis and dermis, including fibroblasts, keratinocytes and dendritic cells (5). Initial replication is followed by an expansion into lymph nodes and circulatory system (5). ZIKV infection has been described as most often asymptomatic (~80%), but can also manifest as a mild fever, arthralgia, maculopapular rash, conjunctivitis and a range of other, less common symptoms, usually lasting no more than a week (4,8,11). There is a considerable overlap with illness caused by Dengue virus (DENV) and chikungunya virus (CHIKV), with a degree of serological cross-reactivity, which further complicates accurate diagnosis (6,7,12,13). Major ZIKV outbreaks have only been confirmed in the 21st century. A turning point for scientific and clinical interest in ZIKV was marked by November 2015, when the Brazilian government reported a possible new association between ZIKV and a serious, congenital disorder of microcephaly in fetuses and neonates. Rising cases of microcephaly in Brazil lead the World Health Organization (WHO) to declare a Public Health Emergency of International Concern, and became a subject of an extensive debate. Since June 2016 and especially in 2017, ZIKV infections decreased significantly (14). At that time, direct evidence for causality was limited and the mechanism behind possible ZIKV-induced microcephaly not definitely established. The scientific discourse also focused on concordance of results across different studies, tracking vertical viral transmission between mother and fetus, the stratification of cases as well as the very criteria of fetal and neonatal microcephaly (7,15). Although subsequent research shed new light on the clinical significance of ZIKV infection for fetal neurodevelopment, answers to some of the key underlying mechanisms remain to be discovered. This review aims to outline the current state of knowledge of ZIKV epidemiology and its outbreaks, and summarise the evidence on the ZIKV-microcephaly association with a particular focus on the events that occurred in Brazil, nine years after initial reports emerged.

Initial Outbreaks

ZIKV constitutes a prime example of a pathogen which, despite being known to the scientific and medical communities for several decades, has only recently been brought to global attention on account of novel clinical findings. Over several decades since its discovery, ZIKV has been isolated in multiple African and South Asian countries. An early serosurvey conducted in 1950s in Uganda, where ZIKV was first isolated, revealed 6.1% seropositivity for anti-ZIKV antibodies, while a more recent survey in Ethiopia found 27% anti-ZIKV antibodies (16,17). Serological evidence also pointed to an early ZIKV presence in Southeast Asia - at least since the 1950s, although it was first isolated in 1966 and the first report of infection in humans appeared in 2010 (18). A survey in Thailand showed 70% prevalence for neutralizing antibodies against ZIKV (18). Two major families of ZIKA strains have been identified – African and Asian (19). Before 2007 only about 14 documented cases of ZIKV-caused illness (including 7 from Java) have been reported and none outside of Africa and Asia (20–22). The condition was generally described as mild and self-limiting, with fever, headache, general weakness, and some less frequent symptoms like dizziness or stomach ache, or a skin rash (6,21,22).

Prior to the epidemic of 2015, two ZIKV outbreaks have been widely reported in the literature - in 2007 and in 2013. In 2007, Micronesian Yap Island experienced an increase in symptomatic Dengue-like febrile illness. The illness was mild and presented itself as a combination of symptoms such as macular/papular rash (90% cases), mild fever (65%), conjunctivitis (55%), arthralgia and arthritis (~65%), myalgia (48%), headache (46%), retro-orbital pain (36%), oedema (19%), among others (7,23). These symptoms differed to an extent from those previously described in the literature as typical of ZIKV infection in that arthralgia, rash and conjunctivitis was either rare or had not previously been reported. On Yap Island some patients were initially also found to be positive for anti-DENV IgM antibodies, however due to the presentation, unusual for Dengue fever, 71 patient samples were sent to CDC in the USA, where subsequently 10 (14%) were found to carry ZIKV RNA by reverse transcriptase polymerase chain reaction (RT-PCR), but no other arboviral RNA (see Waggoner and Pinsky (2016) for more information on ZIKV RT-PCR and serological tests) (23,24). A major study surveyed 173 households and 557 persons (23). It found that 74% of sampled individuals had IgM anti-ZIKV antibodies. Out of those, 38% reported having undergone a febrile illness reminiscent of ZIKV infection at the time of the outbreak (23).

This estimate however has to be treated with caution – firstly because 19% of those patients who tested negative for anti-ZIKV IgM antibodies also had a recent history of illness with similar symptoms, secondly because of the cross-reactivity of IgM antibodies to other Flaviviruses. The authors also sampled 185 patients with an acute ZIKV-like disease presentation within 10 days of symptom onset. They found out that 49 (26%) had ZIKV infection confirmed by either the presence of viral RNA or anti-ZIKV IgM (23). Of those 49, 45 were tested for ZIKV RNA and 15 were found to be positive, giving an estimate of ~8% of the total sample (23). Further 59 patients had anti-ZIKV IgM with no detectable RNA present (23). The authors concluded that about 5,000 or ~73% of the island's inhabitants could have been infected with ZIKV, and 18.4% of them experienced clinical symptoms (23). No deaths or hospitalizations were reportedly attributed to ZIKV, and no ZIKV was isolated from mosquitoes, however an association with *Aedes hensilli* was suspected owing to its role in Dengue fever virus transmission. High seroprevalence of IgM was considered as evidence in favour of a first-time exposure of the Yap Island population to a recently introduced virus. It was inferred that frequent presence of Dengue virus IgM in acutely-affected patients could have been a mark of a concomitant or sequential DengueV-ZIKV infection. The sudden increase in virulence lead to speculation on potential emergence of a new, more virulent ZIKV strain (6).

After a 6 year hiatus, new cases of ZIKV-related disease started emerging in French Polynesia by October 2013. Again, symptoms resembled a mild "dengue-like illness" with low fever (below 38°C), general physical weakness, arthralgia, headache as well as maculo-papular rash (25). Some patients had conjunctivitis and other symptoms. Early RT-PCR tests conducted on a panel of 10 patient samples (sourced from different archipelagoes) who had tested negative for DENV NS1 antigen, found that 4 were positive for ZIKV RNA (25). Later, 584 patients were tested for the presence of active infection by RT-PCR, and 294 confirmed positive – giving an estimate of over 50% (a much higher value than estimates obtained from Yap Island data) (25). Overall, 5895 suspected cases were reported based on symptoms with a projected total number of cases reaching 19000. Other estimates of 28 000 and 30 000 (~11% of the total population) were also proposed (6,7,26). Some estimated that between 50 to 66% inhabitants had been infected (27). Once again, concomitant and sequential occurrence of ZIKV and Dengue virus infections was noted and its clinical significance considered. Just like in the case of Yap Island, the source of introduction was not established, although DNA sequencing of the prM/E protein coding

sequences indicated a close relation to ZIKV strain Cambodia 2010-FSS13025, and also the Yap Island strains (6,23). The outbreak in French Polynesia also brought to light a potentially new and previously unattested complication of ZIKV infection in the form of Guillain–Barré syndrome (GBS) in adults. Against a baseline of 3-10 cases reported annually between 2009 and 2012, 42 GBS cases were reported during the epidemy (25,28–30). All patients were diagnosed after a contracting a disease consistent with ZIKV infection (29,31). A follow up study found that 41 of those patients (98%) had anti-ZIKV IgM or IgG (against 55% in the control group), but interestingly none tested positive for ZIKV RNA by RT-PCR (25,29). Also 25 patients with other neurological complications including encephalitis, meningoencephalitis, paresthesiae, facial paralysis and myelitis were found, and 7 with autoimmune and other conditions like immune thrombocytopenic purpura, ophthalmologic and cardiac complications (32).

Polynesian ZIKV strain subsequently spread to several other Pacific Islands in 2014-2015, including New Caledonia (~1385 lab-confirmed cases, ~0.8% of the population), the Cook Islands (905 reported cases, 49 confirmed), Easter Island (50 cases confirmed), Vanuatu, the Solomon Islands, Samoa and Fiji (5,6,33).

Brazilian outbreak of 2015

An outbreak of ZIKV was declared in 2015 in Brazil with cases at first localized to Northeastern part of the country – a region also known to be affected by Dengue fever. In March, during a local spike in febrile illness, ZIKV RNA was found in 7 out of 24 pre-selected patients at Santa Helena Hospital in Camaçari, in the state of Bahia (34). Reported symptoms mainly included fever, maculopapular rash, arthraliga, myalgia, conjunctivits and headache (34). Co-circulation and possible co-infection with DENV and CHIKV was considered. In May 2015, ZIKV was also identified in Natal, in state of Rio Grande, which was reported by the WHO and has been often cited as the first reported finding of ZIKV in Brazil (35). Earlier in 2015, rising numbers of patients presenting a "dengue-like" infection with mild fever, pain, maculopapular rashes (most frequent symptom), conjunctivitis, arthralgia (mostly hands and ankles) and myalgia (but also lymphadenopathy, distal oedema, headaches, retroorbital pain, vertigo and digestive disorders) were registered in Natal (35). Symptoms occurred at frequencies similar to those in the Yap outbreak (7). No associated deaths and complications were reported. 21 acute-phase serum samples were sent for lab

analysis. All tested negative for DENV and CHIKV by RT-PCR, however 8 (38%; 7 female) showed presence of a ZIKV strain, resembling closely the Asian ZIKV isolates of H/PF/2013, CK-ISL 2014 and FSS13025 (35). By March 2016, over 50 000 suspected ZIKV infections have been reported (7). Ultimately, governmental estimates indicated that between 440 000 to 1 300 000 suspected ZIKV disease cases occurred in 20 out of 27 Brazilian states by January 2016 (6,7). By October 2015, ZIKV was reported from Colombia and, by late 2015, from several other South American and Caribbean states (6). This lead the WHO to declare a public health emergency of international concern in February 2016. Viral strains isolated during the American outbreak belonged to the Asian genotype, resembling primarily the Yap, Cambodia, Thailand, and French Polynesia subvarieties (5,6,35). In a similar development, reminiscent of the Polynesian outbreak, increase in GBS occurred throughout 2015 in Brazil, El Salvador and Venezuela (6). Of note, for the first time ocular complications in adults were reported (6). A study of 29 ZIKV-infected pregnancies found a considerable range of ocular abnormalities in 10 out 29 newborns (35%) (36).

Microcephaly association

The most interesting fact about the Brazilian outbreak, setting it apart from the previous ones, was an accompanying dramatic increase in microcephaly cases among infants (by the Brazilian Health Ministry's definition of head circumference ≥2 standard deviations below the mean for sex and gestational age at birth), which was identified by the Brazilian government in September-October 2015. Microcephaly is a condition characterized by decreased brain volume and is also frequently associated with neurological, intellectual and psychomotor developmental delay and disabilities (37–40), with a prevalence of ~2-12 per 10,000 live births in the US (41,42). However, the exact definition varies and lack of single accepted criteria hamper accurate reporting, cross-comparison and monitoring of cases (7). The condition may be caused by a multiple factors including in utero infections (43). While typically first trimester infections are most likely to affect fetal development resulting in malformations and decrease in growth, which can result in microcephaly (as was commonly observed during ZIKV outbreaks), microcephaly may also develop at a later stage, after the first trimester – as a result of infection stopping normal brain development and causing fetal brain disruption (44). Interestingly, some early microcephaly reports from Brazil were indicative of fetal brain disruption sequence (7)

At first, the state of Pernambuco (one of the most affected Brazilian states, located in-between Bahia and Rio Grande) reported an increase in cases of microcephaly among the newborns in October 2015. There were 26 cases recorded within three weeks in October alone, and 39 since the beginning of 2015 (45). Further 54 cases were reported by Pernambuco in the first week of November and similar reports also came from several other states in North-Eastern Brazil (45). In November the Ministry of Health acknowledged a possible association to ZIKV outbreak. By the end of 2015, Brazil reported 4180 cases of suspected fetal (56 cases were stillbirths and spontaneous abortions) and neonatal microcephaly in 21 out of 27 states, which was over 10 in 10 000 live births, and accounted for 1% of all pregnancies in the most affected state (45). These numbers were much higher than some previous estimates of about 0.5/10,000, established by birth certificate reviews, albeit some level of underestimation has to be taken into account as expected levels of microcephaly were reported at 1–2 cases per 10,000 live births by other authors, and could potentially be even higher given data from other countries (42,44). Using data from cases recorded in 2015 and 2016, Jaenisch et al. (2017) estimated a highly variable absolute risk of reported microcephaly at 0.03% to 17.1% - depending on the source area, adopted criteria of microcephaly and assumed infection rate (46). Later, between November 2015 – July 2016, 8301 cases of microcephaly were reported and 1655 were confirmed, of which 255 (15%) were found to be ZIKV-associated by RT-PCR tests (47). This is in contrast to less than 200 annually, reported before (15). Altogether, the geographical distribution as well as the timing of both the ZIKV outbreak and the increase in fetal microcephaly was highly suggestive.

Early research

The events in Brazil sparked global interest and intensified research. Soon a substantial body of evidence linking ZIKV to microcephaly started emerging. Some of the first studies were initiated by October 2015, by the Brazilian government, who established a taskforce (SBGM-ZETF) with the aim of investigating potential links to the ZIKV outbreak (48). A cohort of 37 affected neonates born between August and October 2015 in eight Brazilian states was selected (48). 35 have been considered probable ZIKV-associated cases, of which 25 had severe microcephaly (head circumference >3 SD below the mean for sex and gestational age). All mothers had documented residence or travel to endemic ZIKV outbreak areas, and 26 reported a rash during pregnancy. 17 babies had some neurological abnormality – mostly hypertonia, hyperreflexia and irritability, while neuroimaging (by CT and ultrasound)

revealed a range of abnormalities in 27 out 27 studied cases (48) . All 35 infants tested negative for a panel of infectious agents. Overall, these abnormalities were considered consistent with complications arising on account of a viral infection. A subsequent work, and an extension of the Schuller-Faccini et al. (2016) study, looked at 83 infants (including 27 cases from said study) born between July 2015 and March 2016 (49) . They were selected from a cohort of 200 suspected microcephaly cases by criteria of having a head circumference of equal or less than 33 cm in addition to a CT/MRI brain scan consistent with ZIKA infection (49) . All had various neurological abnormalities confirmed by neuroimaging. 12 out of 14 tested infants had anti-ZIKV IgM antibodies present in the cerebrospinal fluid (CSF) (fetal CSF should remain free from maternal IgM antibodies, which do not cross the brain-blood barrier) (49). The authors emphasised that all of the cases were phenotypically similar to each other, regardless of their CSF anti-ZIKV IgM status. TORCH and CMV infection were excluded in 71% infants. The authors concluded that, under the assumption of causality, the observed clinical outcome most likely arose as a result of congenital ZIKV infection and produced a pattern of variable yet recognizable anomalies, with a broad degree of severity, but often life-changing. Typically, in addition to microcephaly, scalp redundancy and abnormal shape of skull was observed in 70% infants, indicative of Fetal Brain Disruption Sequence (49) . 96% revealed a variable array of dysmorphic facial features, ~42% had low length and 19% low weight at birth. Most had abnormal neurologic findings with generalized hypertonia being most common (74%). Other abnormalities, suggestive of fetal immobility, were also reported from the same cohort including dimples, distal contractures, feet malpositions and arthrogryposis (49) . Some cases had milder microcephaly or even a normal head circumference (HC). Specific neurotropism of congenital ZIKV infection was noted.

While these early studies provided serological evidence for the ZIKV-microcephaly association (in addition to the spatial-temporal overlap), their main shortcoming was the lack of testing for the presence of viral RNA in mothers and neonates.

The issue of Brazilian ZIKV infections lacking lab-confirmation and being diagnosed clinically was noted by the authors of the earliest and most widely cited (2419 citations at the time of writing of this review) prospective study (conducted September 2015-May 2016), in which Brasil et al. (2016) recruited pregnant patients with a very recent history of ZIKV illness, confirmed by clinical examination as well as by RT-PCR tests on blood or urine samples, as well as ZIKV-negative pregnancies (50) . Eventually, they examined the outcome of 125 ZIKV-affected pregnancies and 61 unaffected. CHIKV infection was discovered in 42% ZIKV-negative and 3% ZIKV-positive women. They reported adverse outcomes in 49

(42%) out of 117 live-born infants among cases, almost all affecting the CNS, most typically cerebral calcifications, atrophy, ventricular enlargement, hypoplasia of cerebral structures and parenchymal brain hemorrhages, in contrast to just 5.3% in the control group (50). 31 out of 49 also had abnormal neurologic findings like hypertonicity, hyperreflexia, contractures, spasticity and seizures (50). The authors identified microcephaly in 4 infants (3.4% of the live-born infants from ZIKV-exposed pregnancies; 2 had disproportionate microcephaly), and none in control group. In three cases, microcephaly was accompanied by intra-uterine growth restriction. In general, fetal growth restriction was observed in 9% cases and 5% controls. Pregnancy loss was similar in both groups. The authors noted that the associated illness was mild with some symptoms being more typical of ZIKV infection (mainly conjunctival injection and maculopapular pruritic rash). They concluded that ZIKV infection during pregnancy carried substantial risk of severe outcomes. Interestingly, the adverse results were independent of the trimester during which the infection had occurred (50). By including ZIKV-negative pregnancies and testing for viral RNA in mothers the authors provided their findings with extra specificity. Nonetheless, it was later noted that the work lacked appropriate population controls (41). Of interest may be the diagnosis of fetal or intrauterine growth restriction (IUGR) in 3 out of 4 infants with microcephaly. IUGR and small gestational age (SGA) can be a feature of ZIKV affected pregnancies, with estimates ranging between 10% and 20% (51–53).

Another prospective study, without a control group, was conducted between February and October 2016, and followed 54 pregnant women with a history of a suspected ZIKV illness and confirmed by RT-PCR to be positive for the viral envelope (E) gene (54). The authors reported no microcephaly albeit a some abnormalities were identified in over ~28% of newborns (15 out of 54). They however, differed from those reported in the previous studies and included, among others, lenticulostriate vasculopathy, subependymal cysts, auditory and ophthalmologic disorders, bilateral cranial bleed, chorioretinitis and premature birth. There were no miscarriages and ZIKV RNA was found in umbilical cord blood or urine samples of 18 out of 51 newborns tested, including 8 of 15 with abnormalities (53%) vs 10 out of 36 without (28%), which was considered as evidence for intrauterine, congenital transmission. The authors noted that their observations of congenital ZIKV syndrome were mild and differed from those of other authors, with some of the adverse outcomes observed by others being rare or “nonexistent” in their cohort (54). Aside from the lack of microcephaly (which could be attributed to small sample size), no instances of macular hypoplasia and abnormal neurologic test results were identified. The authors concluded that ZIKV-related fetal and

neonatal abnormalities reveal high heterogeneity and a definitive link to ZIKV cannot be established nor discarded.

At the same time, Araujo et al. (2016) recruited 32 neonate cases with microcephaly and 62 healthy controls between January and May 2016 (55). 13 (41%) cases and zero controls had ZIKV RNA or anti-ZIKV IgM present in CSF or serum. Authors reported that 11 out of 27 (41%) cases who had brain examined using a CT scan, had CNS abnormalities (most typically cerebral calcifications – 7 individuals, and ventriculomegaly – 5 individuals), including 9 out of 13 ZIKV-positive cases (69%). 84% cases were small for their gestational age (vs 6% among controls) (55). Most mothers did not report a rash during pregnancy, however out of those that did, 54,5% noticed it during the 1st trimester. Contradictory to data given in Table 1, in their conclusion, the authors state that only 7 cases had brain abnormalities on neuroimaging (27%) – a finding they acknowledge is at odds with those of some other authors (55). The same group released their final report in 2018. Eventually, 91 cases and 173 controls were followed between January and November 2016 (56). 32 cases (35%) had lab-confirmed ZIKV infection. 69 (83%) out 83 cases with available data were considered hypotrophic for their gestational age (vs just 5% in the control group). 10 in 79 (~13%) cases with neuroimaging available were ZIKV-positive and showed CNS abnormalities, 11 were ZIKV-negative with abnormalities, while 13 were ZIKV-positive without any abnormalities (56). Consequently, among the infants with microcephaly and brain scans available, about 26.5% had accompanying changes to brain structure typical of CZS (like calcifications, ventriculomegaly, abnormal cortical development).

Altogether the Brazilian studies yielded a strong argument in favour of ZIKV-microcephaly association and, to an extent also causality, yet the global picture grew increasingly discrepant when fine detail was considered. Because of the issue of cross-reactivity and environmental overlap with other Flaviviruses, one common limitation to some of those early studies was the frequent use of alternative criteria of either confirmation of virus-specific IgM antibodies (usually in serum) or a positive RT-PCR result, without making it immediately obvious how many individuals underwent the nuclear acid tests.

Congenital Zika Syndrome

As a consequence of early research, a picture of variable yet mostly repeatedly registered neonatal abnormalities and other clinical features begin to emerge, accompanying and possibly caused by congenital ZIKV infection. These included a broad range of clinical findings: microcephaly, abnormal brain development and structure, brain tissue lesions/damage, altered or impaired neurological function, auditory impairment and ocular lesions, but also other, less common symptoms like aberrant skull shape, scalp redundancy, dysmorphic facial features and other orofacial anomalies (49,50,57–65) . Consequently, a "Congenital Zika Syndrome" (CZS) term was proposed as a broad umbrella-term describing the most common clinical findings in neonates who had in-utero exposure to ZIKV (57). The authors who introduced the term, noted that they found Zika virus RNA in "only a fraction of microcephaly cases", which they argue could be due to its distribution being restricted to specific tissues or early clearing of the viral infection in utero. They also concluded that reported numbers of microcephaly in Brazil suffered from over-ascertainment and thus were likely inflated "due to other causes" (57). The current picture of CZS, with its broad range of symptoms and variable presentation, has been thoroughly discussed and delineated by Pomar et al. (2019), and also by Gaetano et al. (2023), who noted that, according to studies of ZIKV-infected pregnant mothers, the risk of the infant developing CZS is higher (5-14%) than the risk of microcephaly (4-6%), and highest during first trimester infections (5,62) . They also mentioned similarity to FOXG1 syndrome, which may hint at the mechanism by which ZIKV causes some of congenital defects (5,62,66) . Studies show that not all infants exposed to ZIKV during pregnancy develop complications, especially in the absence of microcephaly and symptoms of CZS (67–69) . In fact, a study of 114 children from mothers infected by ZIKV during pregnancy as well as 120 healthy controls found no difference in rate of neurodevelopmental delay (67,70) . This observation was generally shared by other groups (71–73) . Although some developmental and behavioral differences may still occur in comparison to ZIKV-unexposed children (74–77). In contrast, children affected by the CZS or microcephaly typically show growth deficits and differences in cognitive development, and may require a nurturing care approach (78–80).

In-utero vertical transmission of ZIKV

Another new observation revealed by the Brazilian outbreak was the fact that prior to 2016 no vertical mother-to-fetus ZIKV transmission had been reported, although two cases or perinatal infection at birth, resulting in a mild disease in neonates, had been documented (6) . Also no member of the Flaviviridae family had been known to affect fetal development during pregnancy (5,13) . By 2016 two new instances of pregnant patients were described when Melo et al. (2016) reported the first widely cited cases of in utero infection demonstrated by the presence of ZIKV RNA in amniotic fluid (81) . Both patients carried fetuses with microcephaly and reported symptoms resembling ZIKV infection, however tested negative for ZIKV in blood serum. Most significantly, their amniotic fluid samples were found positive for ZKIV RNA by RT-PCR, favouring the vertical mother-to-fetus transmission during pregnancy hypothesis. This was replicated by Calvet et al. (2016) who confirmed presence of ZIKV genome in amniotic fluid samples from two pregnancies in which fetuses had microcephaly (82). Both mothers had symptoms typical of ZIKV infection. ZIKV was absent from urine and serum. Similarity to strains found in French Polynesia in 2013 was noted. By targeting protein 5 and envelope genes and by immunohistochemistry, Martines et al. (2016) were the first to isolate ZIKV RNA from brain tissue of two miscarried fetuses as well as deceased newborns with microcephaly, from pregnancies with a history of clinical symptoms resembling ZIKV illness (83) . This was reported in 2015 by the CDC and also further replicated (58,84). Subsequently, ZIKV RNA and in some cases, complete genomes and other particles were isolated from brain tissue of abnormal fetuses in three cases of ZIKV-infected pregnant patients in Slovenia, Finland and Thailand (85–87) . ZIKV RNA was also isolated from products of conception, amniotic fluid and placenta of three pregnancies suspected of ZIKV infection (88).

Experimental research

ZIKV outbreaks instigated a new wave of functional studies with the aim of elucidating molecular mechanisms behind the viral infection and its clinical effects (89) . Experimental evidence from animal studies and cell cultures had indicated that ZIKV may be neurotropic and capable of infecting brain tissue and promoting neuronal necrosis and degeneration -

directly and through other mechanisms like inflammasome activation (16,63,90). One study concluded that the virus hijacks the innate mechanism of autophagy using it for its propagation and may be altering neurodevelopment through its effects on centrosome formation and segregation, mitosis and chromosomal stability (91). Also, some viral proteins, like the envelope protein may have an adverse effect on neural differentiation (92). Another potential mechanism by which ZIKV may cause developmental abnormalities and brain damage may be inflammation (93). ZIKV has been demonstrated to be able to utilize canonical proinflammatory pathways (3). Tang et al. (2016) demonstrated that ZIKV can enter human neural progenitor cells (which are more susceptible than mature neurons), which produce infectious ZIKV virions and die more frequently as a result (94). Others proposed and provided evidence for the virus infecting glial cells via the Axl receptor (95,96). Microcephaly may therefore arise as a consequence death of progenitor cells or direct infection and death of neurons. It has been speculated that ZIKV infects fetuses via an intermediate stage of placentitis, whereby it replicates within the trophoblasts and endothelial cells of maternal–fetal interface (5,58). Placentitis may lead to hypoperfusion and pregnancy loss (5,97). Proposed mechanisms of ZIKV transmission and infection, also based on cell culture and animal models, were reviewed by other authors (4,98).

Further research

Brazilian ZIKV epidemic prompted some researchers to look at other countries, including those where the virus had established itself long before, and re-evaluate available evidence. In the USA, it was initially shown that out of 442 pregnancies with lab evidence of ZIKV infection (by RT-PCR or serology), congenital abnormalities were present in 6% or 26 cases (21 out of 395 live births), including 4 with microcephaly, 14 with microcephaly and brain abnormalities, and 4 with brain abnormalities only (calcifications, atrophy, ventriculomegaly, hydrocephaly, and other abnormalities) (99). The proportion of CZS grew to 11% if infection occurred during the 1st trimester (99).

Subsequently, based on the data from pregnancy and infant registries from January 2016 – April 2017, the CDC reported 2549 pregnancies completed in the USA (2464 livebirths and 85 pregnancy losses) with molecular evidence of ZIKV infection (by RT-PCR or serologic evidence) and 1508 with RT-PCR-confirmed recent ZIKV infection (in maternal or

infant/fetal tissue) (100). 61% women reported symptoms, while 38% remained asymptomatic. There were 122 or 5% infants/fetuses showing potential abnormalities, including 108 (4%) with microcephaly and/or other brain abnormalities (100). The associated risk of birth defects in infants was 6% (8% in the subset confirmed by nucleic acid testing), 5% and 4% - depending on the trimester in which infection occurred. The authors state that 18% pregnancies were likely infected during the first trimester (100).
Another study showed birth defects present in 5% (51 cases with a history of exposure in 16 different countries) of a cohort of 972 infants with a history of possible ZIKV-infection during pregnancy (with normal prevalence of birth defects being ~2.9 per 1,000 live births) (101). The estimate grew to nearly 10% (24 in 250) in a subset with ZIKV RNA or anti-ZIKV IgM confirmed in maternal or fetal tissue (101). In concordance with previous findings, the authors pointed out that the highest percentage of abnormalities was present in the infants from pregnancies infected during the 1st trimester. Microcephaly or brain structure anomalies accounted for 84% of all the birth defects, with authors concluding that some changes occur without evident microcephaly and therefore neuroimaging is necessary in diagnostics of ZIKV-related infant abnormalities (101). A higher estimate was given by Rice et al. (2018) who looked at a cohort of 1450 children from ZIKV-exposed pregnancies born in the USA and concluded that 203 (14%) had a ZIKA-associated birth defect or neurodevelopmental abnormality, or both (102).

These works confirmed the ZIKV-microcephaly association, but at the same time failed to replicate some of the previous findings, especially the severe, phenotypic presentations and high incidence of CZS symptoms reported from Brazil.

Cauchemez et al. (2016) found that in French Polynesia, between September 2013 and July 2015, 8 cases of microcephaly were reported (5 from abortions and 3 in newborns) (103). The birth rate was 4,182 per year (for 2013–2014) (103). Although the number is within the bounds of the reported incidence of microcephaly, the authors point to temporal clustering suggestive of an association to ZIKV outbreak, and estimate the risk microcephaly at 1% for ZIKV-exposed 1st-trimester pregnancies. The authors compared it to risk of congenital anomalies associated with other viral infections in-utero (13% for cytomegalovirus infections, 38-100% for congenital rubella syndrome and 10% for parvovirus B19), but state that low fetal risk may have been amplified by high rates of infection and exposure in a previously naïve population (103). Other authors pointed out that by counting newborns only, there was a

single case of microcephaly during the Polynesian ZIKV outbreak (104). Jouannic et al. (2016) retrospectively tested amniotic fluid from 6 pregnancies with microcephaly from French Polynesia (out of a total of 13 with cerebral abnormalities reported in 2014), and found that 4 were RT-PCR positive for ZIKV (26).

Another group reported on Thailand, a country where ZIKV been first detected in 1963, and where an earlier study found 8% seropositivity for ZIKV-neutralising antibodies in Bangkok (although, as the authors point out, caution has to be taken because of cross-reactivity with anti-DENV antibodies) (105). In Thailand, 1612 ZIKV infections were recorded between 2016 and 2017, as well as 115 pregnant women with confirmed infection, 2 ZIKV-related cases of microcephaly and 1 case of CZS (105). Subsequently, Kuadkitkan et al. (2020) looked at South-East Asia, where the virus had been first isolated in 1966, in Malaysia, and concluded that very few cases of ZIKV-related illness had been documented (first one verified in 2010) (106). The most compelling evidence was provided by the Ministry of Public Health in Thailand. 150 pregnant women with confirmed ZIKV infection were identified in the years 2016-2018. 132 births were reported including 4 cases of microcephaly (3%; 2 in 13 months), without confirmatory RT-PCR (106). An additional screening of 330 newborns revealed 3 cases of CZS (106). More recently, there were 234 ZIKV infections in pregnant women in Thailand between 2016 and 2022 (and 11 miscarriages, with 4 attributable to ZIKV) (107). Also, screening of 2217 microcephaly cases in infants, lead to identification of 15 cases of CZS (107).

Using a sample of 135 healthy adults, Sornjai et al. (2018) estimated that 70% of the Thai population have anti-ZIKV neutralizing antibodies, which may effectively reduce the transmission (18). More recently, another group of researchers investigated seroprevalence for anti-ZIKV antibodies in Thailand in pregnant women from May to October 2019 (12). They found that out of a cohort of 650 pregnant women (39.42% first, 52.26% second and 7.36% third trimester) about 31% had anti-ZIKV IgG antibodies and 40% anti-ZIKV neutralizing antibodies. The authors try to explain the difference in results by timing and sampling (12).

In India, the first confirmed ZIKV infection in humans was described in 2016 and occurred in Gujarat, even though seropositivity for anti-ZIKV antibodies was estimated at 17% in 1954 already (108). Since then, the country registered further infections in 2017, in Tamil Nadu, and experienced two local outbreaks in 2018 with about 159 and 127 known cases in two different states, including 105 pregnant women (109). No clinical outcomes have been reported and the literature is scarce. There is evidence that ZIKV has widened its range in India, with reports of infections from 16 states between 2017 and 2021 (109).

Between 2016-2017, a group of researchers conducted a prospective study in the French territories in the Americas, following 561 symptomatic and PCR-confirmed, ZIKV-infected pregnant women (51). The group reported 11 miscarriages, 11 terminations, 6 in utero deaths or stillbirths, and 527 live births (with 13% considered small for gestational age). Neurological and ocular defects were detected in 39 cases (7%) including 28 newborns (5%). 32 fetuses and newborns had microcephaly (~6%; including 25 out of 527 live-born), including severe microcephaly (1.6%) (51) . Severe microcephaly or other brain defects typical of CZS were observed in 3.1% cases. Additional defects were present only in 1 out of 23 infants with moderate microcephaly. The risk of congenital abnormalities was estimated to be highest during the 1st trimester. The authors note that while their findings are in concordance with those of other authors, with neurologic birth defects at 7% (compared to 5-6% in the USA), they are different to those reported from Brazil (42%) (51). In terms of risk of microcephaly in ZIKV-exposed pregnancies, they consider their estimate comparable to others (~6% in the current study vs ~4% in the USA and 3.4% in Brazil) (51).

Finally, two studies from Brazil and French Guiana followed up on the course of ZIKV infections in the area affected by the 2015-2016 outbreak. Pomar et al. (2018) investigated clinical outcomes in 300 pregnant mothers who tested ZIKV-positive by RT-PCR and 305 fetuses and neonates born in French Guiana in January-July 2016 (62) . Out of 291 fetuses exposed to the virus, 210 (72%) had no symptoms, 31 had mild-to-moderate symptoms, potentially related to CZS, while 26 had serious complications and 12 pregnancy losses occurred. 76 (26%) fetuses/newborns tested positive for ZIKV, indicative of vertical transmission (62) . In that group only 45% had no complications (versus 87% in ZIKV-negative group). 27 newborns, out of 273 (10%) with available measurements, had microcephaly including 4 with severe phenotype (head circumference below 3 standard deviations) (62). The report prompted two replies (in the form of so called “Rapid responses” to the British Medical Journal) from the journal’s professional medical readers, which offered insight into important limitations and methodological pitfalls. It was pointed out that selection bias could have possibly been introduced during sampling as most serious cases were more likely to get hospital referrals, which likely influenced the CZS risk and vertical transmission estimates. In addition, serological tests, which suffer from cross-reactivity, were used for ZIKV status classification of some individuals, instead of RT-PCR, possibly elevating the number of false positives. An alternative conclusion based on odds ratios calculated from the data reported in the study, taken from 69 ZIKV-positive (by RT-PCR) mothers and associated

pregnancy outcomes, provided limited evidence for an association between ZIKV infection during pregnancy and congenital abnormalities. Finally, it was pointed out that some of the patients may come from a genetically distinct population.

Another group of researchers investigated the outcome of 190 ZIKV-infected pregnancies and 193 neonates referred to a tertiary hospital in Rio de Janeiro between March 2016 – April 2017 (52). Both mothers and infants were confirmed to be ZIKV-positive by RT-PCR testing. 37% newborns/fetuses had congenital defects and 22% were considered small for gestational age. 21% had microcephaly. The authors note that their findings appear much higher than some of the previous estimates (for microcephaly: 1% in French Polynesia or 13% in Bahia, Brazil; for congenital abnormalities 5-7% in the USA and French Polynesia) and suggest ascertainment bias as a likely explanation (52).

Overall, the results of most of the studies discussed above yielded variable, yet to extent reasonably concordant estimates. Critical evaluation of available data lead to some important conclusions on discordant results and limitations of adopted methods of research.

Current situation

The WHO reports that as of July 2019, 87 countries and territories experienced ZIKV infections (110). 8 years after the last major epidemic, ZIKV continues to be present globally and a consensus on several epidemiological and clinical features of the virus seems to be emerging. Nonetheless, there is still missing evidence and variable findings. Some authors listed key "knowledge gaps" about the Brazilian outbreak, most important of which could be summarised as follows: unknown cause of unusually rapid ZIKV propagation in Brazil, unknown biological link between ZIKV and microcephaly, reasons for lack of prior reports on fetal microcephaly in places affected by previous ZIKV outbreaks (111) . Two final points touch upon, perhaps the most important issue of discordance in the estimated risk of microcephaly for pregnancies exposed to ZIKV. With estimates ranging usually from 1% to over 20%, as shown above, a possible explanation may lie in methodological approach variability as well as limitations and biases, which some of the authors acknowledged. The same knowledge gaps are also relevant to the other ZIKV-associated clinical conditions like CZS, their variability and incidence (112).

Several authors identified limitations to observations made during the Brazilian outbreak and in its aftermath. Writing already in 2016, Johansson et al. (2016) pointed that ZIKV infection reports represent a fraction of the number of actual transmissions, which is difficult to

establish reliably, without additional studies or modelling (113). They also noted that variable criteria and difficulties in microcephaly confirmation, case overreporting and an uncertain baseline level of microcephaly rate (2 to 12 cases per 10,000 births) could all potentially distort final results – the reported number of ZIKV illness cases, and in particular the estimated risk of microcephaly in newborns associated with ZIKV exposure during pregnancy (113). These concerns were also voiced by Shapiro-Mendoza et al. (2017) who mentioned multiple possible sources of bias and limitations: ZIKV infection rate underestimation, misclassification of microcephaly leading to over- or underreporting, ignoring alternative etiologies for the observed phenotypes (whether genetic or infectious, or other), inadequate postnatal neuroimaging resulting in omission of some phenotypes (100).

Other authors also mentioned possible selection bias inflating the numbers of microcephaly cases at the reporting stage, but also potential underestimation, especially of infants with mild microcephaly, as well as possible recall bias of pregnancies with a suspected ZIKV infection (47,48). Ascertainment bias in particular could play a critical role in some of the more discrepant reports, especially since the selection process and criteria for inclusion were not always explained in detail, yet could potentially have a profound effect. For instance, del Campo et al. (2017) stated that out of 200 candidate patients with registered head circumference of ≤ 33 cm, only those with neuroimaging "consistent with ZIKV prenatal infection" were included in their study (49). Most were subsequently excluded due to actually having a normal circumference, normal postnatal clinical presentation or due to other causes having been suspected or established. However, the authors were well aware of and acknowledged their study was biased towards the more extreme spectrum of clinical case.

Victora et al. (2016) pointed to the risk of overreporting given raised awareness, more testing and different definitions of microcephaly itself, which alone could account for a considerable discrepancy in numbers of suspected microcephaly in Brazil (600 000 vs 3000 cases) (15). They concluded that the diagnostic criteria adopted by the Brazilian Ministry of Health had low specificity and likely inflated the number of cases.

In addition to the variability in case number estimates mentioned above, there is also the issue of establishing ZIKV causality. IgM tests may not always yield reliable results due to cross-reactivity between the members of the Flaviviridae family (114). Particularly relevant is cross-reactivity with DENV in regions where concomitant DENV outbreaks can occur (13). Flaviviridae-Togaviridae cross-reactivity was also reported (12). Consequently, the incidence of ZIKV illness may be difficult to establish because of unknown level of false positive results, especially in areas where DENV and other Flaviviruses are endemic (6). RT-PCR

tests are more reliable, but they also suffer from potential RNA stability issues. In one of the earlier Brazilian studies, del Campo et al. (2017) stated the main limitation of their work was the absence of detectable ZIKV in all the 14 microcephalic cases, in either blood or CSF (49). This may be due to the fact that ZIKV RNA levels in serum may decrease significantly beyond the 5th day after symptoms onset, although in some pregnancies ZIKV RNA is detectable up to 100 days after the initial infection, presumably because of viral replication in placenta and fetus (49). ZIKV RNA persistence may vary significantly depending on the tissue/source (115). As a result, it has not always been clear how many infections, affected pregnancies and neonatal outcomes may reliably and unequivocally be linked to ZIKV. A similar concern was voiced by Hennessey et al. (2016), who stated that despite a significant increase, it is unknown how many infants born with microcephaly could be linked to ZIKV infection at pregnancy, due to the lack of adequate molecular testing (116). Symptoms-based diagnosis and serological tests are substandard to RT-PCR, yet some studies used them on par and without clear separation of those mothers and infants who were tested by IgM assays only. This is especially important because of possible spatial-temporal overlap with other viruses, including DENV and CHIKV, known to cause similar types of illnesses and known to elicit ZIKV cross-reactive antibody responses (116). Not all studies made the distinction and stratified their samples accordingly. Also, amniotic fluid or placental samples may not always be as reliable as fetal tissue, especially CSF for congenital ZIKV infection diagnosis (61,117,118). On the other hand however, RNA may be cleared from CSF early, limiting the detection window (61)

Concern about this limitation was also expressed by Teixeira et al. (2016) when stating, in 2016, that the number of reliable, lab-confirmed instances of ZIKV illnesses had been inadequate to for the purpose of predicting expected microcephaly cases in Brazil and the Americas (45). Perhaps more importantly they also pointed to the fact that within a panel of 500 reported Brazilian cases of microcephaly, 44% were confirmed to be true by MRI imaging. This, they argue, constitutes evidence for overreporting and by these estimates the true number of cases reported up until January 2016 should be about 1672 instead of 4180 (45).

Some authors proposed alternative causes to explain the Brazilian increase in microcephaly, among them - Pyriproxifen, an insecticide and a mosquito control measure, used extensively in parts of Brazil since the final quarter of 2014 (104). They drew a comparison between Brazil on one hand, and French Polynesia and Colombia (where 12,000 Zika infections were confirmed by March 2016) on the other, by which the discrepancy between the number of

microcephaly cases exceeds two orders of magnitude, and stressed the fact that approximately 12% of Brazilian newborns with microcephaly were laboratory-confirmed to have had a ZIKV infection. A similar estimate of 15.4% (255 out of 1656 confirmed cases, with 8301 reported in total between November 2015–July 2016) was also given by other researchers (47). In addition, Parens et al. (2017) pointed to other discrepancies between various studies and regions (including within Brazil), including rates of ZIKV infection, birth defects, aborted pregnancies and stillbirths (104). They conclude that ZIKV was likely not the only factor, and probably not even the primary factor behind the Brazilian neonate microcephaly increase. Some of their observations, including significant differences in microcephaly rate across different Brazilian states were also shared by Magalhães-Barbosa et al. (2016) (47).

A recent, large meta-analysis conducted on data from 8341 children born with microcephaly (out of 10 250 994 newborns from three continents) looked at the ZIKV-microcephaly association (119). It was concluded that the association was marginal, with risk ratio (RR) being 2.12 (95%-CI 1.01–4.48, *p*-value of 0.048), while other in utero infections showed RR of 15.24 (95%-CI 1.74–133.70 at *p*-value = 0.014), and teratogens RR of 3.43 (95%-CI 2.69–4.38 with *p*-value of < 0.0001) (119). The authors point to the fact that while the association holds for Brazil, it does not in Africa. They conclude that other factors (like socioeconomic status, poverty, other in utero infections, teratogens, alcohol and drug abuse) were likely involved in the Brazilian outbreak (119).

When looking at the global picture of past and present, the 2016 ZIKV outbreak in Brazil was in many ways different to the previous ones. This may be explained by a combination of multiple factors – many of which were discussed previously – including lax and variable definitions and selection criteria, overreporting and sampling bias, inadequate molecular testing, emergence of new, virulent strains in naïve populations, specific genetic susceptibilities as well as environmental co-factors (social class, lifestyle, smoking, substance abuse, pesticides etc.). A suggestion was made about the 2013 Pacific strain gaining virulent properties thanks to the S139N mutation or adaptive mutations occurring in codons of NS1 protein (109,120). Longer history of exposure and higher levels of pre-existing neutralizing antibody seroprevalence, may have decreased outbreak severity and limited transmission and exposure in places where ZIKV had been circulating for decades (12). This may the reason for which ZIKV illness (and CZS) has been very rarely reported in many South-East Asian countries in “stark contrast” to South America and Oceania (7). Potentially, co-circulation and co-infection with a combination of DENV, CHIKV and ZIKV, or even other infectious agents, could also have played a role in the Brazilian ZIKV outbreak and the

observed, associated outcomes (7) ; (other infectious causes of microcephaly have been reviewed by Devakumar et al. (2018)) (43). Didier Musso, director of the Emerging Infectious Diseases Unit at Institut Louis Malardé in French Polynesia speculated that co-circulation with other viruses may have exacerbated the outcome in populations naïve to ZIKV, contributing to higher incidence of CNS malformations (121).

Conclusion

Despite numerous studies and a decade of intense research, the exact mechanism by which ZIKV infection occurs and causes congenital abnormalities is still not fully understood, although considerable progress has been made. Strong evidence for vertical transmission and fetal neurotropism has been provided. Main scientific postulates for future research, outlined by authors like Fauci and Morens (2016) or Teixeira et al. (2016) still hold, including the need to establish evidence for congenital infection risk during pregnancy as well as for a causal link to microcephaly, including both case-control studies and animal models (13,45).

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