Universidade de São Paulo – Faculdade de Saúde Pública

Infecção Vertical Pelo Vírus Zika e suas Repercussões Feto-Maternais: Achados de uma Coorte de Gestantes em Jundiaí, São Paulo

Nuria Sánchez Clemente

Tese apresentada ao Programa de Pós Graduação em Epidemiologia para obtenção do título de Doutor em Ciências.

Área de Concentração: Epidemiologia

Orientadora: Profa. Dra. Marcia Furquim de Almeida

São Paulo
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APROVAÇÃO DO PARECER CONSUBSTANCIADO DO CEP

O projeto foi aprovado pela CEP da Faculdade de Medicina de Jundiaí, sob número 1446577.

Diante do exposto, o presente Projeto encontra-se em conformidade com a resolução 466/12 do CNS.

PARECER CONSUBSTANCIADO DO CEP

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I dedicate this work to my husband Harry and daughter Luna who accompanied me to see the first babies of the cohort and whose unwavering support has ultimately made this all possible.

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A CAPES, FAPESP e ao London School of Hygiene and Tropical Medicine, pelo suporte financeiro, imprescindível para realização deste projeto.

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ABSTRACT

Prior to its arrival in The Americas in 2014, Zika Virus was thought to cause only mild disease. Following the outbreak in Brazil and the declaration of a Public Health Emergency of International Concern by WHO in February 2016, epidemiological and biological evidence has been published which supports a causal link between prenatal Zika Virus (ZIKV) infection and congenital brain abnormalities including microcephaly.

This doctoral thesis uses data from The Jundiaí Zika Cohort, a prospective pregnancy and birth cohort which was set up in The State of São Paulo in 2016 to investigate this causal hypothesis further.

A total of 748 pregnant women were recruited from the high-risk pregnancy clinic at Jundiaí University Hospital in the period March 2016 to August 2017. Baseline sociodemographic and medical data were collected at recruitment. Biological samples (blood, saliva, urine) were collected from women at enrolment and regular intervals throughout pregnancy. Women were asked to report any symptoms consistent with ZIKV infection (as per the WHO clinical case definition) and to attend the hospital to be assessed clinically and for samples to be taken. Further biological specimens (colostrum, umbilical cord, placenta, neonatal blood, saliva, urine and cerebro-spinal fluid when appropriate) were obtained at delivery. Urine samples were processed for ZIKV RT-PCR and the rest of the biological material has been stored in a secure biorepository. Anthropometric measures were obtained from the neonates at birth.

After creating the master database and carrying out the first thorough analysis of the dataset, I created a cohort profile manuscript which thoroughly detailed the creation, the specific methodology and the preliminary findings of The Jundiaí Zika

Cohort. I then employed a prospective cohort study design to investigate the extent to which specific symptoms can be utilized to differentiate ZIKV-infected pregnant women from those with other pregnancy-related problems. Finally, I compared the prevalence of adverse fetal outcomes (prematurity, low birth weight, small-for-gestational-age, fetal death and microcephaly) by prenatal Zika Virus (ZIKV) exposure status.

The main findings are that most pregnant women positive for ZIKV in urine are asymptomatic and do not deliver a baby with microcephaly, that physical symptoms alone do not differentiate between high risk pregnant women positive or negative for ZIKV and that current clinical case definitions have a low sensitivity for detecting ZIKV-positive women living in active ZIKV transmission areas. In addition, ZIKV infection in high risk pregnant women substantially increases the risk of disproportionate microcephaly but not other adverse fetal outcomes.

As a result of this research, we propose clinical case definitions for use in pregnant women living in areas with active ZIKV transmission be revised and that disproportion between head circumference and weight during fetal development should be considered as a diagnostic tool for the presence of a ZIKV-infected fetus.

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PRESENTATION

This doctoral thesis was organized according to directives from the Public Health School of the University of São Paulo, presented under the form of scientific articles. This document comprises an introduction to the theme, the aims and objectives of the investigation and a description of the study design and methods employed. The results and discussion sections are composed of three manuscripts which have been submitted or will be submitted to scientific journals. Each one is formatted according to each journal's respective specifications. The first manuscript, entitled "Cohort Profile: The Jundiai Zika Cohort (JZC)" describes the context, methodology and preliminary results of the cohort. The second manuscript, entitled "Can Zika Virus Infection in Pregnant Women be Differentiated of the Basis of Symptoms?" describes clinical manifestations of ZIKV infection, the proportion of asymptomatic infections and whether ZIKV positive pregnant women can be differentiated on the basis of their symptoms from ZIKA negative women with high-risk pregnancies. The third manuscript, entitled 'Zika Virus Infection and Adverse Fetal Outcomes: A Prospective Pregnancy Cohort Study in Sao Paulo State Brazil' compares the prevalence of prematurity, low birthweight, small-for-gestational-age, microcephaly and fetal death in ZIKV-exposed and unexposed newborns. The final considerations bring together the salient conclusions of each of the manuscripts, thereby summarising the major findings of the study.

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ABBREVIATIONS

CDC	Centre for Disease Control
CMV	Cytomegalovirus
CSF	Cerebrospinal Fluid
CZS	Congenital Zika Syndrome
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAPESP	A Fundação de Amparo à Pesquisa do Estado de São Paulo (São Paulo State Research Foundation)
FBDS	Fetal Brain Disruption Sequence
IUGR	Intra-uterine Growth Restriction
JZC	Jundiai Zika Cohort
LBW	Low Birthweight
LMP	Last Menstrual Period
PAHO	Pan-American Health Organisation
PRNT	Plaque Reduction Neutralisation Test
RT-PCR	Real-Time Polymerase Chain Reaction
SGA	Small-for-gestational Age
TORCH	Toxoplasmosis, Other (Parvovirus B19, Syphilis, Varicella Zoster), Rubella, Cytomegalovirus, Herpes Simplex Virus)
UNICEF	United Nations Children's Fund
USS	Ultrasound Scan
WHO	World Health Organisation
ZIKV	Zika Virus

1 INTRODUCTION

1.1 The Discovery of Zika Virus and its Propagation Around the World

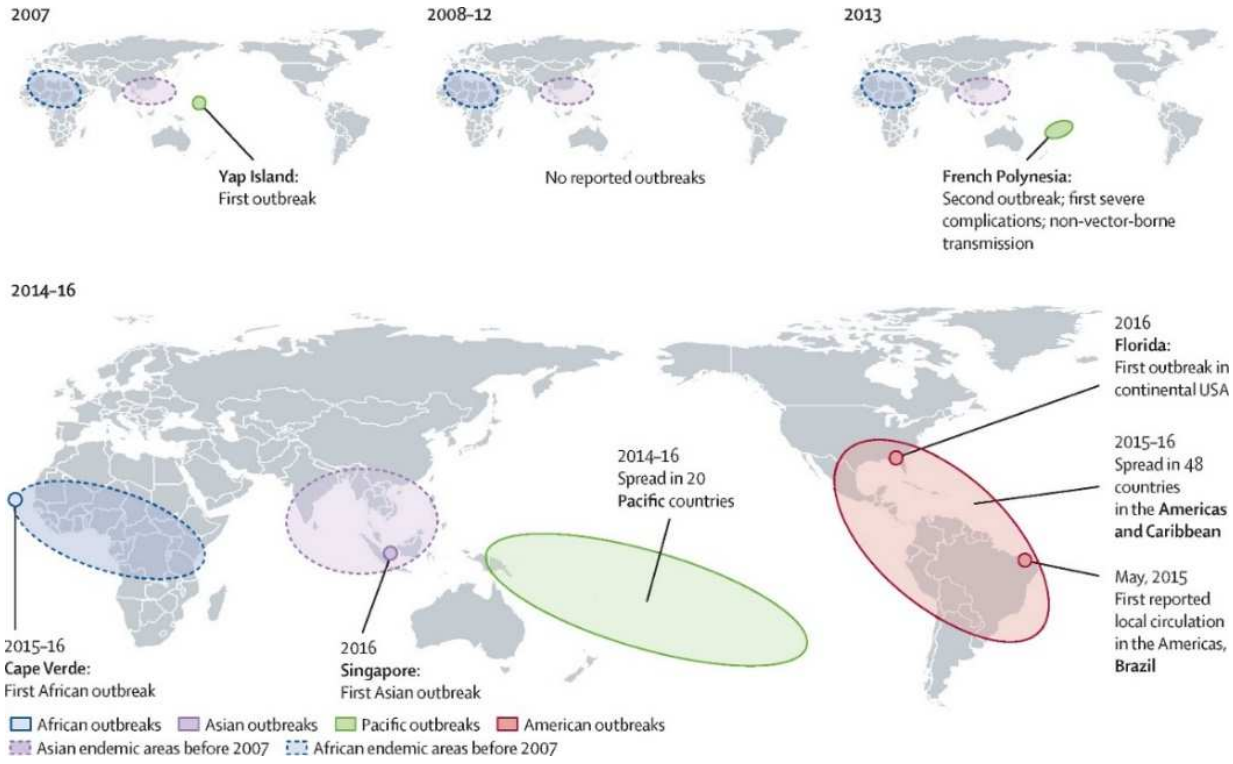
Zika Virus (ZIKV) was discovered incidentally by scientists GWA Dick and AJ Haddow, during routine surveillance for yellow fever[1] in the Zika Forest in Uganda first in 1947 in captive sentinel rhesus monkeys, and thereafter in 1948 in *Aedes (Stegomyia) africanus* mosquitoes.[2] The first bona-fide case of natural infection in humans[3] was reported by Simpson, who described his own course of disease whilst investigating Zika in Uganda between 1962 and 1963.[4] Due to its relatively benign presentation and similarity to dengue and chikungunya in its clinical signs and symptoms, ZIKV spread almost silently through Africa and Asia (see Figure 1) with fewer than 20 confirmed infections in 60 years.[5,6]

Figure 1. The origin and spread of ZIKV in Africa and South East Asia[1]

The first documented outbreak of ZIKV, and the first evidence that ZIKV was circulating outside of Africa and Asia, occurred in 2007 on Yap Island, Federated States of Micronesia in the western Pacific.[7] During this outbreak, an estimated 73% of the population of the island was infected with the virus. Although attack rates were reported to be higher in older adults compared to children, the study did not report if

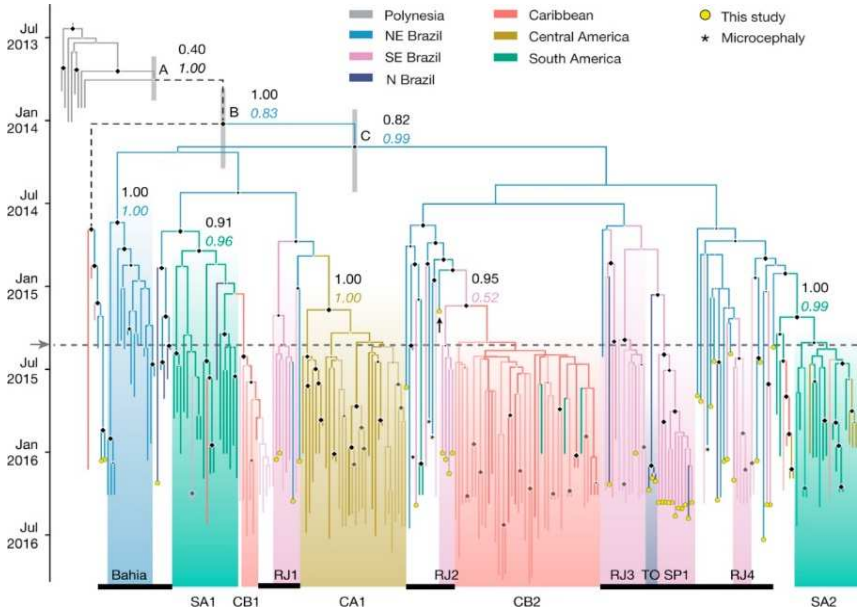
any of the women surveyed were pregnant. Prior to arriving in The Americas, ZIKV appeared in French Polynesia in 2013 causing an even larger outbreak affecting an estimated 28,000 people (11% of the population).[8] Thereafter, smaller outbreaks were reported between 2014 and 2016 in New Caledonia, the Cook Islands and Easter Island[9] in the Pacific (see Figure 2).

Figure 2. ZIKV outbreaks from 2007 to 2016[6]



The earliest reports of an outbreak of a new, distinct, exanthematous infection (later to be identified as ZIKV) in Brazil are from December 2014.[10] However, molecular clock phylogeny techniques (ZIKV genome analysis) indicate that ZIKV was present in northeast Brazil as early as February 2014 (see Figure 3).[11,12] From May 2015, laboratory confirmation of autochtonous ZIKV transmission was established first in the north-eastern states of Pernambuco, Rio Grande do Norte and Bahia, and later in other states of the central-west and south-eastern regions of Brazil.

Figure 3. Phylogeography of ZIKV in the Americas[12]



1.2 The Concomittant Microcephaly Epidemic in Brazil

The occurrence of the ZIKV outbreak in Brazil coincided with a rare phenomenon identified by physicians working in the north-east of the country. In October 2015, two neuropaediatricians, Dr. Vanessa Van der Linden Mota and her mother Dr. Ana van der Linden, working in Pernambuco, examined a cluster of newborns with severe microcephaly associated with a specific and exceptional phenotype that did not seem to conform to any known cause of congenital microcephaly.[13] Around the same time, fetal medicine specialist Dr. Adriana Melo, in the state of Paraiba antenatally diagnosed two cases of severe microcephaly of unknown cause in women who had experienced ZIKV-like symptoms earlier in pregnancy. These findings were reported to public health officials at the Brazilian ministry of health who subsequently authorised the collection of amniotic fluid in the two cases in Paraiba. Both ZIKV RNA and anti-

ZIKV IgM were detected in the amniotic fluid of the two pregnant women demonstrating that ZIKV infection could occur through transplacental transmission.[14] By 30th April 2016, 174,000 suspected and 78,500 confirmed cases of ZIKV infection had been reported in Brazil, 5,500 of which were in the state of São Paulo[15] and 1912 cases of microcephaly had been notified in the country.[16] In the absence of an alternative explanation for the temporal clustering observed (see figures 4 and 5), it was hypothesized that there was a causal association with ZIKV infection during pregnancy[17,18] and the Brazilian Ministry of Health declared a Public Health emergency in November 2015.[19] Subsequently, retrospective analysis of the data collected during the French Polynesia outbreak in 2013 revealed similar congenital brain malformations and the presence of ZIKV RNA in the stored amniotic fluid of four out of six affected newborns, providing more evidence for an association.[20,21] As a result of accumulating proof of the link between ZIKV and congenital birth defects, as well as the spread of ZIKV to 48 countries in the Americas and Caribbean,[6] including Florida and Texas in the United States,[22,23] the WHO declared a Public Health Emergency of International Concern in February 2016.

Figure 4. Spatial diffusion of ZIKV in Brazil from 2014 to 2016 according to case reports and epidemiological data produced by the federal ministry of health and state secretaries of health[10]

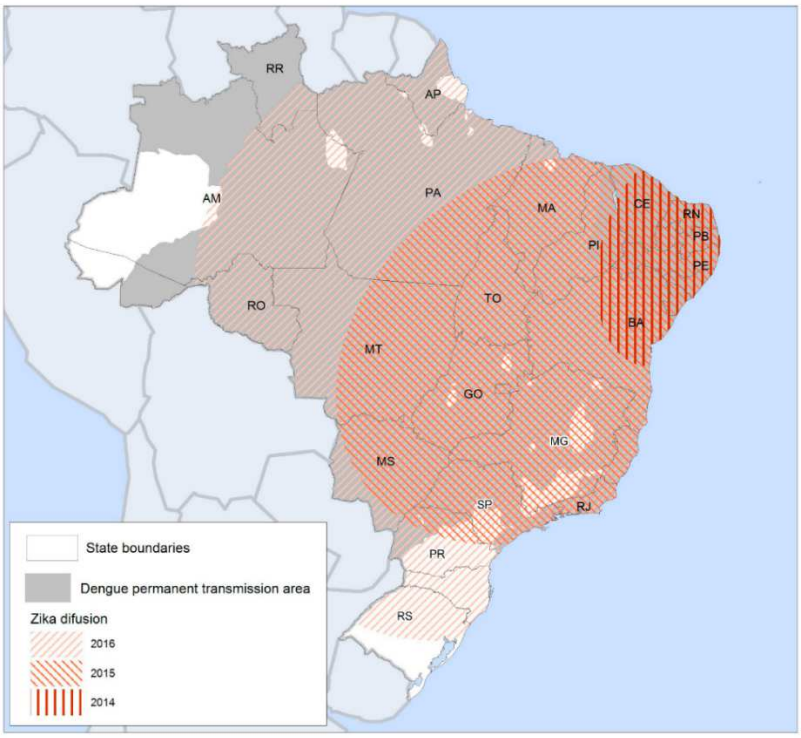
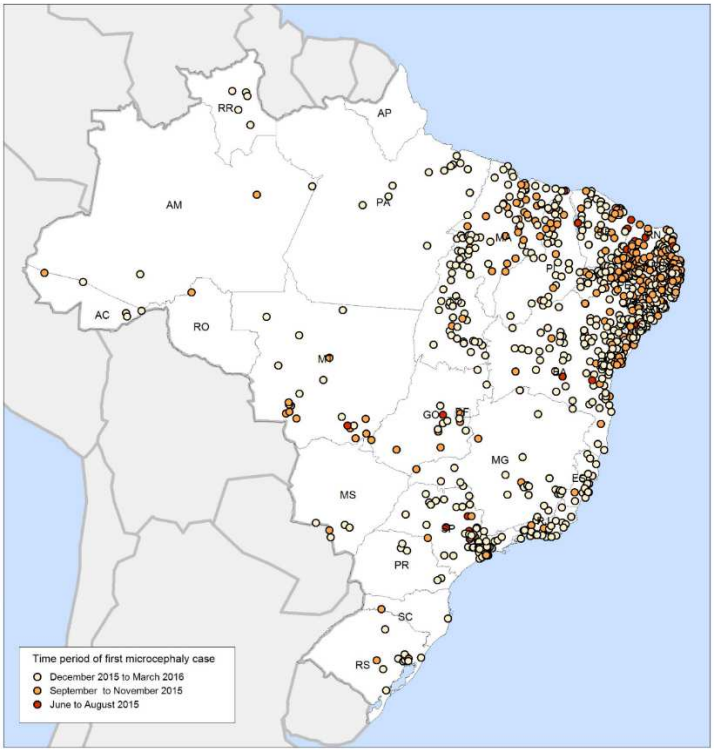


Figure 5. Spatial diffusion of microcephaly in Brazil, June 2015 to March 2016. Data obtained by Lowe et al from the public health events registry (RESP) provided by the Brazilian Ministry of Health[10]



1.3 Viral Characteristics and Transmission

Zika virus is a single-stranded RNA virus member of the *Flaviviridae* family, genus *flavivirus.*[24] Genetic studies have revealed that Zika virus has evolved into three distinct genotypes: West African (Nigerian cluster), East African (MR766 prototype cluster), and Asian.[25] Sequence analyses at the start of the Brazilian outbreak confirmed that the virus isolated was most closely related to a 2013 isolate from French Polynesia, within the Asian clade.[26] In contrast to contemporary Asian strains of the virus, African ZIKV strains have not yet been associated with congenital neurological malformations.[27]

1.3.1 Vector-borne transmission

The rapid expansion of ZIKV in the Americas has largely been due to the biology and behaviour of its principal vector, *Aedes aegypti.*[28] This mosquito, which also transmits the two flaviviruses, dengue virus[29] and yellow fever virus,[30] and the alphavirus, chikungunya virus,[31] is highly domesticated, living in close association with humans in urban households.[28,32] Its eggs are desiccation resistant, and the larvae develop rapidly in subtropical and tropical environments.[28] A potential secondary vector is *Aedes albopictus*, which, like *Aedes aegypti*, is in the subgenus *Stegomyia* and is ecologically similar to *Aedes Aegypti*.[28] *Aedes albopictus* distribution has expanded dramatically into temperate regions of the planet and it is now found on every continent except Antarctica.[33] Even though evidence is lacking to confirm its role in the Brazilian ZIKV outbreak in 2015 to 2017,[34] it is certainly possible that *Aedes albopictus* may become an important vector of ZIKV in the future, particularly if the virus were to adapt to it through genome microevolution, as occurred with CHIKV in La Réunion during the Indian Ocean outbreak in 2005-2006.[35] This potential for vector adaptation also

potentially applies to the many other mosquito species that have shown the potential to be infected by ZIKV including several species of *Aedes, Anopheles coustani, Mansonia uniformis, and Culex perfuscus*.[34,36]

1.3.2 Non-vector-borne transmission

Following acute infection, ZIKV RNA is detectable in plasma, urine, saliva, semen and to a lesser extent vaginal secretions.[37] The time to clearance of the virus is highest in urine and semen where viral clearance times have been reported to be around 24-39 and 25-69 days respectively.[38-40] Evidence that sexual transmission of ZIKV occurs is available from returning travellers who have infected their partners,[41,42] but how many cases of ZIKV are acquired in this way in endemic areas is still unknown and will be difficult to ascertain because in endemic areas, the possibility of mosquito-transmitted infection can never be ruled out. Likewise, it will be challenging to define the role of breast-feeding in the transmission of ZIKV from mother to lactating infant. Although theoretically possible (ZIKV has been detected in breastmilk and reported in a number of case studies),[43,44] no studies have been able to quantify the risk that breastfeeding poses to infants of ZIKV-positive women.

Figure 6. Duration of ZIKV detection in different body fluids[45]

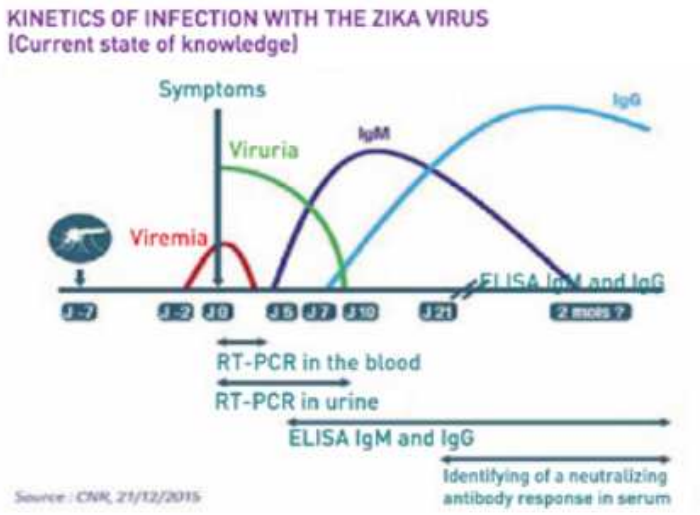
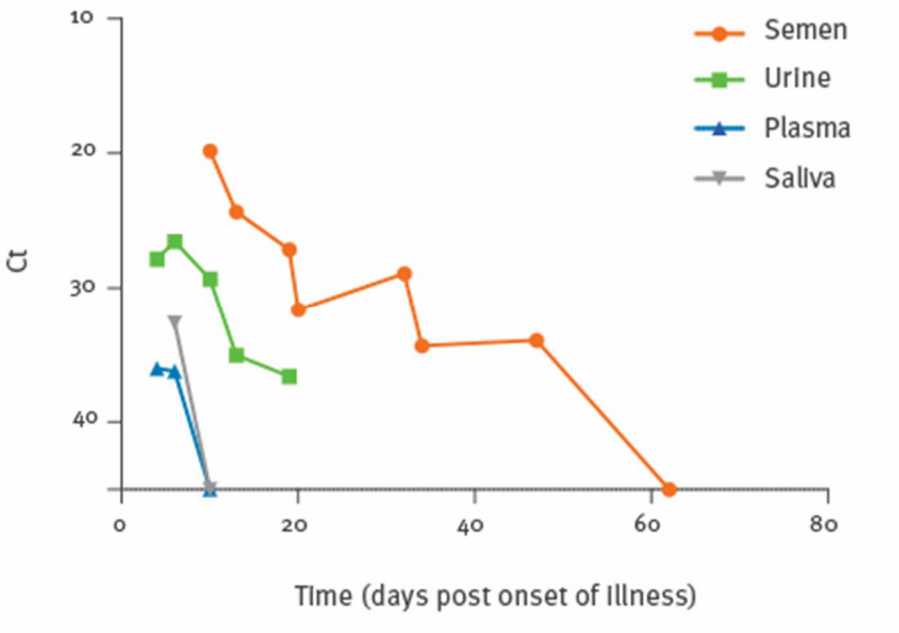


Figure 7. Semi-quantitative kinetics of Zika virus RNA loads in various types of clinical samples according to time post-disease onset, in a Dutch traveller returning from Barbados, March 2016[46]



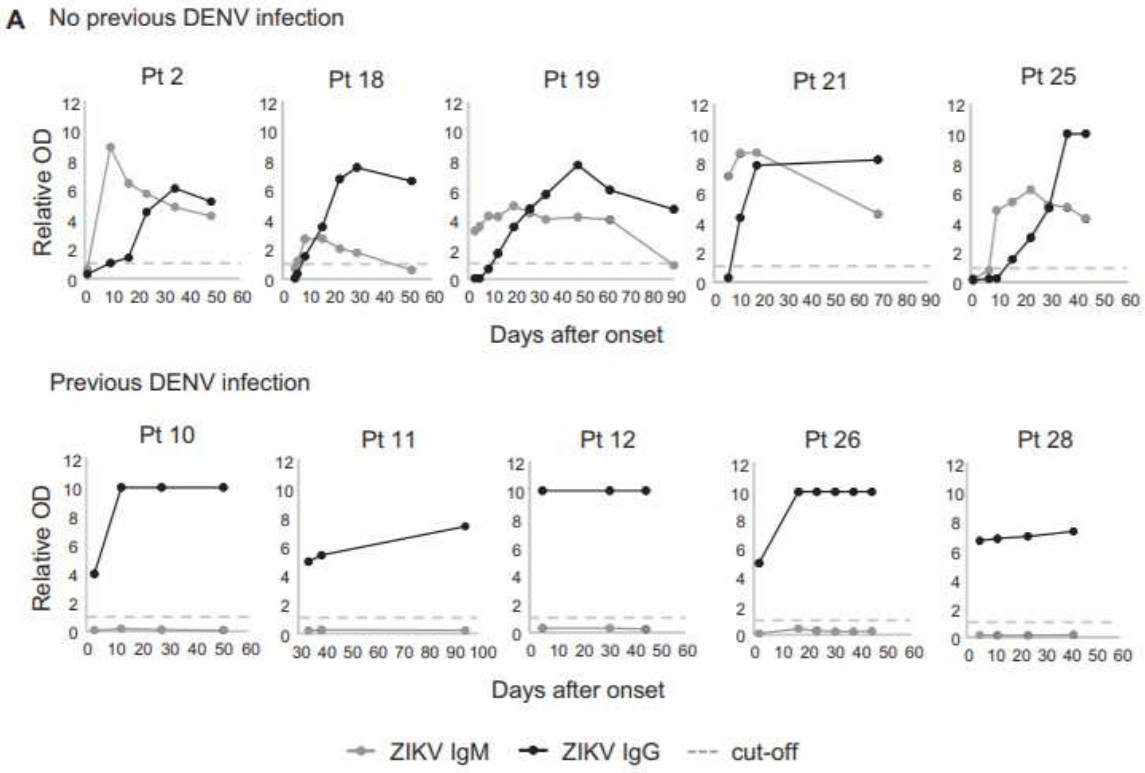
1.4 Diagnostic techniques

In the acute phase, the only ZIKV diagnostic test available relies on the molecular detection of ZIKV RNA via real-time polymerase chain reaction (RT-PCR) tests. The clinical samples of choice for this are urine and blood. The former has been shown to be associated with an increased detection rate and extended time window for detection compared to serum.[40,47] Whereas in blood samples, RNA detection is higher in whole blood rather than serum.[48] RT-PCR in the acute phase of ZIKV infection is currently the gold-standard diagnostic test,[49] however a negative test does not exclude infection due to the aforementioned narrow time frame in which ZIKV circulates in human body fluids.

After the acute phase, the diagnosis of past ZIKV infection relies on the detection of antibodies to the virus. Enzyme-linked Immunosorbent Assay (ELISA) for IgM can detect IgM antibodies as early as 4 to 5 weeks post infection and up to 12 weeks thereafter.[6] IgG responses lag behind and it is not certain how long they remain detectable for. However, the serological diagnosis of ZIKV presents several challenges. The ELISA for ZIKV IgM and IgG can cross-react with antibodies against other flaviviruses (such as dengue and yellow fever virus). This poses a particular challenge in populations living in areas with frequent circulation of Dengue virus and who may be vaccinated against Yellow Fever. Plaque reduction neutralisation tests (PRNT), which are expensive, labour intensive[50] and not available in most labs in endemic areas, can discriminate between different primary flavivirus infections but patients who have secondary infections (those previously vaccinated against or exposed to another flavivirus infection) present a challenge. These individuals usually do not mount an IgM response[38] (see figure 6) and if they do, their PRNT may be equivocal.[7] In addition, although WHO employ a cut-off of $PRNT_{90}$ ≥20 as being a

positive zika neutralizing antibody response (i.e., a 1:20 or higher dilution of serum neutralises 90% of the input virus), PRNT ratios reported have varied significantly between studies from $PRNT_{50}$ ≥10,[51] to $PRNT_{90}$ ≥10,[52] to $PRNT_{90}$ ≥20.[53]

Figure 8. Antibody response to Zika virus (ZIKV) infection[38]



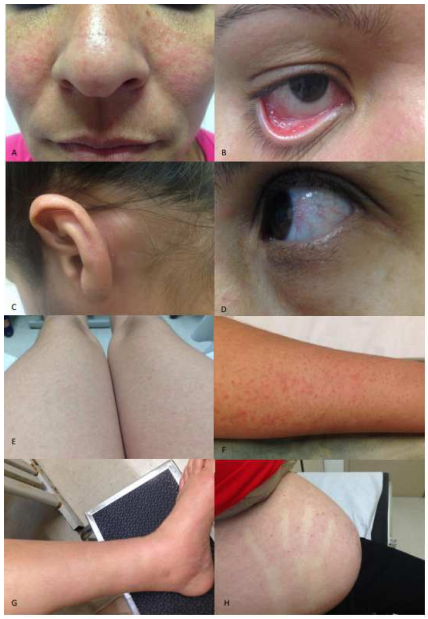
During pregnancy, women with suspected infection should have molecular and serological tests carried out. However, the diagnosis of fetal infection is not straightforward.

Detection of ZIKV RNA in blood, urine and amniotic fluid can be negative despite proven vertical infection.[54] Conversely, pregnant women with detectable virus can have newborns without any congenital malformations. The sensitivity, specificity and positive and negative predictive values of amniotic fluid ZIKV RT-PCR are unknown, making antenatal diagnosis challenging.[6]

1.5 Clinical Features of Zika Virus in Pregnant Women

Early accounts of ZIKV outbreaks included descriptions of the main clinical manifestations of ZIKV infection (rash, fever, arthralgia, conjunctivitis)[7] (see figure 6) and identified that a significant majority (around 80%) of individuals in the general population infected by ZIKV remained asymptomatic.[7]

Figure 9. Clinical features of ZIKV infection in pregnant women[55]



The clinical manifestations of ZIKV are similar, in many ways, to two other *Aedes*-transmitted viruses, dengue and chikungunya. However, subtle differences exist (see table 1).[56] In contrast to dengue and chikungunya, fever is often absent and generally low-grade in ZIKV infection.[57] Arthralgia (joint pain) and arthritis (joint swelling/inflammation) are seen frequently,[7,58] like in chikungunya infection (although not as severe), and headache is a prominent feature of all three infections.[56]

Table 1. Clinical Manifestations of Dengue, Zika and Chikungunya Viruses[56]

Subtle variations

Some clinical signs help to distinguish the infection caused by Zika from dengue and *chikungunya* infections

Symptoms	DENGUE	ZIKA	CHIKUNGUNYA
Fever	Over 38°C (100.4°F) for 4 to 7 days	Absent or up to 38°C for 1 to 2 days	Over 38°C (100.4°F) for 2 to 3 days
Red spots on the skin (rash)	Appear by the fourth day in 30% to 50% of cases	Appear on the first or second day in over 90% of cases	Appear between the second and fifth day in 50% of cases
Muscle pain	Very frequent	Frequent	Infrequent
Joint pain	Infrequent and mild	Frequent and from mild to moderate	Very frequent and from moderate to intense
Swollen joints	Rare	Frequent and mild	Frequent and from moderate to intense
Conjunctivitis	Rare	Occurs in 50% to 90% of cases	Occurs in 30% of cases
Headache	Very frequent and very intense	Frequent and of moderate intensity	Frequent and of moderate intensity
Itch	Mild	Moderate to severe	Mild
Hypertrophy of the ganglia	Mild	Intense	Moderate
Tendency to bleed	Moderate	Absent	Mild
Neurological involvement	Rare	More frequent than in dengue and *chikungunya*	Rare (occurs mainly in newborns)

SOURCE CARLOS BRITO/UFPE – *IN* SURVEILLANCE AND RESPONSE PROTOCOL TO THE OCCURRENCE OF MICROCEPHALY RELATED TO INFECTION BY THE ZIKA VIRUS, 2015

It is not yet known however, whether the clinical presentation of ZIKV differs in pregnant women compared to the general population and the majority of ZIKV epidemiological studies carried out to date have not been able to shed light on this issue as they have only recruited symptomatic women with suspected ZIKV infection. Likewise, little is known about the asymptomatic rate of infection among pregnant women; whether there is any association between levels of viraemia and the presence of symptoms, whether asymptomatic infections are just as likely to lead to vertical transmission of ZIKV infection, and whether, like in dengue, the presence of clinical symptoms and severity of disease are related to the risk of adverse fetal outcomes.[59,60]

1.6 Clinical and Laboratory Case Definitions for ZIKV During Pregnancy

The first case definition of ZIKV infection in pregnancy adopted by the Brazilian Ministry of Health was presented in a protocol for the surveillance and response of

ZIKV microcephaly in 2015.[56] It included, for a suspected case, any pregnant woman with acute exanthematous disease in which other infectious and non-infectious differential diagnoses had been excluded. Confirmed cases required laboratory diagnostics through RT-PCR, however, at the start of the outbreak this was not available in most Brazilian states.[56] In 2016, the WHO developed standardised case definitions to categorise suspected, probable and confirmed ZIKV cases based on clinical signs and symptoms and laboratory criteria (see Table 1).[61] However, these were not written specifically for pregnant women in endemic areas (the target population) and their utility (i.e., sensitivity and specificity for detecting ZIKV infected individuals) has not yet been assessed.

Table 2. WHO ZIKV Interim Case Definitions, February 2016[61]

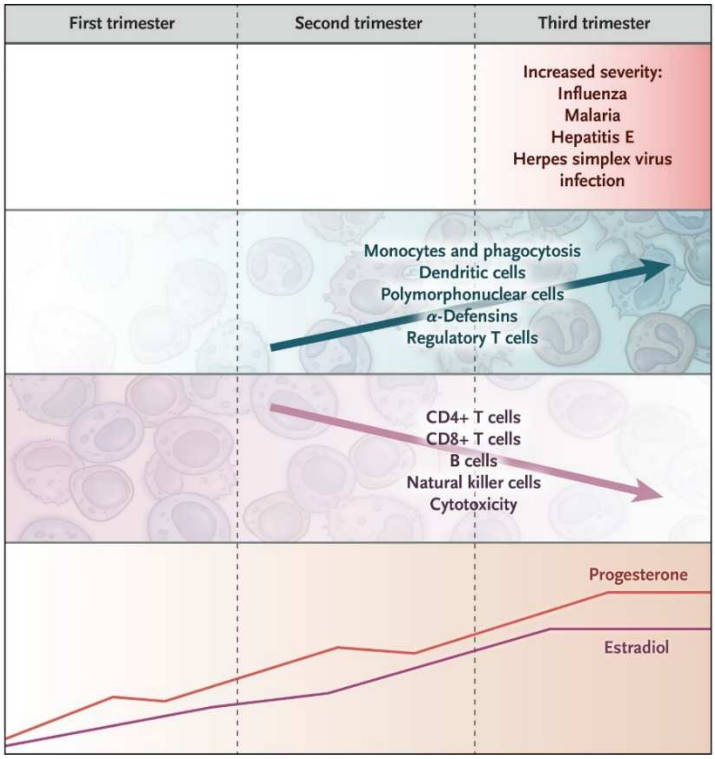
Suspected case	Probable case	Confirmed case
A person presenting with rash and/or fever and at least one of the following signs or symptoms: • Arthralgia; or • Arthritis; or • Conjunctivitis (non-purulent/ hyperaemic)	A suspected case with presence of IgM antibody against Zika virus[1] and an epidemiological link.	A person with laboratory confirmation of recent Zika virus infection: • Presence of Zika virus RNA or antigen in serum or other samples (e.g. saliva, tissues, urine, whole blood); or • IgM antibody against ZIKV positive and PRNT90 for ZIKV with titre ≥20 and ZIKV PRNT90 titre ratio≥4 compared to other flaviviruses; and exclusion of other flaviviruses

1= With no evidence of other flaviviruses, 2= Contact with a confirmed case, or a history of residing in or travelling to an area with local transmission of Zika virus within 2 weeks prior to onset of symptoms.

1.7 Some Immunological Aspects of Infection During Pregnancy

As compared with non-pregnant women, pregnant women are more severely affected by infections with some organisms, including influenza virus, hepatitis E virus (HEV), herpes simplex virus (HSV), and malaria parasites.[62] Immunologic alterations during pregnancy may help explain the altered severity of, and susceptibility to, infectious diseases in pregnant women. These are affected by increasing levels of pregnancy hormones, including oestradiol.[63] In humans, oestradiol can enhance several aspects of innate immunity and both cell-mediated and humoral adaptive immune responses.[63,64] In general, low estradiol concentrations promote CD4+ type 1 helper T-cell (Th1) responses and cell-mediated immunity, and high estradiol concentrations augment CD4+ type 2 helper T-cell (Th2) responses and humoral immunity.[52] Progesterone can suppress the maternal immune response and alter the balance between Th1 and Th2 responses.[65,66]

Figure 10. Changes in Hormone Levels and Immune-System Characteristics during Pregnancy.[62]



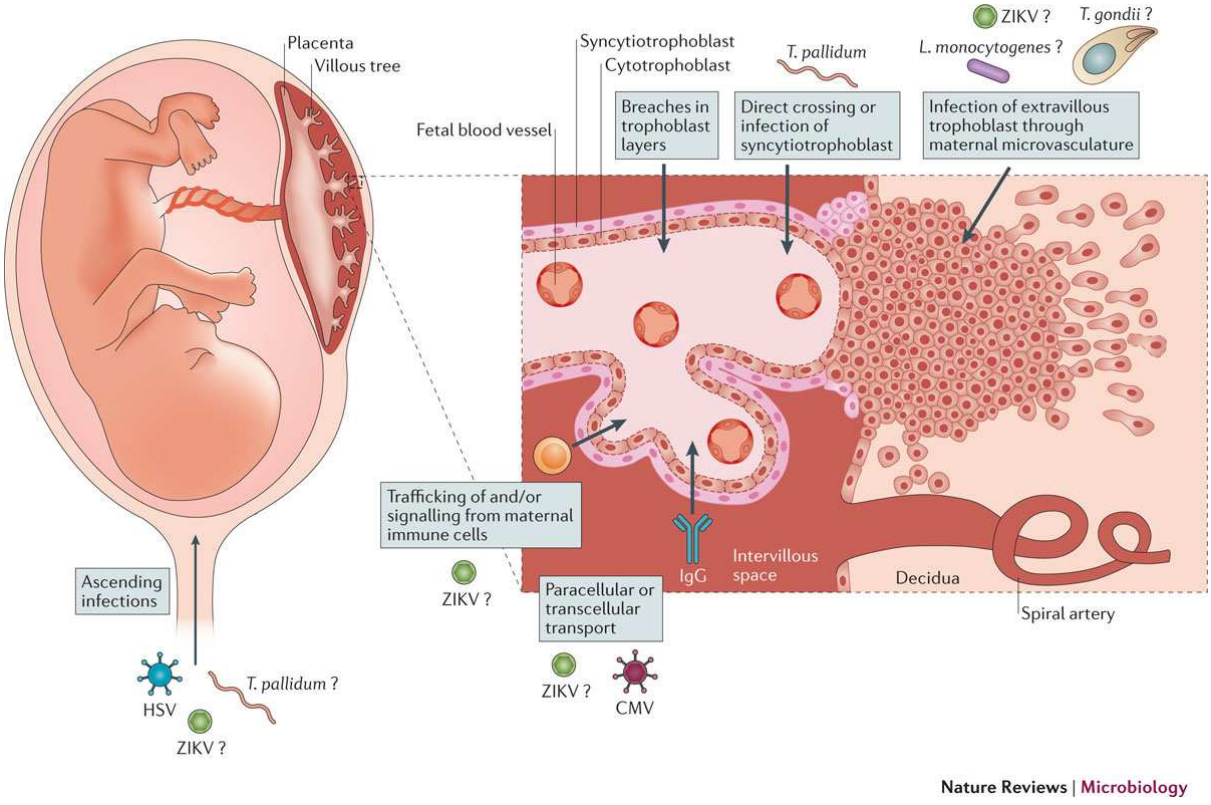
Decreases in adaptive immunity seen in later stages of pregnancy are consistent with the observed increase in the severity of certain infectious diseases during later pregnancy. Decreases in the numbers and function of CD4+, CD8+, and natural killer cells could affect antiviral, antifungal, or antiparasitic responses and delay clearance of the offending microorganism. However, the increases in innate immunity observed during pregnancy may help to prevent acquisition of infection and thus explain the absence of increased susceptibility to infections.[62]

1.8 Congenital Zika Virus infection

The ability of ZIKV to infect and damage the developing fetus implies that the virus can cross and/or bypass the placental barrier.[67] The placenta is characterized by contact between the maternal blood and fetal chorionic villi. Each villus is lined by trophoblasts, which encase the fetal blood supply and placental macrophages (Hofbauer Cells).[68] The pathophysiology of vertical ZIKV infection has been explored through animal studies[69,70] and placental pathological examination and experimentation.[71,72] It is known that ZIKV targets tissues that are crucial for fetal development (maternal decidua, fetal placenta and umbilical cord)[73] and that it infects and replicates in primary human placental macropahges, called Hofbauer cells, and to a lesser extent in cytotrophoblasts, resulting in the induction of type I interferon (IFN), pro-inflammatory cytokines and antiviral gene expression.[68] However, unlike other congenitally transmitted infections of the TORCH group (Toxoplasmosis, Rubella, Cytomegalovirus, Herpes simplex, Herpes zoster, Parvovirus B19, syphilis, Listeria), ZIKV appears to transit from the maternal to the fetal circulatory systems, through the placenta producing a range of tissue-destructing abnormalities in the fetal brain but without eliciting an inflammatory response in the placenta itself,[72] (i.e., causing minimal cell death). It is hypothesised that this is either because Hofbauer

cells are programmed to limit the inflammation following a viral infection, or conversely because the paucity of killer T-cells (CD8+ T cells) contribute to a permissive environment for ZIKV infection and replication in Hofbauer cells.[68]

Figure 11: Routes used by TORCH pathogens to overcome the placental barrier.[67]



The exact mechanism by which ZIKV penetrates the placental barrier is unknown, however, the main vertical transmission routes of pathogens from mother to fetus are described in figure 7 and below.[67]

1. Damage to the villous tree and breaks in the syncytiotrophoblast layer
2. Direct infection of the syncytiotrophoblast layer
3. Infection of endothelial cells in maternal microvasculature and spread to invasive extravillous trophoblasts (EVTs), the cells that anchor the villous trees to the uterine wall.
4. Trafficking of infected maternal immune cells across the placental barrier

5. Paracellular or transcellular transport (for example, immunoglobulin-mediated transcytosis) from maternal blood across the villous trees and into the fetal capillaries
6. Transvaginal ascending infection.

The lack of villous necrosis and placental cell death observed in congenital ZIKV infection in human placental studies[68,72] make the latter four transmission mechanisms more probable. These findings are also highly indicative of the impressive selective predilection of ZIKV for cells of the central nervous system.

Studies looking at the vertical transmission of other flaviviruses such as dengue, which is not associated with congenital malformations, propose a significantly different pathophysiology. Dengue infection stimulates increased production of pro-inflammatory cytokines including IL-6, IL-8 and TNF-α[74] which can affect the uterus through the production of uterine activation proteins stimulating uterine contractions and preterm birth.[75] In addition, thrombocytopenia, plasma leakage and a bleeding diathesis can result in damage to the placental circulation with consequences for the fetus, including stillbirth.[76]

Ultimately, it must be remembered that the placenta is an active immunologic site, capable of interacting with, and responding to, pathogens.[65] Placental infection that elicits the production of inflammatory cytokines may activate the maternal immune system and lead to placental damage and miscarriage or preterm labor,[77] as is the case with dengue. In contrast, although a viral infection of the placenta that triggers a mild inflammatory response may not terminate the pregnancy, it can activate the maternal immune system or that of the fetus, potentially promoting an inflammatory

response that may lead to long-term neurodevelopmental or other sequelae,[78] (as in the case of ZIKV).

Congenital infections in both humans and animals are often associated with a window of susceptibility, which corresponds to a time during gestation at which the infectious agent is most likely to transmit to the fetus and have adverse effects. The specific window of susceptibility varies for different infectious agents, and is based on both pathogen and host factors. Host determinants include a complex interplay between cellular, molecular, and anatomic factors at the maternal–fetal interface, which change throughout gestation and influence the relative receptivity of placental and fetal tissues to infection.[79] In the case of mother-to-child transmission of toxoplasmosis, maternal infection later in pregnancy is more likely to result in congenital infection, but severe congenital toxoplasmosis is associated with infection in early gestation.[80] When looking at ZIKV, some studies have shown that the risk of congenital disease is highest if maternal infection occurs at the end of the first trimester.[81-83] However, it is also known that ZIKV can induce fetal damage well beyond the first trimester, as infections even late during pregnancy can result in fetal disease and/or adverse pregnancy outcomes.[55,84] Additionally, not all pregnant women with ZIKV infection will vertically transmit the virus to their foetuses, and not all vertically infected babies show signs consistent with congenital zika syndrome as is also the case with other congenitally transmitted infections such as cytomegalovirus and toxoplasmosis.[85]

Ultimately, we have yet to quantify the exact rate of vertical transmission and the risk of adverse fetal outcomes that result from ZKV exposure at different stages of pregnancy, making prenatal counselling challenging.

1.9 Zika Virus Neurotropism and Congenital Zika Syndrome

In vitro studies have provided evidence that ZIKV has an affinity for infecting cells of neural origin, [86,87] and causes microcephaly by targeting cortical progenitor cells, inducing cell death by apoptosis and autophagy, and impairing neurodevelopment.[88]

Figure 12. Congenital ZIKV syndrome phenotype from study participants in the Paraiba Zika Cohort (left)[89] and from a review of case reports and case series on congenital zika syndrome (right)[90]

The clinical features of Congenital Zika Syndrome (CZS) are a consequence of direct neuronal damage and severe intracranial volume loss[90] (see figure 8). Although many of the features of CZS are shared by other TORCH syndromes, there are five features that are rarely seen with other congenital infections or are unique to congenital ZIKV infection:

1. Severe microcephaly with partially collapsed skull, consistent with fetal brain disruption sequence (FBDS)[91,92]
2. Thin cerebral cortices with subcortical calcifications[93] (rather than periventricular calcifications as seen in CMV and toxoplasmosis)[94]

3. Macular scarring and focal pigmentary retinal mottling[95]
4. Congenital contractures (i.e., arthrogryposis)[96]
5. Marked early hypertonia and symptoms of extrapyramidal involvement[92]

The diagnosis of these brain malformations can be made antenatally by MRI[97] or ultrasound.[98] Postnatally, in neonates with severe microcephaly, MRI is the imaging investigation of choice as the anterior fontanelle is often small or closed,[99] making transfontanellar cranial ultrasound difficult if not impossible.

Although the phenotype of CZS has been described in detail in case reports and case series, the impact of vertical ZIKV infection on the risk of specific adverse fetal outcomes such as fetal death, low birthweight, small-for-gestational-age, prematurity and proportionate and disproportionate microcephaly have not yet been quantified in high-level evidence epidemiological studies.[100]

1.10 Definitions of Microcephaly and the Congenital Zika Syndrome

Microcephaly occurs as a result of any insult that disturbs early brain growth, and it can be caused by genetic variations, teratogenic compounds, or congenital infections (such as cytomegalovirus, rubella, herpes, or toxoplasmosis).[101]

Prior to December 8th 2015, the Brazilian Ministry of Health defined microcephaly as a head circumference less than or equal to 33cm for term neonates (both sexes and all gestations);[102] and for preterm babies, the cut-off was the 3rd centile of the Fenton curve (by gestational age and sex).[103] This definition was revised by the Brazilian Ministry of Health on 8th December 2015 and reduced the head circumference criterion in term neonates to less than or equal to 32cm, whilst maintaining the preterm definition unchanged.[56]

Subsequently, in January 2016, the Pan-American Health Organisation (PAHO) proposed using the fixed cut-offs of less than 31.6cm for girls and less than 32cm for boys based on the 3rd percentile cut-off on the WHO growth standards curve[104] for term newborn babies of 37 weeks and over. For preterm neonates (under 37 weeks), PAHO recommended using the 3rd centile on the Fenton[103] or Intergrowth curves.[105] Researchers questioned the use of fixed microcephaly cut-offs, as they do not take into account gestational age in weeks. This would pose a problem, particularly in Brazil, where 68.1% of term newborn babies are born below 40 weeks gestational age[106] partly due to the very high pre-labour elective caesarean section rate in the country.[107]

Another question that was widely debated at the start of the microcephaly outbreak was concerning the use of the Fenton curve or the Intergrowth Standards. The former is based on a meta-analysis of six pre-existing studies from high-income countries with non-standardised methods, as is the case for most anthropometric charts.[103] Intergrowth is based on a prospective, multicentre study of healthy gestations from eight countries (including Brazil) where women had ultrasound estimates of gestational age using cown-rump length before 14 weeks gestation or biparietal diameter if antenatal care started between 14 weeks and 24 weeks or less of gestation.[105] The Intergrowth study was designed to be fully consistent with the WHO growth standards,[104] which are used throughout the world.

When using Intergrowth standards, there is then the decision of whether to use the cut-off of -2 SD below the mean or -3 SD below the mean for newborn head circumference. This is influenced by the trade-off between specificity and sensitivity. Since there is no effective treatment for congenital microcephaly caused by ZIKV infection, there is a strong argument to prioritise specificity over sensitivity.[106]

However, it could also be argued that in the early stages of a new epidemic about which little is known, a more sensitive definition would mean identifying a higher number of true positives which would then benefit from early intellectual and physical stimulation. Based on case series results, the sensitivity and specificity of these definitions together with historic definitions was compared (see table 3) and this showed that a cut-off of -2 SD had a specificity of 97.8% and a sensitivity of 85% and a cut-off of -3SD had a specificity of 99.9% and a sensitivity of 57% when using the Intergrowth standards.[106] Although a 2013 systematic review found that most neonates with head circumferences measuring between -2 and -3 SD below the mean did not have any evidence of malformation,[108] currently the consensus seems to be a head circumference at birth below -2SD according to Intergrowth standards.[55,106,109]

Table 3. Estimates of sensitivity and specificity of different case definitions of microcephaly employed during the 2015-2017 outbreak in Brazil[106]

	Cutoffs	Specificity*	Sensitivity†	Estimated annual number of suspected cases (thousands)‡	
				Northeast Brazil	Brazil
Brazil's Ministry of Health[6] (up to Dec 8, 2015)	≤33 cm for term newborn babies of both sexes; <–2 SD of Fenton reference[7] by gestational age and sex for preterm babies	79·3%	92%	158	602
Brazil's Ministry of Health[8] (after Dec 8, 2015)	≤32 cm for term newborn babies of both sexes; <–2 SD of Fenton reference by gestational age and sex for preterm babies	93·8%	86%	46	178
Pan American Health Organization[9]	<3rd percentile (WHO child growth standards[10]) for term newborn babies (<31·6 cm for girls and 32·0 cm for boys) and of the Fenton or InterGrowth reference for preterm babies	96·1%	80%	29	114
Below –2 SD, InterGrowth standards[11]	<–2 SD (InterGrowth standards) for gestational age and sex, all newborns	97·8%	85%	18	63
Below –3 SD, InterGrowth standards[11]	<–3 SD (InterGrowth standards) for gestational age and sex, all newborns	99·9%	57%	0·8	3

*Based on applying the InterGrowth standards to the distribution of livebirths by gestational age in Brazil. †Preliminary results based on a case series of 31 newborn babies with radiological evidence of brain abnormalities. ‡Calculated on the basis of sensitivity and the gestational age distribution of Brazilian newborn babies.

Table: **Preliminary estimates of the specificity, sensitivity, and number of suspected cases of microcephaly in Brazil according to different screening criteria**

1.11 Congenital Zika Virus Epidemiological Studies and Specific Fetal Outcomes

A recent review of the ZIKV literature concluded that prospective studies enrolling both ZIKV-exposed and unexposed pregnant women are lacking and therefore further studies are needed to quantify the risk of specific, objective fetal outcomes after maternal ZIKV infection during pregnancy.[110] Below is a review of the available published epidemiological studies that have looked at the risk of specific adverse fetal outcomes following congenital ZIKV infection.

1.11.1 Microcephaly and neurological birth defects

Soon after the start of the microcephaly epidemic in Brazil, the main causal hypothesis was established as ZIKV infection during pregnancy. However, other hypotheses were proposed and epidemiological studies were set up to investigate further. The first alternative hypothesis was the use of pyriproxyfen (a mosquito larvicide) in reservoirs of drinking water to control populations of *Aedes aegypti* which has been implemented by the Brazilian Ministry of Health in 2014.[111] The second was vaccination during pregnancy (because the epidemic followed the introduction of pertussis vaccine to pregnant women).[112,113] A case control study which enrolled 110 babies with microcephaly and 189 gestational-age matched controls in Recife in northeast Brazil,[109] was able to conclude that neither pyriproxyfen nor vaccine administration during pregnancy was associated with microcephaly. The study's main finding was that out of 91 cases with microcephaly, 35% were ZIKV RT-PCR positive compared to no controls (adjusted matched OR = 73.1 (95% CI: 13-∞). However, the authors admit that ZIKV positivity was significantly higher in CSF than in serum (only one serum sample out of 78 tested was ZIKV positive) and that CSF was not tested in controls. In this study, 29% of cases with microcephaly had severe microcephaly but

the authors did not report the proportion of cases with proportionate and disproportionate microcephaly.

The first Brazilian Zika cohort study to report its findings[55,114] and the only cohort to date who has published results comparing Zika exposed and unexposed women was carried out in Rio de Janeiro, and due to its study design was perhaps in a better position to quantify the risk of adverse fetal outcomes associated with congenital ZIKV infections. Although it showed that the prevalence of microcephaly (both proportionate and disproportionate) was not significantly higher among ZIKV-exposed infants compared to unexposed infants, the main finding reported was that 41.9% of ZIKV-exposed neonates compared to only 5.3% of unexposed neonates had adverse outcomes ($p<0.001$). Adverse outcomes were defined as abnormal neurological examination of the infants at birth and/or abnormal imaging studies at birth (the latter of which were only carried out on the ZIKV-exposed newborns). These findings have subsequently been regarded as somewhat of an outlier, with later studies reporting a prevalence of adverse neurological outcomes of around 6-7%.[58,115,116] The prevalence of microcephaly at birth among ZIKV-exposed newborns has been more consistent between cohorts (3.4%-5.8%),[55,58,115] however it is well known that the spectrum of congenital Zika syndrome is not restricted to microcephaly.[55,115,117]

Four studies have been able to comment on the risk of neurological birth defects according to trimester of ZIKV infection.[55,58,115,118] The latter three have shown that the risk is higher when ZIKV infection occurs in the first trimester (8-12.7%), compared to the second (0-5%) or third trimester (0-5.3%) and the first showed that the window of pathogenicity appeared to be throughout pregnancy.[55] Of note, in all these studies, the presence of neurological birth defects was based on postnatal neurological examinations and/or imaging findings that were carried out health professionals who

were not blinded to the child's ZIKV status and criteria for each study varied and have not been standardised.

A last point, for which conclusive explanations are not yet available, is the apparent heterogeneous geographical distribution of microcephaly and Congenital Zika Syndrome (CZS) cases in Brazil. In a study that constitutes the largest CZS case series published using data from the Public Health Events Registry,[119] França and colleagues reveal that the Northeast region of Brazil had the highest number of reported and confirmed cases. The five states with the highest number of notified cases were Pernambuco (21.3%), Bahia (14.3%), Paraíba (9%), São Paulo (8.1%) and Rio de Janeiro (7.8%). Possible hypotheses for this include that this was the entry point of the virus in the country,[12] that climatic conditions in this region favourable for the proliferation of the vector *Aedes aegypti* and poorer socioeconomic conditions in the Northeast compared to the Southeast of the country which have already been shown to be associated with a higher prevalence of microcephaly prior to the ZIKV outbreak.[120]

1.11.2 Disproportionate Microcephaly

As alluded to earlier, disproportionate microcephaly, in the context of congenital Zika Virus Infection, has at best been described briefly in epidemiological studies[55,58] and no studies so far have explored its potential significance in the Congenital Zika Syndrome (CZS).

1.11.3 Small-for-Gestational Age

With regards to small-for-gestational-age (SGA), case-control study evidence from Recife showed that 83% of microcephaly cases and only 5% of controls without micocephaly were small-for-gestational age.[109] However, this wasn't stratified by ZIKV

exposure status and given that only 35% of the microcephaly cases in the study were either ZIKV RT-PCR or IgM positive in cerebrospinal fluid or serum and none of the mothers were ZIKV RT-PCR positive, it could be that some of these babies were proportionately small for other causes.

In the Rio de Janeiro cohort, mean birthweight was the same among ZIKV-exposed and unexposed babies (whose mothers had positive and negative ZIKV RT-PCR respectively in blood or urine) and the prevalence of small-for-gestational-age was not significantly different among ZIKV exposed and unexposed infants.

The differences in findings may be in part due to differences in the background prevalence of SGA between these two geographically distinct populations in Brazil.

Among 527 women with positive ZIKV RT-PCR during pregnancy in the French Territiories of The Americas, 13.1% were small-for-gestational age but there was no unexposed group or background SGA prevalence data available for comparison.[58]

1.11.4 Prematurity

In the Recife case-control study, cases were more likely to be premature than controls (27% versus 12%) which is surprising as the authors state that controls were chosen to be gestational-age-matched. In the Rio de Janeiro cohort the prevalence of prematurity was similar among ZIKV-exposed and unexposed babies.

1.11.5 Fetal Death

Although miscarriage and stillbirth have been reported as outcomes in case series[121] and aforementioned observational studies,[55,122] no epidemiological studies have yet quantified the risk of fetal death among ZIKV-exposed pregnant women compared to unexposed women. Data from a cohort of ZIKV-positive women in the French

Territories of the Americas showed that 3.1% of these pregnancies ended in fetal demise, the vast majority of which occurred in the first trimester.[58] Data from French Guiana show similar figures, with 4% of ZIKV-exposed pregnancies ending in fetal demise.[116] However, without fetal death rates among unexposed women, we are unable to calculate the risk. In symptomatic dengue infection during pregnancy, it is known that the risk of stillbirth is almost doubled and with severe dengue infection the risk of fetal death increases by five times.[60]

In summary, available evidence on the risk of specific fetal outcomes is incomplete. This is in part due to the fact that many studies only enrolled ZIKV exposed pregnant women and therefore weren't able to compare the incidence of negative outcomes in exposed versus unexposed women. Although a few studies have looked at the incidence of microcephaly and fetal death, only two have attempted to quantify the risk of prematurity and small-for-gestational age and none have looked at disproportionate microcephaly as a specific outcome. No studies have further stratified the risk according to trimester of pregnancy at ZIKV infection, type of delivery or according to different pregnancy co-morbidities.

What we can say however, is that we have sufficient published evidence to identify Zika virus as a teratogenic agent. This is based on Shepard's criteria for teratogenicity,[123] which are made up of seven criteria, of which three of the following four are considered essential:

1. Proven exposure to the agent at one or more critical times during prenatal development

2. Consistent findings by ≥2 high-quality epidemiologic studies, with control of confounding factors, sufficient numbers, exclusion of positive and negative bias factors, prospective studies if possible, and relative risk ≥6
3. Careful delineation of clinical cases; a specific defect or syndrome, if present, is very helpful
4. Rare environmental exposure that is associated with rare defect

In a 2016 review of the evidence for causality between ZIKV and birth defects, the Rasmussen and colleagues[124] found that the second Shepard's criterion was only partially met due to the limitations that epidemiological studies had – such as lack of control for confounding factors and relatively small numbers of cases. This highlights the importance of continued efforts to analyse and divulge the data collected by all the Brazilian Zika cohorts and the need for subsequent meta-analysis of all the available data.

However, many questions remain unanswered and scientific consensus is lacking on many issues, as evidenced by the small amount of literature available on the risk of specific fetal outcomes as discussed above. Baseline ZIKV seroprevalence rates in Africa and Asia before the recent outbreaks are unknown as well as the baseline rate of complications including microcephaly. It is unknown whether the African clade of virus is capable of causing complications during pregnancy and whether immunity against the African lineage confers protection against infection with the Asian lineage of ZIKV. It is still unknown why, in Brazil, the cases of ZIKV and their complications were concentrated in the Northeast of the country. It is unclear why ZIKV circulation declined in Brazil in 2017 and whether herd immunity is a plausible explanation. It is also unknown whether ZIKV infection confers lifelong immunity and what background population immunity is required to prevent emergence or re-circulation in the future.

At a feto-maternal level, we don't yet know what the rate of transplacental transmission or the rate of congenital infection resulting from transplacental transmission. There is still no consensus on what the clinical case definition of ZIKV during pregnancy should be and there are still many definitions of microcephaly and congenital Zika syndrome in use. In terms of diagnostic tests, most gold standard technologies recommended by high-income countries are not applicable to low and middle-income countries and the positive and negative predictive values of diagnostic tests are still unknown. The importance of defining clinical and diagnostic antenatal markers for use in endemic countries is paramount, so that women can be correctly and rapidly referred to receive the correct antenatal care, counselling and financial support.

In the eyes of the media, the threat of ZIKV is over and the disease and its associated complications are no longer considered a public health emergency. However, for the women, children, families and communities affected, the 2015-2017 outbreak will have long-lasting social and economic impacts.[10]

2 AIMS AND OBJECTIVES

This thesis will explore pertinent questions regarding Zikz Virus infection in pregnancy through the use of data collected in the Jundiai Zika Cohort (São Paulo, Brazil) between March 2016 and August 2017. The aims of this investigation are:

1. To describe the Jundiai Zika Cohort including: The context behind its creation, materials and methodology, recruitment and follow-up of pregnant women and children and it main results.
2. To describe the full spectrum of clinical manifestations of ZIKV during pregnancy, in this cohort of high-risk pregnant women by:
    a) Quantifying the number of symptomatic and asymptomatic ZIKV infections,
    b) Revisiting and evaluating the utility of the WHO clinical case definition for clinically defining maternal ZIKV, and
    c) Investigating whether there is an association between the presence of symptomatic ZIKV disease during pregnancy and the development of adverse outcomes among affected newborns.
3. To compare the prevalence of five well-defined negative fetal outcomes (i.e., prematurity, low birthweight, small-for-gestational-age, microcephaly and fetal death) in a group of infants exposed and unexposed to ZIKV prenatally in the context of a cohort of high-risk pregnancies.

3 METHODS

The data on which this thesis is based come from the Jundiai Zika Cohort (JZC), a prospective pregnancy and birth cohort which was set up in the city of Jundiai, São Paulo state, Brazil, in March 2016. Details about its inception, historical and geographical context, design, recruitment, follow-up, data and clinical sample collection methods, as well as laboratory procedures and its main results are described in detail in chapter 4 – Cohort Profile: The Jundiai Zika Cohort (JZC).

3.1 Outcome and Variable Definitions

High-risk Pregnancy: The risk factors which constitute a high-risk pregnancy in Brazil can be categorised using the following four broad sub-headings: 1. Personal and sociodemographic characteristics (such as age, drug use, low or high BMI), 2. Previous reproductive history (previous stillbirth, recurrent miscarriages), 3. Current obstetric illness (bleeding, pre-eclampsia), 4. Intercurrent disease during pregnancy (hypertension, epilepsy, infectious disease).[125]

Zika-virus exposed neonates or infants were considered to have been exposed to ZIKV during pregnancy if their mothers had at least one positive ZIKV RT-PCR sample during pregnancy.[49,126]

Congenital Zika-virus infection or vertical ZIKV transmission was assumed if neonates had a positive ZIKV PCR sample within 10 days of birth.[49,126]

Confirmed case of ZIKV infection: A person with laboratory confirmation of recent Zika virus infection with either presence of Zika virus RNA or antigen in serum or other samples (e.g. saliva, tissues, urine, whole blood); or IgM antibody against ZIKV

positive $PRNT_{90}$ for ZIKV with titre≥20 and ZIKV $PRNT_{90}$ titre ratio≥4 compared to other flaviviruses; and exclusion of other flaviviruses.[61]

WHO ZIKV clinical case definition: a person presenting with rash and/or fever and at least one of the following signs or symptoms: arthralgia or arthritis or conjunctivitis (non-purulent/hyperaemic).[61]

Symptomatic ZIKV infection: For the purposes of follow-up, any women in the cohort who developed any symptoms during pregnancy that were included in the WHO case definition were considered symptomatic and had a more intensive antenatal ultrasound schedule

Prematurity/Preterm is defined as babies born alive before 37 weeks of pregnancy are completed. There are sub-categories of preterm birth, based on gestational age:

- extremely preterm (less than 28 weeks)
- very preterm (28 to 32 weeks)
- moderate to late preterm (32 to 37 weeks)[127]

Gestational age was estimated using first trimester ultrasound (USS) when available and by last menstrual period (LMP) when USS was unavailable.

Small-for-gestational age refers to an infant born with a birth weight less than the 10th centile. Small fetuses are divided into normal (constitutionally) small, non–placenta-mediated growth restriction (for example: structural or chromosomal anomaly, inborn errors of metabolism and fetal infection) and placenta mediated growth restriction.[128]

Low birth weight has been defined by the World Health Organization (WHO) as weight at birth of less than 2,500 grams (5.5 pounds). This is based on epidemiological

observations that infants weighing less than 2,500 g are approximately 20 times more likely to die than heavier babies.[129]

Fetal death means death prior to the complete expulsion or extraction from its mother of a product of human conception, irrespective of the duration of pregnancy and which is not an induced termination of pregnancy. The death is indicated by the fact that after such expulsion or extraction, the fetus does not breathe or show any other evidence of life, such as beating of the heart, pulsation of the umbilical cord, or definite movement of voluntary muscles. Heartbeats are to be distinguished from transient cardiac contractions; respirations are to be distinguished from fleeting respiratory efforts or gasps.[130]

Congenital microcephaly is defined as a head circumference at birth that is more than two standard deviations below the mean for gestational age and sex.[131]

Severe congenital microcephaly is present when the head circumference is more than three standard deviations below the mean for gestational age and sex.[131]

Disproportionate congenital microcephaly is defined as a head circumference that is more than two standard deviations below the mean (-2 SD) for gestational age and sex with a birthweight above -2 standard deviations.[132]

3.2 Study Site

Participants of the JZC are residents of the city of Jundiai and the neighbouring towns and cities that together form the Jundiai “microregion.” These include Cabreúva, Campo Limpo Paulista, Itupeva, Jarinu, Louveira and Várzea Paulista and together have a population of 696,334 inhabitants. The city of Jundiai itself has 409,497 inhabitants (IBGE - Brazilian Institute of Geography and Statistics estimate for

2017).[133] The climate in this region is humid subtropical according to the Köppen classification with an annual temperature of 20.9°C. The mean wage of Jundiai residents is 3.4 minimum wages and its Human Development Index of 0.857 places it 4th out of all the municipalities in the State of Sao Paulo and 14th in the whole country.[134] However, in the year 2000, 25,000 people (8.5% of the city's population at the time) were reported by the Jundiai local authority to be living in so-called favelas.[135] At the time of the study the Jundiaí region was not a Yellow Fever compulsory vaccination area,[136] and no cases were reported to the Brazilian Ministry of Health compulsory notification system (SINAN), although four cases were reported between 2015 and 2016 in other parts of the state.[137] Other mosquito-borne viral infections such as Dengue and Chikungunya were also prevalent in the area with 61,600 confirmed cases of dengue reported in Sao Paulo State between 2015 and 2016 and 59 reported and confirmed cases of Chikungunya.[138] The maternity Department at the University Hospital is the only public maternity facility in the municipality and carries out approximately 300-400 deliveries per month, around two thirds of the total births in Jundiaí.[139]

3.3 Sample Size

As the study commenced in the midst of the ZIKV outbreak in Brazil, it was not known what the incidence of microcephaly was in either ZIKV exposed or unexposed pregnant women. The sample size for the cohort was calculated using an estimated prevalence of cases of microcephaly among neonates of ZIKV RT-PCR positive pregnant women of 2%. A final analytical cohort size of n=531 would give us 80% power to detect a crude relative risk of 2 with a probability of type I error (α) of 5%.[140] Although initially the JZC aimed to enrol 500 pregnant women, recruitment continued

for longer than initially anticipated to try to capture possible seasonal differences in the incidence of ZIKV disease.

3.4 Recruitment and Baseline Data Collection

During the recruitment period (1 March 2016 – 23 August 2017), all women attending the high-risk pregnancy clinic (i.e., due to the presence of risk factors threatening the life or health of the pregnant woman or her fetus)[141] at Jundiaí University Hospital at any stage of pregnancy were considered eligible and offered the opportunity to participate in the study. The only exclusion criteria at this stage were women who had life-threatening conditions and women who had severe learning difficulties. The reasons for choosing this study population of high-risk pregnant women were (i) to try to maximise recruitment efficiency and pregnancy follow-up adherence (ii) to provide an appropriate location for the examination of the women and collection of clinical samples and (iii) to optimise newborn data quality by ensuring a large proportion would be born in Jundiai University Hospital. Over the duration of follow-up, clinical teams cared for pregnant women in accordance with the Brazilian Ministry of Health protocols. Recruited women who were asymptomatic at the time of enrolment and remained without ZIKV-like symptoms until delivery were followed up as per the study protocol for Group 1 (asymptomatic) (Fig. 1). Women who were symptomatic at recruitment and/or developed symptoms consistent with ZIKV infection (i.e., defined by WHO as rash and/or fever and at least one of the following symptoms: arthralgia, arthritis or non-purulent conjunctivitis)[61] were followed up as per the study protocol for Group 2 (symptomatic). Detailed demographic, medical and antenatal information, as well as examination findings were gathered by research nurses who interviewed the women and reviewed their antenatal records. Data was initially collected using an in-house data collection tool and subsequently, in August 2016, when a standardised

tool was created by the WHO[142], this was modified and updated accordingly. Data was entered digitally into the Cohort's database (created using Salesforce™ Brasil online platform).

3.5 Follow-up

The follow-up of the women is described in Figure 1 and as follows: Women in both groups had saliva and urine collected by research nurses for ZIKV RT-PCR at the time of recruitment and 2-3 weeks thereafter. Subsequently, sample collection was repeated on a 2-3 monthly basis during routine check-ups. Antenatal ultrasound scanning was carried out in months 3, 5, 7 and 8 in asymptomatic (Group 1) women and monthly in symptomatic (Group 2) women at the São Paulo Radiology Centre by sonographers specialising in fetal medicine and using Voluson 730 Expert/Voluson E6, GE equipment. In addition, during pregnancy, the women received weekly phone calls to check if they had developed any symptoms consistent with ZIKV. At the time of delivery in hospital, the women had blood, saliva and urine collected. The placenta and umbilical cord were stored for subsequent evaluation by a pathologist. Blood, saliva and urine were collected from the neonates. If the mother had a positive ZIKV RT-PCR result during pregnancy or there was microcephaly present at birth, cerebrospinal fluid was also collected from the neonate for ZIKV RT-PCR. All specimens were stored in a biorepository.

3.6 Biological Sample Collection

Blood, saliva and urine were collected from the women and children by nursing staff, healthcare assistants and auxiliary nurses (all registered at the nursing professional registration body of the state of Sao Paulo (CORENSP)) following an in-house standardised protocol. Personal protective measures were taken (gloves and eye

protection were worn at all times) and samples were labelled appropriately with the patient name, date of collection, date of birth and sample number). Saliva samples were collected by swabbing ten times on the inside of each cheek with sterile swabs which were then placed into sterile Falcon® 15ml tubes. Women were asked to provide urine samples into 50ml sterilised collection tubes. For neonatal and infant urine samples, sterile urine collecting bags with adhesive tape were placed over sterilised skin around the penis/vulva. At birth, blood was collected from the umbilical cord and subsequently, in infants and women, blood samples were obtained by venepuncture in the antecubital fossa or in the dorsum of the hand with a maximum of three attempts. Blood was collected into dry and EDTA tubes which were mixed and labelled appropriately and placed in the fridge.

3.7 Real-Time Polymerase Chain Reaction (RT-PCR) for ZIKV detection

ZIKV RNA detection was performed by Real Time PCR (RT-qPCR), as recommended by the WHO, according to the protocol developed by Lanciotti et al.[24]

Initially, the total RNA was extracted from 140 µL of serum, urine and/or salive by using the QIAamp Viral RNA Mini Kit (QIAGEN), following manufacturer's instructions. The final RNA was eluted in 60 µl of ultrapure H2O Nuclease-Free Water (©2018 Merck KGaA,Darmstadt, Germany) and RT-qPCR was performed on the same day. The remaining RNA was stored in a freezer at -80°C. RT-qPCR was performed with GoTaq® Probe qPCR and RT-qPCR Systems (© 2018 Promega Corporation Brasil, Ltd). For the final volume of 20 µl reaction, 8 µl of RNA template was used. The Mix was created with 10 µl of GoTaq® Probe qPCR Master Mix with dUTP [1x], 0,4 µl of GoScript™ RT Mix for 1-Step RT-qPCR [1x], 1µl of Forward primer [10pmol/µL], 1µl of Reverse primer[10pmol/µL], 1µl of probe [10pmol/µL] (Table 1) and of Nuclease-Free Water to complete the final volume. Two sets of primers and probe were used on

the RT-qPCR reaction. The reaction occurred in ABI Prism 7500 SDS Real-Time cycler (Applied Biosystems), where the amplification cycles consisted of: One cycle of 15 minutes at 45°C for reverse transcription; 1 cycle of 2 minutes at 95°C for reverse transcriptase inactivation and for polymerase activation; 40 cycles of 15 seconds at 95°C for denaturation; and 1 minute at 60°C for annealing and extension. The primers and probes used for this quantification are complementary to the gene encoding the NSI protein of ZIKV. The probe contains a fluorescent 6-carboxyfluorescein (FAM) reporter dye at the 5 'end and the fluorescent dye 6-carboxytemethylhydhodamine (TAMRA) at the 3' end. All reactions followed positive and negative controls previously quantified.

3.8 Anthropometry

Neonatal weight, length and head circumference were obtained for all live-born infants, and the equipment used was consistent for all. Weight was assessed using digital scales, length using a recumbent baby length scale and head circumference using a standardised non-elastic tape measure. Z-scores for weight, length and head circumference were determined using the online Intergrowth calculator, which takes into account gestational age and sex.[105,143,144] Gestational age was estimated using first trimester ultrasound (USS) when available and by last menstrual period (LMP) when USS was unavailable.

3.9 Data Curation and Statistical Analysis

Baseline and follow-up information was collected from pregnant women and mothers on paper forms by the JZC team of medical staff and volunteer field workers and later

transferred to the JZC online database (provided by Salesforce Brasil) by IT and medical staff who had received training in the use of the software.

To create the database for this project, longitudinal data was extracted from the online database in Excel format for the women and children of the cohort. This included all sociodemographic and medical information collected at baseline, antenatal follow-up information, lab test results, telephone consultation data, birth information and all the paediatric follow-up consultations. Women who had been recruited between March 2016 and August 2017 were included and second pregnancies in the same women were excluded. Data was linked between the women and their children using a unique identifying number that was also shared by her child(ren). This was carried out using SPSS version 24 software due to the size of the database. The database was cleaned and redundant variables dropped or merged to create a database with a more manageable number of variables. This database was then transferred to STATA version 12 software using Stat Transfer software. Once this baseline database was formed, secondary databases were formed based on the requirements of each research question. For example, for objective 2, women who did not have PCR results or Zika symptom data were excluded from this analysis. Subsequently, descriptive tables were constructed comparing baseline sociodemographic and pregnancy characteristics among exposed and unexposed individuals. Chi-squared test was used to compare categorical variables except where there were less than 5 in any cell, in which case Fisher's exact test was used. Measures of association (Crude Risk Ratios) and their 95% confidence intervals were calculated directly by comparing the prevalence of the outcome among exposed and unexposed individuals.

3.10 Ethical Considerations

The Jundiai Zika Cohort study received ethical approval by the research ethics committee of Jundiaí Medical School, protocol number 1446577. Participating women provided written, informed consent for themselves and for future follow-up of their child.

Cohort Profile: The Jundiaí Zika Cohort (JZC), A Pregnancy and Birth Cohort in São Paulo State, Brazil

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ABSTRACT

Purpose: The Jundiaí Zika Cohort (JZC) is a prospective pregnancy and birth cohort set up in the State of São Paulo, Brazil, to investigate the epidemic of cases of microcephaly and other neurological disorders, presumed to be associated with Zika Virus (ZIKV) infection.

Participants: A total of 748 women with high-risk pregnancies were recruited in the period March 2016 to August 2017.

Findings to Date: Baseline sociodemographic and medical data were collected at recruitment. Biological samples (blood, saliva, urine, cerebro-spinal fluid, colostrum, umbilical cord and placenta) were obtained and are now contained in a secure biorepository. Antenatal and postnatal imaging studies and neonatal anthropometry were carried out. Important findings have been reported including placental histological case reports, dizygotic twin studies, and fetal outcome results.

Future Plans: The JZC provides a unique dataset which will continue to be explored to study the effects of pregnancy comorbidities on Zika Virus infection during pregnancy, the long-term outcomes of children with congenital Zika infection, and how physiotherapy and group interventions can improve outcomes for congenitally infected children. All women in the cohort have reached the end of their pregnancy and currently the oldest children are two years old. The study will continue until all the children reach their 3rd birthday (April 2021).

Strengths and Limitations of the Study

- The JZC is one of the few prospective Zika cohort studies that has recruited both asymptomatic and symptomatic pregnant women and therefore benefits from having a large control group.
- The high-risk profile of the pregnant women provides a unique opportunity to study co-morbidities that may contribute to, or potentially be protective for, the development of negative sequelae associated with ZIKV exposure.
- The prevalence of ZIKV RT-PCR positivity was relatively low in this study population resulting in small numbers for estimating absolute and relative risks.
- Because high-risk pregnant women are at higher risk of developing some of the outcomes of interest, this may have reduced the power to detect differences between Zika-exposed and unexposed dyads.

INTRODUCTION

The Jundiaí Zika Cohort (JZC) is an ongoing multidisciplinary longitudinal study which is following a cohort of pregnant women and their children to study the effects of prenatal Zika Virus (ZIKV) exposure. The cohort was set up in response to the clusters of cases of severe microcephaly and associated neurological disorders that were reported in areas affected by ZIKV in the Northeast of Brazil in October 2015[1] and that provoked the Public Health Emergency of International Concern (PHEIC) declared by the WHO on 1st February 2016.[2] At this point in time, The São Paulo Research Foundation (FAPESP) encouraged all researchers who had been part of the Brazilian Genome Project to submit thematic research project proposals addressing the potential causes of the cluster of cases of microcephaly.

Laboratory confirmation of autochtonous ZIKV transmission in Brazil was first established in the north-eastern states of Pernambuco, Rio Grande do Norte and Bahia, and later in other states of the central-west and south-eastern regions of Brazil. When the JZC was set up, there was no published data about the ZIKV epidemic in São Paulo state. It was not known how the differences in climate and socioeconomic status between this south-eastern state and the poorer and more tropical north-eastern regions, where the ZIKV epicentre was focussed, would influence the epidemic. The study site of Jundiai in the south-eastern state of São Paulo was therefore chosen in order to explore these variations.

This cohort profile aims to describe the Jundiai Zika Cohort including: The context behind its creation, materials and methodology, recruitment and follow-up of pregnant women and children as well as some of its preliminary results.

COHORT DESCRIPTION

Study Site

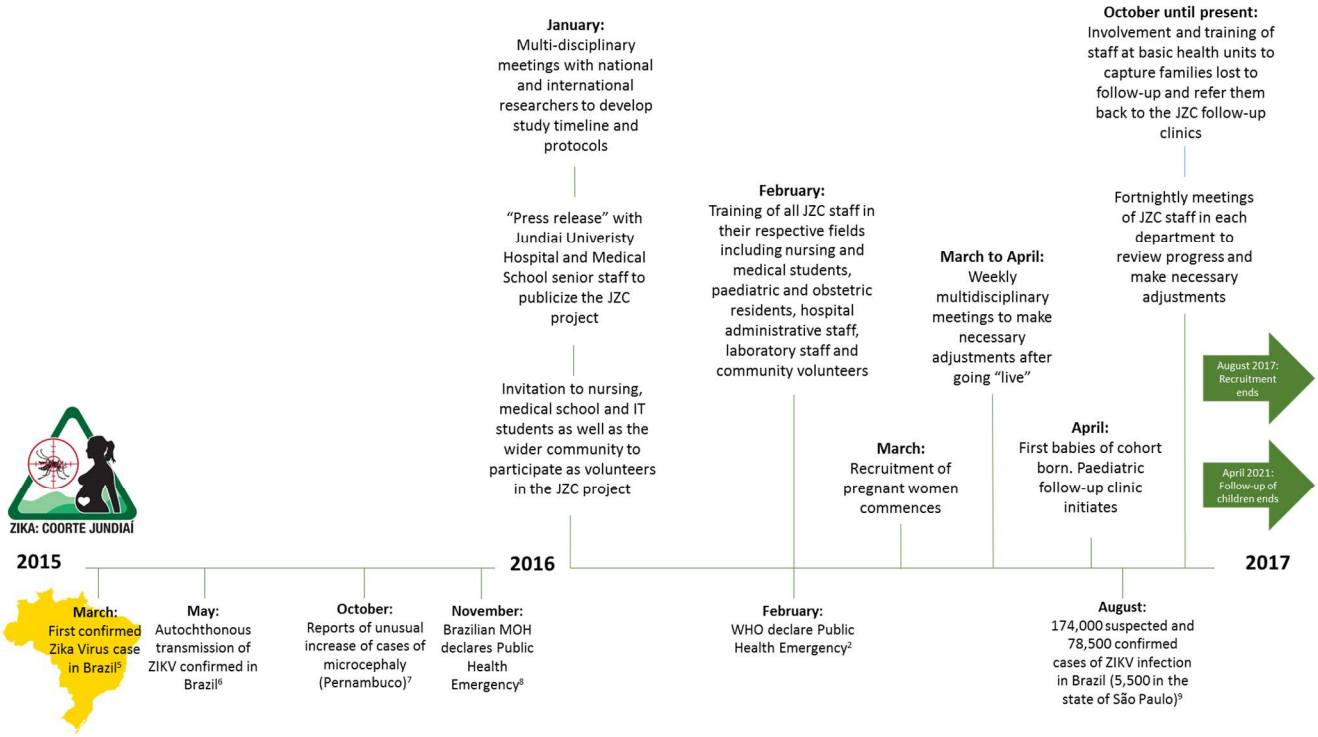
The JZC is housed in the Paediatric department of the Jundiaí Medical School in the city of Jundiaí, São Paulo state. Jundiaí is 50km Northwest of the city of São Paulo and has a population of 405,740 inhabitants.[3] It has a relatively high Human Development Index ranking 11th out of the total 5,565 municipalities in the country.[3] The climate in the area is humid subtropical, according to the Köppen classification, with a mean annual temperature of 20.9°C. The majority (64%) of the land in the municipality of Jundiaí is considered rural and 31% of this is made up of the Japi Mountain, a Biosphere Reserve of Atlantic Forest recognised by UNESCO since 1994.[4] Jundiaí University Hospital is the only public maternity facility in the municipality of Jundiaí and is the local referral centre for high-risk pregnancies.

Figure 1. Map showing the location of the municipality of Jundiaí in the State of São Paulo, Brazil[5]

Given the severity of the phenotype assumed to be associated with prenatal exposure to ZIKV and the urgent need to establish a cause, the Jundiaí Zika Cohort, like many other Zika Cohorts in Brazil, commenced without any formal funding on the 1 March 2016. It later received seed funding from The London School of Hygiene and Tropical Medicine prior to obtaining formal FAPESP funding for a thematic research project.

Following ethical approval for the study from the research ethics committee of Jundiaí Medical School (protocol number 1446577), written informed consent was obtained from participating women for themselves and for future follow-up of their child.

Figure 2. The context of the initiation of the Jundiai Zika Cohort presented as a timeline of events related to the introduction of ZIKV in Brazil in the period 2015-2017 and beyond.[2,6-10]



Eligibility and Recruitment

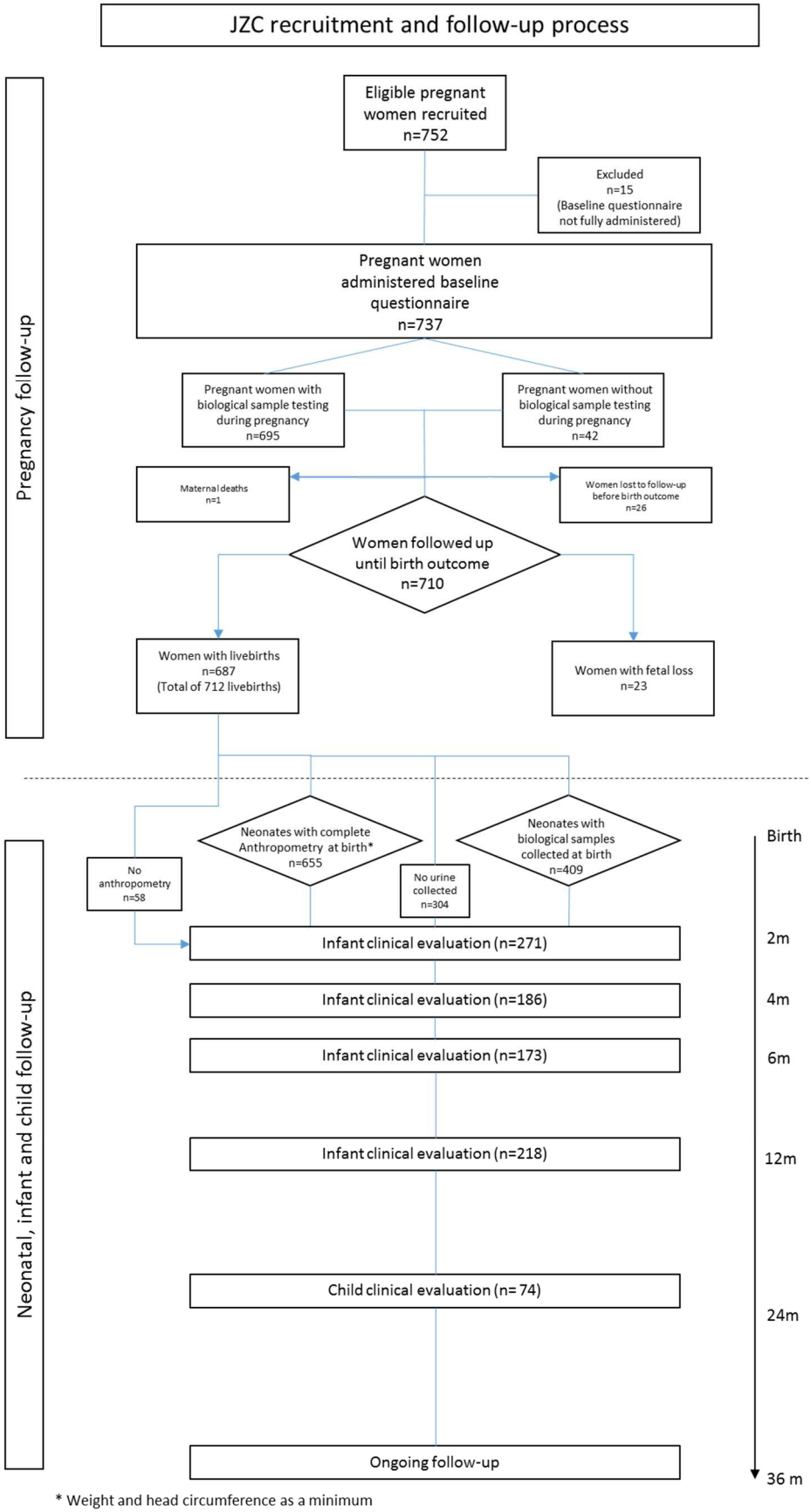
All pregnant women attending the high-risk pregnancy clinic at Jundiaí University Hospital (referred from one of the 38 primary care clinics in the Jundiaí area due to the presence of risk factors that could affect the well-being of themselves or their unborn child, for example diabetes, hypertension, twin pregnancy, adolescent pregnancy etc.)[11] between 1st March 2016 and 23rd August 2017 were invited to participate. Information regarding the number and characteristics of women who chose not to participate in the study are unavailable. The only exclusion criteria at this stage were women who had life-threatening conditions and women who had severe learning difficulties. The reasons for choosing this study population of high-risk pregnant women were (i) to try to maximise recruitment efficiency and pregnancy follow-up adherence (ii) to provide an appropriate location for the examination of the women and collection of clinical samples and (iii) to optimise newborn data quality and care by ensuring a large proportion would be born in Jundiaí University Hospital where adequate tertiary neonatal care services are available. Over the duration of follow-up, clinical teams cared for pregnant women in accordance with the Brazilian Ministry of Health protocols.

As the study commenced in the midst of the ZIKV outbreak in Brazil, and seroprevalence studies had not been carried out, it was not known what the true incidence or prevalence of ZIKV infection was in the Jundiai area; or indeed what the prevalence of microcephaly was in either ZIKV exposed or unexposed pregnant women. In 2016, in the State of Sao Paulo, 9,845 cases of ZIKV infection were reported to the Brazilian Notifiable Disease Registry (SINAN) of which 5,056 were considered probable, giving a crude incidence of 11.3 cases of ZIKV per 100,000

inhabitants in the state of Sao Paulo in the year 2016.[12] However, this is likely to be a gross underestimation.

The sample size for the cohort was calculated using an estimated prevalence of cases of microcephaly among neonates of ZIKV RT-PCR positive pregnant women of 2%. A final analytical cohort size of n=531 would give us 80% power to detect a crude relative risk of 2 with a probability of type I error (α) of 5%.[13] Although initially the JZC aimed to enrol 500 pregnant women, recruitment continued for longer than initially anticipated to try to capture possible seasonal differences in the incidence of ZIKV disease.

Figure 3. Flow diagram showing participants of the Jundiai Zika Cohort at each stage of the study, recruitment period: 01 March 2016 – 23 August 2017, Jundiai, São Paulo, Brazil



Study Participant Characteristics

During the recruitment period, 752 women were enrolled in the study (see figure 3). Of these, 15 were excluded as the baseline questionnaire was not fully administered. The mean age of women was 27.5 years (13 – 46); 53.7% of the women were of white ethnicity, 34.7% mixed race or brown (known as “parda” in Portuguese), 9.6% black, 1.6% Asian and 0.3% indigenous; 77.2% of the women were married or living with their partner (see table 1). A significant proportion of women had diabetes (30.3%) and a smaller fraction (17.3%) had hypertension during pregnancy. Of the 737 who had full baseline information collected, 94.3% (n=695) had biological sample testing for ZIKV RT-PCR during pregnancy. Of note, due to financial constraints it was opted to prioritise the detection of ZIKV in urine samples, by reverse transcriptase-polymerase chain reaction (RT-PCR) and to store blood and saliva for future analysis. Studies have shown that ZIKV RNA is unlikely to be detected in serum after the first week of illness whereas it can be detected in urine for at least 2 weeks after symptom onset.[14-16] There were 26 women who were lost to follow-up during pregnancy (before the birth outcome), and there was one first trimester maternal death. Of the 710 women who were followed until the birth outcome, 23 had fetal losses and 687 had livebirths, of which 25 were twin pregnancies. Of the 712 livebirths, 52.8% were female (see table 2). Of these, 655 had anthropometry at birth (a minimum of weight and head circumference measured at birth); the mean birth weight among livebirths was 3001g (590g – 4525g), mean length at birth was 47.5cm (28.5cm - 58.5cm), and mean head circumference was 33.7cm (22cm – 38.5cm). The number of infants who had biological samples collected within seven days of birth for ZIKV RT-PCR was 409. Of the 712 livebirths, 271 babies were seen in the JZC paediatric clinic between 0-2 months of age, 186 between 3-4 months of age, 173 between 5-6 months of age, 218

between 7-12 months of age and 74 between 13-24 months of age. So far, no infants have been followed up beyond 2 years of age.

Table 1. Description of Women in the Jundiai Zika Cohort

Pregnant Women (n=737)		
Age in years (mean and range)		27.5 (13-46)
Ethnicity	White	53.7% (n=368)
	Mixed race	34.7% (n=238)
	Black	9.6% (n=66)
	Asian	1.6% (n=11)
	Indigenous	0.3% (n=2)
	Unknown	7.1% (n=52)
Education	>12 years	15.4% (n=106)
	12 years	44.4% (n=305)
	9-11 years	23.7% (n=163)
	≤8 years	16.4% (n=113)
	Unknown	6.8% (n=50)
Married/living with partner		77.2% (n=569)
Diabetes	Yes	30.3% (n=208)
	No	69.7% (n=478)
	Unknown	6.9% (n=51)
Hypertension	Yes	17.3% (n=118)
	No	82.7% (n=563)
	Unknown	7.6% (n=56)
Biological sample tested for ZIKV RT-PCR during pregnancy		94.3% (n=695)
Lost to follow up before birth outcome		3.5% (n=26)
Maternal deaths		0.1% (n=1)
Women with livebirths (of those followed up)		97.6% (n=687)
Fetal deaths		3.2% (n=23)

Note: Percentages for all categories were calculated with exclusion of those with missing data from the denominator

Table 2. Description of Liveborn Infants in the Jundiai Zika Cohort

Liveborn Infants (n=712)		
Twin pairs		3.5% (n=25)
Sex (female)		52.8% (n=376)
Delivery method	Vaginal	47.1% (n=330)
	Caesarean section	50.3% (n=352)
	Forceps	2.6% (n=18)
	Unknown	1.7% (n=12)
Weight at birth in grams (mean and range)		3001 (590-4525)
Length at birth in cm (mean and range)		47.5 (28.5-58.5)
Head circumference at birth (mean and range)		33.7 (22-38.5)

Note: Percentages for all categories were calculated with exclusion of those with missing data from the denominator

EXTERNAL VALIDITY AND POSSIBLE PARTICIPATION BIASES

The recruitment of only high-risk pregnant women brought advantages, as discussed earlier, in both logistics and maximisation of follow-up rates, however, these advantages also introduced limitations in external validity when generalising JZC findings to the general pregnant population of Brazil. For example, in our cohort, a significant number of women had diabetes and hypertension (30% and 17% respectively). Moreover, as recruitment was carried out in a specific population of pregnant women that were users of a particular health service, it is possible that there are systematic differences between those recruited and those not recruited, which we were not able to measure, and these may have introduced bias. However, when comparing the socio-demographic profile of the pregnant women in our cohort and the profile of pregnant women living and using public maternity facilities in the state of São Paulo at the time of the study,[17,18] we can see that they are quite similar. For example, around half of the women were white, around half had vaginal deliveries, and the majority had finished high school and were co-habiting and/or married to their partner (see table 1).

FOLLOW-UP

PREGNANT WOMEN

Women who at enrolment reported not having had any symptoms consistent with ZIKV infection during their pregnancy (and who were currently asymptomatic), according the WHO ZIKV clinical case definition,[19] were followed up as per Group 1 and women who were symptomatic at any point during pregnancy were followed up as per Group 2 (see figure 3). Women were asked to contact the research team and attend the hospital if they experienced any symptoms consistent with ZIKV infection at any point in their pregnancy so that biological samples could be collected as detailed below. In addition, trained volunteers carried out weekly telephone follow-up consultations at pre-arranged times that were convenient for the women until the time of birth to ask specifically about the occurrence of any ZIKV symptoms and any women who had experienced symptoms were advised to go to the hospital.

Regardless of symptom occurrence, women in both groups were seen 14-21 days after enrolment for biological sample collection and then in 2-3 monthly intervals thereafter (sample collection details and laboratory procedures are described below). Antenatal ultrasound scanning was carried out in months 3, 5, 7 and 8 in asymptomatic (Group 1) women and monthly in symptomatic (Group 2) women at the São Paulo fetal medicine centre (CPMF). Additionally, where malformations, signs of congenital ZIKV infection or intra-uterine growth restriction (IUGR) were found, ultrasound scanning was carried out weekly at Jundiaí University Hospital or CPMF.

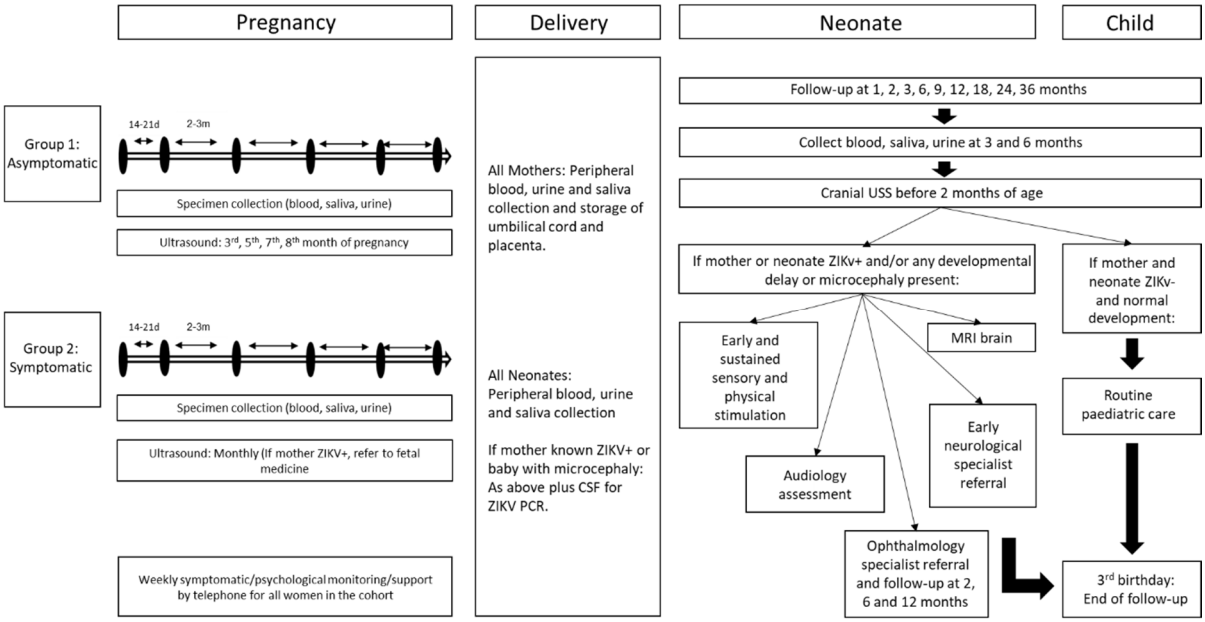
NEONATES, INFANTS, CHILDREN

Women whose birth outcomes resulted in a livebirth were invited to come to the JZC paediatric follow-up clinic within the neonate's first month of life. Subsequently,

mothers were asked to bring their children back monthly in the first 12 months of life and at three-monthly intervals thereafter until they completed 36 months.

At each stage of follow-up there was a significant non-response rate (see figure 3). Pregnant women and mothers who did not attend a scheduled follow-up appointment were contacted by phone, mobile, or facebook. In addition, JZC teams visited and spoke to staff at the basic health units in Jundiaí and requested them to refer on any children belonging to the cohort. Despite these efforts, for many families, a complex and precarious social situation precluded them from being able to attend the follow-up appointments.

Figure 4. Full follow-up protocol for Jundiai Zika Cohort Study (01/03/2016 – 23/08/2017), Jundiaí, São Paulo, Brazil



DATA COLLECTION

The JZC is a multidisciplinary study containing a rich range of information regarding the follow-up and outcomes of the JZC mothers and children, both exposed and unexposed to ZIKV during pregnancy. The main health, medical and laboratory data collected to date are listed in table 1.

The initial baseline questionnaire administered to the pregnant women was designed by the JZC researchers before any internationally standardised collection tool had been developed. In August 2016, the WHO created a standardised questionnaire in order to streamline data collected by all the Zika cohorts[20]; new questions which were not already in the initial JZC tool (mainly related to environmental exposures, for example mosquito repellent and bednet use, type of housing and water sources) were added.

Four specialist fetal medicine doctors carried out the antenatal ultrasound scans at the São Paulo fetal medicine centre (CPMF) with Voluson S10®, Voluson E6® e Voluson E8® GE Healthcare® equipment. Fetal anthropometry was carried out and gestational age estimated using the Hadlock 4 formula.[21]

Anthropometry was carried out in neonates in the first hour of life unless their condition was unstable. Head circumference was measured using a non-elastic tape measure placed between forehead and occiput, weight was measured using digital scales and length using a recumbent baby length scale. Z-scores for these measurements were then calculated using the Intergrowth-21st curves[22-24] which take into account the sex and gestational age of the neonate. Gestational age was calculated using first-trimester ultrasound when available and LMP when not available.

Table 3. The Jundiai Zika Cohort, summary of health, medical and laboratory data collected from women and their children.

Phase	Measurements
Pregnant women at enrolment	Baseline questionnaire - Sociodemographic details, past medical history, family history, past obstetric history (parity, miscarriages, mode of delivery, malformations), current obstetric history (if pregnancy was planned, use of tobacco, alcohol, drug and medications and vaccinations received), presence of symptoms/signs consistent with ZIKV infection (fever, rash, non-purulent conjunctivitis, arthritis/arthralgia, lymphadenopathy, myalgia, headache) at any point throughout pregnancy, the woman's environment (type of housing, number of rooms, number of people per household), preventative measures (use of repellent, protective clothing, window or bed nets, barrier contraception) and their knowledge of ZIKV and its forms of transmission as well as what their sources of information were
Pregnant women follow-up 14-21 days after enrolment	Sample collection (blood, saliva, urine) for ZIKV RT-PCR and IgG/IgM Symptoms questionnaire - presence of symptoms/signs consistent with ZIKV infection (timing, duration, intensity, action taken)
Pregnant women subsequent 2-3 monthly follow-ups	Sample collection (blood, saliva, urine) for ZIKV RT-PCR and IgG/IgM Symptoms questionnaire - presence of symptoms/signs consistent with ZIKV infection (timing, duration, intensity, action taken) Antenatal ultrasound at Sao Paulo fetal medicine centre (CPMF)
Pregnant women weekly phone follow-up	Symptoms questionnaire - presence of symptoms/signs consistent with ZIKV infection (timing, duration, intensity, action taken)
Birth (mother and neonate)	Sample collection (blood, saliva, urine) for ZIKV RT-PCR and IgG/IgM. Colostrum (mother) and cerebro-spinal fluid (CSF) (neonate exposed to ZIKV and/or with microcephaly) for ZIKV PCR. Anthropometry – weight, length, head circumference (neonate) Placenta and umbilical collection - pathology
Neonatal, infant and child follow-ups	Sample collection (blood, saliva, urine) for ZIKV RT-PCR and IgG/IgM in months 1, 3, 6 and 15 for neonates. (Women found to be ZIKV RT-PCR positive during pregnancy also had blood, saliva and urine collected during paediatric follow-up appointments). Paediatric follow-up questionnaire – problems, significant events, feeding, vaccinations, developmental milestones reached, review of lab test results (including heel-prick test) Anthropometry – weight, length, head circumference Paediatric physical examination – general, cardiovascular, respiratory, gastrointestinal, neurological, developmental Physiotherapy assessment Speech and language assessment
ZIKV exposed infants and/or with microcephaly/other neurological abnormalities	Opthalmology assessment (with Teller-CAT Cambridge Colour Test, fundoscopy and extrinsic ocular motility tests) Specialist neurodevelopmental assessment (using Bayley-III developmental scales) Specialist audiology assessment Gastrointestinal assessment Imaging – Cranial ultrasound and CT brain at birth and 12 months

CLINICAL SAMPLES AND LABORATORY PROCEDURES

Blood, saliva and urine were collected from the women and children by nursing staff, healthcare assistants and auxiliary nurses (all registered at the nursing professional registration body of the state of São Paulo (CORENSP)) following an in-house standardised protocol. Personal protective measures were taken (gloves and eye protection were worn at all times) and samples were labelled appropriately with the patient name, date of collection, date of birth and sample number). Saliva samples were collected by swabbing ten times on the inside of each cheek with sterile swabs which were then placed into sterile Falcon® 15ml tubes. Women were asked to provide urine samples into 50ml sterilised collection tubes. For neonatal and infant urine samples, sterile urine collecting bags with adhesive tape were placed over sterilised skin around the penis/vulva. At birth, blood was collected from the umbilical cord and subsequently, in infants and women, blood samples were obtained by venepuncture in the antecubital fossa or in the dorsum of the hand with a maximum of three attempts. Blood was collected into dry and EDTA tubes which were mixed and labelled appropriately and placed in the fridge.

Enzyme-Linked Immunosorbent Assay (ELISA) for identification of anti-ZIKV immunoglobulins (Igm and IgG)

Detection of anti-ZIKV antibodies (IgM and IgG) was performed by ELISA using commercial Zika IgG and Zika IgM ELISA kits Euroimum (Euroimmun BR© 2015), approved by the National Sanitary Surveillance Agency (ANVISA), the regulatory agency for the Brazilian Ministry of Health, for the diagnosis of ZIKV.

Serum, blood or plasma were diluted 1: 101 in sample Buffer. For IgM detection, the buffer contains an IgG / RF absorbent (preparation of anti-goat IgG). After, 100 µL of this dilution was applied to each well of the plates. The plates were covered with

protective film and incubated for 60min at +37°C. After the first incubation, the plates were washed with 400 µl of wash buffer [1X]. One hundred microliters of the enzyme conjugate (peroxidase-labeled human IgG and IgM) were applied to each well, followed by incubation for 30 minutes at room temperature. Another wash was performed, under the same conditions described previously. In each well 100 µL of the substrate/chromogen was added, followed by incubation for 30 minutes at room temperature, protected from light. After washing, 100 µL of Stop solution was added and the plates were read in Automatic Biochemical Analyzer, model prietest™ TOUCH (©2009 ROBONIK India), in 450mn absorbance.

For the detection of IgG, 3 calibrators were used plus the negative and positive controls contained in the kit. For IgM detection a calibrator plus the positive and negative controls were used. The cut-off was calculated by the ratio between the absorbance of the controls and that of the calibrators. Samples with a cut-off <0.8 and positive samples with a cut-off of ≥1.1 were considered negative. The samples with a cut-off between ≥0.8 and 1.1 were considered equivocal.

Real Time PCR for ZIKV detection

ZIKV RNA detection was performed by Real Time PCR (RT-qPCR), as recommended by the WHO, according to the protocol developed by Lanciotti et al.[14] on maternal and neonatal urine samples.

Initially, the total RNA was extracted from 140 µL of urine using the QIAamp Viral RNA Mini Kit (QIAGEN), following manufacturer's instructions. The final RNA was eluted in 60 µl of ultrapure H2O Nuclease-Free Water (©2018 Merck KGaA,Darmstadt, Germany) and RT-qPCR was performed on the same day. The remaining RNA was stored in a freezer at -80°C. RT-qPCR was performed with GoTaq® Probe qPCR and RT-qPCR Systems (© 2018 Promega Corporation Brasil, Ltd). For the final volume of

20 µl reaction, 8 µl of RNA template was used. The Mix was created with 10 µl of GoTaq® Probe qPCR Master Mix with dUTP [1x], 0,4 µl of GoScript™ RT Mix for 1-Step RT-qPCR [1x], 1µl of Forward primer [10pmol/µL], 1µl of Reverse primer[10pmol/µL], 1µl of probe [10pmol/µL] (Table 2) and of Nuclease-Free Water to complete the final volume. Two sets of primers and probe were used on the RT-qPCR reaction. The reaction occurred in ABI Prism 7500 SDS Real-Time cycler (Applied Biosystems), where the amplification cycles consisted of: One cycle of 15 minutes at 45°C for reverse transcription; 1 cycle of 2 minutes at 95°C for reverse transcriptase inactivation and for polymerase activation; 40 cycles of 15 seconds at 95°C for denaturation; and 1 minute at 60°C for annealing and extension. The primers and probes used for this quantification are complementary to the gene encoding the NSI protein of ZIKV. The probe contains a fluorescent 6-carboxyfluorescein (FAM) reporter dye at the 5 'end and the fluorescent dye 6-carboxytemethylhydhodamine (TAMRA) at the 3' end. All reactions followed positive and negative controls previously quantified.

Table 4. Primer and probe sets used for RT-qPCR ZIKV detection

Primer/probe set	Primer and/or probe	Genome position	Sequence (5’ – 3’)
Set 1	ZIKV 835	835–857	TTGGTCATGATACTGCTGATTGC
	ZIKV 860-FAM	860–886	CGGCATACAGCATCAGGTGCATAGGAG
	ZIKV 911c	911–890	CCTTCCACAAAGTCCCTATTGC
Set2	ZIKV 1086	1086–1102	CCGCTGCCCAACACAAG
	ZIKV 1107-FAM	1162–1139	AGCCTACCTTGACAAGCAGTCAGACACTCAA
	ZIKV 1162c	1107–1137	CCACTAACGTTCTTTTGCAGACAT

Primers designed by Lanciotti et al., 2008 (Based on ZIKV MR 766 GenBank accession no. AY632535)

The placenta and umbilical cord were collected and stored in formaldehyde and sent to the pathology laboratory where they were examined by specialist placental pathologists.

FINDINGS TO DATE

The following, unless otherwise stated, have been published in conference proceedings and/or submitted to peer-reviewed journals

ZIKV Clinical Features During Pregnancy

The clinical features of ZIKV infection among pregnant women in the JZC have been described. They have been used to assess the sensitivity of the current standard clinical case definitions, and to investigate whether adverse fetal outcomes are more likely to occur among pregnant women with symptomatic ZIKV infection during pregnancy compared to asymptomatic infection.

Fetal Outcomes After Congenital ZIKV Exposure

This includes studies comparing the incidence of negative fetal outcomes, namely low birth weight, small-for-gestational age, prematurity and fetal death among ZIKV-exposed and unexposed women. Studies have also looked at the prevalence of Chikungunya IgG among mothers who had fetal losses.

Congenital Zika Syndrome (CZS)

The spectrum of congenital Zika Virus syndrome in the JZC children has been explored as well as visual acuity alterations among ZIKV exposed babies, including among dizygotic twins.

The Placenta

Placental histological findings among ZIKV exposed infants with microcephaly have been reported as well as placental histological findings among mothers with Chikungunya and Dengue infection.

Environmental Risk Factors, Prevention, Educational and Vector Control Activities

These studies have included investigations into the environmental risk factors for ZIKV infection; assessment of the peri-domicile environment of women in the JZC and identification of favourable conditions for replication; evaluation of educational activities for children to help combat the proliferation of *Aedes aegypti*; and assessment of the knowledge around the modes of transmission and prevention of ZIKV infection as well as the practice of preventative measures among pregnant women in the cohort.

Susceptibility of ZIKV Infection of Neural Progenitor Cells Among Dizygotic Twins

Analysis of neural progenitor cells (NPCs) of dizygotic twins discordant for Congenital ZIKV Syndrome (CZS) have shown that the development of CZS depends on the intrinsic susceptibility of the NPCs. (Caires-Júnior LC et al. Discordant congenital Zika syndrome twins show differential in vitro viral susceptibility of neural progenitor cells. Nature Comm **vol** 9 (475) (2018). https://www.nature.com/articles/s41467-017-02790-9)

Seroepidemiological Arbovirus Studies

Studies quantifying the seroprevalence of ZIKV, Chikungunya, and Dengue IgG antibodies among pregnant women in the JZC have been carried out.

STRENGTHS AND LIMITATIONS

The JZC is one of the few prospective Zika cohort studies that has recruited both asymptomatic and symptomatic women and therefore benefits from having a large control group. It also provides the necessary study population to carry out analyses on ZIKV symptomatology. Women were recruited over more than a one-year period of time (March 2016 to August 2017) and therefore seasonality can be explored. The diversity and frequency of biological samples collected from the women during pregnancy (and after), as well as their children, mean that JZC now has a rich and invaluable biorepository of clinical material. The high-risk profile of the pregnant women also provides an additional unique opportunity to study other factors that may contribute to, or potentially be protective for, the development of negative sequelae associated with ZIKV exposure. The JZC implemented the use of standardised WHO research method tools, as soon as they were available, and therefore has placed itself in an optimal position to collaborate in Brazilian and international consortia that will ultimately be aiming to perform meta-analyses on all Zika Cohort study data.

The limitations of the JZC in part relate to the pressing nature of the ZIKV and microcephaly epidemic and the urgency to start the investigation. The JZC, like many other Zika studies, commenced without any formal funding. Recruitment and data collection commenced in paper form, before formal data management systems could be put in place. For example, the resources were not available in order to collect information about women who chose not to participate in the study. In addition, as WHO standardized research protocols were produced after the start of the investigation, some variables contained in the WHO protocol were not in our original questionnaire and therefore some of this data is missing for the earliest recruits in our

cohort. Even after the procurement of formal funding, the cost of some important laboratory tests, and the infrastructure required to perform them, continue to be out of reach for many institutions in endemic countries. These include IgG and IgM assays and PRNT. The choice of study population (high-risk pregnant women) had several advantages as stated above. However, there are also a few drawbacks that should be highlighted. Firstly, because women were not recruited based on a suspicion of having been exposed to ZIKV, the prevalence of ZIKV RT-PCR positivity was relatively low, and this equated to small numbers for estimating absolute and relative risks. Furthermore, because high-risk pregnant women are at higher risk of developing some of the outcomes of interest, this may have reduced the power for us to detect differences in frequency of outcomes between Zika-exposed and unexposed dyads. The differential follow-up of women who were symptomatic for ZIKV infection in terms of a more intensive antenatal scanning schedule may have also had consequences in terms of the pick-up of problems in the antenatal period.

Collaboration

The JZC headquarters is at the Jundiai Medical School under the direction of Professor Saulo Duarte Passos. The cohort has a facebook page: https://www.facebook.com/zikacoortejdi/ which contains information about the functioning and organisation of the JZC, fundraising events, support for Zika affected families and media coverage. Any researcher wanting to use JZC data must apply to the Jundiai Zika Cohort Group via Professor Passos (sauloduarte@uol.com.br).

Funding Declaration

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Contributorship Statement

NSC, MR, APP, REG, DV: Conceptualization, data curation, formal analysis, methodology, investigation, validation, visualization, writing -original draft and review & editing.

EBB: Methodology, resources, supervision, validation, visualization, writing- review & editing.

MFA: Formal analysis, funding acquisition, methodology, resources, supervision, validation, visualization, writing- review & editing.

SDP: Conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation.

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Can Zika Virus Infection in High Risk Pregnant Women be Differentiated on the Basis of Symptoms?

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ABSTRACT

Zika virus (ZIKV) infection in pregnancy is associated with congenital neurological abnormalities. Our understanding of the full clinical spectrum of ZIKV infection is incomplete.

Using data from this prospective cohort study consisting of 650 women attending a high risk pregnancy clinic during the Zika virus outbreak in Brazil, we investigated the extent to which specific symptoms can be utilized to differentiate ZIKV-infected pregnant women from those with other pregnancy-related problems. All were tested for ZIKV in urine by RT-qPCR. Demographic and clinical data including physical symptoms during follow-up were recorded and analysed with respect to Zika virus exposure status.

Forty-eight (7.4%) women were positive for ZIKV by RT-qPCR. The majority (70.8%) were asymptomatic, and only four ZIKV-positive women (8.3%) reported symptoms during pregnancy that met the WHO case definition. Among the 602 ZIKV-negative women, 28 (4.7%) reported symptoms that met the WHO case definition, and a further 147 (24.4%) reported at least one symptom consistent with the definition. Zika-positive and negative women reported similar frequencies of ZIKV-like symptoms (as per the WHO definition): fever (16.7% vs. 13.6%), arthralgia/arthritis (10.4% vs. 11.3%), rash (4.2% vs. 5.3%), and conjunctivitis (2.1% vs. 3.2%). Two women, one with a rash and one asymptomatic and both PCR-positive, gave birth to newborns with microcephaly.

Most pregnant women positive for ZIKV in urine are asymptomatic and do not deliver a baby with microcephaly. Physical symptoms alone did not differentiate between high risk pregnant women positive or negative for ZIKV.

INTRODUCTION

More than three years after the appearance of Zika Virus (ZIKV) in the Americas,[138] our understanding of the clinical presentation and consequences of ZIKV infection in pregnant women is still incomplete. The proportion of ZIKV-infected pregnant women who display symptoms, identification of the most common symptoms of ZIKV in pregnancy and their sensitivity and specificity and whether symptomatic ZIKV-infected pregnant women are at greater risk for delivering an infant with congenital abnormalities remain to be definitively determined.

The incidence of asymptomatic ZIKV infections in pregnant women is widely quoted to be 80% based on estimates from household survey data on Yap Island, Micronesia in 2007.[15] Systematic reviews to update this figure have used subsequently published epidemiological studies; however, this has proved difficult due to the paucity of studies looking specifically at the clinical spectrum of ZIKV infection in pregnancy, marked heterogeneity in the reported asymptomatic rates of disease and small sample sizes.[139] For example, in a recent WHO systematic review looking at the proportion of asymptomatic ZIKV infections in a number of population sub-groups, the proportion of asymptomatic infections in pregnant women varied from 10% to 83%.[139] Data from a countrywide pregnancy cohort study in French Guiana[4] showed striking intra-study heterogeneity in the asymptomatic infection rate of ZIKV-infected pregnant women, related to a number of socio-demographic factors. For example, although 77% of pregnant women overall were asymptomatic, those living in the urbanised coastal areas reported significantly more symptoms than those living in the remote interior (35% versus 17%, p=0.001), and women over 30 were also more likely to report symptoms compared to younger women (28% versus 20%, p=0.03).

The frequency of individual symptoms reported by pregnant women with confirmed ZIKV infection also varies according to different reports and is hard to assess because entry criteria for many cohort studies are defined around the presence of being positive for certain symptoms at recruitment.[5-8] Furthermore, areas that are endemic for ZIKV are also endemic for other flaviviruses; co-infection is possible and complicates the clinical picture.[9] The current standard clinical case definition for ZIKV infection proposed by the World Health Organization (WHO)[10] was last updated in 2016. No ZIKV studies to date have evaluated its sensitivity or specificity in the detection of clinical ZIKV infection during pregnancy.

There is also no consensus on whether newborns are more likely to exhibit negative sequelae at birth if their ZIKV-infected mothers were symptomatic during pregnancy. A recent review of 9 case-series performed in Brazil and Colombia and 3 cohort studies from the USA concluded that the ratio of symptomatic versus asymptomatic maternal ZIKV infections that resulted in adverse fetal outcomes was 1:1.[11] However, other publications have hypothesised that women with a symptomatic infection may have a higher viral load than do women with asymptomatic infections and this may translate to a higher probability of birth defects in their offspring.[4,12,13]

The aims of the present study are to determine, in a region with active ZIKV transmission, the relative percentage of ZIKV-positive pregnant women who are asymptomatic and whether symptoms of ZIKV in RT-PCR positive pregnant women can be differentiated from symptoms present in women with high risk pregnancies who are ZIKV-negative.

MATERIALS AND METHODS

Study Design and Participants

The data reported in this prospective cohort study originated from the Jundiaí Zika Cohort, initiated in March 2016 at Jundiaí University Hospital in São Paulo State, Brazil. During the recruitment period (1 March 2016 – 23 August 2017) all women attending a high-risk pregnancy clinic, due to the presence of risk factors threatening the life or health of the pregnant woman or her foetus[14] at any stage of pregnancy, were considered eligible and offered the opportunity to participate in the study. The only exclusion criteria were women with life-threatening conditions or who could not provide informed consent. Research nurses who interviewed the women at enrolment and reviewed their antenatal records gathered detailed demographic, medical and antenatal information. In addition, all participants were specifically asked if they had experienced the following symptoms during their pregnancy: fever, rash, conjunctivitis, joint pain or swelling, headache, vomiting, lymphadenopathy, bleeding, myalgia or pruritus. Women who were symptomatic at recruitment and/or who developed symptoms consistent with the WHO definition of suspected ZIKV infection (rash and/or fever and at least one of the following symptoms: arthralgia, arthritis or non-purulent conjunctivitis, (see Table 1)[10] were noted.

Table 1. WHO 2016 Interim Case Definitions for Confirmed and Suspected Zika virus Infection

Case definition	Description
Confirmed case	A person with laboratory confirmation of recent Zika virus infection: • Presence of Zika virus RNA or antigen in serum or other samples (e.g. saliva, tissues, urine, whole blood); or • IgM antibody against ZIKV positive $PRNT_{90}$ for ZIKV with titre≥20 and ZIKV $PRNT_{90}$ titre ratio≥4 compared to other flaviviruses; and exclusion of other flaviviruses
Suspected case	A person presenting with rash and/or fever and at least one of the following signs or symptoms: • Arthralgia; or • Arthritis; or • Conjunctivitis (non-purulent/hyperaemic)

Research nurses collected blood, saliva and urine from all subjects at the time of enrolment, 2-3 weeks after recruitment and subsequently on a 2-3 monthly basis during routine check-ups. Trained volunteers carried out pre-arranged weekly follow-up telephone interviews and inquired whether they had experienced any new symptoms consistent with ZIKV infection. If symptoms were reported, the women were advised to attend the hospital for clinical review and blood, saliva and urine were collected at this time as well.

Of note, the methods above describe the recruitment, enrolment and follow-up of all women in the cohort. At the analysis stage, some individuals or dyads had to be excluded from the analysis, mainly due to missing information pertaining to risk factors or outcomes of interest. This will be described in detail later.

Laboratory Procedures

All laboratory procedures were performed on de-identified samples. Due to financial constraints it was opted to prioritise the detection of ZIKV in urine samples by reverse transcriptase-polymerase chain reaction (RT-PCR) and to store blood and saliva for future analysis. Studies have shown that ZIKV RNA is unlikely to be detected in serum after the first week of illness whereas it can be detected in urine for at least 2 weeks after symptom onset.[15-17]

Total RNA was extracted from urine by commercial QIAamp Viral RNA Kit (Qiagen®), following the manufacturer's instructions and stored at -80°C until used. Reverse Transcription (RT) and qPCR were performed with GoTaq® 1-Step RT-qPCR System (Promega®) on ABI Prism 7500 SDS Real-Time cycler (Applied Biosystems). The primers and probes designed by Lanciotti and colleagues[15] are complementary to the nonstructural 5 Protein (polymerase). The RT cycle consisted of a 10 minute cycle at 50°C and a 15 minute cycle at 95°C. The PCR consisted of forty cycles of 15 seconds at 95°C and a 1 minute cycle at 60°C. Three positive controls (RNA extracted from positive ZIKV samples) and two negative controls (H_2O) were included. We considered positive those samples that presented with a threshold cycle (Ct) less than 38.5, as per Lanciotti and colleagues.[18] In cases where the results were inconclusive, repetitions were performed with serial dilutions.

Neonatal categorisation

Sonographers specialised in fetal medicine performed antenatal ultrasound scanning in months 3, 5, 7 and 8 in asymptomatic women and monthly in symptomatic women at the São Paulo Radiology Centre using Voluson 730 Expert/Voluson E6, GE equipment. Anthropometric measures at birth (i.e., neonatal weight, length and head

circumference) were obtained for all live-born infants, and the equipment used was consistent. Weight was assessed using digital scales, length using a recumbent baby length scale and head circumference using a standardised non-elastic tape measure. Z-scores for weight, length and head circumference were determined using the online Intergrowth calculator, which takes into account gestational age and sex.[19-21] Gestational age was estimated using first trimester ultrasound (USS) when available and by last menstrual period (LMP) when USS was unavailable. Microcephaly was defined as a head circumference z-score of less than −2, determined using the online Intergrowth-21st calculator which takes into account gestational age and sex.[20]

Statistical Analysis

The sample size for the cohort was calculated using an estimated prevalence of cases of microcephaly among neonates of ZIKV RT-PCR positive pregnant women of 2%. A final analytical cohort size of n=531 would give us 80% power to detect a crude relative risk of 2 with a probability of type I error (α) of 5%. Categorical variables were compared among women with and without symptoms; and symptoms were compared between different case definitions using the Chi-square test except where there were less than 5 in any cell in which case Fisher's exact test was used. For the calculation of sensitivity and specificity of the standard clinical case definition, ZIKV RT-PCR was used as the 'gold standard' diagnostic test. All statistical analyses were carried out using STATA™ version 15.1 software.

Sensitivity and Specificity

The sensitivity and specificity of the current WHO case definition were calculated using the standard formulas (see figure 3) using only women that had at least one symptom during pregnancy. Asymptomatic individuals were removed for the calculation of

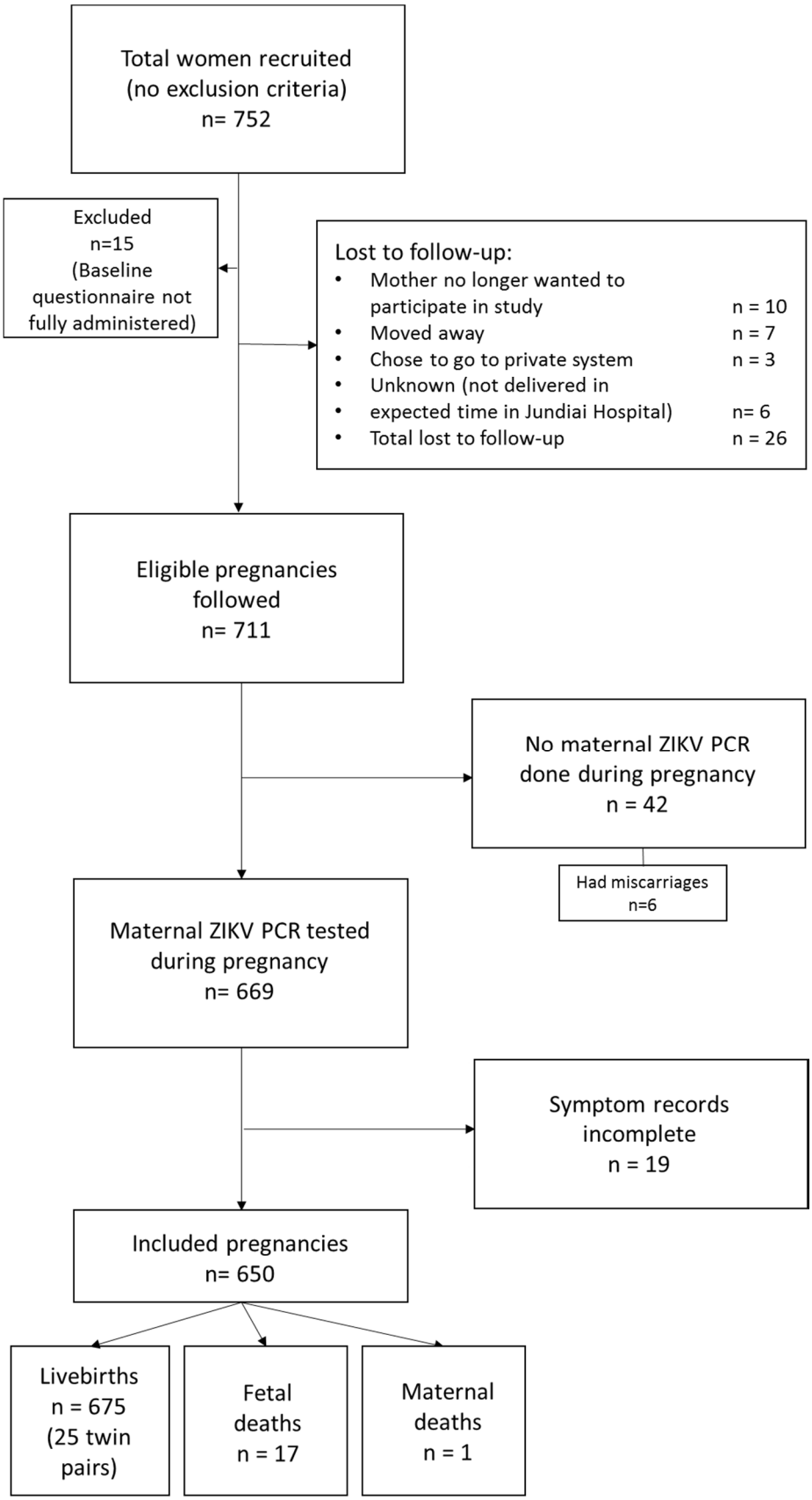
sensitivity and specificity. In other words, true positives were defined as symptomatic ZIKV RT-PCR positive women correctly identified by the WHO case definition and false negatives were defined as symptomatic ZIKV RT-PCR positive women that did not meet this case definition. True negatives were defined as ZIKV RT-PCR negative women who were symptomatic but did not meet the full WHO symptomatic case criteria and false positives were defined as symptomatic ZIKV RT-PCR negative women that met the WHO suspected clinical case criteria and the. With one caveat, that as the current most optimal diagnostic test in areas with active ZIKV transmission is ZIKV RT-PCR and this test has a narrow window for detection of the virus, we cannot assume that any women were are truly negative and therefore the number of false positives may be overestimated and the number of true positives underestimated.

RESULTS

Participant Characteristics

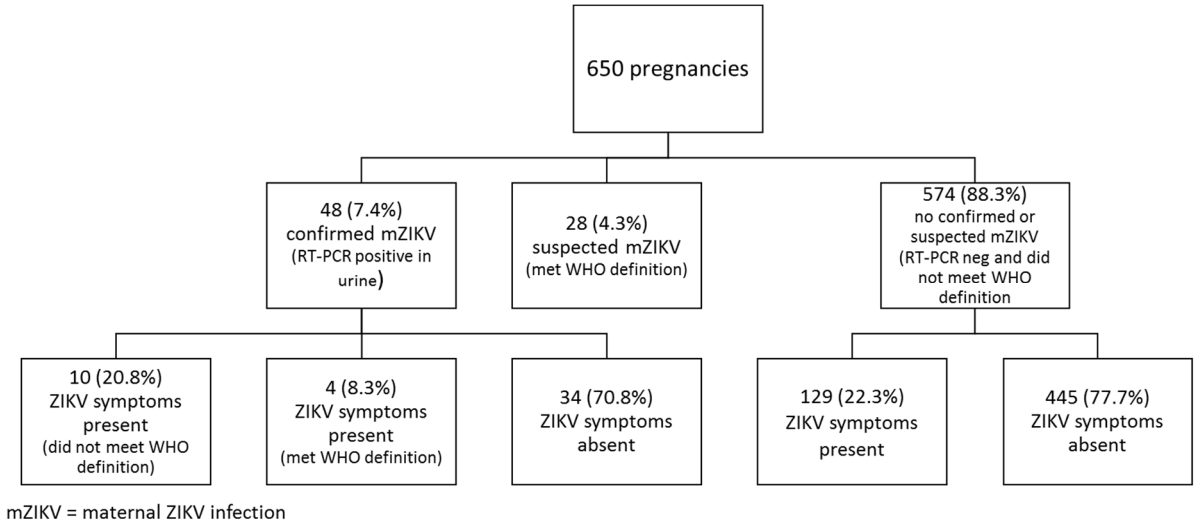
A flow diagram of study participants is shown in Fig. 1. A total of 752 women were initially enrolled in the study between March 2016 and August 2017; 26 were eventually lost to follow-up. Women with missing data pertaining to risk factors and outcomes of interest were also excluded. Forty two women did not have a ZIKV RT-PCR sample taken during pregnancy. An additional 19 women did not have information on symptoms during pregnancy. Among the resulting remaining 650 women, there were 675 live births (including 25 twin pairs), 17 fetal deaths, and one maternal death.

Figure 1. Flow diagram for the selection of study participants



Of the 650 pregnant women in the final cohort, 48 (7.4%) were ZIKV-positive by RT-PCR, 28 (4.3%) were positive by the WHO criteria but were ZIKV-negative and 574 (88.3%) were both negative by RT-PCR and symptoms (Fig. 2).

Figure 2. Distribution of pregnant women according to WHO ZIKV case definitions



Only 4 (8.3%) of the 48 ZIKV-positive women met the WHO definition of a symptomatic case, 10 (20.8%) had at least one ZIKV-like symptom during pregnancy but did not meet the WHO definition and 34 (70.8%) did not report any symptoms compatible with ZIKV infection during pregnancy. Of the 574 pregnant women who did not meet the definition for either a confirmed or a suspected case, 129 (22.3%) had at least one symptom that was consistent with ZIKV infection and 445 (77.7%) never reported any symptoms during pregnancy.

Socio-demographic characteristics of the study population are shown in Table 2. There were no differences in maternal age, level of education, race, co-habitation and delivery by cesarean section between symptomatic and symptomatic women. The time of symptom initiation was known for 13 of the 14 women with confirmed

symptomatic ZIKV infection. Six (46.2%) had symptoms in the first trimester, 4 (30.8%) in the second trimester and 3 (23.1%) in the third trimester. In 25 women who met the WHO case definition but who were ZIKV RT-PCR negative, 11 (39.3%) had symptoms in the first trimester, 11 in the second trimester and 3 (21.4%) had symptoms in their third trimester. Of note, the mean number of separate urine RT-PCR results available for each woman throughout their pregnancy was 2.02 among ZIKV positive women and 1.95 among ZIKV negative women.

Table 2. Maternal characteristics of participants in the Jundiaí Zika Cohort

Variable	ZIKV symptoms present* (n=171)	ZIKV symptoms absent (n=479)	p-value
Age			
13-19 years	32 (18.7%)	65 (13.6%)	0.07
20-34 years	112 (65.5%)	305 (63.7%)	
35-46 years	27 (15.8%)	109 (22.8%)	
Missing	0	0	
Education			
≤8 years	30 (17.8%)	78 (16.6%)	0.96
9-11 years	40 (23.7%)	106 (22.6%)	
12 years	74 (43.8%)	210 (44.8%)	
>12 years	25 (14.8%)	75 (16.0%)	
Missing	2 (1.2%)	9 (1.9%)	
Ethnicity/race			
White	94 (56.3%)	246 (52.5%)	0.86[$]
Mixed race	55 (32.9%)	165 (35.2%)	
Black	15 (9.0%)	49 (10.5%)	
Other (Asian/indigenous)	3 (1.8%)	9 (1.9%)	
Missing	4 (2.3%)	10 (2.1%)	
Relationship with partner			
Married/co-habiting	129 (76.3%)	361 (76.7%)	0.93
Single/divorced/widowed	40 (23.7%)	111 (23.4%)	
Missing	2 (1.2%)	8 (1.7%)	
Type of delivery			
Vaginal/forceps	75 (51.4%)	200 (50.4%)	0.84
C-section	71 (48.6%)	197 (49.6%)	
Missing	2 (1.4%)	1 (0.25%)	
ZIKV RT-PCR status			
Positive in urine	14 (8.2%)	34 (7.1%)	0.64
Negative in urine	157 (91.8%)	445 (92.9)	

*At least one symptom compatible with ZIKV infection as per the current WHO standard clinical case definition

Note: Percentages for all categories were calculated with exclusion of those with missing data from the denominator

$ All p-values calculated using Chi^2 test except for those labelled with $ which were calculated using Fisher's exact test. The 'missing' category was not included as a category when the p-value was estimated.

Of the 48 women who were ZIKV RT-PCR positive, 4 (8.3%) reported symptoms during pregnancy that met the WHO definition of a clinical case. Twenty eight (4.3%) ZIKV-negative women also had symptoms consistent with the WHO definition. Among

ZIKV-positive cases, 25 (52.1%) complained of a headache, 8 (16.7%) reported fever, 5 (10.4%) had arthralgia/arthritis, and 2 (4.2%) had a rash. Among the Zika-negative women, 32 (5.3%) experienced a rash during pregnancy, 68 (11.3%) had arthralgia/arthritis, 82 (13.6%) had fever and 320 (53.2%) complained of headache. The results are summarized in Table 3. None of these differences reached statistical significance.

Table 3. Symptoms in women with and without PCR-confirmed ZIKV Infection

Signs/symptoms		No. (%) positive PCR-positive n=48	PCR-negative n=602	p-value**
WHO criteria	Fever	8 (16.7)	82 (13.6)	0.55
	Arthralgia/arthritis	5 (10.4)	68 (11.3)	0.85
	Rash	2 (4.2)	32 (5.3)	0.73
	Conjunctivitis	1 (2.1)	19 (3.2)	0.68
Other symptoms	Myalgia	4 (8.3)	74 (12.3)	0.42
	Headache	25 (52.1)	320 (53.2)	0.89
	Lymphadenopathy	1 (2.1)	33 (5.5)	0.31
Total symptomatic	Fulfilled required WHO criteria*	4 (8.3)	28 (4.7)	0.20
	Did not fulfill WHO criteria	10 (20.8)	129 (21.4)	0.20
	Total symptomatic	14 (29.2)	157 (26.1)	0.69
No symptoms		36 (75)	445 (73.9)	0.64

*A person presenting with rash and/or fever and at least one of the following signs or symptoms: Arthralgia; or Arthritis; or Conjunctivitis (non-purulent/hyperaemic)
** Chi2 test was used unless there was < 5 in any cell in which case Fisher's exact test was used

As our cohort is made up of women with high risk pregnancies, and as headache is also a common symptom among pregnant women with hypertension or pre-eclampsia, we compared the prevalence of these two pregnancy complications among confirmed ZIKV-positive cases compared to non-cases. The prevalence of hypertension/pre-eclampsia among confirmed cases with headache was 4/25 (16%) compared to 61/295 (20.6%) among non-cases with headache.

Figure 3. Sensitivity, specificity, positive and negative predictive values of the WHO Standard Clinical Case Definition for ZIKV Infection applied to symptomatic pregnant women in The Jundiai Zika Cohort

		Symptomatic women with ZIKV infection		
		ZIKV RT-PCR positive	ZIKV RT-PCR negative	
Have symptoms that fulfil WHO standard case definition	Yes	4 (TP)	28 (FP)	PPV =TP/(TP+FP) = 12.5%
	No	10 (FN)	157 (TN)	NPV =TN/(TN+FN) = 94.0%
		Sensitivity = TP/(TP+FN) = 28.6%	Specificity = TN/(FP+TN) = 84.9%	

TP = true positive; TN = true negative; FP = false positive; FN = false negative; PPV = positive predictive value; NPV = negative predictive value

Additional Findings

Although not part of the main objectives of this study, we studied the head circumference at birth of neonates in the cohort as well as the presence of microcephaly as markers of adverse neurological outcomes. Head circumference z-scores of the cohort of newborns at birth were analysed and stratified by maternal case definition. The infants of suspected cases, as per the WHO standard case definition, had head circumference z-scores that ranged from -1.05 to 2.71 with a mean of 0.63; and infants of RT-PCR confirmed cases had head circumference z-scores that ranged from -2.77 to 2.58 with a mean of 0.51 (p=0.34 using two-sample t-test). Of the two women with RT-PCR confirmed ZIKV infection who had newborns with microcephaly (head circumference z-score<-2) at birth, one was asymptomatic and the other reported having had a rash in the first trimester of pregnancy.

DISCUSSION

Among a population of high risk pregnant women in which 7.4% were RT-PCR positive for ZIKV during pregnancy, most of those positive for ZIKV infection in urine (70.8%) were asymptomatic. In addition, ZIKV-positive women could not be differentiated from ZIKV-negative women on the basis of symptoms. Lastly, the WHO case definition of suspected ZIKV-positive cases had low sensitivity (28.6%) for detecting RT-PCR confirmed ZIKV cases but a high specificity (82.2%). Fever was the most frequently reported symptom in ZIKV-infected women (16.7%) but was also reported by 13.6% of women without suspected or confirmed ZIKV infection. The lack of specificity of fever as a symptom for ZIKV infection has also been reported by others.[4,5]

Our results are consistent with a prior report from French Guiana where only 2.4% of Zika-positive women had symptoms that met the WHO standard clinical case definition for ZIKV disease.[4] The prevalence of asymptomatic ZIKV infections in our cohort (73.9%) is also comparable to reports from Yap Island, Micronesia during the outbreak in 2007[15] and French Guiana in 2016[4] where 81% and 77% of the cohorts were asymptomatic, respectively. In our cohort, rash was infrequently reported in ZIKV-positive women (4.2%). This is in stark contrast to the high prevalence of rash (90%) among 31 patients with confirmed ZIKV during the Yap Island outbreak in 2007.[15] Differences between these two groups include the fact that in our study women were pregnant and, therefore, immunological responses are different, perhaps causing distinct clinical manifestations.[22] Another factor which has been discussed previously,[4] is the difference in skin pigmentation between various study populations which may cause rash to manifest in different ways or be more or less likely to be reported. Under-reporting may have also played a part, particularly during the start of

the outbreak, as the phenotype of the rash was not well recognised by patients or health workers. This Arthritis and arthralgia were present in 10.4% of our confirmed ZIKV-positive women and 11.3% of women negative for ZIKV infection. This manifestation appears at the top of the list of symptoms in many ZIKV studies,[2,4,5,7,,23] as does headache[2,4,5,7,8] which, of note, also forms part of the CDC ZIKV clinical case definition.[24] In our study, headache was frequently reported by women with confirmed ZIKV-positive infection (52.1%) as well as by ZIKV RT-qPCR negative women (53.2%). Thus, it may be especially difficult to differentiate between Zika-positive and negative women on the basis of symptoms when analysing high risk pregnancy populations.

Given the low sensitivity of the current WHO clinical case definition for the detection of clinical ZIKV infection in pregnant women, we propose incorporating arthritis/arthralgia into the major symptoms and adding headache to the minor symptoms. This change would increase the sensitivity to 50% in our study population. Although still far from ideal (as we would still be missclassifying 50% of true positives as false negatives), these additions may be of particular value in pregnant women in regions with active ZIKV transmission where even a presentation with fever and headache (which is otherwise non-specific) would be sufficient to alert a healthcare worker to test for ZIKV.

Our study has several limitations. It cannot be ruled out that an unknown proportion of women in our cohort who were ZIKV-negative in their urine may, nevertheless, have been positive for this virus. The results of ZIKV serological analysis, which would have confirmed viral exposure, are not available for this cohort for the time being. In this study, most women were recruited in the 2nd or 3rd trimester of pregnancy due to time lags including time to diagnosis of high-risk pregnancy and appointment waiting time.

Therefore, women who were infected with ZIKV in the first trimester of pregnancy might have been missed by RT-PCR done at recruitment. It is also possible that some women entered the study having previously been exposed to ZIKV and that some women who tested ZIKV RT-PCR positive on recruitment were in fact infected prior to pregnancy but still shedding virus in the urine. If this was the case, these women may not have reported symptoms occurring prior to pregnancy and therefore symptom status may have been incorrectly classified. As prolonged shedding of ZIKV in the urine is known to occur, there was no limitation set between the onset of symptoms and ZIKV RT-PCR positivity. In other words, a woman who was symptomatic but tested RT-PCR positive in the urine several weeks later was considered to be symptomatic with confirmed ZIKV infection. In addition, the small number of neonates with adverse outcomes negates our ability to address possible associations between symptomatology and fetal pathology. Lastly, the possibility of recall bias cannot be ruled out. A proportion of the subjects reported symptoms retrospectively and so it is possible that women who were ZIKV-positive may have been more likely to report symptoms than ZIKV-negative women. With all these limitations our conclusions are in need of verification by subsequent studies.

In conclusion, we suggest that institutions re-visit clinical case definitions proposed at the start of the ZIKV outbreak and, using available evidence, come to a consensus regarding the most appropriate definition. A more sensitive case definition for pregnant women living in areas with active ZIKV transmission should be considered that includes arthralgia/arthritis as a major symptom and headache as a minor symptom.

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Zika Virus Infection in Pregnancy and Adverse Fetal Outcomes in São Paulo State, Brazil: A Prospective Pregnancy Cohort Study

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Keywords: Zika virus, flavivirus, pregnancy, microcephaly, low birth weight, prematurity, fetal, outcomes, small-for-gestational age

ABSTRACT

Background: Robust epidemiological and biological evidence supports a causal link between prenatal ZIKV infection and congenital brain abnormalities including microcephaly. However, estimates of the risk of negative fetal outcomes have substantial inter-study variability. This prospective cohort study in a group of high-risk pregnant women in Jundiaí, São Paulo aims to compare the prevalence of adverse fetal outcomes (prematurity, low birth weight, small-for-gestational-age, fetal death and microcephaly) by prenatal Zika Virus (ZIKV) exposure status.

Methods: During the recruitment period (01 March 2016 – 23 August 2017), all women attending the high-risk pregnancy clinic at Jundiaí University Hospital were considered eligible. Clinical samples were collected for ZIKV RT-PCR from the women at recruitment and from the neonates after delivery. Anthropometric measures were obtained from the neonates at birth.

Results: Of the 574 women in the analytical cohort, 7.7% had a positive ZIKV RT-PCR during pregnancy. Of the 409 neonates tested, 4.6% had a positive ZIKV RT-PCR in the first 10 days of life. Two (10.5%) neonates who were ZIKV RT-PCR positive at birth had microcephaly compared to 8 (2.1%) ZIKV negative neonates. The risk of microcephaly among ZIKV-positive neonates was five times the risk compared to ZIKV-negative neonates (RR 5.1, 95% CI 1.2-22.5). The risk of disproportionate microcephaly in ZIKV-positive neonates was 10 times the risk compared to ZIKV-negative neonates (RR 10.3 95% CI 2.0-52.6). There was no significant difference between other adverse fetal outcomes by ZIKV RT-PCR status.

Interpretation: In this cohort of high-risk pregnancies, ZIKV infection did not appear to increase the risk of prematurity, low birth weight, small-for-gestational-age or fetal

death. However, these results provide new evidence that ZIKV exposure during pregnancy is strongly associated with disproportionate microcephaly. Disproportion between neonatal head circumference and weight may be a useful screening tool for congenital microcephaly associated with ZIKV infection.

AUTHOR SUMMARY

The majority of data published from Zika pregnancy cohorts have reported a greater proportion of adverse infant outcomes in Zika Virus (ZIKV) exposed infants compared to unexposed infants. However, estimates of the proportion of newborns affected vary from 6% to 42%. Epidemiological studies quantifying the risk of ZIKV exposure on specific, well-defined fetal outcomes including prematurity, low birth weight, small-for-gestational-age, microcephaly and fetal death are lacking. The prospective nature of this investigation enabled us to quantify the risk of microcephaly in infants exposed to ZIKV during pregnancy and to identify that disproportionate microcephaly is more strongly associated with ZIKV exposure compared to microcephaly alone. Furthermore, we were able to ascertain that the strength of association is higher when stratifying by neonatal ZIKV RT-PCR status at birth rather than maternal ZIKV RT-PCR status during pregnancy. This study also shows that in women who already have high-risk pregnancies, ZIKV exposure is not associated with an increased risk of preterm birth, low birth weight or small-for-gestational-age. We propose that disproportionate microcephaly could be used as a screening tool by clinicians and epidemiologists working with ZIKV-exposed pregnant women.

INTRODUCTION

Since the 1950s, Zika Virus (ZIKV) has been known to circulate in Africa and Southeast Asia[1,2] This mosquito-borne infection, which was previously considered to cause only mild disease,[3] spread to the Americas in 2015.[4] By August 2016, Brazil had reported 174,000 suspected and 78,500 confirmed cases of this 'new' flavivirosis, 5,500 of which were in the state of São Paulo.[5-7] Zika virus is a single-stranded RNA virus member of the *Flaviviridae* family, genus *flavivirus.*[8] The rapid expansion of ZIKV in the Americas has largely been due to the biology and behaviour of its principal vector, *Aedes aegypti*[9] however, ZIKV has also been shown to be transmistted sexually[10,11] and has the potential to be transmitted by breast milk[12,13].

The association between maternal flavivirus infection and negative birth outcomes is recently becoming more evident and discussed in the literature. Case-control data have demonstrated that maternal symptomatic dengue infection is associated with an increased risk of stillbirth[14] and a recent meta-analysis[15] also reported a higher risk of preterm delivery and low birthweight associated with prenatal dengue exposure.

In the case of Zika, robust epidemiological and biological evidence supports a causal link between prenatal ZIKV infection and congenital brain abnormalities including microcephaly[8,16-20]. Case control study evidence from Recife in the Northeast of Brazil has shown that intra-utero ZIKV exposure is associated with microcephaly[21,22] and prospective data in symptomatic women with suspected Zika in Rio de Janeiro have shown an association between congenital ZIKV infection and abnormal neurological findings or brain imaging at birth.[23,24] Microcephaly occurs as a result of any insult that disturbs early brain growth,[25] and in the case of Zika has been found to be associated with fetal brain disruption sequence (FBDS)[26,27] a condition arising

from a disturbance in brain tissue formation during the second or third trimester of pregnancy with subsequent fetal skull collapse resulting from decreased intracranial hydrostatic pressure.[28] Differentiation between proportionate and disproportionate microcephaly has been made in some ZIKV studies [24,29], however its importance in helping to characterise the Congenital Zika Syndrome has still not been established.

Therefore, as this apparent causal relationship is still being recognised and described,[16] the scientific community and the WHO[19] have called for urgent analysis of data from all ZIKV pregnancy cohorts to further understand the full spectrum of adverse outcomes associated with ZIKV infection in pregnancy.

This prospective cohort study in Jundiaí, São Paulo, aims to compare the prevalence of five well-defined negative fetal outcomes (i.e., prematurity, low birthweight, small-for-gestational-age, microcephaly and fetal death) in a group of infants exposed and unexposed to ZIKV prenatally, as evidenced by a positive maternal ZIKV RT-PCR result during pregnancy, in the context of a cohort of women with high-risk pregnancies.

METHODS

Study Design and Participants

The Jundiaí Zika Cohort was initiated in March 2016 at Jundiaí University Hospital in São Paulo State, Brazil and recruited 752 pregnant women. Details about the participant characteristics are described in a previous article titled 'The Jundiai ZIka Cohort (JZC): Cohort Profile.'

The municipality of Jundiaí, located 60km to the northwest of São Paulo, has 409,000 inhabitants[30] and one of the highest Human Development Indices of all the municipalities in the state.[31] The maternity Department at the University Hospital is the only public maternity facility in the municipality and carries out approximately 300-400 deliveries per month, around two thirds of the total births in Jundiaí.[32]

As ZIKV seroprevalence studies have not been carried out, the true incidence and prevalence of ZIKV infection in the Jundiai area during the study period is unknown. However, in 2016, in the State of Sao Paulo, 9,845 cases of ZIKV infection were reported to the Brazilian Notifiable Disease Registry (SINAN) of which 5,056 were considered probable, giving a crude incidence of 11.3 cases of ZIKV per 100,000 inhabitants in the state of Sao Paulo in the year 2016.[33] Nevertheless, this is likely to be a gross underestimation of cases as this figure only represents cases that accessed health services, therefore those most severely affected (which represent the minority of ZIKV cases), and those that were reported by health professionals.

During the recruitment period (1 March 2016 – 23 August 2017), all women attending the high-risk pregnancy clinic (i.e., due to the presence of risk factors threatening the life or health of the pregnant woman or her fetus)[34,35] at Jundiaí University Hospital at

any stage of pregnancy were considered eligible and offered the opportunity to participate in the study. Over the duration of follow-up, clinical teams cared for pregnant women in accordance with the Brazilian Ministry of Health protocols. At the time of enrolment, women of any stage of pregnancy were eligible to participate. The only exclusion criteria at this stage were women who had life-threatening conditions and women who had severe learning difficulties.

The sample size for the cohort was calculated using an estimated prevalence of cases of microcephaly among neonates of ZIKV RT-PCR positive pregnant women of 2%. A final analytical cohort size of n=531 would give us 80% power to detect a crude relative risk of 2 with a probability of type I error (α) of 5%. Although initially the Jundiai Zika Cohort aimed to enrol 500 pregnant women, recruitment continued for longer than initially anticipated to try to capture possible seasonal differences in the incidence of ZIKV disease. Criteria for the final selection of women to be included in the analytical cohort for this particular study question were women with high-risk pregnancies who had a ZIKV RT-PCR result and whose babies had anthropometric measures at birth.

At enrolment, detailed demographic, medical and antenatal information, as well as examination findings were gathered by research nurses who interviewed the women and reviewed their antenatal records. Data was initially collected using an in-house data collection tool for n=379 women and subsequently, in August 2016, when a standardised tool was created by the WHO[36,37], this was modified and updated accordingly and put into use straight away. For the new variables that were added from the WHO protocol, pregnant women who had been enrolled and had their baseline questionnaire administered prior to August 2016 were asked to answer any new questions, where possible, during follow-up visits. Data was entered digitally into the Cohort's database (created using Salesforce™ Brasil online platform).

The follow-up of the women is described in Figure 1 and as follows: Women had blood, saliva and urine collected by research nurses for ZIKV RT-PCR at the time of recruitment and 2-3 weeks thereafter. Subsequently, sample collection was repeated on a 2-3 monthly basis during routine check-ups. Trained volunteers carried out pre-arranged weekly follow-up telephone calls and asked the women whether they had experienced any symptoms consistent with ZIKV infection in that time. If symptoms were reported, the women were advised to attend the hospital for clinical review and blood, saliva and urine were collected at this time as well. Antenatal ultrasound scanning was carried out in months 3, 5, 7 and 8 in women who remained ZIKV RT-PCR negative and who did not experience any symptoms in keeping with ZIKV infection during pregnancy and monthly in women who had a positive ZIKV RT-PCR or developed ZIKV-like symptoms during pregnancy according to the WHO definition of a symptomatic case.[38] All women in the cohort had antenatal scans carried out at the São Paulo Radiology Centre by sonographers specialising in fetal medicine and using Voluson 730 Expert/Voluson E6, GE equipment. At the time of delivery in hospital, the women had blood, saliva and urine collected again and the placenta and umbilical cord were stored for subsequent evaluation by a pathologist. All specimens were stored in a biorepository.

For the purposes of the current investigation, the reported data will pertain to the follow-up period including pregnancy and the immediate perinatal period only. However, a detailed description of the entire follow-up programme for the babies and children belonging to the Jundiaí Zika Cohort is included for reference in Appendix Figure 1.

Laboratory Procedures

All laboratory procedures were performed on de-identified samples. Due to severe financial constraints from a lack of funding at the start of the cohort, it was opted to prioritise the running of urine samples for ZIKV RT-PCR. Studies have shown that Zika virus RNA is unlikely to be detected in serum after the first week of illness whereas in the urine it can be detected for at least 2 weeks after onset of symptoms.[8,39,40] Serological tests for ZIKV were not carried on all women initially due to restricted availability and high cost of the ELISA test kits. Therefore, we report only the results of ZIKV RT-PCR in urine n this study.

Total RNA was extracted by commercial QIAamp Viral RNA Kit (Qiagen®), following the manufacturer's instructions and stored at -80°C until use. Zika Virus specific reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) were performed with GoTaq® 1-Step RT-qPCR System (Promega®) on ABI Prism 7500 SDS Real-Time cycler (Applied Biosystems). The Zika virus specific primers and probes designed by Lanciotti and colleagues[8], are complementary to the nonstructural 5 Protein (polymerase). The RT cycle consisted of a 10 minute cycle at 50°C and a 15 minute cycle at 95°C. The PCR consisted of forty cycles of 15 seconds at 95°C and a 1 minute cycle at 60°C. Three positive controls (RNA extracted from positive ZIKV samples) and two negative controls (H_2O) were included. We considered positive the samples that presented a threshold cycle (Ct) greater than 38.5 (as per Lanciotti and colleagues[41]). In cases where the results were inconclusive, repetitions were performed with serial dilutions (1:10 – 1:20 – 1:30 – 1:300).

Outcome and variable definitions and categorisation

Infants were considered to have been exposed to ZIKV during pregnancy if their mothers had at least one positive ZIKV RT-PCR sample during pregnancy and to have vertical ZIKV transmission if they had a positive ZIKV PCR sample within 10 days of birth.[42,43] Women were considered to be symptomatic for ZIKV if they had experienced any of the symptoms compatible with the WHO case definition for suspected ZIKV,[38] defined as a person presenting with rash and/or fever and at least one of the following signs or symptoms: arthralgia or arthritis or conjunctivitis (non-purulent/hyperaemic).

Maternal age was stratified into three groups: 19 years and under, 20 to 34, and 35 and over. Ethnicity in Brazil is self-declared and the standardised categories are as follows: white, black, mixed race, indigenous, and Asian. Due to small numbers, the Asian and indigenous categories were grouped together. The mother's level of education was categorised as follows: ≤8 years is equivalent to incomplete elementary school studies, 9-11 years is equivalent to incomplete high school studies, 12 years is high school completed and >12 years is higher education.

Anthropometric measures at birth (i.e., neonatal weight, length and head circumference) were obtained for all live-born infants, and the equipment used was consistent for all. Weight was assessed using digital scales, length using a recumbent baby length scale and head circumference using a standardised non-elastic tape measure. Z-scores for weight, length and head circumference were determined using the online Intergrowth calculator, which takes into account gestational age and sex.[44-46] Gestational age was estimated using first trimester ultrasound (USS) when available and by last menstrual period (LMP) when USS was unavailable. If neither USS nor LMP-estimated gestational ages were available, infant sex was missing, or

anthropometric measures were not recorded at birth (i.e., if the neonate was delivered outside of the clinic), the infant and mother were excluded from the analytical cohort. Preterm birth was defined as any baby born alive before 37 completed weeks of pregnancy.[47] Low birth weight was defined as a birthweight of less than 2500g. Small-for-gestational-age (SGA) was defined as infants with birthweight z scores of less than −1.28 at birth (equivalent to 10th percentile) and extreme SGA as a birthweight of less than -1.88 z-scores (equivalent to the 3rd percentile).[48] Microcephaly was defined as a head circumference z-score of less than -2.[49] Proportionate microcephaly was defined, as per the National Birth Defects Network definition[50], as a head circumference z-score of less than −2 with a proportionally low birthweight z-score of less than -2. Likewise, disproportionate microcephaly was defined as a head circumference z-score of less than −2 with a birthweight z-score of more than -2.[50] Fetal loss was defined as the death of a product of conception before the expulsion or complete extraction from the body of the pregnant woman at any gestation as per WHO/ICD-10 [51]. The risk factors which constitute a high-risk pregnancy in Brazil can be categorised using the following four broad sub-headings: 1. Personal and sociodemographic characteristics (such as age, drug use, low or high BMI), 2. Previous reproductive history (previous stillbirth, recurrent miscarriages), 3. Current obstetric illness (bleeding, pre-eclampsia), 4. Intercurrent disease during pregnancy (hypertension, epilepsy, infectious disease)[35] (see appendix for full list).

Statistical Analysis

Categorical variables were compared among ZIKV exposed and unexposed women using Chi-squared test except where there were less than 5 in any cell in which case Fisher's exact test was used. As infant ZIKV RT-PCR data was not complete for the full cohort, a subgroup analysis was carried out for the 409 dyads who had known

neonatal ZIKV RT-PCR status. Measures of association (Crude Risk Ratios) and their 95% confidence intervals were calculated directly by comparing the prevalence of negative fetal outcomes in the ZIKV exposed and unexposed groups. All statistical analyses were carried out using STATA™ version 15.1 software.

Ethics & Role of the Funding Source

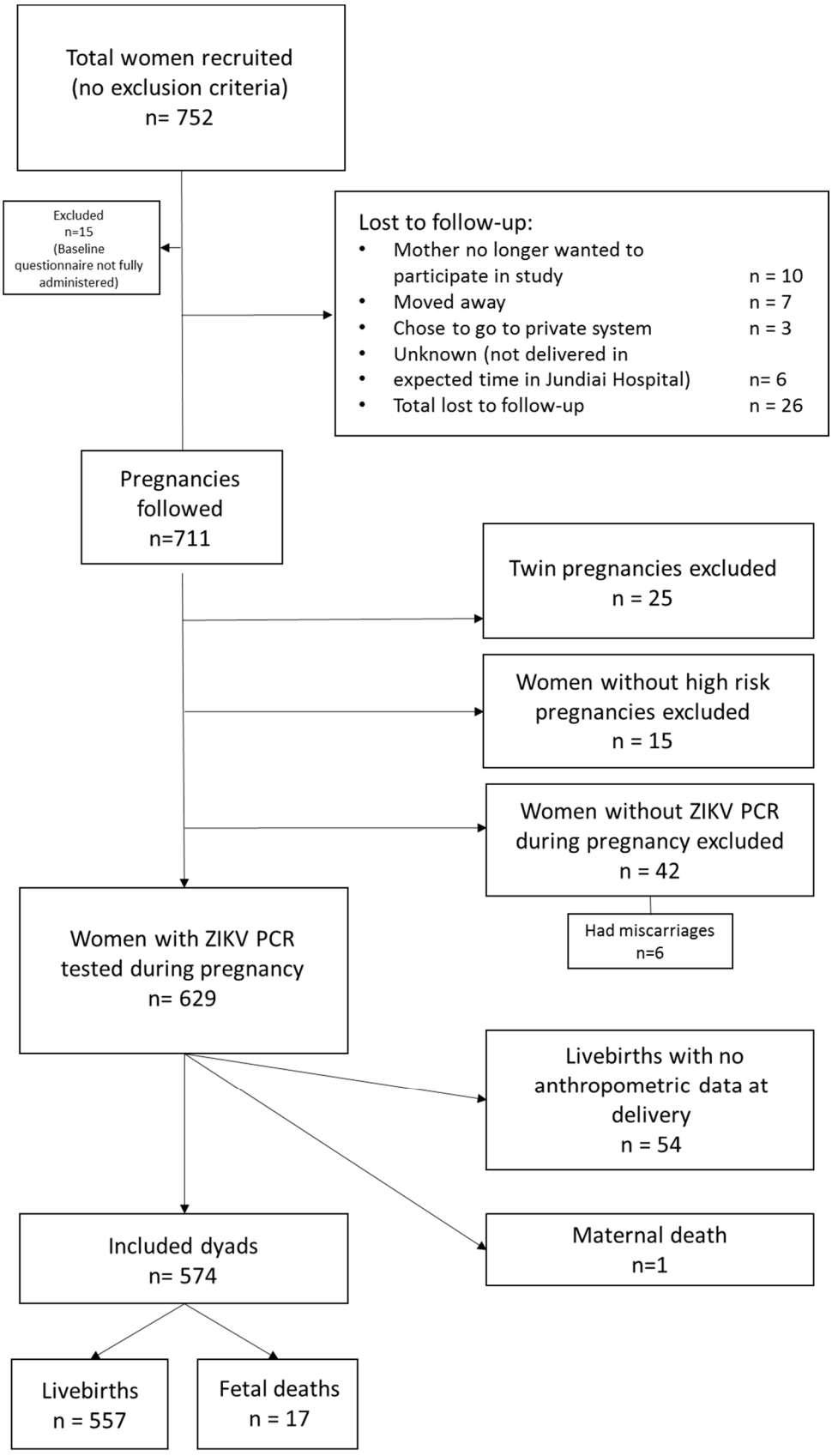
This study received ethical approval by the research ethics committee of Jundiaí Medical School, protocol number 1446577. Participating women provided written, informed consent for themselves and for future follow-up of their child.

RESULTS

Participant Characteristics

A total of 752 women were enrolled in the study between March 2016 and August 2017. Fifteen women who did not have the full baseline questionnaire administered were excluded and 26 women were lost to follow-up (see Figure 1). For the purposes of answering this particular study question, twin pregnancies and 15 women who were recruited because of suspected ZIKV infection but who did not have high-risk pregnancies were excluded from the analysis to ensure all women had similar baseline risk for negative fetal outcomes. Women who had not had a ZIKV RT-PCR sample taken during pregnancy (n=42), of which six had a miscarriage, were also excluded from the analytical cohort. This resulted in 629 eligible pregnancies. Of these, 54 of their infants did not have anthropometric information collected at birth and there was one maternal death. This resulted in a total of 574 dyads (557 livebirths and 17 fetal deaths) in the analytical cohort.

Figure 1. Flow diagram showing participants of the Jundiai Zika Cohort at each stage of the study, recruitment period: 01 March 2016 – 23 August 2017, Jundiai, São Paulo, Brazil



During the 126.4 person-years of pregnancy follow-up time (mean 11.5 weeks, maximum 34.4 weeks) 44 women (7.7%) had a positive ZIKV RT-PCR during pregnancy. The majority (61.4%, n=27) tested positive in the 3rd trimester (36.4%, n=16 in the 2nd trimester, 2.3%, n=1 in the 1st trimester).

The majority of women were aged 20-34 years and had completed high school but had not undertaken any higher education studies (Table 1). The ethnic make-up of the cohort was as follows; around half the mothers described themselves as being white, a third mixed race, 10% black and around 2% indigenous or Asian. The women were married or lived with their partner in around 80% of ZIKV-positive cases and 76% of ZIKV-negative cases although 60% reported that the pregnancy was unplanned. The cohort Caesarean section rate was around 50%

Table 1. Maternal characteristics of participants of the Jundiaí Zika Cohort, March 2016 to August 2017, Jundiaí, São Paulo, Brazil

Variable	ZIKV RT-PCR Pos Women (n=44)	ZIKV RT-PCR Neg Women (n=530)	P-value
Age			
13-19 years	10 (22.7%)	84 (15.9%)	0.47
20-34 years	26 (59.1%)	331 (62.5%)	
35-46 years	8 (18.2%)	115 (21.7%)	
Missing	0	0	
Education			
≤8 years	9 (20.9%)	90 (17.5%)	0.76
9-11 years	12 (27.3%)	118 (22.9%)	
12 years	16 (36.4%)	229 (44.5%)	
>12 years	6 (14.0%)	78 (15.2%)	
Missing	1 (2.3%)	15 (2.8%)	
Ethnicity/race			
White	23 (52.3%)	278 (53.8%)	0.97*
Mixed race	16 (36.4%)	177 (34.2%)	
Black	4 (9.1%)	52 (10.1%)	
Other (Asian/indigenous)	1 (2.3%)	10 (1.9%)	
Missing	0	13 (2.5%)	
Relationship with partner			
Married/co-habiting	35 (79.6%)	395 (76.0%)	0.60
Single/divorced/widowed	9 (20.5%)	125 (24.0%)	
Missing	0	10 (1.9%)	
Type of delivery			
Vaginal/forceps	23 (52.3%)	257 (50.7%)	0.86
C-section	21 (47.7%)	250 (49.3%)	
Missing	0	23 (4.3%)	
Trimester when recruited			
1st	2 (4.7%)	26 (5.0%)	0.97*
2nd	15 (34.9%)	186 (35.8%)	
3rd	26 (60.5%)	308 (59.2%)	
Missing	1 (2.3%)	10 (1.9%)	
Diabetes			
Yes	14 (32.6%)	165 (32.7%)	0.99
No	29 (67.4%)	340 (67.3%)	
Missing	1 (2.3%)	25 (4.7%)	
Hypertension			
Yes	8 (18.2%)	102 (20.2%)	0.75
No	36 (81.8%)	403 (79.8%)	
Missing	0	25 (4.7%)	

Note: Percentages for all categories were calculated with exclusion of those with missing data from the denominator *All p-values calculated using Chi2 test except for those labelled with asterisk which were calculated using Fisher's exact test. The 'missing' category was not included as a category when the p-value was estimated.

Fetal outcomes

In this cohort of women with high-risk pregnancies, there was no significant difference in gestational age at birth between the ZIKV exposed and unexposed infants (Table 2). Premature birth occurred in 9.1% of the ZIKV exposed group and 13.3% of the unexposed group. A slightly larger proportion of early term births (between 37-38 weeks) was found in but this difference was not significant.

Table 2. Gestational age at birth (in weeks) of infants (livebirths) born in the Jundiaí Zika Cohort of mothers exposed and unexposed to ZIKV, March 2016 to August 2017, Jundiaí, São Paulo, Brazil

Gestational age (completed weeks)	ZIKV exposed (n=44)	ZIKV unexposed (n=513)	Crude RR (95% CI)
<37 (Preterm)	4 (9.1%)	68 (13.3%)	0.7 (0.3-1.8)
37-38 (Early term)	21 (47.7%)	222 (43.4%)	1.1 (0.8-1.5)
≥ 39 (Term and post-term)	19 (43.2%)	222 (43.4%)	1.0 (0.7-1.4)
Missing	0	1 (0.2%)	

Note: Fetal deaths excluded

The outcome low birthweight (birth weight < 2500g) occurred in 9.1% of infants exposed to ZIKV during pregnancy and 11.1% in the unexposed (Table 3). Using the parameter SGA, 9.1% of infants exposed to ZIKV during pregnancy met the definition criteria (birth weight <10th percentil, or z score <−1.28) versus 9.7% of unexposed infants. One explanation for the fact that the proportion of low birthweight neonates among exposed mothers was lower than among unexposed mothers is due to the fact that there were less preterm neonates among exposed women compared to unexposed women.

Table 3. Birth weight related outcomes of infants born in the Jundiaí Zika Cohort from March 2016 to August 2017, Jundiaí, São Paulo, Brazil

	Birth weight	ZIKV exposed (n=44)	ZIKV unexposed (n=513)	Crude RR (95% CI)
Birthweight	<1500g (VLBW)	1 (2.3%)	10 (1.9%)	1.2 (0.2-8.9)
	1500-2499g (LBW)	3 (6.8%)	47 (9.2%)	0.7 (0.24-2.3)
	2500-4000g (Normal)	39 (88.6%)	435 (84.8%)	1.0 (0.9-1.2)
	>4000g (Large)	1 (2.3%)	21 (4.1%)	0.6 (0.1-4.0)
	Total LBW	**4 (9.1%)**	**57 (11.1%)**	**0.8 (0.3-2.1)**
SGA	Extreme SGA (z score <−1.88)	2 (4.5%)	15 (2.9%)	1.6 (0.4-6.6)
	SGA (-1.88 <z score <−1.28)	2 (4.5%)	36 (7.0%)	0.6 (0.2-2.6)
	Not SGA	40 (90.9%)	462 (90.3%)	1.0 (0.9-1.1)
	Total SGA	**4 (9.1%)**	**51 (9.9%)**	**0.9 (0.4-2.5)**

VLBW = very low birthweight, LBW = low birthweight, SGA=small for gestational age (birthweight<-1.28 z-scores). Note: Fetal deaths excluded

Twelve infants (2.2%) were born with microcephaly, defined as a head circumference z-score of less than -2, and two (0.9%) of the infants had severe microcephaly (z-score <-3). Microcephaly occurred in 4.5% of ZIKV exposed infants (maternal ZIKV PCR positive during pregnancy) compared to 1.9% of ZIKV unexposed infants (RR 2.3, 95% CI 0.5-10.3) (Table 4). Although our cohort and the sample of mothers used for this study was not specifically designed to compare different microcephaly sub-groups, we looked at disproportionate microcephaly (defined as microcephaly with a birthweight z-score > -2) as an additional outcome of interest. It occurred in two (4.5%) of the ZIKV exposed infants versus five (1%) of the unexposed infants (RR 4.7 95% CI 0.9-23.3). Of the 17 mothers whose pregnancies ended in miscarriage or stillbirth, all had negative ZIKV RT-PCR during pregnancy, and it was not possible to obtain fetal tissue to carry out ZIKV RT-PCR. Overall, adverse fetal outcomes occurred in ten (22.7%) ZIKV exposed infants and 129 (24.3%) ZIKV unexposed infants.

Table 4. Incidence and relative risk of negative outcomes among infants exposed (maternal ZIKV PCR positive) and unexposed to Zika Virus during pregnancy and infants with congenital ZIKV infection (neonatal ZIKV RT-PCR positive) in the Jundiai Zika Cohort March 2016 to August 2017, SP, Brazil.

	Mother ZIKV RT-PCR positive during pregnancy (n=44)	Mother ZIKV RT-PCR negative during pregnancy (n=513)	Crude RR (95% CI)
All negative outcomes	10 (22.7%)	129 (24.3%)	0.9 (0.5-1.6)
SGA	4 (9.1%)	51 (9.9%)	0.9 (0.3-2.5)
LBW	4 (9.1%)	57 (11.1%)	0.8 (0.3-2.1)
Microcephaly	2 (4.5%)	10 (1.9%)	2.3 (0.5-10.3)
Disproportionate	2 (4.5%)	5 (1.0%)	4.7 (0.9-23.3)
Proportionate	0	5 (0.8%)	-
Preterm	4 (9.1%)	68 (13.3%)	0.7 (0.3-1.8)
Fetal death	0	17 (3.3%)	-
	Infant ZIKV RT-PCR positive at birth n=19	Infant ZIKV RT-PCR negative at birth n=390	Crude RR (95% CI)
All negative outcomes	4 (21.1%)	86 (22.1%)	1.0 (0.4-2.3)
SGA	2 (10.5%)	42 (10.8%)	1.0(0.3-3.7)
LBW	2 (10.5%)	38 (9.7%)	1.1 (0.3-4.1)
Microcephaly	2 (10.5%)	8 (2.1%)	5.1 (1.2-22.5)
Disproportionate	2 (10.5%)	4 (1.0%)	10.3 (2.0-52.6)
Proportionate	0	4	-
Preterm	1 (5.3%)	48 (12.3%)	0.43 (0.1-2.9)

Note: Categories are not mutually exclusive. Microcephaly was defined as infants with head circumference z scores of less than - 2 at birth. Severe microcephaly was defined as head circumference z score of less than - 3 at birth. Proportionate microcephaly was defined as infants with both head circumference and birthweight z scores of less than -2 at birth and disproportionate microcephaly as head circumference z score of less than -2 with birthweight z-score of more than -2. SGA=small for gestational age (birthweight<10th percentile for sex and gestational age or -1.28 z-scores). LBW=low birthweight (birthweight<2500g)

A subgroup analysis for the 409 dyads who had known neonatal ZIKV RT-PCR status was carried out. Of these infants, 19 (4.6%) had a positive ZIKV RT-PCR in the first 10 days of life. This subgroup analysis showed that the risk of microcephaly among

infants with positive ZIKV RT-PCR at birth was five times the risk compared to infants with negative ZIKV RT-PCR (RR 5.1, 95% CI 1.2-22.5). Additionally, the risk of disproportionate microcephaly among infants with positive ZIKV RT-PCR at birth was ten times the risk compared to infants with negative ZIKV RT-PCR (RR 10.3, 95% CI 2.0-52.6). There was no statistical difference between the outcomes preterm, low birth weight and SGA according to infant ZIKV RT-PCR status at birth in this high-risk pregnancy cohort. Overall, negative outcomes occurred in 4 (21.1%) infants with positive ZIKV RT-PCR after birth compared to 86 (22.1%) infants with negative ZIKV RT-PCR after birth.

DISCUSSION

In this analysis of 574 pregnant women in the State of Sao Paulo, the incidence of maternal ZIKV infection was 7.7%. Among 409 of their neonates tested, the incidence of congenital ZIKV infection was 4.6%. The results of this cohort study show that neonates exposed to ZIKV congenitally (as evidenced by positive ZIKV RT-PCR in the neonate after birth) have five times the risk of developing microcephaly (RR 5.1, 95% CI 1.2-22.5) and 10 times the risk of developing disproportionate microcephaly (RR 10.3, 95% CI 2.0-52.6) compared to infants unexposed to ZIKV. There is no significant difference between the outcomes prematurity, low birthweight and SGA in infants with and without antenatal ZIKV exposure in this population of women with high-risk pregnancies.

The prospective nature of this investigation enabled us to quantify the risk of microcephaly in infants exposed to ZIKV during pregnancy and to identify that disproportionate microcephaly is more strongly associated with ZIKV exposure compared to microcephaly alone. Although the authors admit that due the urgent nature of the investigation and the lack of published data on microcephaly in the context of congenital ZIKV infection at the time, the power calculation was not specifically designed to compare microcephaly sub-groups.

Our data support the findings of both the Rio de Janeiro cohort and Recife case-control study[22,24] which demonstrate an association between prenatal ZIKV exposure and microcephaly at birth. This is in contrast to recent data published by the São José do Rio Preto pregnancy cohort in the State of São Paulo where 54 of the 216 symptomatic pregnant women (25%) were RT-PCR positive during pregnancy and none of the exposed neonates were born with microcephaly.[52]

As for prematurity, similar to our findings, the Rio de Janeiro cohort[24] also did not find an association with ZIKV exposure and in the Recife case control study[22], cases and controls were matched for gestational age so this was difficult to assess.

In terms of birthweight, in the Rio de Janeiro cohort, there was no difference between median birthweight in the ZIKV exposed and unexposed groups and although SGA seemed to occur more frequently in the exposed group this wasn't statistically significant. In the case control study in Recife, 83% of cases with microcephaly were also SGA compared to 5% of controls without microcephaly, however direct comparison of results with this study is difficult as outcomes were not presented stratified by ZIKV exposure status.

In our study, fetal deaths only occurred in the ZIKV unexposed group and to our knowledge, an association between fetal death and ZIKV exposure during pregnancy remains to be clearly demonstrated in the literature.

As Hoen and colleagues point out in their study looking at pregnancy outcomes after ZIKV infection in French territories in the Americas[29], the reported prevalence of congenital neurologic defects related to Zika virus (ZIKV) infection has ranged from 6 to 42% in various reports. For this investigation, we chose to focus on objective, well-defined adverse fetal outcome measures rather than congenital neurological abnormalities based on imaging or neurological examination findings which may be prone to more inter-study variability.

Congenital ZIKV research will always be challenging due to rare outcomes and small study populations. In our case, this meant that we were only able to calculate crude relative risks and did not have a sufficient number of ZIKV-positive cases in order to adjust our results for possible confounders such as maternal co-morbidity, trimester of

ZIKV RT-PCR positivity etc. Additionally, an inherent difficulty with pregnancy cohorts is recruitment and adhesion in often hard-to-reach populations of women who may not access antenatal care. In this study, this challenge was circumvented by recruiting women from a high-risk antenatal clinic but this brought with it the challenge of having a study population already at higher risk for negative fetal outcomes. Despite the exclusion of twins from the analysis (as they are known to have a particularly higher risk of negative birth outcomes) this likely introduced some bias and reduced the ability to detect a difference between negative outcomes in the ZIKV exposed and unexposed groups. Data published recently looking at preterm birth, LBW and fetal death in the context of high-risk pregnancies in the State of São Paulo[53] showed that the risk of negative fetal outcomes are doubled in women who have had a hospital admission during pregnancy. A further study looking at microcephaly in the pre-ZIKV era also found that maternal smoking was associated with microcephaly.[54]

Differing study population characteristics will also pose a challenge when comparing results from different epidemiological studies. The profile of the Jundiaí cohort not only differs from a comorbidity perspective to other other Zika cohorts but also likely from a sociodemographic and ethnic profile point of view. This study was carried out in a region with relatively high socioeconomic status where the majority of study participants were white, had completed high school and had a support network. Most of the other studies carried out in Brazil to date have been in poorer communities judging by the characteristics of the study populations which reflect a lower sociodemographic status. Data published looking at the spatial distribution of cases of microcephaly in Recife have shown that residing in areas with precarious living conditions is associated with a higher prevalence of microcephaly.[55]

Until cheaper, more widely available diagnostic tools are available, ZIKV studies will be hampered by a gross underestimation of cases. As RT-PCR has a narrow window for the detection of ZIKV RNA,[42] undoubtedly there are many women who are exposed during pregnancy but aren't detected. In this study, most women were recruited in the 2nd or 3rd trimester of pregnancy due to time lags including time to diagnosis of high-risk pregnancy and appointment waiting time. Therefore, the majority of women who were infected with ZIKV in the first trimester of pregnancy would have been missed by RT-PCR done at recruitment. Neonatal ZIKV RT-PCR testing at birth gets around some of these issues, and in this study was more predictive of negative fetal outcomes, however only 72% of the neonates were tested and this was mainly because the mother declined. Similarly, due to difficulties with consent, in the cases of miscarriage and stillbirth, it was not possible to obtain fetal tissue to carry out RT-PCR analysis. Due to severe financial constraints from a lack of funding at the start of the cohort, it was opted to prioritise the running of urine samples for ZIKV RT-PCR. Studies have shown that Zika virus RNA is unlikely to be detected in serum after the first week of illness whereas in the urine it can be detected for at least two weeks after onset of symptoms.[8,39,40] However, since ZIKV RT-PCR was only tested in one sample type, this may also have led to an underestimation of cases. Ultimately, it is likely our results have been biased by an underestimation of the number of women truly ZIKV RT-PCR exposed among our cohort. This erroneous placement of women in the unexposed group will have likely attenuated our results and it is possible that true measures of association are higher than those estimated.

An additional limitation to this analysis is the current unavailability of toxoplasmosis, rubella, cytomegalovirus, Herpes virus, syphilis, and parvovirus B19 (TORCH)

antibody screen results in all the mothers in the cohort to confirm that microcephaly was not due to other causes. However, a similar study carried out over the same time period by Krow-Lucal and colleagues in Paraíba found a substantial attributable risk of microcephaly due to ZIKV (35–87% of microcephaly occurring during the time of the investigation was attributable to ZIKV).[56] Also, in the sub-analysis of neonates that had ZIKV RT-PCR testing after birth, we can be more certain that in those neonates with positive ZIKV RT-PCR, that microcephaly was due to ZIKV.

In summary, our results show that, in women who already have risk factors during pregnancy, exposure to ZIKV has no effect on negative fetal outcomes namely prematurity, low birth weight and SGA. However, in line with other evidence published to date, microcephaly, and more specifically disproportionate microcephaly, is associated with ZIKV exposure during pregnancy even in the presence of other risk factors. Disproportion between head circumference and weight should be considered a useful screening tool for congenital microcephaly associated with ZIKV infection.

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Study conducted in Jundiaí, São Paulo, Brazil at Jundiaí Medical School, Paediatrics Department

SUPPORTING INFORMATION

S1 Checklist: STROBE Checklist

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract (Lines 1-3)
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found (Lines 42-51)
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported (Lines 89-101)
Objectives	3	State specific objectives, including any prespecified hypotheses (Lines 102-105)
Methods		
Study design	4	Present key elements of study design early in the paper (Line 108)
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection (Lines 109-118)
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up (Lines 115-155)
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable (Lines 157-199)
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group (Lines 157-167 (lab) and 181-186 (anthropometrics))

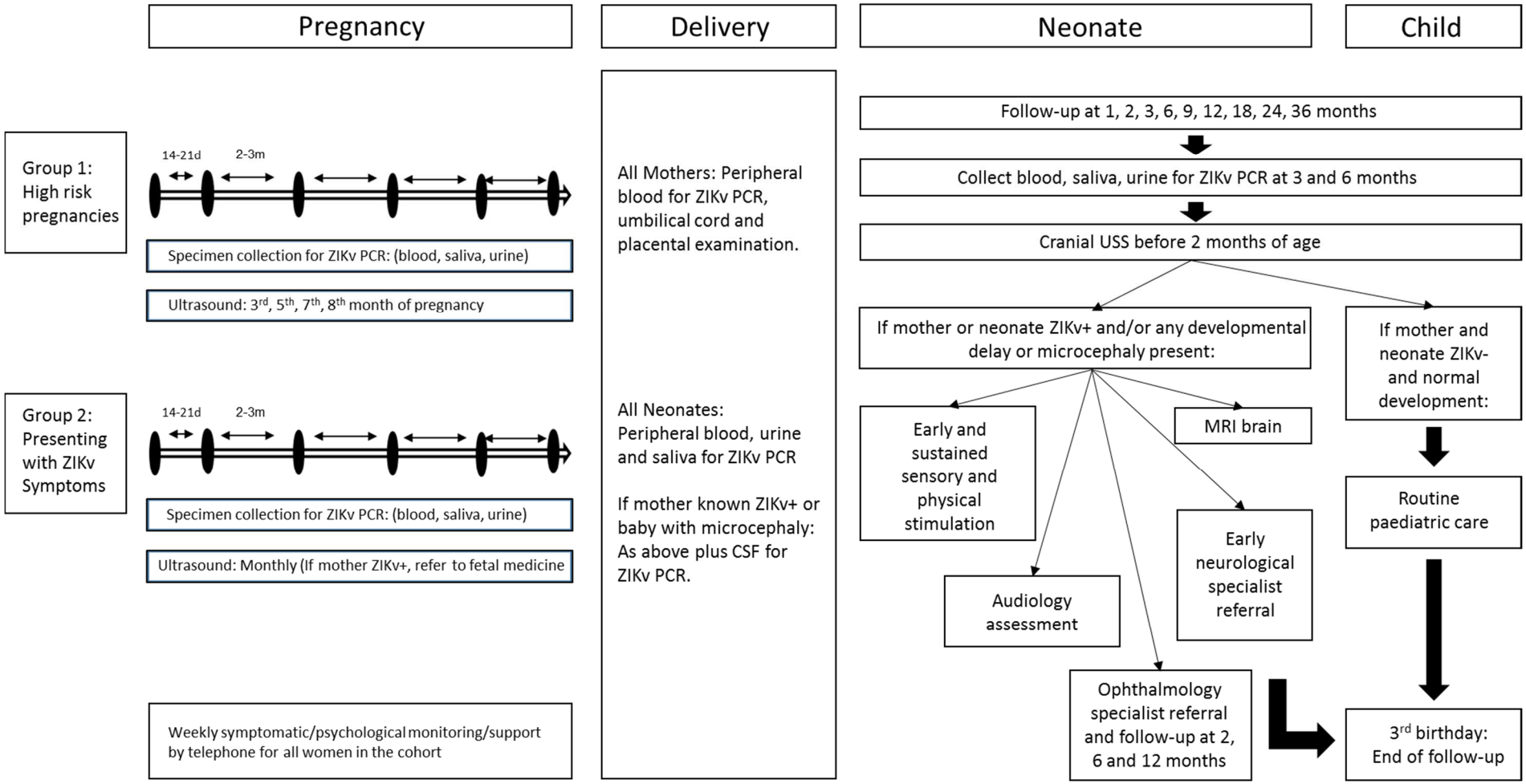
Bias	9	Describe any efforts to address potential sources of bias (Lines 126-129)
Study size	10	Explain how the study size was arrived at (Lines 201-203)
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why (Lines 169-180)
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding (Lines 201-210)
		(*b*) Describe any methods used to examine subgroups and interactions (Lines 201-210)
		(*c*) Explain how missing data were addressed (See results tables)
		(*d*) If applicable, explain how loss to follow-up was addressed (Figure 2)
		(*e*) Describe any sensitivity analyses (N/A)
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (Lines 217-225 and figure 2)
		(b) Give reasons for non-participation at each stage (Figure 2)
		(c) Consider use of a flow diagram (Figure 2)
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (Table 1)
		(b) Indicate number of participants with missing data for each variable of interest (Tables 1-5)
		(c) Summarise follow-up time (eg, average and total amount) (Lines 228-229)
Outcome data	15*	Report numbers of outcome events or summary measures over time (Lines 229-232)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which

		confounders were adjusted for and why they were included (Tables 2-5)
		(*b*) Report category boundaries when continuous variables were categorized (Tables 1-5)
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses (Table 5)
Discussion		
Key results	18	Summarise key results with reference to study objectives (Lines 318-328)
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias (Lines 353-396)
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence (Lines 397-402)
Generalisability	21	Discuss the generalisability (external validity) of the study results (Lines 323, 397)
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based (In the submission form)

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

S2 Figure: Full follow-up protocol for Jundiai Zika Cohort Study (01/03/2016 – 23/08/2017), Jundiaí, São Paulo, Brazil



S3 Table 1. ZIKV RT-PCR results for mothers infant pairs in the Jundiai Zika Cohort

	Mother positive	Mother negative	Total
Baby positive	1	18	19
Baby negative	32	358	390
Baby untested	11	154	165
Total	44	530	574

S4 Table 2. Anthropometry of neonates born with microcephaly in the Jundiai Zika Cohort

Patient ID	Head circumference in cm (z-score)	Length in cm (z-score)	Weight in g (z-score)
80	31 (-2.47)	49.5 (0.07)	2600 (-1.67)
190	30 (-2.71)	44 (0.76)	2660 (-1.03)
197	30 (-2.46)	44 (-2.18)	2215 (-2.18)
199	29.5 (-2.77)	44 (-2.12)	3075 (0.44)
224	31.5 (-2.83)	47 (-2.17)	2595 (-2.35)
314	28.9 (-3.19)	41 (-3.3)	1690 (-2.87)
338	31.5 (-2.63)	47 (-3.07)	2750 (-1.07)
438	28 (-2.46)	40 (-2.52)	1420 (-2.19)
559	28 (-2.32)	42 (-1.4)	1995 (-0.37)
587	32 (-2.12)	47 (-1.87)	2790 (-1.67)
659	26.3 (-3.28)	37.5 (-2.92)	1300 (-1.89)
701	30.5 (-2.78)	45 (-2.38)	2125 (-2.66)

FINAL CONSIDERATIONS

The Jundiai Zika Cohort was set up in March 2016 in the state of Sao Paulo, Brazil in response to, and with the aim to investigate the epidemic of congenital neurological features, including microcephaly, presumed to be associated with prenatal ZIKV exposure. To date, only three epidemiological studies from three distinct study sites have been published looking at this association; two cohort studies[55,58] and one case-control.[109] The Jundiai Zika Cohort is uniquely placed to contribute to that small body of research in that it is truly prospective and has recruited both symptomatic and asymptomatic pregnant women. An additional facet to the study is that women in the cohort have coexisting co-morbidities during pregnancy providing a unique opportunity to explore what effects, if any, these have on maternal and fetal outcomes when occurring concurrently with ZIKV infection. Finally, in the context of the Brazilian Zika epidemic, it is also unique as its setting is in a region with relatively high socioeconomic conditions compared to other Zika research being carried out in Brazil.

Cohort studies present a number of challenges in their execution due to difficulties in retention of study participants and loss to follow-up. However, due to their prospective nature, they also bring many advantages such as the possibility of studying other relatively rarer outcomes.

A review of the current literature revealed that there was striking inter-study variability in the reported asymptomatic ZIKV infection rates, particularly among pregnant women. This was in part due the different definitions employed to define symptomatic cases. However, even when considering the standardised WHO definition, it became apparent that this was developed as an interim definition in 2016 and that this hadn't been validated or its utility evaluated in the context of pregnant women in active ZIKV

transmission areas. In the manuscript “Clinical Presentation of Zika Virus Infection among Pregnant Women – A Prospective Cohort Study in São Paulo, Brazil,” I was able to show that 70.8% of ZIKV RT-PCR positive pregnant women did not have any symptoms compatible with ZIKV disease and that only 8.3% of women reported an illness that fulfilled all the criteria to be classified as a suspected symptomatic case according to WHO. Using the Jundiai Zika Cohort study population, the sensitivity of the current WHO case definition for a suspected symptomatic case was calculated to be 28.6% and the specificity as 82.2% (acknowledging the fact that this is a crude estimate due to the imperfect ‘gold standard’ ZIKV RT-PCR test which, if negative during pregnancy, cannot be fully relied upon to confirm that indeed ZIKV infection did not occur during pregnancy). Using data collected from both Zika-positive and Zika-negative women in the cohort with regards to symptoms during pregnancy, I was able to propose an alternative case definition that, in this study population, would have almost doubled the case detection rates from 28.6% to 50%.

Only three epidemiological studies have reported on the risk of congenital neurological sequelae following prenatal maternal ZIKV exposure, two of which are prospective. Microcephaly is just one of a constellation of features known to constitute the Congenital Zika Syndrome (CZS), however, it is arguably the most objectively measurable. The other features of CZS (thin cerebral cortices with subcortical calcifications,[93] macular scarring and focal pigmentary retinal mottling[95] and congenital contractures[96] and marked early hypertonia and symptoms of extrapyramidal involvement may be quite obvious when severe but may be more complex to define when mild. The risk of other negative fetal outcomes (prematurity, SGA, low birthweight and fetal death) as a result of ZIKV infection during pregnancy had not been explicitly quantified in the aforementioned studies.

I was able to show that, in the context of high-risk pregnancies, ZIKV infection during pregnancy did not increase the risk of prematurity, SGA, low birthweight or fetal death. I was also able to show that in vertically infected newborns (i.e., those that were found to have positive ZIKV RT-PCR at birth), the risk of microcephaly was five-fold greater when compared to uninfected newborns. Furthermore, I demonstrated that the risk of disproportionate microcephaly (i.e., microcephaly with a normal birthweight) ZIKV-positive newborns was 10 times the risk compared to ZIKV-negative newborns. This latter finding was unprecedented as although other studies had alluded to its importance in defining the Congenital Zika Syndrome, this had not been quantified in prospective studies until now.

Both the proposal for a more sensitive clinical case definition for ZIKV infection during pregnancy and the marker of disproportionate microcephaly facilitate earlier detection rates and promote timely referral of the women and families most at risk to receive specialised care and financial support (in the case of Brazil). In the first instance, a more sensitive case definition will alert health professionals to test pregnant women in a timely manner and therefore maximise the chances of viral RNA detection via RT-PCR; and in the second instance, an anthropometric marker that can be used in antenatal ultrasonography to alert health professionals to a fetus at risk.

Lastly, a noteworthy observation is the difference in ZIKV RT-PCR positivity between the women (7.7%) and neonates (4.6%) in the cohort. This has been found in other studies[109,115,145] and has been hypothesised to be related to the timing of infection in pregnancy. Infections later in pregnancy that cross into the fetal circulation have less time to be cleared by the fetus compared to infections earlier in pregnancy. However, studies have also reported that even in infants with phenotypic features of congenital

Zika Syndrome and prenatal evidence of congenital ZIKV infection, it is not uncommon for them to have negative results on cord samples by both PCR and IgM.[115,146]

In addition to these findings, this investigation has provided a unique opportunity to explore highly valuable data in the field of ZIKV research and has been exceptional learning exercise. As a result we make the following public health recommendations:

1. The current standardised case definitions for symptomatic ZIKV case detection should be updated and modified for pregnant women living in areas with active ZIKV transmission.
2. Disproportionate microcephaly should be explored further as a useful screening tool and marker for Congenital Zika Syndrome.
3. The scientific community, biotechnology companies and funders should continue to strive to develop affordable and implementable diagnostic tests with a high positive predictive value for pregnant women in active ZIKV transmission areas.
4. Following dissemination of individual cohort results, ZIKV researchers should continue to work together in consortia to create meta-analyses of available data in order to maximise the potential of this extremely valuable data.

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Eu,_____*(nome), brasileira, com anos,*

_____*(profissão),*_____*(RG)*, estou sendo convidada a participar de um estudo sobre *Infecção Vertical pelo vírus ZIKA e as repercussões na área materno-infantil* " cujos objetivos e justificativas são: saber a qual a freqüência de infecções pelo vírus Zika (ZIKV) na gestantes do Ambulatório de alto risco da FMJ e das gestantes com sintomas semelhantes a ZIKA da microrregião de Jundiaí. Queremos também conhecer qual o risco de ocorrer alterações nos bebês, cujas mães tenham tido infecção pelo ZIKV, inclusive se casos de abortamento e natimorto estão relacionados com a infecção na gestante. Desejamos verificar se o aleitamento materno pode transmitir a infecção e causar doença nos bebês. Também queremos saber se os vírus estão presentes na saliva, sangue e urina das gestantes e crianças infectadas e por quanto tempo.

O (os) procedimento(s) de coleta de dados serão feitos com a aplicação de questionários próprios, a fim de se fornecer informações pertinentes. Para detectarmos o vírus ZIKA serão realizados exames de amostra de sangue, urina e saliva com o objetivo de diagnosticar, monitorar ou acompanhar a infecção pelo vírus ZIKA, nas participantes. Para detectar malformações no feto serão realizados exames de ultrassom. Se você concordar em participar, serão coletadas amostras, no momento que ingressar no estudo, e a cada 2-3meses até o momento do parto. Desta forma saberemos qual foi o período que você contraiu o vírus. Logo após o parto você fará a sua ultima coleta do projeto que será urina, saliva, sangue, quando possível do líquido amniótico e colostro. Neste dia será a primeira coleta do bebe onde serão coletados sangue do cordão umbilical, urina e saliva. Se o seu bebê apresentar qualquer tipo de doença do sistema nervoso central, mesmo uma simples suspeita, o médico poderá indicar a coleta de dois ml de liquido da espinha chamado de liquor ou liquido cefalorraquidiano - LCR. Nestes materiais serão realizadas a pesquisa do vírus e outros exames que o médico achar importante para o seu bebe. Ele dará todos os esclarecimentos que você queira.

O bebe terá acompanhamento regular pediátrico no Ambulatório de Pediatria da FMJ , todo mês até sexto mês; depois a cada três meses até completar um ano de idade e a cada seis meses até 3 anos. Para sabermos quando desaparecem os seus anticorpos ou quando o bebe adquire a

infecção, será coletada os mesmo tipos de amostras do bebe ao nascimento e no 2º, 4º e 6º mês de vida ou se apresentar em qualquer idade durante a pesquisa febre, exantema ou conjuntivite.

Fui avisada que caso ocorra abortamento ou feto natimorto serão realizados exames de sangue, saliva e urina na gestante e coleta do material do aborto ou do natimorto para tentar esclarecer as causas.

Fui advertida que todo o material utilizado na coleta da pesquisa é esterilizado e descartável e toda a coleta será feita dentro dos padrões e normas técnicas. Os exames são colhidos pelos métodos habituais, considerados seguros, por profissionais dentro das normas de segurança. Não será coletado liquor se o bebe não estiver bem ao nascimento, mas o médico assistente dará toda explicação do motivo. Existe um desconforto mínimo por conta da coleta de sangue e líquor, pois se trata de um procedimento invasivo, e algumas podem pessoas podem ficar com dor no local, mas é passageiro. No entanto, esta será supervisionada e realizada por profissionais treinados e capacitados para este fim, sendo que se justifica pelo benefício para o paciente e para a comunidade.

Fui informada de que não serão fornecidos medicamentos, mas ao participar dessa pesquisa, posso esperar alguns benefícios para mim e para a comunidade, tais como: que os profissionais possam entender melhor esta nova infecção e assim contribuir para o desenvolvimento de medidas de prevenção e de controle, e futuramente, outros profissionais possam utilizar estas informações para o desenvolvimento de vacinas e medicamentos. O risco desta pesquisa é mínimo.

Recebi os esclarecimentos necessários sobre os possíveis desconfortos e riscos decorrentes do estudo, levando-se em conta que é uma pesquisa, e os resultados positivos ou negativos somente serão obtidos após a sua realização. Os exames são realizados pelos métodos habituais, considerados seguros.

Estou ciente de que minha privacidade será respeitada, ou seja, meu nome ou qualquer outro dado ou elemento que possa, de qualquer forma, me identificar, será mantido em sigilo.

Estou informada que a coleta e os exames não põem em risco a minha vida nem a das outras crianças e que receberei respostas ou esclarecimentos a quaisquer dúvidas, antes e durante o curso da pesquisa, sobre tudo o que for feito, riscos, benefícios e outros assuntos relacionados com a pesquisa.

Estou ciente de que de que as amostras biológicas coletadas, depois que utilizadas para os devidos fins da pesquisa, serão armazenadas em freezer -80°C em um banco de amostras. Caso sejam necessárias, as amostras poderão ser usadas para novos exames para esclarecer e termos uma melhor compreensão das doenças que transmitem de mãe para o bebe, quando necessários.

Serão respeitados nossos valores culturais, religiosos e morais; e deverei retornar para as avaliações nas datas agendadas.

Entendi que caso ocorra algum dano, ainda que improvável, resultando direta ou indiretamente a minha participação ou a do meu filho (a), se este dano for declarado, imediatamente haverá tratamento médico disponível, garantido o acesso ao HUJ.

Entendi que toda a minha participação gerará custo somente ao hospital e não será cobrado qualquer tipo de valores, pois a pesquisa é inteiramente gratuita.

Também fui informado de que posso me recusar a participar do estudo, ou retirar meu consentimento a qualquer momento, sem precisar justificar, e de, por desejar sair da pesquisa, não sofrerei qualquer prejuízo à assistência que venho recebendo. Além disso, minhas amostras biológicas e as amostras do recém nascido, até então colhidas, serão destruídas a partir deste momento.

O pesquisador responsável pelo projeto é o Prof. Dr. Saulo Duarte Passos, Professor Titular do Depto de Pediatria da Faculdade de Medicina de Jundiaí. Ele esta Inscrito no Conselho Regional de Medicina: 41828 fone 11984564720 e com ele poderei manter contato pelos telefones 011 984564720.

Em caso de dúvidas poderei ser esclarecida pelo pesquisador responsável: Prof. Dr. Saulo Duarte Passos pelo telefone cima citado ou pelo Comitê de Ética em Pesquisa Faculdade de Medicina de Jundiaí, Rua Francisco Telles, 250, Villa Arens, Jundiaí- SP, telefone: 11 4587-1095 e pelo e-mail: cep@fmj.br

O CEP de a FMJ objetiva defender os interesses dos participantes, respeitando seus direitos e contribuindo para o desenvolvimento da pesquisa desde que atenda às condutas éticas.

É assegurada a assistência durante toda pesquisa, bem como me é garantido o livre acesso a todas as informações, inclusive de resultados obtidos e esclarecimentos adicionais sobre o estudo e suas conseqüências, enfim, tudo o que eu queira saber antes, durante e depois da minha participação.

Enfim, tendo sido orientado quanto ao teor de todo o aqui mencionado e compreendido a natureza e o objetivo do já referido estudo, manifesto meu livre consentimento em participar, estando totalmente ciente de que não há nenhum valor econômico, a receber ou a pagar, por minha participação.

Recebi uma cópia deste termo de assentimento e li e concordo em participar da pesquisa.

_____

Jundiaí,_____de_____de _____