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Zika in Infants and Pregnancy (ZIP) study: results from a prospective international cohort study of prenatal Zika virus infection and adverse fetal and infant outcomes

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Abstract

Background Before Zika virus (ZIKV) infections were observed in the Americas, an association between ZIKV and microcephaly or other congenital malformations was not well documented. Initial reports suggested strong associations between ZIKV and congenital malformations, but plausible estimates of causal effects from prospective studies with adequate sample size and covariate data were few.

Methods From 2016–2018, the Zika in Infants and Pregnancy (ZIP) study enrolled pregnant people before 18 weeks gestation or with confirmed symptomatic ZIKV in a prospective cohort across 10 sites in South and Central America, and in Puerto Rico. Pregnancies were followed monthly through delivery and 6 weeks postpartum. Infants were followed quarterly to age 12 months. Prespecified co-primary analyses evaluated the associations between a composite endpoint of adverse fetal, neonatal, and infant outcomes with intrauterine ZIKV exposure overall and with symptomatic intrauterine ZIKV exposure. Secondary analyses separately evaluated the association of intrauterine ZIKV exposure with individual components of the primary endpoint.

Results Six thousand one hundred pregnant participants were included in the primary analysis, including 61 with ZIKV infection during pregnancy confirmed by a ZIKV-specific RNA test. For the primary analyses, the relative risk (RR) for the composite endpoint associated with any ZIKV exposure was 1.64 (95% CI: 0.65, 4.13) and with symptomatic ZIKV exposure 1.08 (95% CI: 0.15, 7.64). Sensitivity analyses provided similar results. Secondary analyses showed significant adjusted RRs [95% CI] for stillbirth (4.28 [1.39, 13.21]), infant death within six weeks (6.20 [1.08, 35.60], and fetal loss before 20 weeks (3.72 [1.82, 7.59]).

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Conclusions The ZIP study identified an elevated but not statistically significant risk of the primary composite outcome with intrauterine ZIKV exposure, and a significantly increased risk of some adverse fetal and infant outcomes with intrauterine ZIKV exposure in secondary analyses. Fewer than expected infections observed during pregnancy, coincident with a waning epidemic, limited study power to evaluate risk. Combining data from multiple cohorts for future meta-analysis may better define the risks of intrauterine ZIKV exposure.

Trial Registration NCT02856984. Registered August 5, 2016. Retrospectively registered.

Keywords Zika virus, Pregnancy, Latin America, Microcephaly, Fetal, Neonatal outcomes

Background

The emergence of Zika virus (ZIKV) in the Americas in 2015 was associated with an increased incidence of congenital microcephaly [1, 2]. Prior to this outbreak, the largest known ZIKV outbreak occurred in French Polynesia in 2013–2014 where researchers observed an association between Guillain-Barré Syndrome and ZIKV infection [3]. The emergence of the 2015 outbreak in the Americas prompted researchers to conduct a retrospective study of the French Polynesian outbreak. They detected an unusual increase in congenital cerebral malformations and dysfunction in fetuses and newborns, identifying 19 cases, including eight with major brain lesions and severe microcephaly [4].

Initially, clinical and case series studies from Northeast Brazil reported the first cases of microcephaly among babies born to pregnant participants with possible ZIKV infection during pregnancy. Subsequent early epidemiological studies provided further evidence of the association between prenatal ZIKV exposure and microcephaly [2, 5–7]. Additional adverse impacts on brain development were detected among children born to individuals with prenatal ZIKV infection, but the extent and frequency of the abnormalities were unknown [6, 8]. Amidst a rapidly expanding epidemic, accelerated development of accurate ZIKV detection assays, and increasing numbers of microcephaly cases, several key questions emerged:

- What adverse fetal and neonatal outcomes were associated with prenatal ZIKV exposure?
- Did the frequency of the outcomes differ by geographical region?
- Did symptomatic Zika infection increase the risk of adverse outcomes?

To address these questions, the United States National Institutes of Health (NIH) launched a multi-national prospective cohort study of pregnant people and their babies, called the Zika in Infants and Pregnancy (ZIP) Study. The primary objective of the ZIP study was to assess the strength of the association between ZIKV infection during pregnancy and adverse fetal and infant outcomes.

Methods

Study setting and population

The ZIP study recruited pregnant people from ZIKV-endemic regions in Brazil (four sites), Colombia, Guatemala, Nicaragua, Puerto Rico (two sites), and Peru between the years 2016–2018. Females were recruited from a variety of clinical environments in both urban and rural settings, including referral and research hospitals, public health facilities, and private maternity clinics, from diverse ethnic and sociodemographic backgrounds. At the beginning of the study in June 2016, initial research suggested that infection during the first trimester would incur the greatest risk. Therefore, pregnant individuals were recruited up to 13 weeks 6 days gestational age. In January 2017, as additional information emerged to suggest that the risk of adverse outcomes may be associated with infection at any time during pregnancy, the protocol was revised to allow enrollment of pregnant individuals up to and including 17 weeks 6 days gestational age. Additionally, to increase the number of ZIKV-infected individuals in the study, any pregnant participant, regardless of gestational age, with acute Zika-like symptoms (rash, fever, arthralgias, pruritus, lymphadenopathy, eye pain, conjunctivitis) confirmed by serology and/or a positive reverse transcriptase polymerase chain reaction (RT-PCR) test was eligible. Potential participants were given information about the study, screened for eligibility, and invited to provide written informed consent if eligible. The study planned to enroll up to 10,000 pregnant participants, a target sample size based on the assumption of 15–25% of enrolled pregnant individuals with laboratory confirmed ZIKV infection during pregnancy. Enrolled pregnant participants were followed longitudinally throughout pregnancy, delivery, and six weeks postpartum. Infants were eligible for enrollment if they were born alive to participants enrolled in the ZIP study and had parental consent. Enrolled infant participants were followed through 12 months of age.

Data and specimen collection and analysis

Questionnaires were administered at the study entry visit, and clinical assessments were performed on pregnant participants. Data collected at entry included date of visit and of the pregnancy test, and gestational age by last menstrual period or fetal ultrasound if available. Other collected data included pre-pregnancy weight, height and weight at visit, hemoglobin level, blood pressure, and temperature, and demographic and socio-economic profile. Medical history data included vaccinations, past infections, and infections or Zika-like symptoms since three months prior to pregnancy; pregnancy history, including complications; and current pregnancy status and prenatal care including dietary supplements. Fetal ultrasound information including gestational age, abnormal findings, and physical measurements was extracted from medical records, if available. Pregnant participants without a prior fetal ultrasound were referred for ultrasound. At study entry and again at each trimester, information was obtained on the pregnant participant and their partner's occupation, household characteristics, health risk factors such as smoking, drinking, and illicit drug use, use of products that typically contain fragrance, and exposure to pesticides and insecticides. At study entry, biological specimens were collected, including blood, urine, saliva, and vaginal swabs for ZIKV testing, blood for toxoplasmosis, rubella, cytomegalovirus (CMV), herpes, and syphilis (TORCH) testing as part of standard of care, and blood for dengue virus (DENV), chikungunya virus (CHIKV), and West Nile virus (WNV) testing as indicated. Pregnant participants were scheduled for monthly study visits at a clinic or hospital, which included clinical examinations and assessment of pregnancy-related adverse outcomes, fetal ultrasound (at least once per trimester), laboratory testing per standard of care, assessment of Zika-like symptoms, and biospecimen collection (blood, urine, saliva, vaginal swab) for Zika testing and storage for future research. In between monthly visits, pregnant participants provided a urine sample, which was collected at home and brought to the next monthly visit or collected at a biweekly visit in the clinic. Pregnant participants were educated about signs and symptoms of acute ZIKV infection and requested to alert the study team and return to the study clinic for blood and urine collection any time they experienced Zika-like symptoms. At the delivery visit, information was obtained about mode and location of delivery; type of provider who conducted the delivery; whether the pregnant participant was diagnosed with hypertensive disease or pre-eclampsia/eclampsia during the pregnancy; outcome of the delivery (i.e., numbers of fetuses/infants, born alive, stillborn, preterm/term, and sex of infant(s)); Zika-like symptoms experienced since the last study visit; results from previous laboratory tests; and written consent for enrollment of the infant. Enrolled infant participants were assessed at birth, three months, six months, and 12 months for signs of neurological abnormalities (irritability, lethargy, seizures, apnea, low tone, decreased reflexes, asymmetry, problems with opening or moving eyes, other problems with movement, dysphagia, continuous crying, arthrogryposis, hypertonia, hypotonia, other), head circumference, and other clinical outcomes. Infants who were observed by study clinicians to have potential neurological problems were referred to a local neurologist per the local standard of care, with findings recorded from completed neurologist visits. At three-, six-, or 12-month visits, audiological and ophthalmological screening was conducted, and infant participants were referred to specialists for additional testing if they failed the screening exam. A previous publication details the study design and procedures [9].

Exposure assessment and definition

Pregnant participants' blood specimens were tested for anti-ZIKV IgM antibodies monthly. [10] If a routine monthly visit blood sample tested positive by ZIKV IgM, the local laboratory performed PCR testing for Zika virus ribonucleic acid (RNA) on samples from the same visit and, if available, samples (urine and/or blood) from the prior month. [11] The primary exposure was defined as Zika infection during pregnancy as demonstrated by one or more positive ZIKV RNA test results in approved specimen types during pregnancy through two days after delivery (see Supplemental Materials). Among pregnant participants who were considered ZIKV-infected, those who reported related symptoms that occurred no more than 84 days before or 14 days after confirmed ZIKV infection were considered symptomatic; those who did not were considered asymptomatic, and negative for the indicator of symptomatic ZIKV infection. In this paper, 'ZIKV infection' refers to infection of the pregnant person during pregnancy and 'ZIKV exposure' refers to in utero exposure of the infant to ZIKV infection of the pregnant person.

Outcome assessment

Outcomes were observed in the second or third trimester ultrasound, at birth, and/or during one or more infant clinical visits in the first year of life during the study visit or at a neurology consultation. Outcomes included fetal loss prior to 20 weeks gestation, fetal demise occurring at 20 or more weeks of gestation (stillbirth), infant death up to six weeks of age, adverse neurological outcomes, and abnormal results on ophthalmological or audiological screening tests. Based on the limited available science on outcomes associated

with ZIKV infection during pregnancy, and a lack of a CZS definition when the study began, the consortium planned to analyze the total adverse outcome risk of prenatal ZIKV infection. Therefore, a primary composite outcome was derived, which included all component outcomes described in Fig. 1, except for fetal loss prior to 20 weeks gestation. We present the results of the primary composite, as well as for each of the component fetal, neonatal, and infant outcome. More details about the definitions for each component outcome are provided in Fig. 1.

Covariates assessment

Covariates that were defined a priori as potential confounders were site of enrollment, pregnant participant age at enrollment, self-reported diabetes at enrollment, Body Mass Index (BMI) category at enrollment, acute CMV during pregnancy, and a set of variables meant to capture socio-economic status (SES) and living conditions, including employment status at enrollment, highest level of education attained, number of people living in the household, and indicators of whether the home had screens on windows, presence of animals, and proximity to open sewage. The statistical analysis prespecified that the set of SES variables could be reduced to include only highest level of education attained in case of issues with model convergence or fit.

Assay methods

Pregnant participants were tested for Anti-ZIKV IgM antibodies using the U.S. Centers for Disease Control and Prevention (CDC) ZIKV IgM MAC-ELISA assay [10] and for Zika RNA using the CDC Trioplex real time RT-PCR assay [11] with Emergency Use Authorization (EUA) approval from the United States Food and Drug Administration (FDA). All sites used the same molecular and

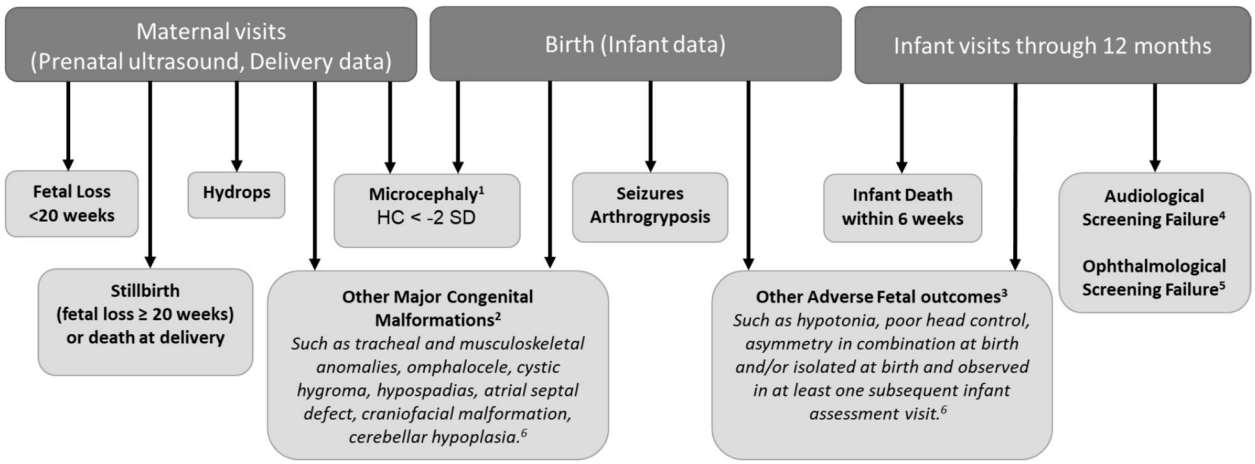

Fig. 1 Primary and secondary outcome definitions by data source and visit

Abbreviations: HC: Head circumference; SD: standard deviation

1 The protocol specified head circumference measurement at birth with confirmation by physical examination between 12 and 24 h by the participating neonatologist. Standard deviation scores (Z-scores) were determined from the Intergrowth reference standard appropriate for sex and gestational age through the birth visit (https://intergrowth21.tghn.org/standards-tools/) and for post-birth visits from WHO child growth standards for sex and age (https://www.who.int/tools/child-growth-standards/standards/head-circumference-for-age)

2 Categorized based on EUROCAT guide 1.4, Sect. 3.5 (https://eu-rd-platform.jrc.ec.europa.eu/sites/default/files/Full_Guide_1_4_version_28_DEC2018.pdf), to determine whether a finding is a major congenital malformation. The definition does not include microcephaly and malformations considered to be minor using the EUROCAT guide

3 Includes observation of a single adverse fetal outcome at birth and at least one subsequent infant assessment visit, or observation of these fetal outcomes in combination at birth; does not include microcephaly or other major congenital outcomes previously defined

4 Infants that failed the audiological screening exam during at least one study visit

5 Infants that failed the ophthalmological screening exam during at least one study visit

6 Data reviewed independently by three clinicians to determine if participant had one or more of the outcomes. Where discordance occurred, meetings were held to come to consensus

serological test kits and standardized protocols. Among pregnant participants, assays to detect antibodies reactive with *Toxoplasma gondii*, rubella virus, cytomegalovirus, herpes simplex virus, and *Treponema pallidum* were performed following local clinical laboratory standard procedures. Assays were performed for detection of DENV and in some cases CHIKV or WNV infection as indicated per local epidemiologic disease surveillance report guidelines.

Additional tests were approved by the FDA for ZIKV testing over the course of the study. Additional ZIKV RNA testing was conducted on stored samples using a diagnostic assay with improved performance characteristics including enhanced sensitivity, the Aptima ZIKV Assay (Hologic, Marlborough, MA) [12, 13]. One stored whole blood and one stored urine specimen from each pregnant participant from each visit time point was shipped to Vitalant Laboratories (San Francisco, CA) and batch tested using the Aptima transcription mediated amplification (TMA) assay.

Statistical methods

Primary analysis

The two primary analyses compared: 1) the composite outcome among ZIKV-exposed infant participants to unexposed, and 2) the composite outcome among infants born to symptomatic ZIKV-infected pregnant participants vs. infants born to uninfected or asymptomatic ZIKV-infected pregnant participants. These two primary analyses were prespecified to be tested simultaneously at alpha $=0.05$ after a Holm's adjustment for multiple comparisons.

The two primary analyses were prespecified in the statistical analysis plan to be performed using targeted maximum likelihood estimation (TMLE). However, lower than anticipated numbers of enrolled infected pregnant individuals and a relatively rare outcome led to concerns about the stability of the TMLE models. Therefore, we present the prespecified alternative analysis estimating the primary results using standardized weighted logistic regression.

Missingness was handled using multiple imputation via the mice package in R, and the resulting estimates from 50 imputed datasets were pooled using Rubin's rules to conduct inference. There was a concern that some fetal losses before 20 weeks might represent induced abortions in response to a ZIKV infection in early pregnancy and fear of negative outcomes; therefore, we specified a priori that fetal losses before 20 weeks gestation were not included in the primary composite outcome. Instead, weighting was used to account for pregnant participants who experienced these losses.

Sensitivity analyses

The statistical analysis plan (SAP) included several prespecified sensitivity analyses of the primary endpoints that would be conducted regardless of the significance of the primary analyses, in order to assess robustness of the conclusions to methodological choices. These included: 1) primary analysis on complete case data only; 2) inclusion of recorded dengue infection during pregnancy as an additional covariate in the model; 3) suspected but not confirmed cases of ZIKV infection set to missing; 4) broader definition of "documented ZIKV infection" including suspected but not confirmed cases (pregnant participants who had one or more positive results on IgM assays only); 5) primary analysis on the subset of pregnancies with documented live birth; 6) the outcome of missing pregnancies set to positive, negative, and removed; 7) outcome of pregnancies lost before 20 weeks set to positive, negative, and removed; 8) modified imputation algorithm for pregnancies lost before 20 weeks based on all exposures before 20 weeks; 9) missing exposure data (ZIKV infection) set to positive, negative, and removed; 10) primary analysis on subset of pregnancies enrolled in first trimester; 11) matched case–control analysis, and 12) complete case unadjusted analysis. See Supplemental Materials for details of the statistical analyses and results of these models.

Secondary analyses

We planned to fit the same models as in the primary analysis with the following variations: 1) evaluating each component of the composite as the outcome rather than the composite; 2) comparing fetal and infant outcomes in asymptomatic ZIKV-infected vs. uninfected pregnant participants, removing symptomatic infections; and 3) restricting analyses to only those with confirmed ZIKV infection, comparing infant and fetal outcomes in symptomatic ZIKV-infected vs. asymptomatic ZIKV-infected, by trimester of ZIKV infection. Like for the primary analysis, standardized generalized linear models were prespecified to be used in addition to TMLE for secondary analysis. However, the smaller numbers of events in these secondary analyses further increased the concern about the stability of the proposed TMLE models and led to unacceptable variability in these models. Therefore, the secondary analyses presented here are based on the standardized generalized linear models.

All participating sites and RTI International obtained Institutional Review Board or Institutional Ethics Committee approval and written informed consent was provided by all participants or legally authorized representatives. Data were cleaned and analyzed by statisticians at RTI and transferred to NIH for analysis.

Table 1 Demographic and pregnancy characteristics of enrolled participants by ZIKV infection status and symptoms[a]

Variables	No ZIKV Infection *N* (%)	ZIKV Infection asymptomatic *N* (%)	ZIKV Infection symptomatic *N* (%)	Missing ZIKV Infection *N* (%)
Number of pregnant participants with defined infection status[a]	5191	42	19	848
Age, mean (SD)	25.6 (6.2)	24.5 (5.8)	26.0 (8.0)	24.9 (6.0)
Self-Reported Maternal Diabetes at Enrolment	100 (1.9)	0 (0.0)	0 (0.0)	14 (1.7)
BMI Category at Enrolment				
< 18.5	300 (5.8)	2 (4.8)	3 (15.8)	50 (5.9)
18.5–25	2356 (45.4)	22 (52.4)	5 (26.3)	418 (49.3)
25–30	1373 (26.4)	9 (21.4)	5 (26.3)	215 (25.4)
30–35	641 (12.3)	7 (16.7)	6 (31.6)	97 (11.4)
35–40	230 (4.4)	1 (2.4)	0 (0.0)	29 (3.4)
> =40	114 (2.2)	0 (0.0)	0 (0.0)	17 (2.0)
Missing	177 (3.4)	1 (2.4)	0 (0.0)	22 (2.6)
Trimester at Enrollment				
1 (up to 13 weeks 6 days)	4112 (79.2)	39 (92.9)	16 (84.2)	721 (85.0)
2 (14 weeks 0 days – 27 weeks 6 days)	1078 (20.8)	3 (7.1)	1 (5.3)	127 (15.0)
3 (28 weeks – 45 weeks)	1 (0.0)	0 (0.0)	2 (10.5)	0 (0.0)
Gestational age at delivery, mean (SD)	37.7 (6.0)	35.2 (8.3)	35.4 (8.6)	39.4 (2.7)
Last year of school				
8 th grade or less	1149 (22.1)	8 (19.0)	1 (5.3)	214 (25.2)
9 th −12th grade	1197 (23.1)	6 (14.3)	4 (21.1)	185 (21.8)
High School diploma or GED	1804 (34.8)	17 (40.5)	5 (26.3)	261 (30.8)
0–2 years college or Associate's degree	705 (13.6)	8 (19.0)	6 (31.6)	139 (16.4)
Bachelor's degree or higher	334 (6.4)	3 (7.1)	3 (15.8)	48 (5.7)
Missing	2 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)
CMV Seroconverter	10 (0.2)	0 (0.0)	1 (5.3)	0 (0)

[a] ZIKV Infection = one or more positive ZIKV RNA test results in approved specimen types during pregnancy through two days after delivery. Among pregnant participants who were considered ZIKV-infected, those who reported related symptoms that occurred no more than 84 days before or 14 days after confirmed ZIKV infection were considered to be symptomatic; those who did not were considered asymptomatic

Results

A total of 6,100 pregnant participants and 4,584 infant participants were enrolled across 10 sites. Participant characteristics are presented by exposure in Table 1. Among these participants, 234 experienced early (before 20 weeks) fetal loss and were only included in the weighting of the primary analyses. Of the remaining 5,866, 841 participants (13.9%) were missing information on their exposure to ZIKV during pregnancy, and 1,991 more were missing data sufficient to assess the primary composite outcome (Fig. 2). Data presented in the tables do not include those records, but they are included in the regression analyses (Tables 2 and 3). Of the remaining 3,034 participants with complete data on exposure and outcome, 90 were missing some or all covariates, leaving 2,944 for the complete case secondary analyses.

Primary analysis

The distributions of infections and outcomes are given in Table 3, separated into uninfected and infected, followed by the number of participants with the outcomes of interest among those with symptomatic ZIKV infection. The RR of having the primary composite outcome associated with intrauterine ZIKV exposure was estimated by standardized weighted logistic regression to be 1.64 for the first primary analysis of all infections (95% CI 0.65, 4.13), with an estimated risk ratio of 1.08 for the second primary analysis of symptomatic infections (95% CI 0.15, 7.64). Confidence intervals for primary analysis results indicate these RR were not statistically significant.

In addition to the standardized and weighted logistic regression models, other sensitivity analyses of the primary endpoint included logistic regression and exact

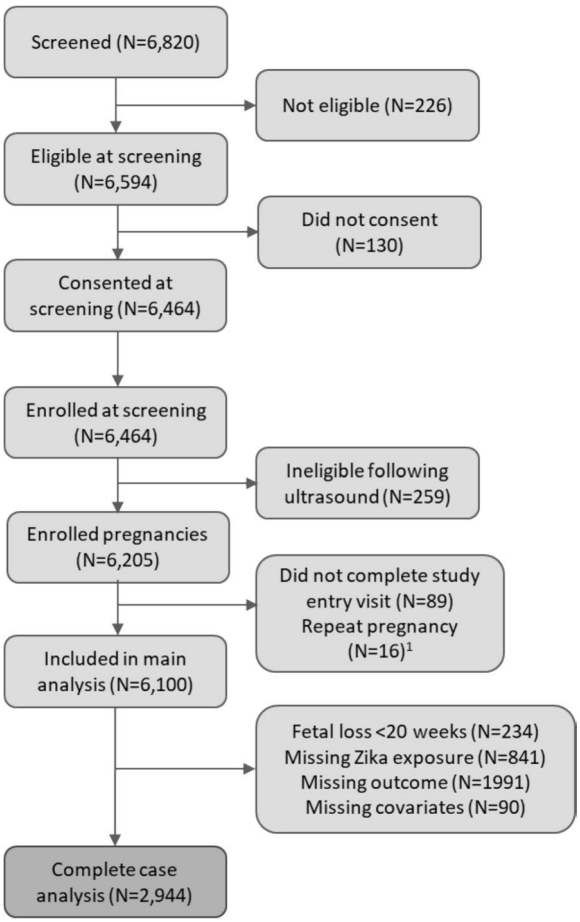

Fig. 2 Disposition of screened individuals. [1]Only one pregnancy per participant was planned for inclusion in analysis

binomial methods of the subset of pregnant participants with no missing data. For the complete case logistic regression, 2,944 pregnant participants were included who had ZIKV infection status ascertained throughout pregnancy, sufficient data to determine the composite endpoint, and known values for the covariates included in the model. The complete case RR was 1.84 (95% CI: 0.74, 4.55) for all infections and 0.91 (95% CI: 0.14, 5.83) for symptomatic infections. For the exact binomial model, 2,961 pregnant participants were included with complete exposure and outcome data, yielding a RR ratio of 2.25 for all infections (95% CI 0.63, 5.18) and an estimated risk ratio of 1.45 for symptomatic infections (95% CI 0.04, 6.52). For further results of the sensitivity analyses, see the Supplemental Materials.

Secondary analyses

Table 3 also presents the results of the secondary analyses examining the association between ZIKV infection and each component of the primary composite endpoint, plus

Table 2 ZIKV infection status and symptoms by site

Sites	No ZIKV Infection	ZIKV Infection asymptomatic	ZIKV Infection symptomatic
	N (%)[a]	*N* (%)	*N* (%)
Total	5191	42	19
Brazil-Pernambuco	873 (98.1)	16 (1.8)	1 (0.1)
Brazil-Rio	686 (98.7)	8 (1.2)	1 (0.1)
Brazil-Salvador	566 (100)	0 (0.0)	0 (0.0)
Brazil-São Paulo	469 (100)	0 (0.0)	0 (0.0)
Colombia	259 (99.2)	2 (0.8)	0 (0.0)
Guatemala	891 (100)	0 (0.0)	0 (0.0)
Nicaragua	580 (99.1)	3 (0.5)	2 (0.4)
Peru	269 (98.9)	1 (0.4)	2 (0.7)
Puerto Rico-North Karst	336 (98.8)	3 (0.9)	1 (0.3)
Puerto Rico-San Juan	262 (92.6)	9 (3.2)	12 (4.2)

[a] Numbers in parentheses represent the percent in that category among the enrolled particpants at that site

fetal loss before 20 weeks. Stillbirth or death at delivery as well as infant death within six weeks were significantly associated with confirmed ZIKV infection, with adjusted RRs of 4.28 (95% CI: 1.39, 13.21) and 6.20 (95% CI 1.08, 35.60), respectively. Fetal loss < 20 weeks was also significantly associated with any Zika infection (RR: 3.72; 95% CI: 1.82, 7.59). None of the other individual components were significantly associated with ZIKV infection or symptomatic ZIKV, after adjusting for baseline covariates.

None of the other secondary analyses yielded statistically significant results after covariate adjustment, including the comparison of symptomatic vs asymptomatic ZIKV restricted to those who tested positive (RR: 0.64; 95% CI: 0.09, 4.75 by the primary definition of positivity; RR: 1.27; 95% CI: 0.40, 4.02 by the broader definition), the comparison of those who tested positive in the first trimester compared to never testing positive (all infections RR 1.38; 95% CI 0.46, 4.21; symptomatic infections only RR: 1.00; 95% CI 0.14, 7.00), and the comparison of those who tested positive in the second trimester compared to never testing positive (all infections RR: 1.19; 95% CI 0.17, 8.21; too few cases to model symptomatic infections) There was an insufficient number of exposed pregnancies to conduct the other planned secondary analyses.

Discussion

In this large international prospective cohort study of pregnant individuals and their offspring, we assessed the risk of adverse fetal, neonatal, or infant outcomes associated with ZIKV infection during pregnancy. The primary

Table 3 Frequencies and estimated adjusted relative risks (RR) of primary and secondary outcomes categorized by ZIKV infection status and symptoms

Outcome Variables	Uninfected	ZIKV Infected	Adjusted[a] RR for comparing confirmed ZIKV infections to all others	ZIKV Infected, symptomatic[b]	Adjusted[a] RR for comparing confirmed symptomatic ZIKV infections to all others	Missing ZIKV Infection
n	5191	61		19		848
Primary Composite Outcome[c]						
No	2802 (54.0)	22 (36.1)		9 (47.4)		95 (11.2)
Yes	206 (4.0)	4 (6.6)	1.64 (0.65, 4.13)	1 (5.3)	1.08 (0.15, 7.64)	3 (0.4)
Missing	2183 (42.1)	35 (57.4)		9 (47.4)		750 (88.4)
Secondary Analyses						
Microcephaly						
No	4754 (91.6)	50 (82.0)		16 (84.2)		364 (42.9)
Yes	50 (1.0)	0 (0.0)	0 (NA)	0 (0.0)	0 (NA)	0 (0.0)
Missing	387 (7.5)	11 (18.0)		3 (15.8)		484 (57.1)
Other Congenital Malformation[d]						
No	4659 (89.8)	51 (83.6)		16 (84.2)		270 (31.8)
Yes	40 (0.8)	0 (0.0)	0 (NA)	0 (0.0)	0 (NA)	1 (0.1)
Missing	492 (9.5)	10 (16.4)		3 (15.8)		577 (68.0)
Seizures						
No	3391 (65.3)	29 (47.5)		11 (57.9)		120 (14.2)
Yes	3 (0.1)	0 (0.0)	0 (NA)	0 (0.0)	0 (NA)	0 (0.0)
Missing	1797 (34.6)	32 (52.5)		8 (42.1)		728 (85.8)
Arthrogryposis						
No	3390 (65.3)	29 (47.5)		11 (57.9)		120 (14.2)
Yes	1 (0.0)	0 (0.0)	0 (NA)	0 (0.0)	0 (NA)	0 (0.0)
Missing	1800 (34.7)	32 (52.5)		8 (42.1)		728 (85.8)
Hydrops						
No	5101 (98.3)	59 (96.7)		19 (100.0)		699 (82.4)
Yes	2 (0.0)	0 (0.0)	0 (NA)	0 (0.0)	0 (NA)	0 (0.0)
Missing	88 (1.7)	2 (3.3)		0 (0.0)		149 (17.6)
Ophthalmological Screening Failure						
No	3853 (74.2)	36 (59.0)		14 (73.7)		153 (18.0)
Yes	29 (0.6)	0 (0.0)	0 (NA)	0 (0.0)	0 (NA)	0 (0.0)
Missing	1309 (25.2)	25 (41.0)		5 (26.3)		695 (82.0)
Audiological Screening Failure						
No	3864 (74.4)	36 (59.0)		14 (73.7)		156 (18.4)
Yes	3 (0.1)	0 (0.0)	0 (NA)	0 (0.0)	0 (NA)	0 (0.0)
Missing	1324 (25.5)	25 (41.0)		5 (26.3)		692 (81.6)
Stillbirth or Death at Delivery						
No	4637 (89.3)	48 (82.8)	4.28 (1.39, 13.21)	15 (78.9)	3.93 (0.56, 27.68)	269 (31.7)
Yes	65 (1.3)	3 (5.7)		1 (5.3)		1 (0.1)
Missing	489 (9.4)	7 (16.7)		3 (15.8)		578 (68.2)
Other Adverse Fetal Outcome[e]						
No	4685 (90.3)	51 (83.6)		16 (84.2)		269 (31.7)
Yes	14 (0.3)	0 (0.0)	0 (NA)	0 (0.0)	0 (NA)	1 (0.1)
Missing	492 (9.5)	10 (16.4)		3 (15.8)		578 (68.2)
Infant Death within 6 weeks[f]						
No	4123 (79.4)	40 (65.6)	6.20 (1.08, 35.6)	13 (68.4)	0 (NA)	173 (20.4)
Yes	16 (0.3)	1 (1.6)		0 (0.0)		0 (0.0)
Missing	1052 (20.3)	20 (32.8)		6 (31.6)		675 (79.6)

Table 3 (continued)

Outcome Variables	Uninfected	ZIKV Infected	Adjusted[a] RR for comparing confirmed ZIKV infections to all others	ZIKV Infected, symptomatic[b]	Adjusted[a] RR for comparing confirmed symptomatic ZIKV infections to all others	Missing ZIKV Infection
Fetal Loss < 20 weeks						
No	4971 (95.8)	37 (88.1)	3.72 (1.82, 7.59)	17 (89.5)	2.99 (0.77, 11.57)	433 (51.1)
Yes	220 (4.2)	5 (11.9)		2 (10.5)		7 (0.8)
Missing	0 (0.0)	0 (0.0)		0 (0.0)		408 (48.1)

[a] RR adjusted for site and maternal age, diabetes, BMI, education level and CMV serostatus

[b] While the counts are not listed specifically for participants with asymptomatic ZIKV infections, these can be calculated by subtracting the number of participants on each row who are symptomatic from the total number infected

[c] Includes at least one of the outcomes in the categories below other than fetal loss < 20 weeks

[d] Includes congenital malformations such as tracheal and musculoskeletal anomalies, omphalocele, cystic hygroma, fetal hydrops, hypospadias, atrial septal defect, craniofacial malformation, and cerebellar hypoplasia; does not include microcephaly

[e] Includes observation of a single adverse fetal outcome (e.g., hypotonia, poor head control, asymmetry) at birth and at one or more subsequent infant assessment visist, or observation of these fetal outcomes in combination at birth; does not include microcephaly or other outcomes listed in this table

[f] Includes participants that may have also experienced other outcomes

composite endpoint, which included adverse fetal outcomes, congenital malformations, infant death before six weeks, and clinical findings of neurological damage, was not significantly more likely to be observed among ZIKV-exposed infant participants than in unexposed infant participants. There were fewer enrolled ZIKV-infected participants, fewer observed outcomes, and more missing data than expected, leading to limited statistical power for planned analyses. Due to the large amount of missing data and resulting uncertainty in our estimates, we conducted several sensitivity analyses under different assumptions, the results of which were generally consistent with our overall findings of no significant evidence of a difference in the primary outcome, for either the analysis of all confirmed infections or only symptomatic infections. Based on the number of participants with no missing data on the primary outcome or exposure, post-hoc analysis showed the study had adequate (~ 80% or higher) power only to detect a relative risk greater than 4.1, a reasonable effect estimate supported by earlier case–control studies [5]. We also conducted secondary analyses to review each component of the primary endpoint as well as fetal loss before 20 weeks, and observed significantly higher risk of fetal loss < 20 weeks, stillbirth, and infant death before six weeks among exposed offspring compared to unexposed.

In early 2016, the NIH and Fiocruz released emergency funds to nine research sites and one coordinating center to rapidly develop and implement the ZIP protocol. The 10th site, in Iquitos, Peru, joined the consortium in August 2017. The ZIKV epidemic in the Americas peaked in early-to-mid 2016 and then declined throughout the study follow-up period [14]. The incidence of Zika infections declined sharply once study enrollment began, limiting both interest in enrolling in the study and eligible people experiencing Zika infection. Though the study enrollment target was initially 10,000 pregnant individuals, recruitment was stopped in 2018 with 6,464 participants enrolled due to little to no circulation of ZIKV in the study areas.

Reports of the proportion of symptomatic infections in Zika infected individuals have varied widely in the Americas depending on the study design and participant population. In a study of pregnant travelers returning to the United States who were screened for ZIKV infection, 83% of 547 ZIKV-positive participants were asymptomatic [15]. Similar to our study, studies of symptomatic and asymptomatic ZIKV infection during pregnancy observed similar risk of birth defects in both groups [16].

We observed only four ZIKV-exposed children with an adverse outcome of interest in this study, resulting in wide confidence intervals for estimates of association. This finding likely under-represents the true frequency of possible outcomes because neuroimaging was only conducted on a small subset of infants who were referred to a neurologist for additional testing and evaluation beyond the basic neurological assessment conducted by a physician at each study visit. The results of the secondary analysis show a significant difference for fetal loss before 20 weeks, for stillbirth, and for infant death before six weeks between the exposed and the unexposed group. However, these results should be interpreted with caution. The ZIP study was particularly well designed to study these outcomes, as pregnant participants were enrolled before 18 weeks of pregnancy. However, there may have been misclassification of induced abortion as fetal loss among participants who knew they had been exposed to ZIKV, especially where induced abortion was not legal or if abortions were reported as fetal loss to avoid stigma.

The ZIP study found 11.8% fetal loss before 20 weeks in the infected group, with an adjusted RR of 3.72 compared to the uninfected group. This frequency was at the high end of that observed in the meta-analysis performed by Martins et al. (2021) [17] who reported values ranging from 0.3% to 10.6% in ZIKV-infected pregnant individuals in 15 studies, while the ZBC-Consortium IPD-MA reported an absolute risk of 0.9% [18]. The risk of stillbirth in the ZIP study was 5.7% among ZIKV-infected participants, with an adjusted RR of 4.28 compared to the uninfected group (1.3%). Martins et al. (2021) reported no stillbirths in four out of nine studies of ZIKV-infected participants, and in five studies there was a frequency of stillbirth varying from 0.78% to 2.4%, while in the ZBC-Consortium individual participant data meta-analysis (IPD-MA) the absolute risk was 0.3% [16, 17]. Neither study included comparison (unexposed) groups. The small number of exposed pregnant individuals and the low frequency of outcomes in the ZIP study make the estimates unstable and limit conclusions that can be drawn from the comparisons. Fetal autopsy was not performed and consequently, the cause of the fetal loss is not known. Because fetal abnormality is a common cause of fetal loss and our findings are inconclusive, this issue needs further exploration in meta-analyses with larger sample sizes and comparison groups.

ZIKV research during the epidemic proved difficult due to the narrow time window in which the molecular tests remain positive, the low sensitivity of serological tests, and the non-specificity of ZIKV symptoms [19, 20]. A strength of the ZIP study was the algorithm that was used to test and identify pregnant individuals with ZIKV infection. While the number of identified infections during pregnancy was small, the serial (in many cases monthly) serological testing of participants throughout pregnancy provides a well-characterized *unexposed* population unmatched in size and scope in the Americas. The data collected in the ZIP study, and in the follow-on study evaluating a subset of ZIP children through 3.5 years of age, provide opportunities for future research.

Comparison of results across studies has been complicated due to the variability in frequency of assessments, length of follow-up, assay type, and tools and methods used for assessing and diagnosing outcomes, leading to heterogeneity between studies. Efforts among the scientific community to overcome some of these problems have led to the creation of national and international consortia which have worked to harmonize data to make it possible to conduct IPD-MA [21–23]. The ZIP study benefited from a willingness by investigators from many institutions to collaborate in earnest without the usual limitations (e.g., prior umbrella funding, existing research partnerships, competition for participant populations, etc.). Procedures and techniques were standardized, and fieldworkers underwent rigorous standardized training across all sites. A standardized study protocol was developed and implemented, producing a large dataset representative of pregnant individuals and their offspring that used the same exposure and outcome metrics and analytical methods across research sites in multiple countries, allowing for comparison of results across diverse research settings. The ZIP study is the largest Zika cohort study to date, and has produced a well-characterized dataset with relevant information on unexposed pregnant participants and children that will be useful in IPD-MA, such as those being conducted by the WHO and by the ZBC consortia [22, 23]. In addition, the ZIP study evaluated a variety of potential outcomes, some of which fall outside typical definitions of congenital Zika syndrome (CZS) [24].

Limitations

Despite the success achieved through a standardized ZIP protocol, the initiation of the study at many sites after the peak of the epidemic highlights the need for preexisting research networks to permit rapid scale up of research activities when epidemics occur. The rapid implementation required in response to a fast-moving epidemic affecting pregnant individuals and their babies requires registries or cohorts that are prepared to track pregnant individuals and their births in the clinical setting and, in many cases, at home. Some ZIP sites had excellent linkages between antenatal care and the birth hospital, while others had missing birth and neonatal data due to a high prevalence of home births or limited capacity within the birth hospital to implement the ZIP study protocol at birth. Overall, close to half of the participants were missing some data on exposure or outcome, which raises questions of selection bias as those with complete data might be systematically different than those without. Our analysis approaches, including multiple imputation and a large number of sensitivity analyses, sought to minimize these concerns, and it is reassuring that the results of these analyses are generally consistent. In addition, the demographic summaries of those with complete data and those missing some data do not show any apparent differences, as shown in Table 1.

Because brain imaging was conducted only among the subset of children who were referred to and evaluated by a neurologist, children with relevant neurological outcomes may have been missed. In any microcephaly study, there is inter- and intra-examiner variability in measurement of head circumference. Lack of standardized head

circumference measurement is of particular concern in the context of ZIKV infection research, given the importance of microcephaly as a major sequela [25]. In the ZIP study, standardized guidance and training for head circumference measurement was provided to all study teams, and longitudinal data were checked extensively for inconsistencies.

Conclusions

To date, associations between intrauterine ZIKV exposure and adverse fetal and infant outcomes have been observed in case–control studies. The ZIP study identified fewer observed ZIKV infections than expected during pregnancy, coincident with a waning epidemic, which limited the power of the study to evaluate risk. There was a numeric but not statistically significant increase in the risk for the primary composite outcome with intrauterine ZIKV exposure, and also when the exposure was limited to symptomatic ZIKV infections only. There was also an increase in some specific adverse fetal and infant outcomes observed in secondary analyses. Combining data from multiple cohorts for future meta-analysis may better define the risks of intrauterine ZIKV exposure. The rigorous methodology used, large overall sample size, and standardized application of a single protocol across multiple sites make the ZIP study dataset an important resource to understand and to quantify the magnitude of the risks of intrauterine exposure by providing unexposed group data for large meta-analyses.

Abbreviations

CDC	Centers for Disease Control and Prevention
CKNV	Chikungunya virus
CMV	Cytomegalovirus
DCC	Data Coordinating Center
DENV	Dengue virus
ELISA	Enzyme linked immunosorbent assay
EUA	Emergency Use Authorization
FDA	Food and Drug Administration
Fiocruz	Fundação Oswaldo Cruz
IPD-MA	Individual participant data meta-analysis
IRB	Institutional Review Board
NIAID	National Institute of Allergy and Infectious Diseases
NICHD	*Eunice Kennedy Shriver* National Institute of Child Health and Human Development
NIH	National Institutes of Health
RPR	Rapid plasma reagin
RT-PCR	Reverse transcription polymerase chain reaction
TMA	Transcription mediated amplification
TMLE	Targeted maximum likelihood estimation
WHO	World Health Organization
WNV	West Nile Virus
ZIKV	Zika virus

Supplementary Information

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Supplementary Material 1

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Authors' contributions

KBS, RX, MMMP, JM, WB, JFC, EG, LAG, ALG, EH, NFK, AIK, DMFS, MELM, ETAM, SSC, CMVV, MN, and CDZ participated in the conception or design of the study. MN, JFL, KBS, RX, MMMP, JM, JFA, AB, WB, JFC, LF, EF, EG, ALG, LAG, AG, EH, CMI, NFK, AIK, DMFS, MELM, DBMF, ETAM, URM, TJO, JEO, SSC, MTC, CAUG, SBMN, CMC, CMVV, MW, and CDZ participated in the acquisition, analysis or interpretation of study findings. MN, JFL, KBS, RX, MMMP, and JM drafted or substantially revised the manuscript. All authors approved the submitted version of the manuscript and any substantially modified version that involved the author's contribution to the study. All authors agreed to be personally accountable for their own contributions and ensure that questions related to the accuracy or integrity of the work, even ones in which they were not personally involved, are appropriately investigated and resolved, and with a resolution documented in the literature.

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Data availability
The protocol (version 1.0, February 1, 2017) for this study is listed on clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT02856984). De-identified data from this study is publicly available on the NICHD Data and Specimen Hub (DASH) platform.

Declarations

Ethics approval and consent to participate
All participating institutions provided ethical review and approval of the study prior to study initiation. Participants provided written informed consent to participate in the study. Age of minority varied by country and sites followed local IRB/IEC requirements for enrollment of minors in the study. If the participant was considered a minor in the participating country, written permission was required from the parent(s)/legal guardian and separate written assent was required from the participating minor. If not possible to obtain written consent, permission, or assent, e.g., due to illiteracy, pregnant participants and their parent(s)/guardian, as appropriate, provided consent, permission, or assent using a fingerprint instead of a signature. The ethics committees of the following institutions provided approval for this study: Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz (Fiocruz Pernambuco); Hospital Geral Roberto Santos; Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (Fiocruz Bahia); Yale University; Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo; University of Alabama at Birmingham; Secretaria Municipal de Saúde do Rio de Janeiro; Instituto Fernandes Figueira, Fundação Oswaldo Cruz (Fiocruz – Rio de Janeiro); University of Wisconsin, Madison; St. Jude Children's Research Hospital; Universidad de Antioquia, Medellín; Red de Salud de Ladera Empresa Social del Estado; University of Colorado, Denver; Instituto de Nutrición de Centro América y Panamá; Ministerio de Salud Pública y Asistencia Social, Guatemala; Centro Nacional de Diagnóstico y Referencia, Ministerio de Salud, Nicaragua; University of California, Berkeley; University of Puerto Rico, Medical Sciences Campus; University of Georgia, Athens; Hospital Iquitos César Garayar Garcia; Hospital Regional de Loreto Felipe Arriola Iglesias; and Universidad Peruana Cayetano Heredia, RTI International.

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Competing interests
The authors declare no competing interests.

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