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Association between microcephaly, Zika virus infection and other risk factors in Brazil, final report of a case-control study

Summary

Background A Zika virus epidemic emerged in Northeast Brazil in 2015 and was followed by a striking increase in congenital microcephaly cases, triggering an international public health emergency declaration. This is the final report of the first case-control study evaluating potential causes of the microcephaly: congenital Zika virus infection, vaccines and larvicides. The published preliminary report reported a strong association between microcephaly and congenital Zika infection.

Methods We conducted a case-control study in public maternities in Recife, Brazil, between Jan to Nov, 2016. Cases were neonates born with microcephaly. Two controls without microcephaly were matched to each case by expected date of delivery and area of residence. Serum of cases and controls and cerebrospinal fluid of cases were tested by quantitative RT-PCR and anti-Zika-IgM. Maternal serum was tested by plaque reduction neutralization assay for Zika and dengue viruses. Matched crude and adjusted ORs were estimated using exact conditional logistic regression.

Findings We included 91 cases and 173 controls. Congenital ZIKV-infection was laboratory confirmed in 32 cases; no controls had confirmed Zika-infection. Approximately 83% (69/83) of cases were small for gestational age, compared to 5% (8/173) among controls. The overall matched odds ratio (mOR) was 73.1 (95%CI 13.0-∞) after adjustments. Neither vaccination during pregnancy nor using the larvicide pyriproxyfen was associated with microcephaly. 37% of the cases (34/91) had either laboratory confirmation of Zika-infection or major cerebral anomalies identified on computed tomography.

Interpretation The association between microcephaly and congenital Zika virus infection was confirmed. We provide the first evidence of the absence of effect of other potential factors such as exposure to pyriproxyfen or vaccines (Tdap, MR, MMR) during pregnancy.
**Funding:** Brazilian Ministry of Health, Pan American Health Organization and Enhancing Research Activity in Epidemic Situation.

**Introduction**

In August 2015, physicians reported a cluster of microcephaly cases in the state of Pernambuco, Northeast Brazil. Microcephaly was a rarely reported abnormality in birth before the Zika virus (ZIKV) epidemic. Microcephaly is a clinical sign that may reflect abnormal brain development, but can be also found in healthy neonates. By definition it is any insult that disturbs early brain growth, and it can be caused by genetic variations, teratogenic agents, or other well-established congenital infections (cytomegalovirus, rubella, herpes, toxoplasmosis).

At the start of this microcephaly epidemic, the main causal hypothesis was ZIKV infection during pregnancy, but other potential causes were proposed; two of these causes were of particular interest because of the potential implications: larvicides use in drinking water reservoirs to control *Aedes aegypti* (since Pyriproxyfen was introduced in 2014, by the Brazilian Ministry of Health), or vaccine administration during pregnancy.

Microcephaly was the first reported postnatal clinical finding at the beginning of the epidemic. However, rapidly accumulating evidence showed that Congenital Zika Syndrome can cause more than isolated microcephaly. In the early months of the marked increase in the prevalence of microcephaly, we designed a case-control study to investigate an association between microcephaly and congenital ZIKV infection and other potential causes. The previously published preliminary report documented a strong association with Zika, we now report the final results assessing the association between microcephaly and congenital ZIKV infection along with a comprehensive investigation of other potential risk factors in an epidemic context in Pernambuco State, Brazil.
Methods

Study design and participants.
We present the final analysis of our case-control study with neonates consecutively recruited at birth. The preliminary analysis included subjects recruited from Jan 15 to May 2, 2016; this analysis includes subjects recruited up to Nov 30, 2016. We conducted this analysis before reaching 200 cases for two reasons: first, we reached the necessary power for statistical analysis because the exposed proportion of controls was lower than expected; second, the epidemic slowed down in Recife and cases became rarer.

The study population consisted of neonates born from women residing in Pernambuco and delivered in eight public maternities in Recife. Cases - neonates with microcephaly (livebirth or stillbirth) – had head circumference (HC) at least 2 SD smaller than the mean for sex and gestational age on the Fenton growth chart. Microcephaly was considered severe when the HC was at least 3SD smaller than the mean. Exclusion criteria were anencephaly, encephalocele, and confirmation of the phenotype of a well-defined congenital syndrome. Controls were live neonates without microcephaly and with no brain abnormalities (by transfontanellar ultrasonography) and no major birth defects by physical examination by the study neonatologist. We selected two controls per case, which were matched by health region of residence and expected date of delivery, to ensure that cases and controls were conceived at the same stage of the epidemic.

Controls were selected from the first neonates born after 8am on the following morning in one of the study hospitals, where a trained female nurse stayed seven days a week, from 8am to 5pm, and listed women admitted. However, we cannot guarantee that all consecutive neonates were screened.

The criteria for matching for the expected date of delivery were specific to the gestational age of the cases. For cases born at term and post-term (37 weeks or more), controls were the next eligible neonates born at 37 weeks gestation or more. For early preterm cases (born at <34 weeks), controls were the next eligible neonates who were
born at less than 34 weeks gestation. For preterm cases born between 34 and 36 weeks gestation, controls were the next eligible neonates born at 34–36 weeks gestation.

**Procedures**

We estimated gestational age by antenatal foetal ultrasonography. If not available, the date of the last menstrual period recorded on the antenatal care card or reported by the mother was used. When both were not available, the Capurro method was used.\(^{15}\) Head circumference was measured in the delivery room with a non-stretch Teflon tape; a second measurement was done 12–24h after birth to confirm microcephaly by the study neonatologists. Cerebrospinal fluid (CSF) was collected from cases. Umbilical cord blood was collected from cases and controls; when necessary, peripheral blood was collected before the neonate left the hospital. Blood specimens were stored at the Virology and Experimental Therapy Department, Fiocruz Pernambuco. Sera of mothers and neonates (cases and controls) and CSF samples (cases) were tested by quantitative real time polymerase chain reaction (qRT-PCR) for detection of the ZIKV genome,\(^ {16}\) and with capture-IgM enzyme-linked immunosorbent assay (ELISA) for IgM antibodies.\(^ {17}\) Macerated tissues (brain, kidney or pooled organs) of stillbirths cases were tested by qRT-PCR. The presence of ZIKV and DENV (1-4) specific neutralizing antibodies was assessed in the sera of mothers and neonates (cases and controls) by Plaque Reduction Neutralization test (PRNT\(_{50}\)), with a 50% cut-off value for positivity. Serum samples were tested for toxoplasmosis, rubella, and cytomegalovirus IgM antibodies, the main infectious causes of congenital microcephaly.\(^ {7}\)

Brain imaging was performed by computed tomography (CT) scan in cases and classified as the presence or absence of major cerebral abnormalities identified on CT by physicians specialized in imaging diagnosis (calcification, ventriculomegaly, malformation of cortical development such as lissencephaly and polymicrogyria and presumed vascular abnormalities). Controls were investigated by transfontanellar ultrasonography. Mothers were interviewed using a standardized questionnaire.
Variables: Laboratory-confirmed ZIKV-infection was defined in a neonate as a positive qRT-PCR and/ or IgM result for ZIKV in any biological specimen (serum, CSF or macerated tissues); neonates were considered to be small for gestational age (SGA) if their birth weight was lower than the 10\textsuperscript{th} percentile for gestational age and sex on the Fenton growth chart.

Information on demographic and socioeconomic factors included the following: mothers’ age, years of schooling and skin colour (self-referenced). The purchasing power of individuals and families was defined using the Brazilian Economic Classification Criteria – CCEB 2015,\textsuperscript{18} which defines eight socioeconomic classes from A (highest) to E (lowest). We also collected data on a family history of microcephaly or malformations; vaccination; self-reported misoprostol ingestion (medical abortion pill), epilepsy treatment or folic acid; the use of recreational drugs, tobacco and alcohol in pregnancy, exposure to Pyriproxyfen (including in any domestic water reservoir) and the use of insect repellent on skin. Vaccination cards were consulted (when available), and we only considered vaccination during pregnancy.

**Statistical analysis**

We investigated the association between microcephaly and potential risk factors, one by one, by conditional logistic regression. The variables associated with microcephaly with a $p \leq 0.10$ were included in the multivariable analysis using a conditional exact logistic regression model. Thus, we calculated matched odds ratio (mOR) for the association between microcephaly (outcome) and ZIKV-infection (exposure) adjusted by smoking during pregnancy, race/skin colour, receiving tetanus, diphtheria, and acellular pertussis vaccine (Tdap) in pregnancy.

Median unbiased estimator for binary data in exact conditional logistic regression was applied to deal with the fact that all controls tested negative for ZIKV.\textsuperscript{19} The model respected matching and included others conditioning variables – “condvars”.\textsuperscript{20}
We estimated the crude mOR and 95% confidence interval (CI) for the association between microcephaly and ZIKV infection for all cases, considering the results in any specimen (serum or cerebrospinal fluid for livebirth or macerated tissues for stillbirth). Additionally, crude mOR were estimated, separately, by sample type (serum or CSF) and microcephaly severity.

We investigated the agreement between qRT-PCR ZIKV-positivity in serum and CSF; and between the IgM positivity in serum and CSF.

We also compared the means of anthropometric parameters (HC, weight, height, Z score-weight for gestational age and sex) in four categories of cases: laboratory(-)/imaging(-); laboratory(+)/imaging(-); laboratory(-)/imaging(+); laboratory(+)/imaging (+) and controls, using analysis of variance (ANOVA) and we use Bonferroni pos hoc test to identify homogeneous subgroups.

Stata version 14.1 software was used for the statistical analyses.

The study was approved by the research ethics committees of the Pan American Health Organization (PAHO-2015-12-0075) and Fiocruz Pernambuco (CAAE: 51849215.9.0000.5190). All mothers provided written informed consent.

Role of the funding source: The funders of the study were involved in data interpretation and writing of the report. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.
Results

The flowchart describes the screened cases and controls. There were 110 eligible cases: 92 livebirths and 18 stillbirths. Ten livebirths were excluded. Among the 18 eligible stillbirths, nine were excluded. Of the 189 eligible controls, there were 16 exclusions: ten due to the presence of cerebral anomalies (one ventriculomegaly, one with calcification, two with hydrocephaly and six with other abnormalities) and six refusals. Our final analyses included 91 cases (82 livebirths and nine stillbirths) of microcephaly and 173 controls.

Cases were female, SGA, and premature, more frequently than controls. Approximately 29% (26/91) of cases had severe microcephaly. There were no difference in the age or schooling of the mothers of the cases and controls. Mothers of cases were slightly more likely to have serologic markers of previous Zika infection by PRNT$_{50}$ than mothers of controls with a borderline p value (p=0.051). (Table 1). All mothers of cases and controls tested RT-PCR negative for ZIKV.

One-third of cases were ZIKV laboratory positive; confirmation of congenital infection by qRT-PCR or specific anti-Zika IgM in CSF was more frequent than in serum, and more cases were confirmed by qRT-PCR than by IgM. (Table 2) There was good agreement between Zika IgM positivity in CSF and in serum (K 0·94, 95% CI 0·82-1·00). Of 28 negative PRNT$_{50}$ mothers of cases, six had a Zika IgM seropositive neonate, and five had a neonate with major cerebral abnormalities on CT.

No neonate tested IgM positive for cytomegalovirus, toxoplasmosis or rubella (data not shown). Of the nine stillbirths, seven were laboratory positive for ZIKV, and six had severe microcephaly. There were three neonatal deaths; all deaths occurred in intensive care unit; CT scan imaging was not performed. Two of the neonates who died were positive for ZIKV and had severe microcephaly; one was negative for ZIKV (data not shown).
Severe cases had a higher usage of Intensive/intermediate care unit (75% or 15/20 livebirths) than the moderate cases (52% or 32/62 livebirths). The proportion of SGA was high for cases either severe or moderate microcephaly. There was no difference between these groups considering the archaic reflexes examined by neonatologists (Suction, Moro, Babkin and neck tonic reflexes).

Zika laboratory tests and brain imaging were performed for 79 cases. Approximately 27% (21/79) of cases had major cerebral anomalies on CT. Among ZIKV-positive cases, 43% (10/23) had major cerebral abnormalities on CT, compared to 20% (11/56) of cases who tested negative (p=0.029) (Table 3). Among the severe cases 67% (12/18) were ZIKV-positive; and 58% (7/12) of them had cerebral anomalies. Of the six severe cases ZIKV-negative three had cerebral anomalies. Considering the moderate cases, 18% (11/61) were ZIKV-positive; among these 27% (3/11) had cerebral anomalies. 16% (8/50) of the moderate cases ZIKV-negative had cerebral anomalies.

When the anthropometric parameters were compared by case categories (lab(-)/img(-); lab(+)/img(-), lab(-)/img(+); lab(+)/img(+)) and controls, the only difference was between the controls and the four categories of cases (ANOVA; p<0.001 for all comparisons; Bonferroni pos hoc test discriminated the controls from cases categories). Cases categories were homogeneous. Specifically, cases with laboratory and imaging negative were similar to other cases categories; however they were significantly different from the control groups.

Most mothers of cases and controls lived in poverty; around half were classified in the two bottom levels of the socioeconomic scale. Only two of the 18 investigated factors (other than Zika) were associated with microcephaly (p<0.05) in the conditional analysis: smoking (OR 3.2; 95% CI 1.5-7.0; p=0.004); being non white (OR 0.3, 95% CI 0.1-0.7; p=0.01), having received Tdap in pregnancy was at borderline level (OR 0.6, 95% CI 0.3-1.0; p=0.06) (Table 4). Only two mothers of controls and no mothers of cases reported having taken misoprostol during pregnancy. There was no increase in
the risk of microcephaly with MMR (measles/rubella/mumps) or MR (measles/rubella) vaccines.

We further explored the association of reported smoking and skin colour with economic class. Smoking was more common among the poorest classes in both cases and controls: 2.4% (1/41) in B2-C1, 3.4% (3/88) in C2, 19.3% (26/135) in D-E. The proportion of reported smoking during pregnancy in the D-E category was higher among cases (29.9%; 15/52) than in controls (13.3%; 11/83) (p=0.044). Skin colour was not associated with economic class (p=0.51). We also explored the association of SGA and mothers’ reported smoking in pregnancy. Among all neonates whose mothers smoked 57% were SGA, versus 27% neonates born from non-smokers mothers. However, among the SGA cases only 22% (15/69) were born from a smoker mother.

In our study, smoking was a potential confounder for the association between congenital ZIKV infection and microcephaly, as it was associated with Zika congenital infection (p=0.046) and microcephaly (p<0.01). The association between Zika congenital infection and microcephaly remained when adjusted for smoking.

The matched association between microcephaly and laboratory confirmation of ZIKV infection was extremely strong (mOR 87); no controls had laboratory-confirmed ZIKV infection. The association remained strong (mOR 73.1) and significant when adjusted by confounders (smoking during pregnancy, skin colour, and receiving Tdap during pregnancy). When controlling for laboratory confirmation of Zika, the association between microcephaly and smoking, being non white and having received Tdap vaccine lost significance (p-values between 0.07 and 0.10). By subgroups these associations were as following: severe cases (mOR 52.4); less severe cases (mOR 33.7). (Table 5)
Discussion

The association between microcephaly and Zika laboratory confirmation by qRT-PCR and/or IgM was strong after controlling for confounders. The association was strong with severe and non-severe microcephaly. None of the other risk factors investigated (other than Zika) was associated with microcephaly in multivariable analysis; these factors include use of the larvicide pyriproxyfen and vaccine administration during pregnancy. We confirm our preliminary analysis that the increase in microcephaly prevalence at birth in the Northeast of Brazil was caused by congenital Zika-infection.\textsuperscript{13}

The proportion of cases with laboratory confirmation was similar to the published in the preliminary results.\textsuperscript{13} Even increasing the number of controls from 62 to 173, none was positive. The magnitude of the mOR remained extremely strong and asymptotically infinite. The mOR point estimate was higher in the final analysis (mOR 87) since the fact that with increased numbers decreased the probability of having missed a positive control due to the sample size. Our study found a high proportion of SGA among the cases, which was also found in a cohort of ZIKV-infected pregnant women in Brazil.\textsuperscript{10}

Consistent with the preliminary analysis, 43.5\% (10/23) of Zika laboratory confirmed cases had major cerebral abnormalities on CT; 47.6\% (10/21) of cases with these abnormalities had laboratory confirmation. The descriptions of children with microcephaly during the early days of the epidemic reported all cases had cerebral lesions by radiologic imaging, but this result may be due to the abnormal imaging criteria being an inclusion criteria in the first case series.\textsuperscript{8,21} Although one typical phenotype of Zika microcephaly has been described,\textsuperscript{22} not all cases of CZS with microcephaly will have that phenotype, and the spectrum of CZS is not restricted to microcephaly.\textsuperscript{10,11,23} An early description of the spectrum of abnormalities found cases with microcephaly with normal imaging and abnormal imaging without microcephaly.\textsuperscript{10} An important finding is that microcephaly with congenital ZIKV
syndrome can be present with normal brain imaging and that cases with typical brain anomalies can be laboratory negative. The low proportion of neonates with laboratory confirmation is not surprising: Zika qRT-PCR is very specific but is less sensitive than IgM, especially if the virus has disappeared from the serum at the end of pregnancy. The duration of persistence of IgM is unknown and might also disappear at birth.\textsuperscript{24}

Our findings showed a higher ZIKV-positivity in CSF then in serum (both qRT-PCR and IgM); this is however no longer recommended, unless there is a specific clinical indication. The good agreement between ZIKV-positive IgM in CSF and serum suggests IgM in serum as an alternative. Positive qRT-PCR in neonates is consistent either with infection late in pregnancy or the virus persisting longer in CSF than in postnatal serum: this is consistent with other evidences suggesting that ZIKV might persist longer in CSF.\textsuperscript{25}

As for laboratory results in mothers, in the context of this study, the time of infection in pregnancy was not known and mothers were tested only after birth, when levels of IgM might have disappeared, so a negative IgM does not exclude maternal infection. Similarly, a negative PCR result cannot exclude infection as it may be due to the short period of virus production and to the low number of viral genomic copies present in bodily fluids.

The timing of the maternal infection indicated by neutralization cannot be identified in a case-control study, as a positive test at delivery does not discriminate whether women were infected before or after they became pregnant. The information is however useful as the presence of typical CZS microcephaly in neonates of mothers with negative PRNT\textsubscript{50} shows limitation of maternal serology.

Our study confirmed the ZIKV PRNT\textsubscript{50} seropositivity (57\%) among mothers of controls—which represents the population—indicating that by December 2016 a large part of the population of Recife (at least of that age group) had had ZIKV-infection. Similar frequencies were observed in Yap Island,\textsuperscript{26} and in the French Polynesia after their outbreaks of ZIKV.\textsuperscript{27} During the study period, the prevalence of microcephaly at birth
among the screened neonates born in the maternities where the study was conducted was 74/10.000 birth (CI-95%: 60 - 90).

At the beginning of the microcephaly epidemic, hypotheses were raised that the microcephaly cases were due to the use of pyriproxyfen, and vaccine administration during pregnancy (this epidemic followed the introduction of Tdap to pregnant women). The hypothesis on pyriproxifen was based on the scarcity of human toxicity data and on its addition to water domestic reservoirs for vector control, in areas of water shortage. Our results provide evidence rejecting both hypotheses, confirming the findings of an ecological study of Pyriproxyfen in the Pernambuco State, and previous studies on the safety of Tdap vaccine administration during pregnancy.

The similarity in socioeconomic conditions between the cases and controls is not surprising because they were matched by area of residence and only women delivering in the public health system were included. Most of mothers were self-classified as non-white and were in the lower levels of the socio-economic scale. Probably because of these restrictions, in our data skin color was not associated with socioeconomic conditions, although this association is well documented in Brazil. Areas of low socioeconomic conditions suffer more environmental degradation and favorable conditions for mosquito breeding and, consequently, transmission of vector-borne infections. Being non-white was associated with microcephaly in the initial stage of the analysis, but lost significance when adjusted for Zika laboratory positivity and other co-variables.

In our study smoking was a potential confounder for the association between congenital ZIKV-infection and microcephaly. It is well know that smoking causes adverse perinatal outcomes, including SGA and other birth defects, none related to microcephaly. However, among the SGA cases less than a quarter were born from a smoker mother, pointing to other physio-pathogenic mechanism such as placental dysfunction caused by congenital ZIKV-infection.
This study has limitations. Cases were neonates with microcephaly and, therefore, the conclusions are not generalizable to the full spectrum of CZS. In addition, few cases that would have been born with microcephaly in the absence of a Zika epidemic would have been recruited in the study. For ethical reasons, CSF was collected of cases but not of controls. If CSF of some controls were positive for ZIKV-infection the strength of the association would have decreased.

We used CT scan to investigate the presence of cerebral abnormalities among cases, which may be a limitation since MRI has a higher resolution to detect minimal anomalies in gyration and myelination. However, both CT and MRI are considered sufficient to identify major typical radiological features of CZS. Some cases were laboratory negative and without detectable brain abnormalities and may be either neonates with mild Zika-associated congenital disease or normal newborns who fall into the <-2 SD category. Although the anthropometric characteristics of this subgroup were more similar to the other cases than to the controls, only longitudinal monitoring of these neonates will identify whether they will develop clinical manifestations compatible with congenital Zika-infection.

Information on exposures during the gestational period were reported by the mothers and, therefore, may be subject to recall bias. Ongoing cohorts of pregnant women will be able to assess properly the timing of the onset of ZIKV-infection and whether cofactors increase the risk of microcephaly and to describe the full spectrum of the adverse outcome of pregnancy.

The recruitment of neonates and the collection of samples at birth in our study ensured that laboratory confirmation resulted from intrauterine ZIKV-infection, rather than postnatal. We used the best available assays for recent ZIKV-infection, however at birth, neonates and mothers may not have detectable viral RNA or IgM antibodies.
In conclusion, this is the first case-control study to confirm the association between congenital ZIKV-infection and microcephaly and to show no association between Microcephaly and with exposure to pyriproxyfen or vaccine intake during pregnancy.

**Contributors**

TVBA, CMTM, LCR, RAAX, and DBM-F participated in all phases of the study. All other authors participated in data interpretation and critical revision of the manuscript. All authors approved the final version and agree to be accountable for all aspects of the work.

**Declaration of interests**

We declare no competing interests.

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Table 1: Characteristics of mothers and neonates
Table 2: Cases by laboratory confirmation with qRT-PCR or Zika-specific IgM in cerebrospinal fluid, serum samples or tissue macerate
Table 3: Cases by laboratory confirmation of Zika infection (qRT-PCR or specific IgM) and brain imaging findings
Table 4: Association between microcephaly and investigated co-factors
Table 5: Association between microcephaly and laboratory confirmation of Zika virus infection
Figure 1: Flow chart of participants of the case-control study of microcephaly and Zika virus infection in pregnancy. Pernambuco, Brazil, 2016

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