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

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## Summary

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

**Methods** **We did a case-control study in eight public maternity hospitals in Recife, Brazil. Cases were neonates born with microcephaly, defined as a head circumference of 2 SD below the mean. Two controls without microcephaly were matched to each case by expected date of delivery and area of residence. We tested the serum of cases and controls and the CSF of cases for detection of Zika virus genomes with quantitative RT-PCR and for detection of IgM antibodies with capture-IgM ELISA. We also tested maternal serum with plaque reduction neutralisation assays for Zika and dengue viruses. We estimated matched crude and adjusted odds ratios with exact conditional logistic regression to determine the association between microcephaly and Zika virus infection.**

**Findings** **We screened neonates born between Jan 15 and Nov 30, 2016, and prospectively recruited 91 cases and 173 controls. In 32 (35%) cases, congenital Zika virus infection was confirmed by laboratory tests and no controls had confirmed Zika virus infections. 69 (83%) of 83 cases with known birthweight were small for gestational age, compared with eight (5%) of 173 controls. The overall matched odds ratio was 73·1 (95% CI 13·0–∞) for microcephaly and Zika virus infection after adjustments. Neither vaccination during pregnancy or use of the larvicide pyriproxyfen was associated with microcephaly. Results of laboratory tests for Zika virus and brain imaging results were available for 79 (87%) cases; within these cases, ten were positive for Zika virus and had cerebral abnormalities, 13 were positive for Zika infection but had no cerebral abnormalities, and 11 were negative for Zika virus but had cerebral abnormalities.**

**Interpretation** **The association between microcephaly and congenital Zika virus infection was confirmed. We provide evidence of the absence of an effect of other potential factors, such as exposure to pyriproxyfen or vaccines (tetanus, diphtheria, and acellular pertussis, measles and rubella, or measles, mumps, and rubella) during pregnancy, confirming the findings of an ecological study of pyriproxyfen in Pernambuco and previous studies on the safety of Tdap vaccine administration during pregnancy.**

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## Introduction

In August, 2015, physicians reported a cluster of cases of microcephaly in the state of Pernambuco, northeast Brazil. Microcephaly is an abnormality in birth that was rarely reported before the Zika virus epidemic.[1] Microcephaly is a clinical sign that can reflect abnormal brain development, but it can be also be found in healthy neonates. By definition, microcephaly is any insult that disturbs early brain growth, and it can be caused by genetic variations, teratogenic compounds, or other congenital infections (such as cytomegalovirus, rubella, herpes, or toxoplasmosis).[2]

At the start of this microcephaly epidemic, the main causal hypothesis was Zika virus infection during pregnancy,[3] but other possible causes were proposed; two of these causes were of particular interest because of their potential implications. The first of these possible causes was larvicide use in reservoirs of drinking water to control *Aedes aegypti*, namely pyriproxyfen, which was introduced in 2014 by the Brazilian Ministry of Health).[4]

## Research in context

### Evidence before this study

We searched PubMed and LILACS using the search terms "Zika" and "case-control study". We searched for articles published up to Sept 30, 2017, including publications in English, Portuguese, and Spanish. The causal link between Zika virus infection and microcephaly, as part of the congenital Zika virus syndrome, is now well established; however, we did not identify any case-control studies of Zika virus infection and microcephaly. The final piece of the puzzle, providing epidemiological evidence, was the preliminary finding of a strong association in a case-control study of Zika virus infection and microcephaly in Recife, Pernambuco (Brazil), the hotspot of the microcephaly epidemic. Other risk factors have been suggested but never investigated at individual level, the more crucial being vaccines during pregnancy and use of the larvicide pyroxifen in containers of drinking water for mosquito control.

### Added value of this study

This is the final report of a case-control study, with a much larger sample size than a preliminary analysis of a subset of these data. This analysis supports the strength of association with Zika virus and, for the first time, investigates other potential risk factors including use of larvicides and vaccination during pregnancy. We confirm the strong association between Zika virus infection and microcephaly at birth and provide evidence that use of larvicides and vaccines during pregnancy did not increase the risk of microcephaly. We also provide information regarding all cases of microcephaly born during the study period: about half had either laboratory confirmation of Zika virus or typical brain image abnormalities. No controls had laboratory-confirmed Zika virus infection. There was some association between laboratory-confirmed Zika virus infection and cerebral abnormalities; 60% of those with brain abnormalities were negative for Zika virus when tested with specific IgM and PCR, and about half of those who were Zika virus-positive had no cerebral abnormalities. A high proportion of cases of microcephaly were small for gestational age. The high prevalence of serological markers of Zika virus infection in the mothers of controls indicate a high transmission of infection in the study area.

### Implications of all the available evidence

This study supports the magnitude of risk of microcephaly associated with congenital Zika virus infection; provides evidence that neither larvicide or vaccinations during pregnancy caused the epidemic; highlights that neither a negative laboratory result for Zika virus nor an absence of cerebral abnormalities alone are sufficient to discard Zika virus as a cause of individual cases of microcephaly.

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See **Online** for appendix

The other possible cause of interest was vaccine administration during pregnancy.[5,6]

Microcephaly was the first postnatal clinical finding to be reported at the beginning of the epidemic.[7–9] However, rapidly accumulating evidence showed that congenital Zika syndrome could cause more than isolated microcephaly.[10–12] In the early months of the marked increase in the prevalence of microcephaly, we designed a case-control study[13] to investigate an association between microcephaly and congenital Zika virus infection and other potential causes. The previously published preliminary report[13] documented a strong association with Zika virus; we now report the final results, with the aim of assessing the association between microcephaly and congenital Zika virus infection, along with a comprehensive investigation of other potential risk factors in an epidemic context in Pernambuco, Brazil.

## Methods

### Study design and participants

We present the final analysis of our case-control study of neonates who were consecutively recruited at birth. The protocol can be accessed online.

The study population consisted of neonates born from women residing in Pernambuco, Brazil, and delivered in eight public maternity hospitals in Recife. Cases—neonates with microcephaly (livebirths or stillbirths)—had head circumferences at least 2 SD smaller than the mean for their sex and gestational age on the Fenton growth chart.[14] Microcephaly was considered severe when the head circumference was at least 3 SD 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 (determined from transfontanellar ultrasonography) and no major birth defects, determined from 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 0800 h on the morning after the birth of a case in one of the study hospitals, where a trained nurse stayed 7 days a week (from 0800 h to 1700 h) and listed the women who were 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

For the **study protocol** see http://www.cpqam.fiocruz.br/merg

cases born between 34 and 36 weeks' gestation, controls were the next eligible neonates born at 34–36 weeks' gestation.

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.

## Procedures

We estimated gestational age by antenatal fetal ultrasonography. If ultrasounds were not available, we used the date of the last menstrual period recorded on the antenatal care card or reported by the mother. When both ultrasounds and the date of the last menstrual period were not available, we used the Capurro method.[15]

Head circumference was measured in the delivery room with a non-stretch Teflon tape; a second measurement was done 12–24 h after birth to confirm microcephaly by the study neonatologists. At this second measurement, the neonates had a complete clinical examination by the study neonatologist, which included the assessment of reflexes. CSF was collected from cases around 48 hours after birth (but longer in infants who were in an intensive care unit). 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 (Recife, Brazil).

Serum samples of mothers and neonates (cases and controls) and CSF samples (cases) were tested by qRT-PCR for detection of the Zika virus genome,[16] and by capture-IgM ELISA for IgM antibodies.[16,17] Macerated tissues (from the brain, kidney, or pooled organs) of stillbirth cases were tested by qRT-PCR. The presence of Zika virus and dengue virus (1–4)-specific neutralising antibodies was assessed in the serum samples of mothers and neonates (cases and controls) by the plaque reduction neutralisation test ($PRNT_{50}$),[13] with a 50% cutoff value for positivity. Serum samples were tested for toxoplasmosis, rubella, and cytomegalovirus IgM antibodies, the main infectious causes of congenital microcephaly.[7]

In cases, brain imaging was done by CT scan and was classified as the presence or absence of major cerebral abnormalities, identified by physicians who were specialised in imaging diagnosis. Abnormalities included calcification, ventriculomegaly, malformation of cortical development (such as lissencephaly and polymicrogyria), and presumed vascular abnormalities. Controls were investigated by transfontanellar ultrasonography. Mothers were interviewed with a standardised questionnaire to determine several demographic and socioeconomic factors.

Laboratory-confirmed Zika virus infection was defined in a neonate as a positive qRT-PCR or an IgM result for Zika virus in any biological specimen (serum, CSF, or macerated tissues). Neonates were considered to be small for gestational age if their birthweight was lower than the teth percentile for gestational age and sex on the Fenton growth chart.[14]

Information that was recorded on demographic and socioeconomic factors included mother's age, number of years of schooling, and skin colour (self-reported). The purchasing power of individuals and families was defined by use of the Brazilian economic classification criteria[18] of 2015, which defines eight socioeconomic classes from A (highest) to E (lowest). We also collected data on the family history of microcephaly or malformations; vaccination status of the mother; self-reported ingestion

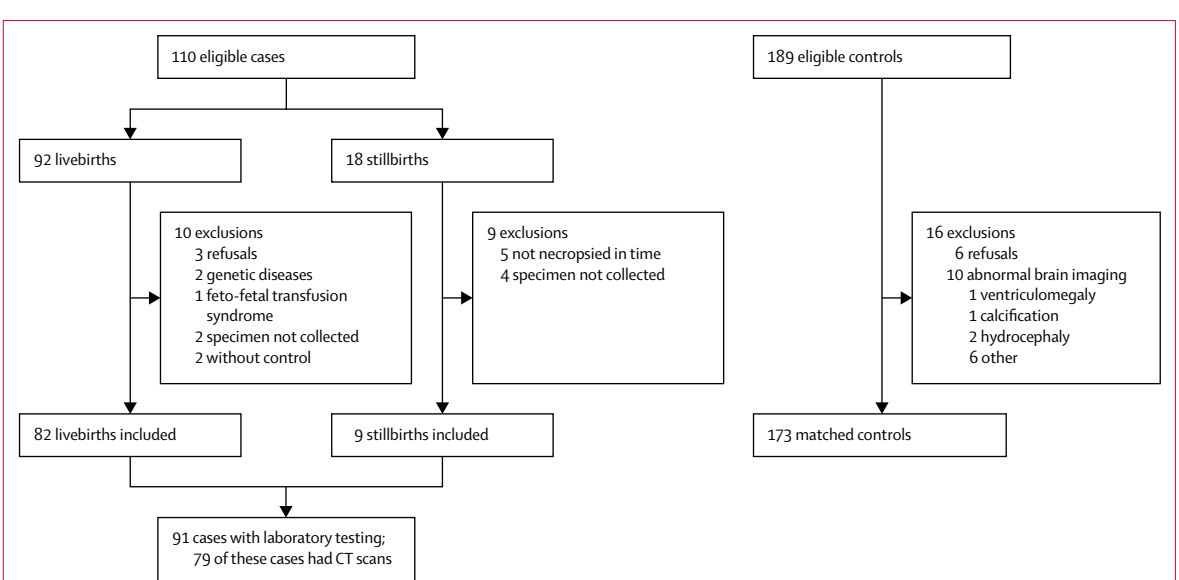


*Figure:* **Study profile**

of misoprostol (medical abortion pill), epilepsy medication, or folic acid; maternal use of recreational drugs, tobacco, and alcohol during 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 each potential risk factor by conditional logistic regression. We included the variables associated with microcephaly with a p value less than or equal to 0·10 in the multivariable analysis by use of a conditional exact logistic regression model. Thus, we calculated matched odds ratio (mOR) for the association between microcephaly (outcome) and Zika virus infection (exposure), adjusted by smoking during pregnancy, skin colour, and having received the tetanus, diphtheria, and acellular pertussis vaccine (Tdap) during pregnancy.

We applied a median unbiased estimator for binary data in exact conditional logistic regression to control for the fact that all controls tested negative for Zika virus.[19] The model respected matching and included other conditioning variables: "condvars".[20] The study originally aimed to include 200 cases and 400 controls to have 90% power and 95% precision to detect an association with an odds ratio of 2 or greater, assuming that 67% of cases were exposed.

We estimated the crude mOR and 95% CI for the association between microcephaly and Zika virus infection for all cases, considering the results in any specimen (serum or CSF for livebirths, or macerated tissues for stillbirths). Additionally, crude mORs were independently estimated by sample type (serum or CSF) and microcephaly severity. We also investigated the agreement between qRT-PCR Zika virus-positivity in serum and CSF and between the IgM positivity in serum and CSF.

We also compared the means of anthropometric variables (head circumference, weight, height, and *Z* score–weight for gestational age and sex) in four categories of cases. These categories were negative for Zika virus (laboratory tested) and negative for cerebral abnormalities (determined by CT imaging of the neonates' brains); positive for Zika virus and negative for cerebral abnormalities; negative for Zika virus and positive for cerebral abnormalities; and positive for Zika virus and positive for cerebral abnormalities. These anthropometric variables were also recorded in controls, and these groups were compared with analyses of variance and Bonferroni post-hoc test to identify homogeneous subgroups. A $\chi^2$ test was used to compare the characteristics of mothers and neonates, the frequency of abnormalities between neonates who were positive and negative for Zika virus, and smoking between mothers from different socioeconomic classes. Stata (version 14.1) software was used for the statistical analyses.

| | Cases (n=91) | Controls (n=173) | p value |
|---|---|---|---|
| **Mothers** | | | |
| Age, years | .. | .. | 0·11 |
| 13–24 | 44 (48%) | 95 (55%) | .. |
| 25–34 | 29 (32%) | 60 (35%) | .. |
| ≥35 | 18 (20%) | 18 (10%) | .. |
| Number of years in education | .. | .. | 0·13 |
| ≤4 | 17 (19%) | 20 (12%) | .. |
| 5–9 | 36 (40%) | 60 (35%) | .. |
| 10–12 | 33 (36%) | 87 (50%) | .. |
| ≥13, higher education | 5 (5%) | 6 (3%) | .. |
| Reported rash during pregnancy | .. | .. | 0·10 |
| No rash | 66 (73%) | 139 (80%) | .. |
| First trimester | 7 (8%) | 10 (6%) | .. |
| Second trimester | 13 (14%) | 10 (6%) | .. |
| Third trimester | 5 (5%) | 14 (8%) | .. |
| $PRNT_{50}$ result | .. | .. | 0·051 |
| Zika virus-positive | 62 (70%) | 99 (57%) | .. |
| Zika virus-negative | 27 (30%) | 74 (43%) | .. |
| Testing not done | 2 | 0 | .. |
| **Neonates** | | | |
| Sex | .. | .. | <0·0001 |
| Girls | 61 (67%) | 84 (49%) | .. |
| Boys | 29 (32%) | 89 (51%) | .. |
| Intersex | 1 (1%) | 0 | .. |
| Gestational age | .. | .. | <0·0001 |
| Term (≥37 weeks) | 66 (73%) | 153 (88%) | .. |
| Premature (≤36 weeks) | 25 (27%) | 20 (12%) | .. |
| Birthweight, g | .. | .. | <0·0001 |
| ≥2500 | 21 (23%) | 159 (92%) | .. |
| 1500–2499 | 52 (57%) | 14 (8%) | .. |
| <1500 | 18 (20%) | 0 | .. |
| Weight for gestational age | .. | .. | < 0·0001 |
| Normal | 14 (17%) | 165 (95%) | .. |
| Small for gestational age | 69 (83%) | 8 (5%) | .. |
| Not available* | 8 | 0 | .. |

Data are n (%). $PRNT_{50}$=plaque reduction neutralisation test. *Not available in eight stillbirths.

***Table 1:*** **Characteristics of mothers and neonates**

### 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 preliminary analysis included participants recruited from Jan 15 to May 2, 2016;[13] this Article includes participants recruited up to Nov 30, 2016. We did this analysis before reaching 200 cases for two reasons: first,

we reached the necessary power for statistical analysis because the proportion of controls who were exposed to Zika virus was lower than expected (as evidenced by the absence of Zika virus infection in all controls); second, the epidemic slowed down in Recife and cases became rarer. We screened 110 eligible cases, which included 92 livebirths and 18 stillbirths (figure). Our final analyses included 91 cases (82 livebirths and nine stillbirths) of microcephaly and 173 controls. We initially screened 13 624 neonates (13 531 livebirths and 93 stillbirths) from the study maternity hospitals during the study period; the prevalence of microcephaly at birth was estimated to be 101 (91 cases included in the study and ten excluded livebirths), resulting in an estimated prevalence of 74 cases of microcephaly per 10 000 births (95% CI 60–90).

Cases were more likely to be female, small for gestational age, and premature than controls (table 1). 26 (29%) of 91 cases had severe microcephaly. There were no significant differences in the age or number of years that the mothers of the cases and controls had spent in education. Mothers of cases were slightly more likely to have serological markers of previous Zika virus infection (judged by $PRNT_{50}$) than mothers of controls, with a borderline p value (p=0·051). All mothers of cases and controls tested negative for Zika virus by qRT-PCR testing.

Approximately a third of cases were positive for Zika virus infection (32 [35%] of 91 cases); confirmation of congenital infection by qRT-PCR or anti-Zika virus IgM ELISA was more frequent in CSF than in serum samples, and more cases were confirmed to have a Zika virus-positive result by qRT-PCR than by capture-IgM ELISA (table 2). There was good agreement between Zika virus IgM positivity in CSF and in serum (OR 0·94, 95% CI 0·82–1·00). Of 27 $PRNT_{50}$-negative mothers of cases, six had a neonate who was seropositive for Zika virus IgM, and five others had a neonate with major cerebral abnormalities on CT; none of these neonates was stillborn.

No neonate tested IgM positive for cytomegalovirus, toxoplasmosis, or rubella (data not shown). Of the nine stillbirths, seven were positive for Zika virus, and five had severe microcephaly. There were three neonatal deaths; all deaths occurred in the intensive care unit and CT scan imaging was not done for these neonates or the stillbirths. However, two of the neonates who died were positive for Zika virus and had severe microcephaly, and the other was negative for Zika virus (data not shown).

Cases with severe microcephaly had a higher usage of intensive or intermediate care units (15 [75%] of 20 livebirths) than did the cases with moderate microcephaly (32 [52%] of 62 livebirths). The proportion of neonates who were small for gestational age was high for cases with severe or moderate microcephaly. 69 (83%) of 83 cases with known birthweight were small for gestational age, compared with eight (5%) of 173 controls. Archaic reflexes did not differ between groups when examined by neonatologists (who assessed suction, Moro, Babkin, and neck tonic reflexes).

Laboratory tests for Zika virus and brain imaging for cerebral abnormalities were done for 79 cases. 21 (27%) of 79 cases had major cerebral anomalies on CT (table 3). Ten (43%) of 23 cases that were positive for Zika virus had major cerebral abnormalities on CT, compared with 11 (20%) of the 56 cases who tested negative for Zika virus (p=0·029; $\chi^2$ test comparing the frequency of abnormalities between neonates who were positive and negative for Zika virus). Among the 26 cases with severe microcephaly, 19 (73%) were Zika virus-positive and seven (37%) of these 19 cases had cerebral anomalies. Seven of these 19 cases who were Zika virus-positive did not have CT imaging done (five were stillborn and two died as neonates). Of the seven severe cases who were Zika virus-negative, three had cerebral anomalies and one did not have CT imaging done. In the moderate cases, 13 (20%) of 65 were Zika virus-positive and, of these cases, three (23%) of 13 had cerebral anomalies. Eight (15%) of the 52 moderate cases who were Zika virus-negative had cerebral anomalies. Four of these 52 moderate cases did not have CT imaging done, of which two cases were Zika virus-negative (one was stillborn, one died as a neonate) and two cases were Zika virus-positive and were stillborn.

When we compared the anthropometric variables between case categories (positive or negative for Zika virus and for cerebral abnormalities) and against controls, the only difference in anthropometric variables was found between the controls and the four categories of cases (p<0·0001 for all comparisons

| | qRT-PCR | Zika virus-specific IgM | Either test |
|---|---|---|---|
| CSF | 17/70 (24%) | 10/70 (14%) | 25/71 (35%) |
| Serum | 1/78 (1%) | 9/79 (11%) | 10/79 (13%) |
| Tissue macerate (stillbirth) | 7/9 (78%) | .. | .. |
| Any specimen | .. | .. | 32/91 (35%) |

Data are number of positive tests/total number of tests (%), assessed by qRT-PCR or ELISA for Zika-specific IgM. Cerebrospinal fluid, or serum, or both were not collected for nine stillbirths (in which tissue macerate was collected instead), two of the three neonatal deaths, and in 11 cases for other reasons.

***Table 2:*** **Proportion of cases with laboratory confirmation of Zika virus infection**

| | Positive for Zika virus infection (n=23) | Negative for Zika virus infection (n=56) | Total | p value |
|---|---|---|---|---|
| Abnormalities present | 10* (43%) | 11† (20%) | 21 (27%) | 0·029‡ |
| Abnormalities absent | 13 (57%) | 45 (80%) | 58 (73%) | .. |

Data are n (%). *Five cases with calcification and ventriculomegaly; one case with calcification, ventriculomegaly, and malformation of cortical development; one case with ventriculomegaly; one case with calcification and malformation of cortical development; one case with malformation of cortical development; and one case with vascular abnormality. †One case with calcification; two cases with ventriculomegaly; one case with ventriculomegaly and vascular abnormality; one case with ventriculomegaly and malformation of cortical development; two cases with malformation of cortical development; and four cases with vascular abnormality. ‡$\chi^2$=4·74; comparing frequency of abnormalities between cases who were positive and negative for Zika virus.

***Table 3:*** **Association of cerebral abnormalities with Zika virus infection in cases**

with an analysis of variance; a Bonferroni post-hoc test identified the controls as different from the case categories). The case categories were not significantly different. Specifically, cases that were Zika-negative and showed no cerebral abnormalities were similar to the other case categories but significantly differed from the control groups.

Most mothers of cases and controls lived in poverty; around half were classified in the two lowest levels of the socioeconomic scale (table 4). Only two of the 18 investigated factors (other than Zika virus infection) were associated with microcephaly (p<0·05) in the conditional analysis: smoking (OR 3·2, 95% CI 1·5–7·0; p=0·004) and having skin colour that was not white (3·5, 1·3–9·5; p=0·013). The association between microcephaly and Tdap vaccination in pregnancy was at borderline level (0·6 [0·3–1·0]; p=0·06). 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 the measles, mumps, and rubella or measles and rubella vaccines.

The matched association between microcephaly and Zika virus infection was extremely strong (mOR 87·0, 95% CI 15·6–∞); no controls had laboratory-confirmed Zika virus infection (table 5). The association remained strong (73·1, 13·0–∞) 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, having a skin colour that was not white, and having received Tdap vaccine lost significance (p values between 0·07 and 0·10). By subgroups, these associations were mOR 52·4 for severe cases and mOR 33·7 for less severe cases.

We further investigated the association of self-reported smoking and skin colour with economic class. Smoking during pregnancy was more common among the poorest classes in both cases and controls: one (2%) of 41 women in B2–C1, three (3%) of 88 women in C2, and 26 (19%) of 135 women in D–E reported smoking during pregnancy ($\chi^2$=17·3; p=0·0002). The proportion of mothers who reported smoking during pregnancy in the D–E category was higher for cases (15 [29%] of 52) than for controls (11 [13%] of 83; p=0·044). Skin colour was not associated with economic class (p=0·51). We also explored the association of small for gestational age and mothers' reported smoking in pregnancy. Among all 30 neonates whose mothers smoked, 17 (57%) were small for gestational age, compared with 63 (27%) of 234 neonates born from mothers who did not smoke. However, among the small for gestational age cases, only 15 (22%) of 69 had a mother who smoked.

In our study, smoking was a potential confounder for the association between congenital Zika virus infection and microcephaly, since smoking was associated with Zika virus congenital infection (p=0·046) and microcephaly (p<0·004). The association between congenital Zika virus infection and microcephaly remained when adjusted for smoking.

| | Case (n=91)* | Control (n=173)* | Odds ratio (95% CI) | p value |
|---|---|---|---|---|
| Mother is not white | 84 (92%) | 141 (82%) | 3·5 (1·3–9·5) | 0·013 |
| Family per-capita income, US$ | | | | |
| ≤56·0 | 24 (26%) | 43 (25%) | 1·0 | (Ref) |
| 56·0–96·9 | 22 (24%) | 39 (23%) | 1·0 (0·5–2·1) | 0·97 |
| 97·0–168·6 | 19 (21%) | 43 (25%) | 0·8 (0·4–1·7) | 0·63 |
| >168·6 | 19 (21%) | 44 (25%) | 0·8 (0·4–1·6) | 0·47 |
| Unknown | 7 (8%) | 4 (2%) | .. | .. |
| Economic class (ABEP)[18] | | | | |
| D–E | 52 (57%) | 83 (48%) | 1·0 | (Ref) |
| C2 | 28 (31%) | 60 (35%) | 0·7 (0·4–1·3) | 0·26 |
| B2–C1 | 11 (12%) | 30 (17%) | 0·6 (0·3–1·3) | 0·17 |
| Siblings with malformation (including microcephaly) | | | | |
| No | 53 (96%) | 89 (98%) | 1·0 | (Ref) |
| Yes | 2 (4%) | 2 (2%) | 1·7 (0·2–16·4) | 0·62 |
| Had no siblings | 36 | 82 | .. | .. |
| Familial history of microcephaly or other malformation | 26 (29%) | 49 (28%) | 1·0 (0·6–1·8) | 0·96 |
| Maternal use of folic acid in pregnancy (self-reported) | | | | |
| Yes, regularly | 56 (63%) | 120 (69%) | 1·0 | (Ref) |
| Yes, occasionally | 15 (17%) | 18 (10%) | 1·7 (0·8–3·4) | 0·18 |
| No | 18 (20%) | 35 (20%) | 1·1 (0·6–2·1) | 0·79 |
| Unknown | 2 | 0 | .. | .. |
| Maternal use of medication for epilepsy (self-reported) | 4 (4%) | 9 (5%) | 0·8 (0·3–2·5) | 0·77 |
| Vaccinated | | | | |
| Tetanus, diphtheria, and acellular pertussis | 45 (57%) | 107 (70%) | 0·6 (0·3–1·0) | 0·058 |
| Measles and rubella | 3 (4%) | 6 (4%) | 0·9 (0·2–3·3) | 0·90 |
| Measles, mumps, and rubella | 3 (4%) | 5 (4%) | 1·1 (0·3–5·0) | 0·88 |
| Maternal risk behaviours during pregnancy | | | | |
| Smoking | 18 (20%) | 12 (7%) | 3·2 (1·5–7·0) | 0·004 |
| Drinking alcohol | 16 (18%) | 22 (13%) | 1·6 (0·8–3·3) | 0·21 |
| Recreational drugs | 3 (3%) | 1 (1%) | 5·2 (0·5–50·3) | 0·16 |
| Exposure to other substances | | | | |
| Larvicides at the water storage site | 49 (54%) | 92 (53%) | 1·0 (0·6–1·8) | 0·89 |
| Larvicides elsewhere in the house | 14 (15%) | 22 (13%) | 1·3 (0·6–2·8) | 0·45 |
| Daily use of insect repellent on the body | 9 (10%) | 13 (8%) | 0·9 (0·4–2·0) | 0·83 |
| Occupational exposure to pesticides | 4 (4%) | 4 (2%) | 1·9 (0·5–7·7) | 0·39 |

*Data are n (%), unless otherwise indicated. Vaccination status was unknown for measles and rubella and measles, mumps, and rubella in 19 cases and 37 controls, and for tetanus, diphtheria, and acellular pertussis in 12 cases and 21 controls.

***Table 4:*** **Association between microcephaly and investigated cofactors**

## Discussion

The association between microcephaly and Zika virus infection, confirmed by qRT-PCR, capture-IgM ELISA, or both, was strong after controlling for confounders. The association was strong with severe and non-severe microcephaly. None of the other risk factors investigated was associated with microcephaly in multivariable analyses; these factors include the use of the larvicide,

| | Cases* | Controls* | Matched odds ratio (95% CI) |
|---|---|---|---|
| **Serum, CSF samples, or macerated tissue** | | | |
| Zika-positive, of total cases or controls | 32/91 (35%) | 0/173 | 87·0 (15·6–∞) |
| Zika-positive, of total cases or controls, adjusted† | .. | .. | 73·1 (13·0–∞) |
| **Cases, categorised by severity of microcephaly‡** | | | |
| Severe | 19/26 (73%) | 0/51 | 52·4 (9·1–∞) |
| Not severe | 13/65 (20%) | 0/122 | 33·7 (5·6–∞) |

*Data are the number of all cases or controls who were positive for Zika virus, assessed by qRT-PCR or Zika virus-specific IgM/total number of patients (%). †Odds ratio when adjusted by smoking during pregnancy, maternal vaccination against tetanus, diphtheria, and acellular pertussis during pregnancy, and skin colour. ‡Severe is defined as a head circumference of more than 3 SD smaller than the mean for their sex and gestational age.[10,14] Not severe was defined as a head circumference of 2–3 SD smaller than the mean for their sex and gestational age. Matched odds ratios in this subgroup are crude because of small numbers.

*Table 5:* **Association between microcephaly and Zika virus infection**

pyriproxyfen, and vaccine administration during pregnancy. These data support our preliminary findings that the increase in microcephaly prevalence at birth in northeast Brazil was caused by congenital Zika virus infection.[13]

The proportion of cases with laboratory-confirmed Zika virus infection was similar to that published in the preliminary results.[13] Even after increasing the number of controls from 62 to 173, none of these neonates was Zika virus-positive. The magnitude of the mOR remained extremely strong and asymptotically infinite. The mOR point estimate was higher in the final analysis (mOR 87·0) because increased group size decreased the probability of having missed a positive control because of the sample size. Our study found a high proportion of small for gestational age neonates among the cases, which was also found in a cohort of Zika virus-infected pregnant women in Brazil.[10]

Consistent with the preliminary analysis, ten (44%) of 23 Zika virus-positive cases had major cerebral abnormalities on CT; ten (48%) of 21 cases with these abnormalities had laboratory confirmation of Zika virus. The descriptions of children with microcephaly during the early days of the epidemic reported that all cases had cerebral lesions, as determined by radiological imaging, but this result could be due to use of abnormal imaging standards within the inclusion criteria in the first case series.[8,21] Although one typical phenotype of Zika-related microcephaly has been described,[22] not all cases of congenital Zika syndrome with microcephaly will have that phenotype, and the spectrum of congenital Zika syndrome is not restricted to microcephaly.[10,11,23] An early description of the spectrum of abnormalities found cases with microcephaly with normal cerebral structures and with cerebral abnormalities, but without microcephaly.[10] An important finding is that microcephaly with congenital Zika virus syndrome can be present with normal cerebral structures, and that cases of microcephaly with typical cerebral anomalies can be Zika-negative on laboratory tests. The low proportion of neonates with laboratory confirmation of Zika virus infection 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.[24]

Our findings showed a higher Zika virus-positivity in CSF than in serum (by both qRT-PCR and IgM); however, testing CSF is no longer recommended, unless there is a specific clinical indication. The good agreement between Zika virus-positive IgM in CSF and serum suggests tests of IgM in serum as an alternative method of analysis. Positive qRT-PCR in neonates is suggestive of infection late in pregnancy or of the virus persisting longer in CSF than in postnatal serum, which supports previous evidence suggesting that Zika virus might persist for longer in CSF.[25]

In the context of this study, the time of infection in pregnancy was not known and mothers were tested only after birth, when concentrations of IgM might have disappeared, so a negative IgM does not exclude maternal infection. Similarly, a negative PCR result cannot exclude infection because the time between viral replication and PCR testing might be too short, or due to the low viral load present in bodily fluids.

The timing of the maternal infection, indicated by neutralisation assays, cannot be identified in a case-control study, since a positive test at delivery does not show whether women were infected before or after they became pregnant. However, information about maternal infection with Zika virus is useful because the presence of typical congenital Zika syndrome microcephaly in neonates of mothers with negative $PRNT_{50}$ shows the limitation of maternal serology.

Our study confirmed Zika virus $PRNT_{50}$ seropositivity (57%) among mothers of controls (a group that represents the population) indicating that, by December, 2016, a large proportion of the population of Recife (at least, of that age group) had been infected with Zika virus. Similar frequencies were observed in Yap Island,[26] and in French Polynesia after their outbreaks of Zika virus.[27] During the study period, the prevalence of microcephaly at birth among the screened neonates born in the maternity wards where the study was done was 74 per 10 000 births (95% CI 60–90).

At the beginning of the microcephaly epidemic, hypotheses were made that the microcephaly cases were due to the use of pyriproxyfen[4] or vaccine administration during pregnancy (because this epidemic followed the introduction of Tdap to pregnant women).[28] The hypothesis on pyriproxyfen toxicity 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.[4] Our results provide evidence to reject both hypotheses, confirming the findings of an ecological study of pyriproxyfen in Pernambuco[4] and previous studies[5,6] 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 mothers self-reported as non-white and were in the lower levels of the socioeconomic scale. Probably because of these restrictions, in our data, skin colour was not associated with socioeconomic conditions, although this association is well documented in Brazil.[29] Areas of low socioeconomic conditions have more environmental degradation and favourable conditions for mosquito breeding and, consequently, transmission of vector-borne infections.[30] Being non-white was associated with microcephaly in the initial stage of the analysis, but lost significance when adjusting for Zika virus positivity and other covariables.

In our study, smoking was a potential confounder for the association between congenital Zika virus infection and microcephaly. It is well known that smoking causes adverse perinatal outcomes,[31] including small for gestational age and other birth defects, none related to microcephaly.[32] However, among the small for gestational age cases, fewer than a quarter were born from a mother who smoked, suggesting the involvement of other pathogenic physiological mechanisms, such as placental dysfunction caused by congenital Zika virus infection.[33]

This study has limitations. Cases were neonates with microcephaly, so the conclusions cannot be generalised to the full spectrum of congenital Zika syndrome. Additionally, a few cases that would have been born with microcephaly in the absence of a Zika virus epidemic would have been recruited in the study. For ethical reasons, CSF was collected from cases but not from controls. If CSF of some controls was positive for Zika virus infection, the strength of the association would have decreased.

We used CT scans to investigate the presence of cerebral abnormalities among cases, which might be a limitation since MRI has a higher resolution to detect minimal anomalies in gyration and myelination.[33] However, both CT and MRI are considered sufficient to identify major typical radiological features of congenital Zika syndrome.[34] Some cases showed negative laboratory results for Zika virus infection and had no detectable brain abnormalities and so could be either neonates with mild Zika-associated congenital disease or healthy newborn babies who had head circumferences of less than 2 SD under the mean. 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 consistent with congenital Zika virus infection.

Information on exposures during the gestational period was reported by the mothers and, therefore, might be subject to recall bias. Ongoing cohorts of pregnant women will be able to properly assess the timing of the onset of Zika virus infection, to determine whether cofactors increase the risk of microcephaly, and to describe the full spectrum of the adverse outcomes of Zika virus on pregnancy.

The recruitment of neonates and the collection of samples at birth in our study ensured that laboratory confirmation resulted from intrauterine Zika virus infection, rather than postnatal infection. We used the best available assays for recent Zika virus infection; however, at birth, neonates and mothers might not have detectable viral RNA or IgM antibodies.

In conclusion, to our knowledge, this Article is the first case-control study to confirm the association between congenital Zika virus infection and microcephaly and to suggest no association between microcephaly and exposure to pyriproxyfen or vaccine administration during pregnancy.

#### Contributors

TVBA, RAAX, CMTM, LCR, and DBM-F participated in all phases of the study. All other authors participated in data interpretation and critical revision of the report. 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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