

Summary and conclusions of Documents 1-5

This document was produced by the Latin American Collaborative Study of Congenital Malformations (ECLAMC) and seeks to summarize previous documents (1-5) analyzing microcephaly occurrences, in the ECLAMC database and Brazil, and present a conclusion regarding the reported increase in microcephaly cases in Northeastern Brazil in 2015.

ECLAMC was notified of the suspected increase in microcephaly cases in Northeastern Brazil in late October of 2015 and has since reviewed the microcephaly data in ECLAMC from 1967 to 2015 and the Live Birth Information System (SINASC) from 2013 to 2015, and revised the literature regarding all causes of microcephaly.

Some of the important points in this study were:

- The definition of microcephaly is based on a cut-off measurement of a continuous variable that is the head circumference. The ECLAMC's definition is: *congenital small head defined by a head circumference more than three standard deviations below the average in appropriated charts for sex and age. If another definition or different limits are used (e.g. 2 SD), specify the used criteria. Microcephaly associated with anencephaly or encephalocele must be excluded.* Although there is global consensus accepting microcephaly as the condition of decreased head circumference below two standard deviations of the average in the population in question and considering age and gender, the small brain size is the underlying defect, and the decreased brain growth rate must be the relevant criterion to confirm microcephaly.
- To estimate the expected number of cases of congenital microcephaly in Pernambuco and other Northeastern states is not trivial. The

SINASC database records about 1% of birth defects in live births for an expected 3%. Any active search for a specific defect will result in an increase of at least 2/3 in the number of cases usually recorded.

- Correcting by secular and seasonal trends and by the effect of hospitals with extreme prevalence rates, the ECLAMC estimate of the prevalence of congenital microcephaly at birth in Brazil is 1.98/10,000, IC 95% (1.48 to 2.27).
- There is no secular increase trend in hospitals with typical microcephaly prevalence. However, there is a seasonal variation in these hospitals, with a peak between July and September, and therefore, increased microcephaly rates are expected in this period.
- This ECLAMC estimated rate in Brazil (1.98/10,000) may be an underestimation for the Northeastern region where the prevalence of microcephaly was always higher than that of hospitals in the rest of Brazil.
- This estimated ECLAMC congenital microcephaly rate does not differ significantly from that observed by EUROCAT in Europe as 2.85 (2.69 - 3.02).
- The number of cases of congenital microcephaly observed in the northeastern states in the second half of 2015, even considering only those recorded at SINASC, exceeds the expected number in most states by more than three times.
- A total of 1,153 microcephaly cases had been reported in Pernambuco by 12/26/2015. The estimated number of cases in Pernambuco for 147,597 births in 2015 would be 45 based on the upper limit microcephaly prevalence reported by EUROCAT [2.85 (95% CL 2.69 - 3.02)]. The relative risk in Pernambuco is 25.6 times (1,153/45) over

the European baseline. Viral infection must have been present in 100% of all pregnant women to explain 1,108 microcephaly cases that would have been caused by the virus ($1153 - 45 = 1108$). If the exposure of these pregnant women was similar to that observed in women in Micronesia in 2007 (0.0179), only 353 cases would be related to this cause, and 755 cases would not have an explanation.

- This high rate resulting from exposure during any trimester of pregnancy has never been verified as due to any chemical, physical, or biological agent, which makes this correlation highly unlikely. The observed excessive number of cases is probably due to active search and over-diagnosis.
- The wide media coverage of the possible microcephaly epidemic caused by the Zika virus (ZIKV) should lead to increased number of microcephaly cases in all Brazilian states resulting from improved notification to SINASC derived from mandatory reporting of cases of microcephaly to the Epidemiological Surveillance Service.
- The current data, affected by the change of criterion determining the measurement of head circumference to suspect microcephaly do not allow to assess whether:
 1. a real increase in microcephaly prevalence at birth occurred in Northeastern Brazil
 2. what was the magnitude of this increase
 3. was this increase due to exposure to the ZIKV or increased exposure to one or more environmental causes of microcephaly (STORCH, alcohol, prematurity, diabetes, etc.).
- ECLAMC estimates chromosomal and single-gene causes of congenital microcephaly in 38%. Because of the short diagnostic period in ECLAMC, the actual proportion should be above 50% meaning that there is a genetic

diagnosis that can be confirmed through laboratory examination in half of the microcephaly cases.

The Health Surveillance Secretary of the Ministry of Health in Brazil updated the Protocol for surveillance and response to the occurrence of microcephaly related to ZIKV infection on December 9, 2015. This document states that on November 28, 2015, the Ministry of Health has recognized the relationship between infection by this virus and occurrence of microcephaly cases and correlated deaths.

In addition, this document stated that this recognition is unprecedented in the national and international literature based on the presentation of eleven evidence facts supporting the recognition of the relationship between the infection by the ZIKV and occurrence of microcephaly and deaths.

The main basis of this evidence is an increase in cases of microcephaly in Northeastern Brazil. Because to prove this increase of cases of microcephaly is difficult for several reasons, we will analyze each evidence presented:

1. Findings show that the distribution patterns of suspected cases of post-infectious microcephaly present characteristics of dispersion and do not indicate spatial concentration;
 - The mothers of microcephaly cases could have had a prenatal infection caused by other viruses. Spatial concentration would also not be expected in other causes of microcephaly such as "STORCH".
2. Findings show that the first months of pregnancy of women with children with microcephaly match the strongest period of the ZIKV circulation in the Northeast;
 - This evidence implies that there is an increase in cases. If only expected microcephaly cases were recorded and identified after a search driven by an initial Rumor, the coincidence of the first months of pregnancy with the

strongest period of virus circulation would be a consequence of the process that led to that search. This feedback mechanism of initial suspicion has always existed in other suspected birth defects epidemics in Brazil that were discarded later.

3. Findings show that after an epidemiological investigation of medical records and interviews with more than 60 pregnant women who reported rash illness during pregnancy, and whose children were born with microcephaly, there was no genetic disease history in the family and/or imaging exams suggestive of an infectious process;

- This finding also requires further explanation because the lack of specification of time, location, type of records, type of interview, and sequence of events hinders the possibility of sorting spontaneous information from induced information. What is the reference population for the 60 cases? How many children without microcephaly have been exposed to the ZIKV? The current explanation does not provide evidence of the speculated correlation because the current data could be an entirely normal outcome for the population.

4. Evidence of a change in the pattern of occurrence of microcephaly in SINASC (Live Birth Information System) presenting a clear excess in the number of cases in various parts of Northeastern Brazil;

- There is an underreporting of about 2/3 of the existing birth defects to SINASC. Any active search for congenital microcephaly triggered after the starting Rumor correlating the defect to prenatal infection by the ZIKV could increase the prevalence of microcephaly in SINASC in up to 2/3 of the cases usually recorded. However, the increase in 26 times over the estimated risk seen in Pernambuco far exceeds the record of all congenital microcephaly based on previously existing causes. The change in the diagnostic criterion for the determination of microcephaly cases based on head circumference smaller than 33 cm could perhaps explain the excessive over-diagnosis. The

confirmation of microcephaly diagnosis in survivors, exceeding the expected, could confirm an actual increase.

5. Observations of experts in various fields of medicine (infectious diseases, pediatrics, pediatric neurology, gynecology, genetics, etc.) show that there is alteration in the individual clinical pattern of these cases with compromised characteristics in the Central Nervous System, similar to those present in congenital infections by arboviruses in animals as described in the literature;

- We believe it is difficult to state that cases considered suspect of embryopathy caused by the ZIKV have a characteristic pattern; it must be demonstrated that the cases mentioned had no other known cause. Several genetic syndromes can mimic viral infections. Furthermore, the literature studies in animals with viral teratogenesis emphasize i) the lack of pathognomonic defects in each type of viral infection, and ii) that there is a great etiological heterogeneity of defects that may also have genetic causes.

6. Evidence in the literature that the ZIKV is neurotropic, demonstrated in an animal model and by the increased frequency of neurological symptoms reported in the French Polynesia and Brazil after infection by the ZIKV was confirmed in Pernambuco after the virus isolation in patients with acute neurological syndrome;

- It is likely that the ZIKV is neurotropic as other arboviruses and flaviviruses. The neurological cases that occurred in the French Polynesia are not published and difficult to understand.

7. Identification of microcephaly cases also in the French Polynesia after Brazil's notification to the World Health Organization;

- The report on cases in the French Polynesia is not a scientific publication, which would not be a major problem if it was not a report that is difficult to

understand as stated above. In addition, this report only appears after Brazil declared a state of emergency, and is, therefore, not an independent event.

The cases are not described individually. Initially, there were 18 cases that subsequently became 12, of which 9 were abortions. It is not clear how many were microcephaly cases or were related to some significant CNS malformation. The description of hydrocephalus, cerebellar hypoplasia, esophageal atresia, and akinesia signs appear in three born alive cases. However, individual descriptions are not presented, and it is unlikely that all three cases showed the same defects. They report seven cases as negative for CMV but do not clarify the denominator or how many cases were positive for CMV. Were there 11 cases positive for CMV ($18 - 7 = 11$)? A careful description of each case could clarify whether there was an increase in cases of microcephaly as suggested.

8. Findings show the correlation between the ZIKV infection and severe cases and deaths based on the identification of cases who died in different states and both with identification of ZIKV viral RNA and negative results for other known viruses such as dengue and chikungunya among others;

- This consideration does not seem to support the establishment of a relationship between maternal infection with ZIKV and microcephaly. Moreover, the related deaths would need to be quantified in order to have some meaning. Apparently, ZIKV viral infection is generally not severe.

9. Identification of the ZIKV in the amniotic fluid of pregnant women whose fetuses presented microcephaly, in the countryside of Paraíba;

- This evidence shows that mothers were infected with the ZIKV. The chance to observe it will depend on the virus exposure rate in the population. The existence of microcephaly in these two cases also needs to be validated. We could by chance expect two cases of both, microcephaly and maternal infection with the ZIKV if 60,000 births occurred in Paraíba in 2015 with an expected microcephaly rate of 0.000302 and a presumed rate of 11% of

pregnant women infected with the ZIKV prenatally, if these were events independent. In Micronesia, the Yap Island had presented 73% of the population above three years infected by ZIKV in 2007.

10. Identification of newborn deaths with malformations and pattern suggestive of infection in the State of Rio Grande do Norte and other States;

- The death rate at birth in congenital microcephaly is usually high because 6% of cases are stillborn, and 24% have neonatal death. Therefore, these death rates are expected.

11. Identification of a newborn in the State of Ceará diagnosed with microcephaly during pregnancy and positive for ZIKV infection, having evolved to death in the first five minutes of life.

- The presence of perinatal death is expected in 1/3 of congenital microcephaly cases. The probability of the presence of microcephaly after a pregnancy with ZIKV infection as independent events will depend on both, microcephaly prevalence and ZIKV infection rate in the population of Ceará. Using the prevalence rate of 0.000302 and the exposure rate of 11% mentioned above, we would expect between 132,255 births in Ceará in 2015 and 4 cases by chance involved with these two events, that is, four more than the observed number.

Rumors are extremely important to identify environmental or genetic causes that can trigger an increase in the frequency of a certain condition in a population. Epidemics or endemics of a particular birth defect are possible when the causes are environmental. The increase in cases of genetic origin, in general, leads to endemics of a particular defect.

The process of validating rumors varies according to the specific problem. The validation process of the Rumor for increased frequency of

congenital microcephaly by maternal infection with the ZIKV should be conducted as it has been already programmed by the Ministry of Health.

However, we observed that the studies usually indicated to select pregnant women infected by the ZIKV present some technical problems such as the lack of adequate serological test due to the similarity between the Dengue virus and ZIKV. RT-PCR reactions to identify the ZIKV viral genome are also inefficient due to the reduced time of virus circulation in the body, which makes the material collection time crucial for obtaining the correct diagnosis.

Thus, the establishment of epidemiological studies to analyze the effect of prenatal infection with the ZIKV would only be prospectively feasible because of the retrospective difficulty of determining the exposure of a case with certainty.

The ability to analyze microcephaly cases of survivors beyond the perinatal period to identify known causes of microcephaly would lead to partial results. The serological determinations already made in the mother and child would probably indicate TORCH embryopathies. Permanent maternal diabetes would also be of simple detection. Cases with common chromosomal abnormalities and severe non-chromosomal holoprosencephaly would have already died, and their causes can only be suspected through clinical examination data. The same will occur with the most lethal monogenic syndromes.

Summary and suggestions

In summary, when we ask ourselves if there is a microcephaly epidemic in Brazil, or if there is a causal relationship between maternal infection with the ZIKV and children born with microcephaly, we face problems in all epidemiological steps to clarify the Rumor.

- **the definition of outcome (microcephaly):** microcephaly, as a result of failure or decreased brain growth is not a defect of simple identification through the measurement of head circumference; the threshold suggested between defect and normality at around 33 cm or 32 cm head circumference in a continuous distribution curve includes necessarily a large number of normal individuals.
- **the definition of exposure (ZIKV infection):** the problems in identifying the ZIKV through serology are significant because this virus is very similar to the Dengue virus, which co-circulates in the Brazilian population. Moreover, the ZIKV RNA identification by RT-PCR is hindered by the short period in which the virus is present in the blood or other tissues.

Thus, without a well-defined outcome and uncertain exposure, the questions mentioned above cannot be answered.

Suggestions:

There is no possibility to detect maternal infection with the ZKV at the delivery of a child with microcephaly and even, in most cases, when the detection of microcephaly is prenatal. Therefore, we indicate that a case-cohort study with pregnant women who present rash and/or other signs of infection could identify cases, with and without microcephaly at birth, which could then be compared to the frequency of ZKV infections that had been confirmed at the onset of viral infection.

CASE-COHORT STUDY IN RIO DE JANEIRO OR IN ANOTHER STATE WHERE ZKV CIRCULATION IS INCREASING

- Conduct a case-cohort study to evaluate whether there is a causal relationship between prenatal exposure to the ZKV and microcephaly beginning on November 1, 2015, including pregnant women being studied for exposure to ZKV and those who present signs of viral infection. Cases of

microcephaly will be identified by the evaluation of prenatal ultrasound exams and/or at delivery. The controls will be selected randomly from the cohort studied for the exposure factor.

- Define Confirmed Exposure when the ZKV RT-PCR test is positive in the blood and/or urine of pregnant women.
- Define Unconfirmed Exposure when the ZKV RT-PCR is negative in the blood and/or urine of pregnant women.
- Follow-up all pregnant women with ZKV viral infection until delivery, with confirmed and unconfirmed exposure, to determine the presence of microcephaly in the newborn, whether if it is miscarried, stillborn, or born alive.
- The presence of microcephaly must be determined by evaluating the measurement of head circumference considering age and gender, and using brain MRI and newborn follow-up in questionable cases. Aborted and stillborn cases should go to autopsy examination for the evaluation of defects in the brain and other organs and systems.

These are the basic points in a case-cohort study and should show whether there is a causal relationship between ZKV infection and microcephaly.

The sample size required for different values of ZKV exposure in order to detect a relative increasing risk of at least three times higher than the baseline for the studied population, including a test power of 80%, a significance level of 5%, and four controls per case.

Exposure	Cases	Controls
0.01	498	1992
0.05	113	452
0.10	66	264
0.25	40	160
0.50	42	168
0.75	76	304
0.90	182	728

The calculation of number of cases and controls for the case-cohort study to evaluate whether the presence of microcephaly is related to confirmed maternal infection with the ZKV can be performed with 42 pregnant women who gave birth to microcephalic children and 168 pregnant women who did not give birth to microcephalic children considering the ZKV infection rate of 50% and a relative risk of 3 times over the baseline risk for the studied population.

For example, 36 cases would be expected in two months in the State of Rio de Janeiro. (See ECLAMC document 3).

CASE STUDY OF MICROCEPHALY

- In parallel with the case-cohort study, we suggest that the tests necessary for evidencing a microcephaly case include examinations to detect the genetic causes, which are prevalent in cases with microcephaly.

- STORCH serology should be performed in all infants born to mothers with suspected ZIKV infection, with confirmed or unconfirmed exposure.

- Blood should be collected in EDTA-containing tubes from all newborns with microcephaly, with confirmed or unconfirmed exposure to the ZIKV. DNA extracted from this blood samples will be used in diagnostic tests for chromosomal microanomalies and monogenic syndromes using methods such as array-CGH, microarrays, next generation sequencing, Trusight One Panel, or specific microarray panels for microcephaly. The DNA may also be used to study numerical chromosomal abnormalities using QF-PCR when results from cell culture karyotyping were not obtained.

- Blood should be collected in heparin-containing tubes for karyotyping purposes only from newborns with microcephaly and other birth defects, with confirmed or unconfirmed exposure to the ZIKV.

- Tissue samples from abortions and stillbirths with microcephaly, collected for the identification of the ZKV according to the Ministry of Health protocol, may have their DNA extracted for further etiological studies.

The flowchart for the case study:

Jorge S. Lopez-Camelo - ECLAMC, CEMIC, INAGEMP
Ieda Maria Orioli - ECLAMC, UFRJ, INAGEMP

