INVESTIGATION PROJECT

CLINICAL COHORT OF CHILDREN PRESENTING MICROCEPHALY IN PERNAMBUCO

MICROCEPHALY EPIDEMIC RESEARCH GROUP – MERG

RECIFE - 2015

Study context: Between August and October 2015 the Brazilian Ministry of Health identified a notable increase in cases of microcephaly initially in the state of Pernambuco, northeastern Brazil. Until November 28th, 2015, 1,248 suspected cases of microcephaly in 311 municipalities in 14 Brazilian federal units had already been reported. The largest number of cases was registered in Pernambuco with identification of 646 suspected cases of microcephaly from the early 2015, followed by states in Paraíba (248 cases) and Rio Grande do Norte (79 cases). On November 10th, 2015 the Ministry of Health decreed Public Health Emergency of National Importance to give greater agility to the investigations. In late November, a group of researchers at the Universidade de Pernambuco, Universidade Federal de Pernambuco and Centro de Pesquisas Aggeu Magalhães-Fiocruz-PE formed the MERG group (Microcephaly Epidemic Research Group), for, along with researchers and professionals in the care of several hospitals and supported by the Ministry of Health and State Departments of Pernambuco and the city of Recife, formulate and develop research projects related to this issue.

Primary objective: to characterize the clinical condition and describe the dental and neurocognitive overall growth and development in the first two years of life in a cohort of children identified with microcephaly in the epidemic observed in Pernambuco from August 2015.**Secondary objective:** To characterize the clinical condition and describe the dental and neurocognitive overall growth and development in the first two years of life in a cohort of children considered borderline: with head circumference (HC) between 32 and 33 cm and no changes in the central nervous system (CNS) in baseline transfontanellar ultrasonography (USG-TF) and whose mothers had history of rash or children with HC between 32 and 33 cm with calcifications in the CNS detected by baseline transfontanellar ultrasonography (USG-TF).

Methods

Study design and definition of exposed and not exposed (controls): Study of **clinical cohort**. The **exposed group** will be composed of children born with microcephaly defined as: head circumference (HC) ≤ 32 cm for those born with 37-42 weeks of gestational age (GA) or HC ≤ percentile 3 (2 standard deviations) on Fenton's growth curve and CT findings consistent with congenital CNS infection. The **control group** will be composed of children born with normal head circumference for the GA and no changes in the USG-TF performed after birth. The group of children considered borderline will be composed of those with HC between 32 and 33 cm and no changes in the CNS in baseline USG-TF and whose mothers had history of rash or children with HC between 32 and 33 cm with calcifications in the CNS detected by baseline USG-TF. This group will be considered separately, for analysis purposes.
For evaluation of microcephaly, gestational age of the newborn will be obtained from USG performed in the first trimester (preferably between the 8th and 14th week); as second option, the date of the last menstrual period (LMP) recorded in the pregnant

woman's card or informed by the woman, and finally from USG performed at any time in pregnancy.

Study place, population and collection of information and samples: Children will be monitored in the Pediatric Service of Infectious Diseases at the University Hospital Oswaldo Cruz (DIP Child / HUOC-UPE). Newborns considered cases (with microcephaly) in the case-control study, which is being developed in Pernambuco by MERG and already has financing secured by PAHO/MS-Brazil, will be added to the follow-up cohort, and thus the same examinations will be considered for baseline assessment. Thus, the following information obtained from clinical, laboratory and imaging tests will be part of the baseline assessment: clinical examination, including examination at birth and delivery data, neuropediatrician evaluation, laboratory tests to investigate anemia and other hematological disorders, renal function and hepatic impairment: CBC, GOT, GPT, urea, creatinine. Tests to detect other congenital infections (coinfection) and/or to exclude the hypothesis of other possible causes of microcephaly: VDRL, serology for TORCHES (toxoplasmosis, rubella, cytomegalovirus - CMV, herpes virus and syphilis) and Zika virus - ZikV, Chikungunya - CHIKV, Dengue - DENV, Parvovirus B19. Serologies should be paired with maternal tests, collected at the same time. PCR in umbilical cord blood for ZikV, ChikV, DenV, toxoplasmosis and CMV; PCR in CSF for ZikV, ChikV, DenV; and PCR in urine for CMV. Computer tomography (CT) scan to identify calcifications in the skull and assessment of possible neurological damage. Transthoracic echocardiography (ECO-TT) and USG of total abdomen, to investigate other congenital malformations. At the end of the second year of follow-up, cases will undergo magnetic resonance imaging (MRI), for a final CNS evaluation at the time in which, in normal children, the end of the myelination process occurs.

Other tests that will be added to the baseline investigation and which are not included in the case-control study: Electroencephalogram (EEG) to assess the newborn baseline brain electrical activity and investigate the presence of epileptogenic foci. Ophthalmologic evaluation with additional tests (RetCam, OCT and eye USG). Otologic evaluation will be made in a subsample of 90 children with ABR test.

Collection of biological material and imaging tests will be performed for exposed and controls or comparison groups following the standard operational procedures (SOP) including storage of biological materials for further testing.

Cohort follow-up: For cases where there is positivity of serology at baseline assessment - VDRL and toxoplasmosis - the serial follow-up of serology in children may be required to clarify the diagnosis of coinfection or to indicate specific treatment. In case of positivity for CMV in children, the test should be repeated every two months until it tests negative. EEG will be repeated twice over the follow-up period. Children will also undergo a diagnostic evaluation and dental follow-up in the first months of life.

Follow-up returns for reassessment for cases and healthy controls: After the baseline assessment at birth, children will be reassessed at 3, 6, 12, 18 and 24 months of

age to collect a variety of information of interest to their follow-up, which is detailed in Figure 1.

Figure 1. Intervals between revaluations and activities/exams scheduled to be made each time.

3m	6m	12m
appointment with infectious pediatrician and neuropediatrician. Evaluate growth (anthropometric measurements) and general development and neurological functions and investigate the appearance of convulsions and other complications. EEG. CBC, GOT, GPT, urea, creatinine. Ophthalmologic evaluation: RetCam, eye USG, OCT. Otologic evaluation: OAE and ABR tests	appointment with pediatric dentist, infectious pediatrician and neuropediatrician. Evaluate growth (anthropometric measurements) and general, odontologic development and neurological functions and investigate the appearance of convulsions and other complications. Assessment of neurocognitive development.	appointment with infectious pediatrician and neuropediatrician: to evaluate growth (anthropometric measurements) and general development and neurological functions and investigate the appearance of convulsions and other complications. Assessment of neurocognitive development. EEG. CBC, GOT, GPT, urea, creatinine. Transthoracic echocardiography.
18m	24m	
appointment with infectious pediatrician, pediatric dentist and neuropediatrician: to evaluate growth (anthropometric measurements) and general development and neurological functions and investigate the appearance of convulsions and other complications. Serology for ChikV, ZikV, DenV in cases of positive results at birth.*	appointment with infectious pediatrician, pediatric dentist and neuropediatrician: to evaluate growth (anthropometric measurements) and general development and neurological functions and investigate the appearance of convulsions and other complications. Assessment of neurocognitive development. EEG. CBC, GOT, GPT, urea, creatinine. MRI.	

*The repetition of serological tests is only indicated to children who have positive tests at baseline assessment for diagnostic definition for serial follow-up.

The cohort of cases will be useful to describe the evolutionary dynamics of newly diagnosed clinical syndrome and the onset of new symptoms and complications, considering several possible outcomes. For group comparison purposes, will be considered as primary outcomes: head circumference, visual impairment, hearing impairment, epilepsy and number of hospitalizations and mortality.

Sample size: To calculate the sample and estimate the frequency of outcomes in cases with significance level of 5%, the expected proportions of the following outcomes were considered: mortality (4%), microcephaly (97%), delayed psychomotor development (70%), visual impairment (60%), hearing loss (40%), epilepsy (80%), assuming an error that ranged from 3 to 10%. The largest sample size was 124 cases for microcephaly variable. We assume a total sample of 150 cases.

A **control group** of 60 children will be recruited to verify the frequency of these outcomes in children without microcephaly. For comparative analysis purposes, children diagnosed with infection for the known causes of microcephaly will be excluded from the cohort. To allow that the assessment of mild neuropsychomotor development take into account differences in interventions eventually adopted for the cohort of cases, all interventions aimed at early or late stimulation with physical

therapy, speech therapy, etc., are recorded in the questionnaire. The control group will be evaluated regarding the following outcomes: growth, measured by anthropometric measures and neurocognitive development, evaluated by specific tests.

Data entry: data entry will be made on a platform that will include a structured database; mask for data collection in tablet, where possible, mechanisms for automatic data entry, and for field monitoring, including recruitment of exposed, controls and comparison group; flow of the questionnaires and materials collected and exams and entry of results.

Analysis strategy: initially, a detailed clinical characterization of cases of microcephaly with description of the characteristic phenotype of the clinical syndrome of congenital infection under investigation will be made: head circumference, craniofacial proportion, presence and evolution of pachygyria, prominence of the occipital protuberance, change in neurological functions: presence or absence of irritability, how reflexes behave, muscle tone, hearing and visual function (cochlear-palpebral reflex, eye-gaze, following with the eyes), presence or absence of nystagmus. A description of the general physical examination will be made, also focusing on the description of other malformations. For the investigation of other internal malformations, ultrasonography and echocardiography will be used. This characterization will also include a description of the changes found in EEG tests and CNS, abdomen and chest imaging. Laboratory patterns found for diagnosis of congenital infection for all etiologic agents investigated in the study, including coinfection patterns, will also be described. These parameters will be monitored in periodic reassessments in order to establish and characterize the evolution of this syndrome. At the end of the follow-up period, MRI of cases will be performed in order to evaluate the CNS formation in children with congenital microcephaly at the time in which, in normal children, the end of the myelination process occurs. Some outcomes will have their frequencies compared in the group of cases and controls.

Ethical considerations: The project will be submitted to CONEP for evaluation in an emergency basis in view of the severity and need for quick answers to the congenital microcephaly epidemic and social transcendence of it. Despite such an emergency, the project will follow all ethical procedures recommended for this type of study by Resolution MS/CNS 466/2012. Informed consent will be requested to all mothers or legal guardians of children. Most investigation tests and procedures are in alignment with the Clinical and Epidemiological Protocol of Microcephaly of the State Health Department of Pernambuco (SES), developed in conjunction with experts of the leading reference hospitals for care of microcephaly and set in routine care routine of children. Invasive procedures in newborns and children will be avoided or minimized by adopting strategies, such as collection of umbilical cord blood at birth. In the investigation of CNS changes, the method that exposes the newborn to as little risk as possible will be the one used, considering the clinical indication as a priority. Thus, for the choice of imaging test method of the central nervous system, the research team along with experts chose CT because it is faster and easier to perform compared to MRI,

and this indication is not because of the research but for assistance to cases. For cases, it is assumed that the risk of exposure to the radiation inherent to this test is outweighed by the potential benefits arising from the correct initial assessment of newborns, considering the fact that this is a new clinical syndrome and the need for an imaging test to diagnose malformations, evaluate the degree of impairment and the occurrence of alleged infection of the CNS, usually signaled by the presence of calcifications. In order to not expose the controls to the CT scan, the CNS imaging for this population will be obtained with USG-TF, which although is not the method of choice to investigate calcifications in the CNS of newborns, is considered a suitable, low-risk alternative and with no radiation exposure. At the end of the follow-up period, MRI of cases will be performed, considering the need to evaluate the CNS formation in children with congenital microcephaly at the time in which, in normal children, the end of the myelination process occurs. Even considering the potential risks of the procedure and the need for sedation of these children to perform this test, this risk is lower than that observed among newborns and is justified by the potential benefit that the resulting information can bring to the assessment of prognosis and for monitoring of these children. Cerebrospinal fluid analysis has also been indicated by doctors accompanying newborns with microcephaly and is in the SES-PE protocol for assistance to cases as part of the diagnostic investigation, and the same laboratory collection procedure will meet the needs of obtaining material for research. It is a procedure with potential risk of accident of lesions to the CNS, although this risk is low when the procedure is performed by an experienced specialist. The research protocol provides for the hiring of a skilled and experienced professional for the collection of cerebrospinal fluid, which also meet the demands of the care team. The electroencephalogram (EEG) was added to the baseline investigation protocol by the research team at the suggestion of the neuropediatricians involved in assisting the cases, in order to evaluate the electrical activity of the brain, and to investigate the presence of epileptogenic foci in other times of follow-up of the cohort. The test will be preferably conducted with the child naturally asleep, and only exceptionally with sedation, if necessary. In these situations, there will be expert assistance in order to minimize the risks of depression inherent to the procedure, although these are low.

Budget considerations: The budget submitted in this project proposal includes the follow-up of cases of microcephaly at months 3, 6, 12, 18, and 24. Most of the baseline assessment has already secured funding, since the cases are the same as the case-control study conducted by the same research group. A partial international financing for part of the follow-up tests of the cohort, corresponding to £38,400.00, has already been obtained from the Wellcome Trust. Some tests presented in the follow-up table do not match the total number of patients foreseen in the sample, since they will only be performed in special cases (serology and molecular biology). In return, tests such as CBC, GOT, GPT, urea, creatinine are under the responsibility of the service where the children will be monitored. Pediatric and specialist assessments will also be made in the reference units by professionals enabled and belonging to the staff of the hospitals involved in the study. Serological tests will be performed at the Central Laboratory of

the State Health Secretariat (LACEN) and at the Virology Laboratory of Research Center Aggeu Magalhães - Fiocruz/PE, reference for Dengue and Zika research. Additional financing will be requested for costs with assessment of controls.

Team responsible for preparing the project:

Celina Maria Turchi Martelli - PhD, MD, Visiting Researcher CPqAM/Fiocruz-PE, Fellowship PQ CNPq Level 1C, Epidemiology and Infectious Diseases.

Laura Cunha Rodrigues - PhD, Invited Researcher of CPqAM/Fiocruz-PE and professor of Epidemiology and Infectious Diseases - University of London/NP.

Demócrito de Barros Miranda Filho - PhD, professor of Infectious and Parasitical Diseases, Fellowship PQ CNPq Level 2. FCM/UPE.

Ricardo Arraes de Alencar Ximenes - PhD, professor of Epidemiology and Infectious Diseases, Fellowship PQ CNPq Level 1B. UFPE.

Thália Velho Barreto de Araújo - PhD, professor of Epidemiology. Postgraduate Program in Public Health, UFPE. Researcher in reproductive health area.

Maria Cynthia Braga - PhD, MD, Fellowship PQ CNPq Level 1C, Pediatrician and Epidemiologist of Infectious Diseases. CPqAM/Fiocruz-PE

Maria de Fatima P. Militão de Albuquerque - PhD, researcher, Epidemiology of Infectious Diseases, Fellowship PQ CNPq Level 1D. CPqAM/Fiocruz-PE

Wayner Vieira Souza - PhD, researcher, Fellowship PQ CNPq Level 1C. CPqAM/Fiocruz-PE.

Ernesto Torres Marques - PhD, senior researcher and professor at University of Pittsburg/USA. Vaccinology, Molecular Biology, Molecular Immunology. CPqAM/Fiocruz-PE

Maria Angela Wanderley Rocha, Master's Degree in Tropical Medicine UFPE, pediatric infectious disease physician, professor. Head of Pediatric Infectious Diseases Department of the University Hospital Oswaldo Cruz FCM/UPE.

Regina Coeli Ferreira Ramos - Master's Degree in Health Sciences UPE, pediatric infectious disease physician. Coordinator of the reference clinic of microcephaly HUOC/UPE.

With the participation of representatives of the Executive Secretariat of Health Surveillance of Pernambuco's State Health Department - and of the professor Sinval Pinto Brandao Filho, PhD, director of CPqAM/Fiocruz-PE, Fellowship of Research Productivity (PQ) CNPq Level 2.

References:

1. John CC, Carabin H, Montano SM, Bangirana P, Zunt JR, Peterson PK. Global research priorities for infections that affect the nervous system. Nature. 2015;527:S178-86.
2. Brazilian Ministry of Health. Epidemiological bulletin with zikaV data [in Portuguese]. 2015. Available at <http://portalsaude.saude.gov.br/index.php/situacao-epidemiologica-dados-zika>.
3. Zanluca C, de Melo VCA, Mosimann ALP, dos Santos GIV, dos Santos CND, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015;110:569-572.
4. Brazilian Ministry of Health. Ministry of Health confirms relationship between Zika virus and microcephaly [in Portuguese]. 2015. Available at <http://portalsaude.saude.gov.br/index.php/o-ministerio/principal/secretarias/svs/noticias-svs/21016-ministerio-da-saude-confirma-relacao-entre-virus-zika-e-microcefalia>.
5. Brazilian Ministry of Health. Protocol for monitoring and response to microcephaly occurrence relating to ZikaV infection [in Portuguese]. 2015. Available at <http://portalsaude.saude.gov.br/images/pdf/2015/dezembro/09/Microcefalia---Protocolo-de-vigil--ncia-e-resposta---vers--o-1----09dez2015-8h.pdf>.
6. Pan American Health Organization. Neurological syndrome, congenital malformations, and Zika virus infection. Implications for public health in the Americas. Epidemiological Alert. 2015. Available at <http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&Itemid=270&gid=32405&lang=en>.
7. Brazilian Ministry of Health. Epidemiological Bulletin. Ministry of Health updated microcephaly numbers related to ZikaV [in Portuguese]. 2015. Available at <http://portalsaude.saude.gov.br/index.php/cidadao/principal/agencia-saude/21254-ministerio-da-saude-atualiza-numeros-de-microcefalia-relacionados-ao-zika>.
8. Oehler E, Watrin L, Larre P, Leparc-Goffart I, Lastère S, Valour F, et al. Zika virus infection complicated by Guillain-Barré syndrome – case report, French Polynesia, December 2013. Euro Surveill. 2014;19(9):pii=20720.
9. Besnard M, Lastère S, Teissier A, Cao-Lormeau VM, Musso D. Evidence of perinatal transmission of Zika virus, French Polynesia, December 2013 and February 2014 . Euro Surveill. 2014;19(13):pii=20751.
10. European Centre for Disease Prevention and Control. Rapid risk assessment: Microcephaly in Brazil potentially linked to the Zika virus epidemic. 2015. Available at <http://ecdc.europa.eu/en/publications/Publications/zika-microcephaly-Brazil-rapid-risk-assessment-Nov-2015.pdf >.
11. Besnard M, Mallet HR. Increase of cerebral congenital malformations among newborns and fetus in French Polynesia, 2014-2015, following a Zika virus outbreak. 2015. Available at <https://app.box.com/s/ptvqdargwr10m3ag849rv59c5iditco7>.
12. Fenton TR. A new growth chart for preterm babies: Babson and Benda's chart updated with recent data and a new format. BMC Pediatr. 2003;3:13.

13. Fenton TR, Kim JH. A systematic review and meta-analysis to revise the Fenton growth chart for preterm infants. BMC Pediatrics. 2013;13:59.
14. Pereira AP, Leal MC, da Gama SG, Domingues RM, Schilithz AO, Bastos MH. Determining gestational age based on information from the Birth in Brazil study. Cad Saude Publica. 2014;30:S1-12.
15. Neu N, Duchon J, Zachariah P. TORCH Infections. Clin Perinatol. 2015;42:77-103.
16. Stutterd CA, Leventer RJ. Polymicrogyria: A common and heterogeneous malformation of cortical development. Am J Med Genet Part C Semin Med Genet. 2014;166:227–239.
17. Fenton and Kim: A systematic review and metaanalysis to revise the Fenton growth chart for preterm infants. BMC Pediatrics 2013 13:59. doi:10.1186/1471-2431-13-59.
18. Snijders RJ, Nicolaides KH. Fetal biometry at 14-40 weeks' gestation. Ultrasound Obstet Gynecol. 1994;4: 34-48.
19. ECLAMC - Estudo Colaborativo Latino Americano de Malformações Congênitas. Manual Operacional, Rio de Janeiro, 2009.
20. Wacholder S, Silverman DT, Mclaughlin JK, Mandel JS. Selection of Controls in Case-Control Studies II: Types of Controls. American Journal of Epidemiology 1982; 135 (9): 1029-1041.