MERG
Grupo de Pesquisa da Epidemia de Microcefalia

INVESTIGATION PROJECT

INVESTIGATION OF CASES OF MICROCEPHALY

CASE-CONTROLE STUDY

(MICROCEPHALY EPIDEMIC RESEARCH GROUP – MERG

RECIFE

2015

SUMMARY

1. PRIMARY OBJECTIVE

To investigate factors associated with microcephaly.

2. SECONDARY OBJECTIVES

- To identify the association between potential risk factors and microcephaly: infections in pregnancy (Zika, Chicungunya, Dengue, ToRCHeS) and others, use of medicines (including vaccines) in pregnancy, environmental exposures (including larvicides and insecticides), radiation, drugs and alcohol abuse, parental consanguinity, family history of malformations and other classic teratogenic agents.
- To explore whether the effect of the identified factors varies with gestational age during exposure, and whether there is interaction between the effects of co-infections and between infections and medications.
- To describe the phenotype and clinical, laboratory and imaging characteristics of newborns with microcephaly.
- To identify congenital abnormalities present in cases identified of microcephaly.

3. STUDY CONTEXT

In October 2015, the Brazilian Ministry of Health identified a notable increase in cases of microcephaly 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.

4. METHOD

Study design:

A case-control study, with prospective recruitment of newly born cases and concurrent controls.

5. STUDY POPULATION AND PLACE

The place of the study is the metropolitan region of Recife. Recife (PE) is one of the oldest capitals of the Brazilian Northeast region, and is embedded in dense metropolitan areas, with high levels of inequalities[1], living proof of the accelerated process of urbanization that has taken place in Brazil in recent decades. Other centers may be incorporated if necessary.

The population of the study are newborns from mothers living in Pernambuco and born in the metropolitan region of Recife in the maternities included in the study.

Study maternities: Maternities included in the study are listed in attachment 1. During pilot study and during the conduction of the study, recruitment will be monitored and other hospitals, other geographical areas, can be posteriorly included if necessary.

6. CASE DEFINITION

Case: Newborn (dead or alive)[2] with a head circumference below the defined threshold in accordance with appropriate standards for gestational age and sex in the appropriate chart. The head circumference will be measured at birth and confirmed by physical examination, between 12 and 24 hours, per neonatologist physician participating in the research. Newborns will be included if they have a head circumference less than or equal to the 3rd percentile (bellow two standard deviations from the mean) in Fenton Growth Chart (Fenton and Kim, 2013), Attachment 2. Live newborns with a gestational age of less than 22 weeks will be included if they have a head circumference less than or equal to the 5th percentile in Table developed by Snijders & Nicolaides (Snijders & Nicolaides, 1994).

[1] Gini index in 2000: Recife: 0.68; Salvador: 0.66; and São Luís: 0.65 (Recife City Hall.) Human Development Atlas in Recife, 2005. http://www.recife.pe.gov.br/pr/secplanejamento/pnud2006/.

[2] **Live births** are all newborns, regardless of birth weight and length of pregnancy, showing any sign of life (crying, breathing movement, heartbeat, umbilical cord pulsation, or effective movement of voluntary muscles), whether or not the umbilical cord was cut and whether or not the placenta was detached (ICD-10, WHO). **Stillbirths** are all newborns with no sign of life, if the pregnancy was longer than 22 weeks, or the fetus has body weight higher than 500 grams or height equal to or higher than 25 cm (ICD-10). **Stillbirths** under 500 grams in weight and/or gestational age less than 22 weeks that are considered abortions (ICD-10) will be excluded.

The HC was confirmed in a second measurement 12-24 after birth by a neonatologist, the neonate was considered eligible for the study.

Exclusions: Anencephaly and encephalocele.

Gestational age: 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[3].

7. CONTROL DEFINITION

Controls: Live newborns without microcephaly (both to live births or stillbirths)[4]. Two controls will be selected for each case.

8. RECRUITING CASES AND CONTROLS

CASES: All births, dead or alive, occurred in the hospitals included in the study will be clinically examined in order to identify microcephaly. The cases will be identified prospectively. The study will only include cases of microcephaly born during the study period in the hospitals selected for the study.[5]

[3] In the absence of USG in the first trimester and information on LMP, standards specified in the Standard Operational Protocol of fetal medicine will be considered.

[4] Live controls were chosen because they are representative of the population that produced the cases. Stillbirths are not the best control group because they died and therefore are matched by death. Thus, newborns from other causes would be less representative of newborns without microcephaly in cases reference population. Since they are also fetal deaths, there would be more frequently between them newborns having characteristics and expositions associated with such conditions and causes of death, i.e., stillbirth controls represent a special sample in non-cases in the study base. (Wacholder et al, 1992).

[5] Cases of newborns with microcephaly born in other hospitals and sent for clinical investigation and/or clinical care will not be considered cases of this study.

CONTROLS: For each case, two controls were selected, from the first neonates born from the following morning in one of the study hospitals with normal HC, matched by expected time of delivery and mother's home area.[6]

Approximate date of delivery: The approximate date of delivery will be calculated from the gestational age.

Matching by approximate date of delivery: Controls will be matched to cases by the approximate date of delivery, so the case and its controls will have been conceived in similar periods. For cases born at full term and post-term (37 to < 45 weeks), will be recruited, i.e. the first two full term newborns that are born from 8 am of the following day of the birth of the case.[7] For preterm cases, controls must be matched by approximate date of delivery within defined intervals. The intervals are: < 34 weeks (early preterm) and 34 to < 37 weeks (late preterm). For preterm controls, the first two full term newborns that are born from 8 am of the day defined as the expected date of delivery will be selected, according to the table below:

Gestational age of the case	Selected born controls
37 to < 39 weeks	14 days after the birth of the case
34 to < 37 weeks	28 days after the birth of the case
< 34 weeks	42 days after the birth of the case

Matching by mother's living area: the controls will be selected among newborns of mothers living in the same area where the mother of the case lives, using GERES (Regional Health Areas) as criterion.

The control may not have microcephaly, but does not necessarily need to be healthy; may have some morbidity; however, newborns identified as possible controls that present calcifications or other brain lesions found in imaging exams or other congenital malformation

[6] The matching of controls by area of residence and approximate date of delivery becomes important to ensure comparability between cases and controls for exposures that vary over time, the likeness of the person-time calculation in cohort studies, given that membership in the basis of the study is time dependent.

[7] This criterion will elect the controls before their birth, independent of any characteristic of the selected newborns. This form of recruitment of controls facilitates collection of blood sample from the umbilical cord and other biological material. The control can be born in a different year than the one when the newborn with microcephaly was born.

will be excluded. All data refer to newborns and not the births, hence twin birth newborns will be considered separately (attachment 4).

9. COMPARISON GROUPS FOR STILLBIRTH CASES

In addition to include usual controls for all cases (stillbirths or live births), the study will include comparison groups for stillbirths, to make it possible the comparison of virus isolation in these groups, using not acceptable or impossible investigations in living controls.

Stillbirth cases: A comparison group will consist of a sample of stillbirths referred to post-mortem examination service during the study period. Stillbirth cases and stillbirths of the comparison group (stillbirths without microcephaly) will undergo autopsy and have material collected for investigations.

10. INFORMATION AND SAMPLE COLLECTION

After signing the informed consent, a standard electronic questionnaire will be applied to mothers of cases and controls. Hospital records and prenatal booklets will be reviewed and information collected in a systematic way.

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

	Cases	Controls	Comparison group stillbirth
Collection of umbilical cord blood	X	X	
Collection of mother's blood	X	X	
Autopsy with material collection	Stillbirth cases		X
CSF Collection	X		
CT scan without contrast	X		
Transfontanellar ultrasound	Cases with an open fontanelle	X	
Placenta and umbilical cord fragments	**Sample**	**sample**	
Questionnaire-interview of mothers / prenatal booklet	X	X	
Structured review of medical records	X	X	

Samples of umbilical cord blood and cerebrospinal fluid (CSF) of newborns must be collected at birth, along with blood (serum) of the mother. Regarding the newborn, 10 mL of umbilical cord blood will be collected, of which 5 mL will be sent to LACEN/PE to perform serology for Chikungunya, dengue, cytomegalovirus, rubella, toxoplasmosis and parvovirus B19 and 5 mL will be sent to the virology laboratory Fiocruz/PE for PCR for zika virus, chikungunya, dengue, toxoplasmosis, cytomegalovirus and as aliquot for the biobank. It will be collected 3 mL of urine for research of cytomegalovirus. For newborns with microcephaly, 1 mL of CSF will be collected for investigation of zika virus, chikungunya, dengue, toxoplasmosis and as aliquot for the biobank. Regarding postpartum women, 20 mL of blood will be collected to perform the same serological tests and the same molecular biology tests.

The placenta of a subsample of women for macroscopic and microscopic tests, especially villous and vascular changes, will be collected. A fragment of the placenta for molecular biology test will be removed.

Stillbirths will be referred to post-mortem examination service, histopathological study and molecular biology test. The same tests will be repeated for a stillbirth comparison group without microcephaly referred to post-mortem examination service for other reasons.

For all children with microcephaly, cranial CT scan and ultrasound without contrast will be performed. And for the children without microcephaly only ultrasound will be performed.

All investigative procedures of cases form the standard of care in cases of microcephaly proposed in the clinical research protocol of the State Health Department of Pernambuco State. (http://portal.saude.pe.gov.br/sites/portal.saude.pe.gov.br/files/protocolo_microcefalia_versao02.pdf)

11. DATA ENTRY

It will be prepared 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 cases, controls and comparison group; flow of the questionnaires and materials collected and exams and entry of results.

12. ANALYSIS PLAN

Sample calculation

Sample calculation for detection of the association between Zika virus infection during pregnancy and the occurrence of microcephaly with odds ratio ≥ 2, 90% power, 5% significance level and two-tailed test and the recruiting of two matched controls for each case. Different exposure proportions were considered among cases, resulting in different sample sizes as shown in the table below. In view of these numbers, we decided to include 200 cases and 400 controls.

Proportion among cases	n cases	n controls (2 per case)
67%	136	272
50%	135	270
30%	183	366

* Parameters considered: OR = 2; power = 90%; level of significance = 5%; matched two-tailed test.

Proportion among cases	n cases	n controls (3 per case)
67%	114	456
50%	112	448
30%	150	600

Data preparation

Data will be prepared and verified for consistency in the traditional way.

Descriptive analysis

Characterize cases in accordance with phenotype, congenital malformations, clinical, laboratory and imaging characteristics. If appropriate, define phenotypes groups based on image and neurological tests.

Investigate the frequency of calcifications or other brain lesions found in imaging tests of potential controls. Exclude these newborns from the control group, and if the number is

enough, treat them as a separate group of cases; called newborns with brain injuries without microcephaly.

Compare cases and controls, (and if the number is enough, the third group of newborns with calcification, but without microcephaly) regarding social and demographic information including questionnaire information, medical record, clinical examination and risk factors investigated by the questionnaire or laboratory or imaging tests.

Univariate analysis: Compare findings between cases and controls in relation to risk factors investigated in general and in relation to gestational age at exposure. Compare the proportion of cases and newborns with calcification or others brain anomalies, but no microcephaly, who had rash in pregnancy and the gestational age at rash.

Conditional logistic regression of microcephaly cases identifying risk factors for microcephaly, global and separately, by trimester of occurrence of rash in pregnancy. Explore interactions between rash with characteristics of ZIKA (e) and the presence of antibodies against dengue and chikungunya (ii) and medications during pregnancy (iii).

Non-conditional logistic regression of newborns with calcifications or others brain anomalies and without microcephaly (if the number is enough) compared to controls.

13. ETHICAL CONSIDERATIONS

The protocol 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.

Informed consent will be requested to all pregnant women or legal guardians. All 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 Executive Secretariat of Health Surveillance, Central Laboratory of SES (LACEN), Executive Secretariat of Healthcare, Research Center Aggeu

Magalhães – CpqAM FIOCRUZ-PE and experts of leading reference hospitals for care of microcephaly (neuropediatricians, pediatricians, infectopediatricians and radiologists) and set in routine care routine of mothers and pregnant women.

Invasive procedures in newborns 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 Computerized Tomography (CT) because it is faster and easier to perform compared to Magnetic Resonance Imaging (MRI). For cases, 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. CSF analysis has also been indicated by doctors accompanying newborns with microcephaly 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 CSF, which also meet the demands of the care team.

14. PILOT

A pilot project will be conducted for two weeks. The pilot will assess recruitment procedures for cases and controls, proportion of refusals, acceptance and understanding of the questionnaire, feasibility of collecting biological materials and flow of imaging tests, operation of the data collection platform and monitoring of field procedures, and the frequency of cases and the geographical distribution in the notification system. After pilot analysis, the number of

cases, geographic area, procedures for data production and logistics for carrying out the study will be reviewed.

15. TEAM RESPONSIBLE FOR PREPARING THE PROJECT

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Demócrito Miranda Filho - PhD, professor of Infectious Diseases, Fellowship PQ CNPq Level 2. FCMPE/UPE.

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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/, Fellowship of Research Productivity (PQ) CNPq Level 2 CPqAM/Fiocruz-PE.

16. REFERENCES

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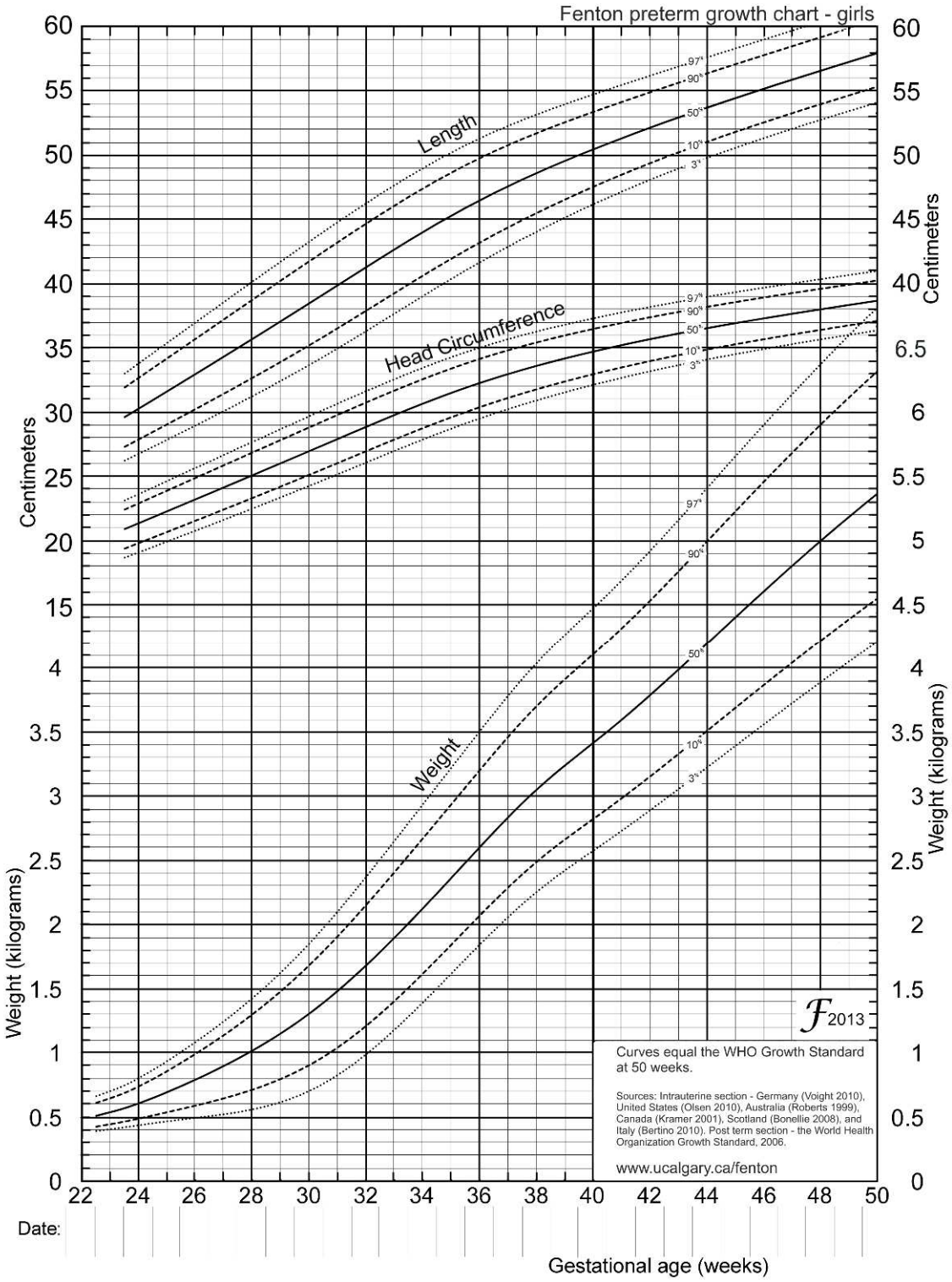
17. ATTACHMENTS

ATTACHMENT 1. STUDY MATERNITIES

NAME OF THE ESTABLISHMENT	CITY	MANAGEMENT
INSTITUTO DE MEDICINA INTEGRAL PROFESSOR FERNANDO FIGUEIRA (IMIP).	RECIFE	BY CONTRACT
UNIDADE MISTA PROF BARROS LIMA	RECIFE	MUNICIPAL
HOSPITAL BARAO DE LUCENA	RECIFE	STATE
HOSPITAL AGAMENON MAGALHAES	RECIFE	STATE
MATERNIDADE SANTA LUCIA	RECIFE	BY CONTRACT
POLICLINICA E MATERNIDADE ARNALDO MARQUES	RECIFE	MUNICIPAL
MATERNIDADE BANDEIRA FILHO	RECIFE	MUNICIPAL
HOSPITAL DAS CLINICAS	RECIFE	FEDERAL
CENTRO INTEGRADO DE SAUDE AMAURY DE MEDEIROS CISAM	RECIFE	STATE
HOSPITAL DO TRICENTENARIO	OLINDA	BY CONTRACT

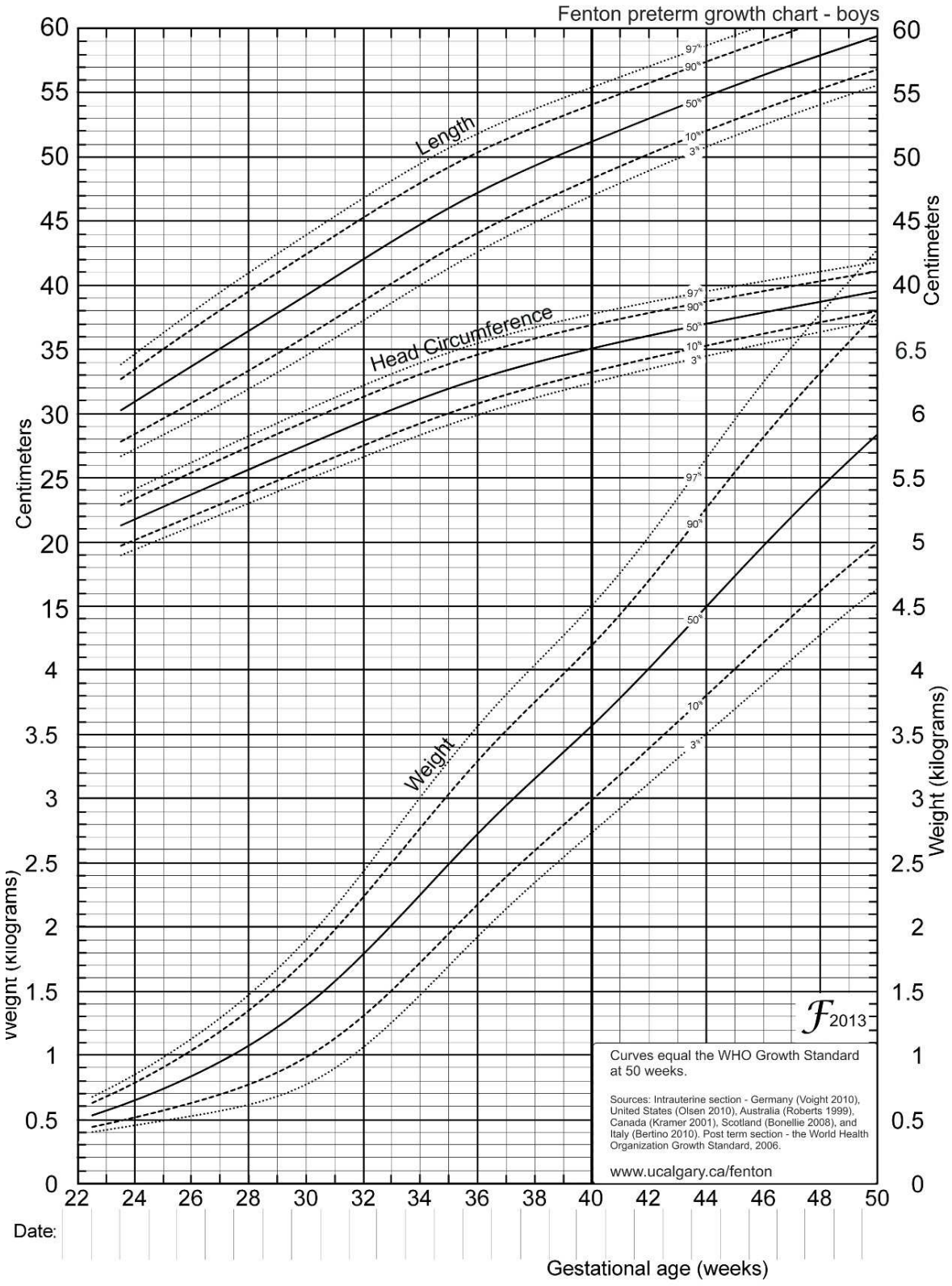
ATTACHMENT 2. FENTON GROWTH CHART

a. Fenton Growth Chart for Girls



Source: http://ucalgaryca/fenton/files/fenton/fenton2013growthchartgirlspdf

a. Fenton Growth Chart for Boys



Source: http://ucalgaryca/fenton/files/fenton/fenton2013growthchartboyspdf

ATTACHMENT 3. PROCEDURE FOR RECRUITING CASES AND CONTROLS

Map of screening for identification of case and control to be filled in each of the recruitment places with:

a) Daily record of the number and list (with name and number of medical record) of pregnant women admitted per delivery from the ward admissions book.
b) Consultation to medical records to identify pregnant women with fetus already diagnosed with microcephaly by prenatal USG. Register the name of the pregnant woman and the medical record number on own research instrument and communicate the field supervision.
c) Monitor the time of delivery through visit to antepartum rooms of the hospitals, in order to ensure the collection of biological material.
d) Verify if there was collection of biological material and send it to the laboratory defined in the research protocol.
e) Identify the gestational age of the newborn with microcephaly and the mother's area of residence in the previous year to inform the selection of controls. Determine whether the controls will be selected among those born in the following day or at a later date (based on matching criteria).
f) Identify the number of controls (depending on the number of cases) to be recruited in the following day, and at later dates, and born to mothers living where, having as reference the criteria for selection of controls.
g) Select controls
h) Organize the collection of biological material of controls.
i) If a stillbirth occurs, obtain the mother's (or guardian's) consent to perform necropsy, fill the referral form and provide the body's removal to the post-mortem examination service.
j) Register the stillbirths in own research instrument, with mother's name, medical record number, date of delivery, record number of referral to the post-mortem examination service, to rescue the necropsy report.
k) Contact women to get the signature of consent prior to the interview.
l) Prepare a map to organize the interviews, with the woman's name, maternity identification, ward and bed number.

In the case of a research protocol to be deployed in the service, logistics will be defined in accordance with the established routine care activities.

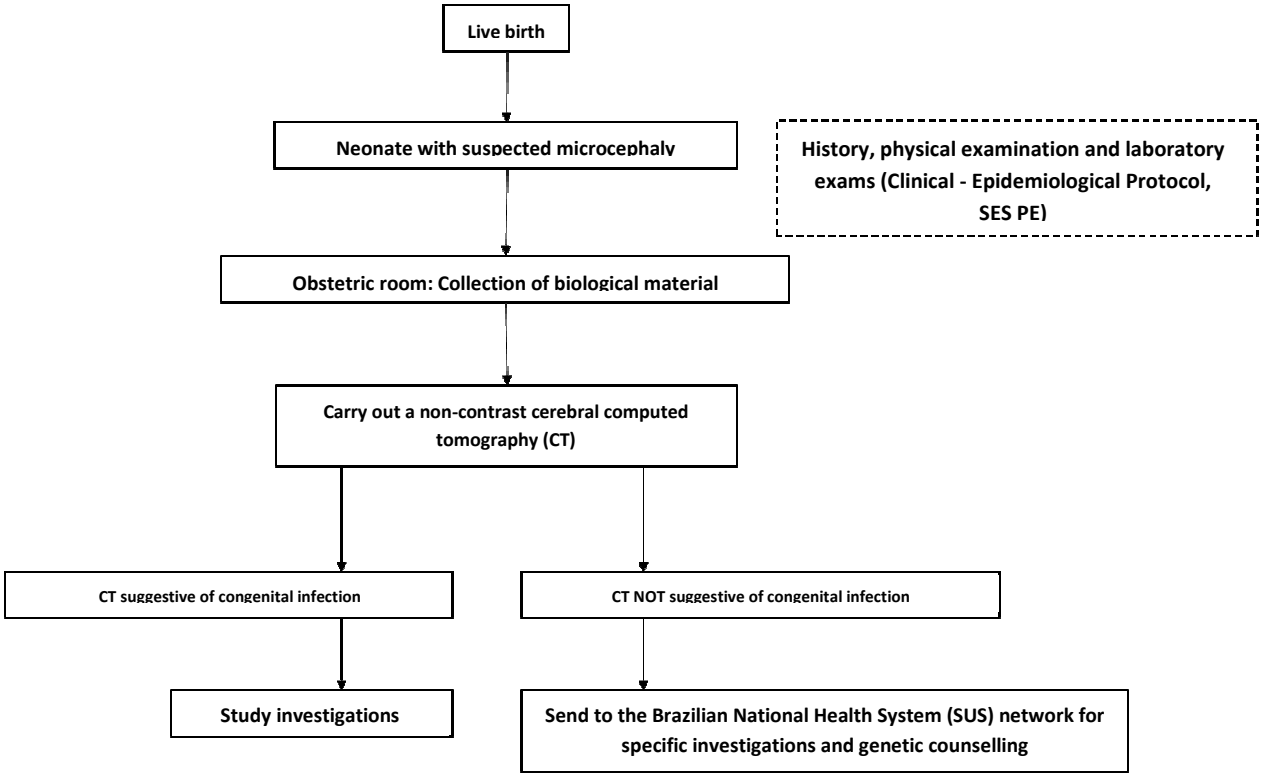


Figure 1 Live birth Algorithm

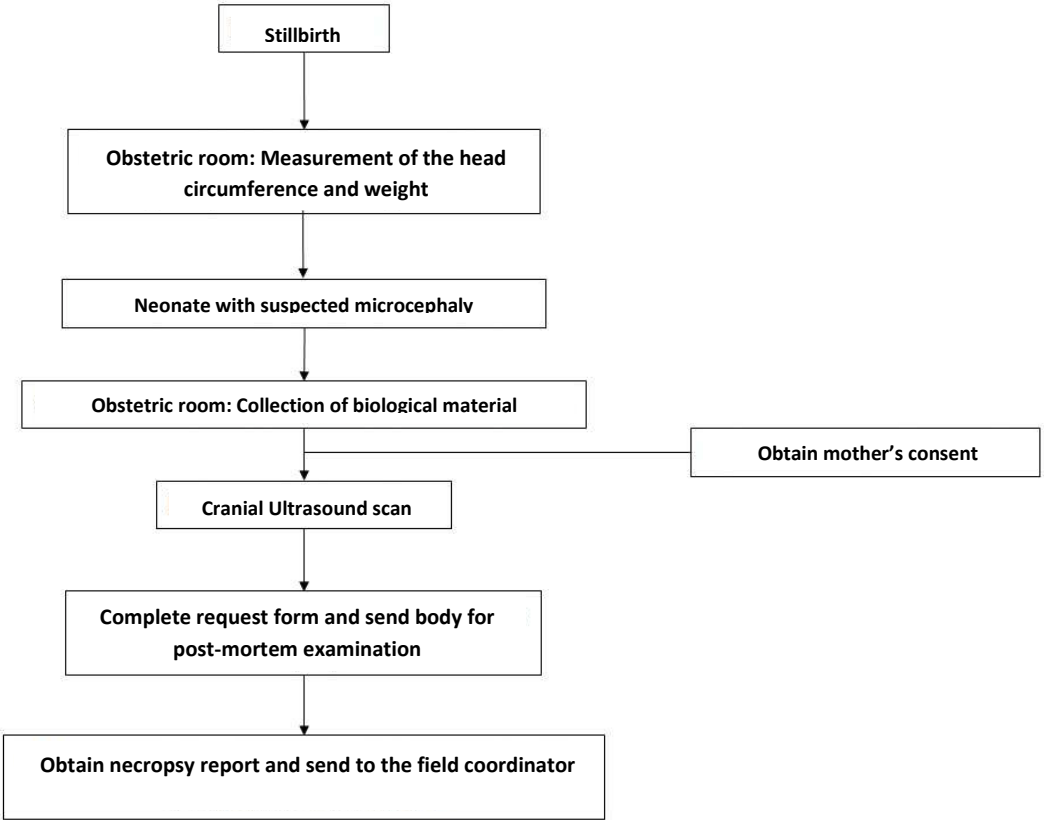


Figure 2 Stillbith Algorithm

ATTACHMENT 4. SELECTION OF CONTROLS IN CASE OF TWINS

1. A pair of twins is born, both with microcephaly and both live births;
   Two controls are selected for each, following the order of birth of the twins.

2. A pair of twins is born, both with microcephaly, one live birth and one stillbirth;
   Two controls are selected for the live birth and two controls are selected for the stillbirth, following the order of birth of the twins.

3. A pair of twins is born, both with microcephaly and both stillbirths;
   Two controls are selected for each, following the order of birth of the twins.

4. A pair of twins is born, one with microcephaly and one without; Two controls are selected for the one with microcephaly.

5. A pair of twins is born, both without microcephaly and both live births. Only one will be included as control, because the inclusion of both would duplicate the same mother in the database. The first newborn will be selected.

6. A pair of twins is born, one with microcephaly and one without, after a newborn with microcephaly born immediately before them. Two following controls are selected for the twin with microcephaly and two for the newborn with microcephaly from the previous birth.
   Attention: the normal twin cannot be control of the newborn with previous microcephaly, as one mother would enter the database twice, as malformed mother and as control mother.