Protocol of the project Barcelona Life Study Cohort -BiSC-

Protocol from pregnancy until the age of 18 months of the child
- Version 2 -

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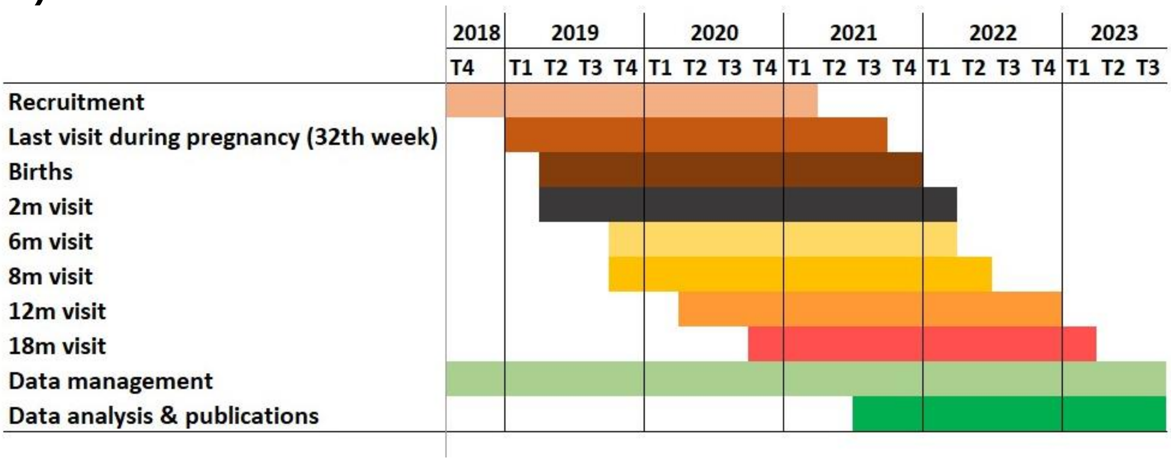
Summary

Title	Protocol of the project Barcelona Life Study Cohort -BiSC- Protocol from pregnancy until the age of 18 months of the child - Version 2 -
Description of the study	A birth cohort study conducted in the city of Barcelona and its metropolitan area to study the effect of pre and postnatal exposure to environmental exposures, particularly air pollution, on fetal growth and on pre- and post-natal development, particularly brain development.
Objectives	Towards the main aim, the project will: **Objective 1.** Establish a new urban pregnancy-birth cohort; **Objective 2.** Measure the pre- and post-natal exposomes; **Objective 3.** Measure fetal and child growth and development including several health domains: fetal and child growth, neurodevelopment and respiratory outcomes; **Objective 4.** Prospectively test the relationship between various pre-natal and post-natal exposures and fetal and child growth and development and health outcomes; **Objective 5.** Investigate the molecular mechanisms linking the exposome with fetal and child growth and development and health outcomes; **Objective 6.** Assess the genetic variation of the mother-father-child trios.
Design	Birth cohort (longitudinal study)
Inclusion and exclusion criteria	**BISC will recruit pregnant women**: with singleton pregnancy, aged between 18-45 years, within weeks 11-15 of their gestation, able to communicate in Spanish/Catalan with the BISC team, resident in the catchment area of the hospitals, initially plan to give birth in one of the BISC recruiting hospitals. The following **exclusion criteria** should be applied: having a severe chronic health condition that could limit the ability to communicate or attend the follow-ups, having a plan to leave the catchment area of the hospitals before delivery or having a plan to leave Barcelona Province after delivery, being classified as high-risk for congenital anomalies at the first routine ultrasound measures.
Treatments or interventions	Does not apply
Variables	See figure below summarizing the data collected
Study population	1100 pregnant women, their partners and their babies
Participant centers	ISGlobal, and three major hospitals, Sant Joan de Déu (SJdD), Maternitat-Clínic and Santa Creu i Sant Pau
Duration	Five years
Duration of the study for the study participant	From pregnancy until the age of 18 months of the child (<27 months)

Figure 1. Calendar of the study and summary of the procedures followed

1a)



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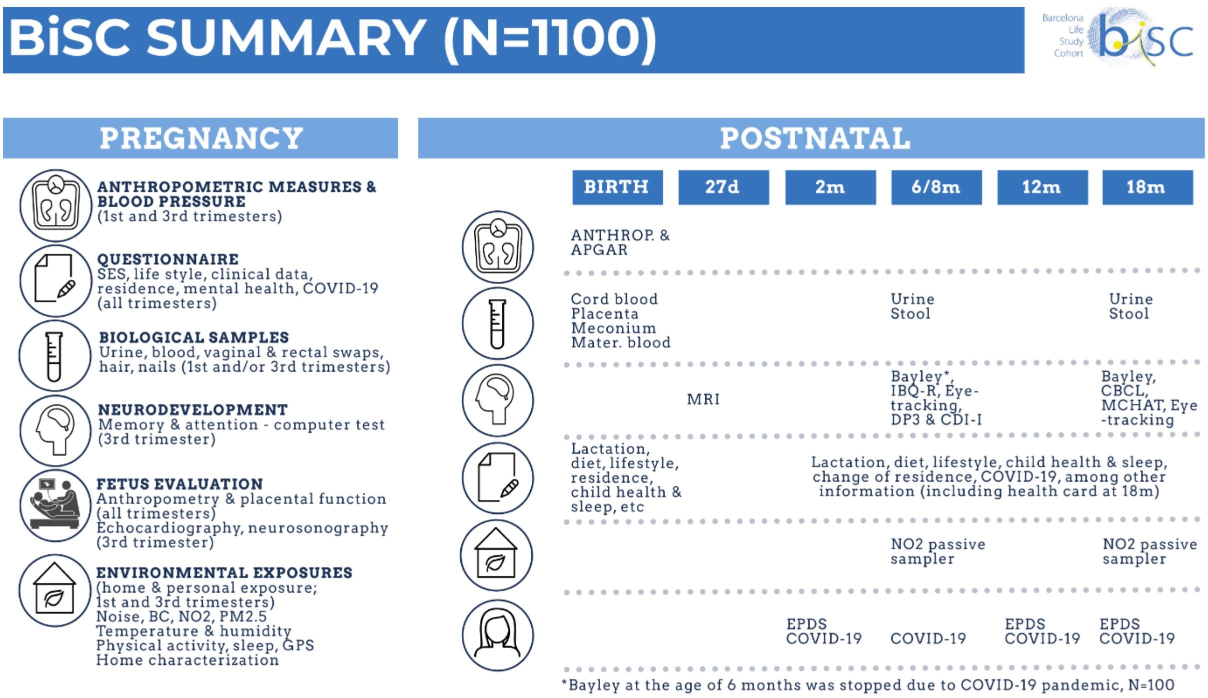

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RECORD OF VERSIONS

Version	Date	Description of the change	Brief justification
V1	18/06/2018	First version	First version
V2 (current version)	12/07/2021	As requested by the CEIm, we have integrated the 15 amendments presented in the last 2.5 years into one single document	There is the need to integrate all the changes that we have conducted in the BiSC project (previously entitled AIR-NB) in order to have a one single and clear protocol. The protocol includes the follow-up of the participants from pregnancy (women and their partners) until the age of 18 months of their children.

1. Justification

It is well established that the fetus and infant are especially vulnerable to the effects of socioenvironmental factors[1] and hence pregnancy is increasingly recognized as a particularly influential period shaping health over the life course[2]. The influence of exposures during pregnancy is not limited to reproductive and childhood outcomes and can extend over a lifetime, a corpus of knowledge embodied by the DOHaD (Developmental Origins of Health and Diseases) concept[3].

The World Health Organization (WHO) has classified air pollution as the biggest environmental health risk[4] and has identified children as particularly vulnerable to its effects[5]. Moreover, air pollution is one of the most studied environmental exposures during pregnancy and one of the top preventable causes of disease over time[6]; more than 20% of stroke, ischemic heart diseases and chronic respiratory diseases could be prevented by reducing particulate pollution to the WHO limits[6]. The direct health costs associated with air pollution exceed 5% of Europe's GDP[7]. Despite the well-known health effects, over 80% of world's population lives in urban areas that have higher levels of air pollution than the limits set by the WHO[4].

Air pollution exposure has been associated with a range of pregnancy complications, such as preeclampsia and gestational diabetes, and adverse pregnancy outcomes, such as congenital anomalies, preterm birth and, more consistently, impaired fetal growth[8–10]. Impaired fetal growth, as characterised by low birth weight, has been associated with childhood morbidity and mortality and with increased risk of noncommunicable diseases (NCDs) such as cardiovascular conditions and diabetes later in life[3]. In addition, strong evidence suggests that environmental toxicants contribute to a global, silent pandemic of brain developmental problems, which can lead to neurobehavioural disorders (i.e. autism spectrum disorder and attention-deficit hyperactivity disorder (ADHD)[11]. Optimal brain development involves multiple complex stages that must be completed sequentially, principally during foetal and early life, and that are uniquely vulnerable to adverse environment interferences[12].

Because of the inadequacy of the available evidence, the potential effects of air pollution on brain development (and cognitive decline) have not been considered to date when estimating the burden associated with air pollution[4]. Pioneering studies in animals show that air pollutants, particularly particles, can lead to a neuroinflammatory response in various brain regions[13]. In humans, researchers have shown that exposure to air pollution *in utero* is associated with increased risk of neuro-developmental delay[14]. Sunyer et al. also found that air pollution exposure during childhood is inversely associated with brain executive trajectories[15], and others have found an inverse association with academic achievement[16]. However, the evidence is still inadequate, given the limitations in measuring air pollution exposure (most studies only used residential levels based on geographical modelling) and brain development (most studies were based on subjective tools), the lack of studies during the most vulnerable stages of brain development (i.e. prenatally and first one year/two years postnatally), and the lack of data on brain anatomical structure and regional connectivity underlying these effects[14].

As mentioned above, one particular interest is the prenatal period, when brain structures are forming and growing, and when the effect of *in utero* exposure to environmental factors may cause permanent brain injury[12]. In humans, there is currently no evidence on the effect of *in utero* exposure to air pollution on foetal and early brain structure and function.

Placenta is the gate between mother and fetus. A mechanism by which air pollution may affect foetal brain development during pregnancy is by impairing placental function and decreasing transplacental oxygen and nutrient transport[17–21]. To our knowledge, there is only one study on the impact of air pollution on placental function that showed negative effects[22]. However, this study did not evaluate whether such an effect can mediate the association between air pollution and fetal growth or brain development. What is known, however, is that that impaired placental function disrupts foetal neurodevelopment in animals and neurobehavioral development in children[18]. Indeed, studies have recently shown that altered placental function is associated with the development of the corpus callosum in the foetal brain[19], and with brain function in neonates and brain connectivity at 1 year of age[20,21].

In addition to air pollution, urban residents are often exposed to higher noise levels, which we and others have shown to detrimentally affect brain development, and to increase the risk of various disorders, such as ADHD[23]. However, the only currently available evidence on the effect of prenatal noise exposure comes from experiments in rodents[24]. Moreover, we found that growing up in urban non-natural built-up environments with less green areas is associated with higher levels of air pollution, higher risk of behavioural problems and poorer cognitive function [25,26]. Another component that could modulate exposure to ambient air pollution is physical activity[27]. These findings demand that any study of the impact of air pollution on brain development needs to address comprehensively the urban context, including early life stressors[28].

The hypothesis of the BiSC project is that prenatal exposure to environmental pollutants during pregnancy impairs foetal and postnatal health and brain development. Specifically focusing on air pollution, we hypothesize that the effects of air pollutants on pre-natal brain development are at least partially mediated by translocation of airborne particulate matter to the placenta and by placental dysfunction, and that prenatal exposure to air pollution impairs foetal brain development independently of pre- natal urban context; and, post-natal brain development independently of post-natal urban context and post- natal exposure to air pollution.

The BiSC project takes advantage of recently improved neuroimaging techniques, such as ultrasound and magnetic resonance imaging (MRI), which provide an unprecedented opportunity to investigate prenatal and early life brain development in children, and the myelination process in particular. In parallel, there are also new techniques, such as magnetometry and electron microscopy, that provide an unprecedented opportunity to track down exogenous particles in human tissues. This development allows us to assess whether part of the effect of air pollution on placental function and ultimately on brain development occurs via direct translocation of the particles to the placenta, and through the placenta to the foetus. Finally, ultrasound has only rarely been used to explore the mechanisms responsible for air pollution-related pregnancy outcomes; in the BiSC project we will use prenatal ultrasonography measures of placental function to understand how this pathway mediates the relationship between air pollution and brain development during pregnancy. Other innovative aspects of the BiSC project are the improvement of the personal exposure assessment to air pollution and noise, and the inclusion of the broader context of the urban environment to address the influence of specific pollutants, noise, greenness, socioeconomic status (SES), and physical activity on the study of the health effects of air pollution on brain development. Finally, the BiSC project will repeatedly collect biological samples in infants (i.e. urine and stool) in order to explore the health effects of exposure to other pollutants in early life (e.g. phenols, pesticides, etc) and assess gut microbiota composition and biological activity, in order to understand the biological pathways of the health effects associated with environmental exposures[29–31]. Genetics and epigenetics will also be explored.

In addition, we took the chance of the having the BiSC birth cohort ongoing in order to conduct the Mood-COVID study, which started after the general lockdown in March 2020, in order to study the mental health effects of the pandemic on the BiSC mothers (see section 4 for further details, including objectives of the study).

2. Objectives

The overarching aim of BiSC project is to evaluate the effect of pre- and postnatal exposure to environmental exposures, particularly urban-related exposures, together with maternal lifestyle habits on pregnancy outcomes and on pre- and postnatal growth and development in offspring, particularly brain development. Towards this aim, the project will:

Objective 1. Establish a new urban pregnancy-birth cohort.

Objective 2. Characterize the pre- and post-natal exposomes, including urban-related exposures, particularly air pollution, noise, greenspace, and temperature, chemical exposures, socio-economic and demographic factors, and lifestyle habits

Objective 3. Measure fetal and child growth development including several health domains: fetal and child anthropometric growth as well as development of nervous, cardiovascular, and respiratory systems. In particular, neurodevelopment will be assessed with brain imaging techniques to evaluate prenatal brain structure, neonatal brain structure, connectivity and function, and with validated neuropsychological tests to assess postnatal motor and cognitive development and behavior.

Objective 4. Prospectively test the relationship between various prenatal and postnatal exposome and fetal and child growth and development and health outcomes, with a particular emphasis on neurodevelopment.

Objective 5. Investigate the molecular an functional mechanisms linking the exposome with fetal and child growth and development and health outcomes, including the methylome, transcriptome, proteome, metabolome and gut metagenome**. Special focus will be put on the mediating role of the placenta**, and its function will be assessed at the physiological level (ie. fetomaternal hemodynamic indicators) and molecular (i.e. methylome) level.

Objective 6. Assess the genetic variation of the mother-father-child trios, using genome-wide methods, analyze their association with molecular and clinical traits, and study their effect modification in the relationships between the exposome and child growth and development and health outcomes.

Objective 7. *This is an additional objective emerged from the COVID19 outbreak in 2020*: to study the effect of COVID19 pandemic on maternal stress and child behavior and the molecular and clinical implications in pregnant women and their children.

3. Materials & methods

3.1 Design

The BiSC project is conducted in the city Barcelona (Catalonia, Spain) and part of its metropolitan area, particulary in the cities of Cornellà and Esplugues de Llobregat. Three major hospitals, Sant Joan de Déu (SJdD), Maternitat-Clínic and Santa Creu i Sant Pau are involved in the study. The study is designed to identify early environmental and genetic causes of normal and abnormal growth, development and health from foetal life until young adulthood.

Therefore, the BiSC project is a prospective cohort study of pregnant women, their offspring and partners, which represent in their-selves three sub-cohorts:

a) Pregnant women, recruited at week 12 of pregnancy and followed up through the whole pregnancy and afterwards.

b) Offspring, recruited at birth.

c) Partners, recruited during pregnancy.

3.2 Study population

Sample recruitment

In total, we aim to recruit 1100 pregnant women in the BiSC project. BiSC participants will be recruited during weeks 11-15 of their pregnancy when they attend the recruitment centers for their first routine ultrasound examination. Given that this routine ultrasound examination asserts whether the pregnancy is singleton and the foetus is not at high risk of congenital anomalies (two inclusion criteria as described below), the participants will be recruited after their ultrasound examination. The following methods will be applied to promote the BISC among the potential participants: i) digital and paper posters presenting BISC displayed in the participating hospitals as well as all the primary healthcare centres in the catchment area of these hospitals, ii) flyers presenting BISC distributed in the participating hospitals as well as all the primary healthcare centres in the catchment area of these hospitals, iii) promotion of the project supplemented with a video describing its objectives and importance. This video will be run on the screens at the waiting areas of the participating hospitals and their referral primary healthcare centres to inform the target audience (i.e. pregnant women). The video will also be promoted via other communication channels and social networks such as hospital websites, iv) the BISC website (http://biscproject.org/) and institutional and personal social media including Twitter, Instagram, and Facebook, v) public lectures and periodical press releases will be applied to promote BISC project among general public. Details on the consent forms for the mothers and their partners, including specific informed consents for genetic and molecular studies, are provided in section 5 (Ethical consideration) and in annexes (section 7).

Inclusion & exclusion criteria

BISC will recruit pregnant women:

a) with singleton pregnancy
b) aged between 18-45 years
c) within weeks 11-15 of their gestation
d) able to communicate in Spanish/Catalan with the BISC team
e) resident in the catchment area of the hospitals (i.e. they live anywhere in Barcelona city or Esplugues)
f) Initially plan to give birth in one of the BISC recruiting hospitals. In case of planning delivery at home, this will be registered in the participant's history to plan the collection of birth data with the midwife who is going to carry out the home delivery.

The following exclusion criteria should be applied:

a) having a severe chronic health condition that could limit the ability to communicate or attend the follow-ups (e.g. severe mental health conditions).
b) having a plan to leave the catchment area of the hospitals **before** delivery or having a plan to leave <u>Barcelona Province</u> **after** delivery (Note: if the potential participant does not know whether she will move out of Barcelona/Barcelona Metropolitan Area, she

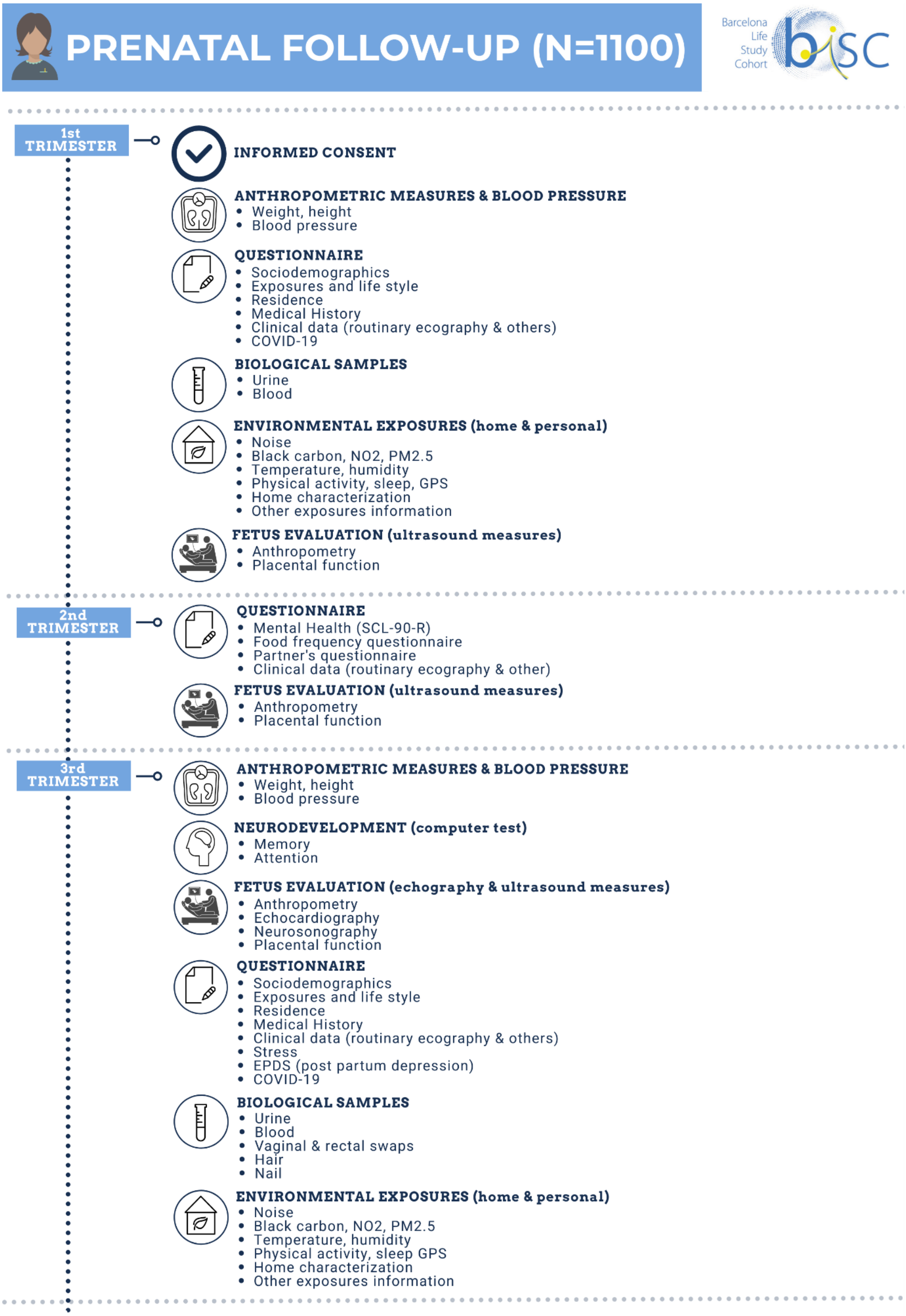


Figure 2. BiSC follow-up during pregnancy.

should **NOT** be excluded. Only having a plan to leave is an exclusion criterion.)

c) Being classified as high-risk for congenital anomalies at the first routine ultrasound measures.

Notes:

- In case of use of artificial reproduction techniques for the current pregnancy, either donated oocyte or sperm, the participant will be included, but this situation will be registered in the participant's history.
- Participants should be able to communicate with the BISC team, which means they need to have a minimal understanding of Spanish/Catalan. An English paper version of the questionnaires will be available for those participants that need it.

3.3 Follow-up during pregnancy

Figure 2 illustrates the follow-up of the pregnant women during their pregnancy. Women will be followed-up three times before giving birth: at week 12th, 20th and 32nd of pregnancy.

3.3.1 Week 12th (1st trimester) of pregnancy

The following tasks are conducted during the first visit and after having obtained the signed consent form from participants:

- Maternal anthropometric measures and blood pressure
- Questionnaire
- Collection of biological samples: urine and blood
- Personal and home exposure assessment to air pollution, noise and other exposures
- Fetus evaluation: anthropometry and placental function
- Collection of biological samples from the father: buccal swab

3.3.1.1. Maternal anthropometric measures and blood pressure: the examination will be carried out by trained personnel, who will follow standardized measurement procedures for anthropometry and blood pressure and appropriate calibrated instruments to ensure the collection of reliable data. The conditions under which blood pressure is measured should be standardized. This includes training to minimize observer biases; equipment factors, subject factors and technique factors. Similarly, the evaluation and interpretation of anthropometric data requires standardized measurement procedures, trained personnel, the use of appropriate, regularly calibrated instruments and the collection of reliable data.

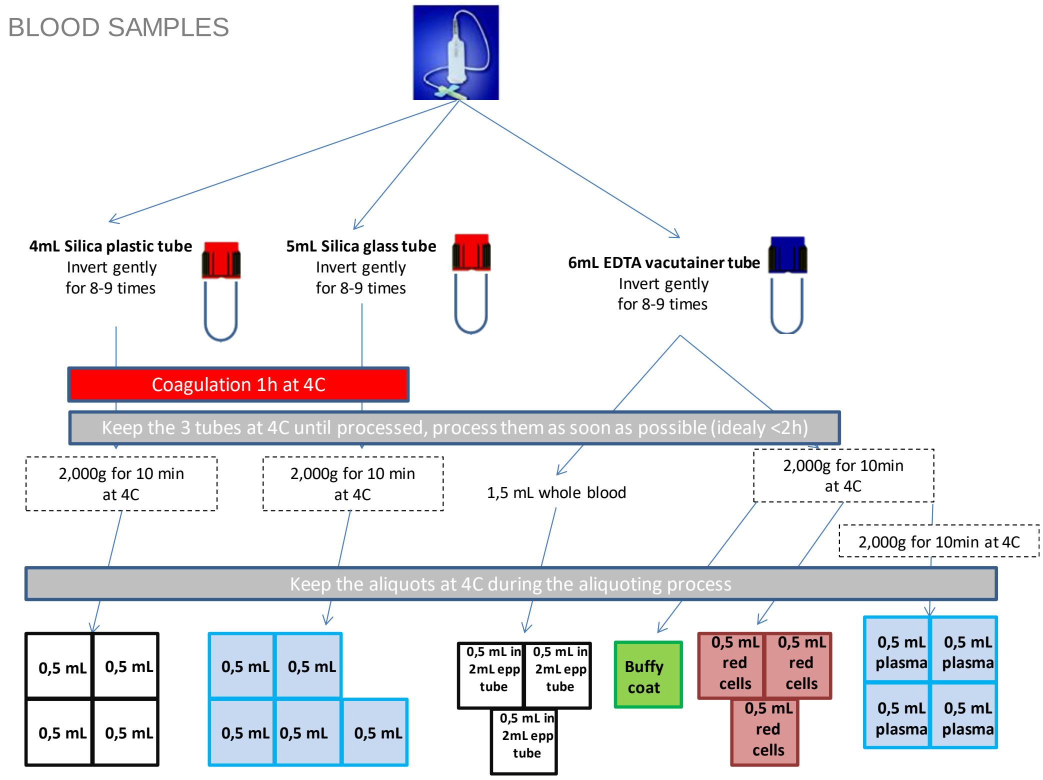
3.3.1.2. Questionnaire: A part of the questions will be administrated in face-to-face interviews by the BiSC nurses or environmental fieldworkers, and the rest will be self-administrated as described below:

- Sociodemographic characteristics (face-to-face interview by the BISC nurses during the enrolment visit).
- Environmental exposure (one part is self-administrated by the participants and the other part is administrated through face-to-face interviews conducted by BiSC environmental fieldworkers).
- Lifestyle (one part is self-administrated by the participants and the other part is administrated through face-to-face interviews conducted by BiSC environmental fieldworkers).
- Home characterization (administrated by the BiSC environmental fieldworkers in the home visit).
- Past medical and obstetrical history and the ongoing medical and obstetrical procedures or conditions.

Questions will be asked both in Catalan and Spanish and will be filled in Clinapsis. After enrolling a participant and assigning her an ID, the BiSC nurse will enter the participant data in Clinapsis and conduct a face-to-face interview on the sociodemopraphic questions. BiSC nurses will then send a user and a password to the participant via email with a link for the participants that will direct her to the environmental exposure and lifestyle questionnaires. Paper versions of the questionnaires are will also be available. Nurses will also collect information on past medical and obstetrical history and the ongoing medical and obstetrical procedures or conditions

3.3.1.3. Biological samples (urine and blood): blood should be collected only by trained personnel using aseptic methods. Sampling location should be an isolated, peaceful area (e.g., a separate room) with all the necessary equipment prepared beforehand. We will take measures to avoid hemolysis (lysis of red blood cells) when collecting blood samples, and to prevent backflow of tube additives from the tube into the individual's arm. A total of 15 ml of blood will be obtained in different tubes and processed in the laboratory to separate different blood fractions (plasma, serum, whole blood, red cells, and buffy coat for DNA), as summarized in Figure 3. After being processed, samples will be stored at -80ºC until analysed in different laboratories to obtain data on genotyping, epigenetics, transcriptomics, metabolomics, proteomics and chemical analyses of the exposome, among others. Time from collection to storage will be recorded.

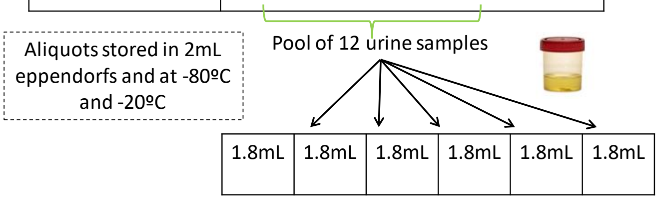
Figure 3. Overview of the protocol for maternal blood sampling and storage at the 12th of pregnancy.



Regarding urine, fieldworkers will bring all the material for sample collection to mother's home on day 1, and mothers will be instructed how to collect the samples. Mothers will collect their morning and bedtime urine samples from day 2 to day 7 (See Figure 4). They will be stored in the freezers (around -20ºC) at home. Samples will be picked up from the houses on day 8 by the field workers and brought to ISGlobal-Campus Clínic (-20ºC). Twice per week samples will be sent from Campus Clínic to Campus Mar, where they will be processed and stored at -80ºC. During processing we will prepare aliquots of individual voids as well as aliquots of weekly pools. They will be used to analyse several chemical compounds a part of different projects.

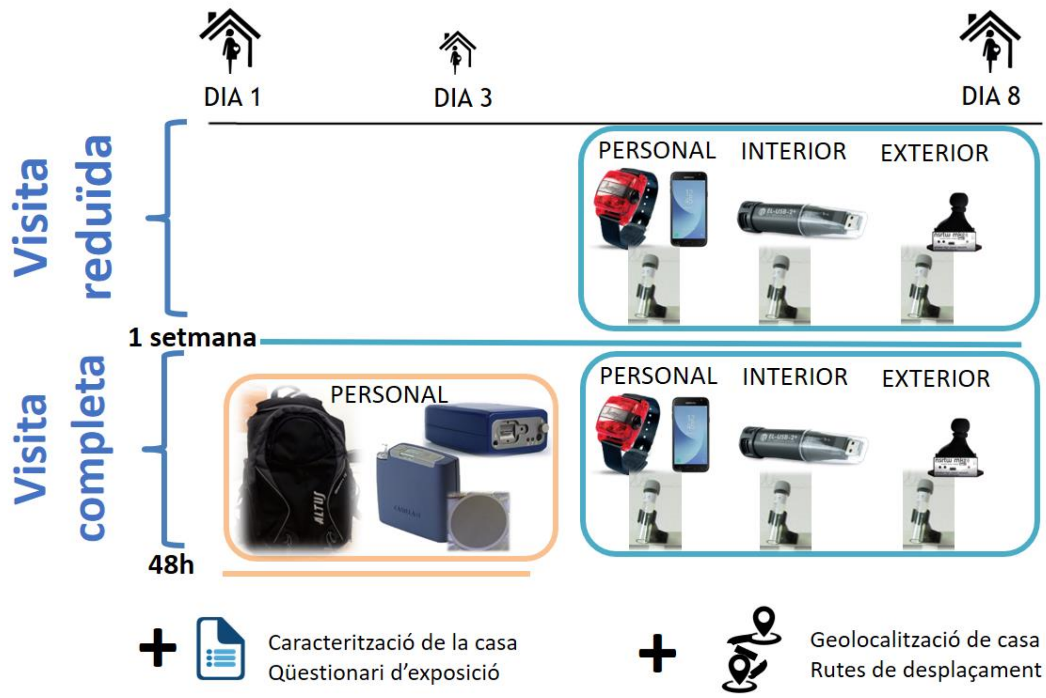
Figure 4. Summary of the collection of maternal urine samples during 12w.

DAY	UM	UN
Day 1	Fieldworkers come to mothers' houses	
Day 2	70mL → 1.8 mL	70mL → 1.8 mL
Day 3	70mL → 1.8 mL	70mL → 1.8 mL
Day 4	70mL → 1.8 mL	70mL → 1.8 mL
Day 5	70mL → 1.8 mL	70mL → 1.8 mL
Day 6	70mL → 1.8 mL	70mL → 1.8 mL
Day 7	70mL → 1.8 mL	70mL → 1.8 mL
Day 8	Fieldworkers come to pick up samples	



3.3.1.4. Personal and home exposure assessment to air pollution, noise and other exposures: figure 5 summarizes the exposure protocol followed in BiSC. Women can choose a reduce or a complete visit, which requires a little bit more of dedication in terms of carrying air pollution samplers. In total 20% of the women are expected to choose the complete visit.

Figure 5. Summary of the exposure protocol followed in BiSC (figure in Catalan)



The following parameters are assessed:

- ***NO_2:*** NO_2 passive diffusion tubes will be worn by the study participants in the first and third trimester of pregnancy to assess personal exposure; tubes will also measure outdoor and indoor exposure to NO_2 at the participant's house. All tubes will be exposed for one week on both the first and third trimesters of pregnancy.
- ***$PM_{2.5}$, including mass and elements:*** personal portable samplers (model Apex2) will be used to assess $PM_{2.5}$ exposure, coinciding with NO_2 measurements. Each subject will carry a backpack with the monitoring pump system during 48h and during her first and third trimesters of pregnancy. Filters will be weighted before and after sampling for gravimetry and will go through determination of absorption coefficient and PM speciation analysis. $PM_{2.5}$ outdoor measurements will be sampled for a subsample of 100 subjects. For those subjects, outdoor measurements will be sampled with an extra pump installed at subjects' home while personal monitoring is in progress.
- ***Black carbon (BC):*** microAeth® AE51, a light portable device (400 g) will be carried in the backpack to measure real-time BC exposure. BC will be monitored 48h in all BiSC study participants, principally during the first trimester of pregnancy; participants unable to do it on the first trimester will be monitored on the third trimester.
- ***Ultrafine particles (UFP):*** this measurement will be done in a subsample of 100 subjects. DISCmini, a portable device, will be carried in the backpack to measure real-time UFPs during 48h in one of the trimesters (if possible, during the first trimester).
- ***Temperature and humidity:*** The EL-USB-2+ device will be placed at the participant's bedroom on the first and third trimesters of pregnancy, for a complete whole week on both occasions.
- ***Noise:*** outdoor sound pressure levels will be recorded at the most exposed facade of the participant's house for one week using a Convergence NSRT_mk2 sound-level meter. The noise meters will be installed next to the NO_2 passive samplers in order to correlate both exposures during data analysis. For some participants, if the bedroom is not in the most exposed facade, noise will be also measured at the bedroom facade.
- ***Geolocation (GPS):*** real-time location data (recorded by GPS) will be used to estimate levels of exposure to these pollutants at residences and workplaces, and the commuting routes between them. A smartphone with the mobile phone application "ExpoApp" and the use of SensorLab software will be used. Geolocation will be monitored using ExpoApp for one complete week in all BiSC study participants (n=1100) during the

both the first and third trimesters of pregnancy. The Smartphone will be carried inside the backpack provided for the first 48h and then in the participant's hand back or where it is more convenient for them. In addition, we will geolocate participant's outdoor monitoring devices, and will also geolocate the commuting of pregnant women during the monitoring week at the 1st trimester and the 3rd trimester visit. In order to represent the route that participants usually to do in the first or the third trimester, fieldworker will register the most regular route of the participant until the day of the visit, independently of the exact week of pregnancy. For the third trimester, the period to consider will be the time between the first trimester visit and the third trimester visit. For each case we will create one layer in the QGIS program: one for the first trimester commuting and one for the third trimester commuting.

- ***Physical activity:*** a physical activity monitor (ActiGraph wGT3X-BT) will be carried by the participants to collect data on the level of physical activity. The ActiGraph will be worn by study subjects on the first and third trimesters of pregnancy, for a complete whole week on both occasions. To support these data, a standardized questionnaire to collect data on physical activity and the average time that each participant spends in each microenvironment (home, work, etc) will be used.
- ***Home characterization:*** fieldworkers will visit the home of the participants to characterize it. This includes collecting information on number of rooms, windows (and their characteristics), whether there are blinds, etc.
- ***Other urban related exposures***: based on the residential address, exposure to green spaces will be assessed using GIS techniques, from which specific indicators can be obtained (e.g. NDVI).

All these measurements will be combined to estimate exposure to air pollution: we will integrate the geolocation data with the direct personal black carbon assessment to estimate exposure in each microenvironment (home, work, and commuting), and we will integrate microenvironmental air pollutant levels with physical activity data to estimate the inhaled dose for each study participant. To do this, we will first calculate the duration and intensity of activity obtained from the personal physical activity monitor for each microenvironment. We will then estimate each participant's inhalation rate in each microenvironment as a function of their age and their average activity intensity in that microenvironment. This estimate will be computed using a regression framework parameterized according to the recommended methodology from the US Environmental Protection Agency (EPA). We will then calculate inhaled doses for $PM_{2.5}$ total, by sources and elements based on the high correlation (r=0.98) between the black carbon in the $PM_{2.5}$ filters and the black carbon in the personal monitors. Finally, we will calculate pollutant-specific total inhaled dose during the different time-windows.

3.3.1.5. Fetus evaluation: participants will undergo their routine ultrasounds each trimester. The following parameters will be assessed in the first trimester:

- *The biparietal diameter (BPD):* is measured on the largest true symmetrical axial view of the fetal head, structures such as the midline third ventricle, interhemispheric fissure and choroid plexuses should be visible.
- *The cranio-caudal distance (CRL):* is measured in a middle sagittal section that includes the fetus in a neutral position.
- *The Uterine arteries:* At first trimester a better flow velocity wave with an optimal insonation angle is obtained with the vaginal route. Transvaginally, the probe is placed in the anterior fornix, the probe is moved laterally to visualize the paracervical vascular plexus, Color Doppler is turned on and the uterine artery is identified as it turns cranially to make its ascent to the uterine body. Measurements are taken at this point, before the uterine artery branches into the arcuate arteries.

3.3.1.6. Biological samples from the father (buccal swab): BiSC nurses will give the kit to the fathers (or to the mothers if the fathers are not at the visit) as well as the instructions. The buccal swab sample can be collected any day of the week at home. The father will fill in a form as well. Fieldworkers will collect the sample during the environmental visit. Samples will be stored at -20ºC. We will extract paternal DNA for genotyping from these samples.

3.3.2 Week 20th (2nd trimester) of pregnancy

The following tasks are conducted during the second visit:

- Fetus evaluation: anthropometry and placental function
- Questionnaire

3.3.2.1. Fetus evaluation: The following parameters will be assessed in the second trimester:

- *Fetal biometry:* fetal growth will be calculated from the next four parameters using the

Hadlock formula.

- *Biparietal diameter (BPD):* measured at a transverse plane at the level of the thalami and the cavum septum pellucidum, from the outer border to the inner border of the skull.
- *Head circumference (HC):* at the same plane of the BPD, with ellipse placing outside of the skull bones.
- *Abdominal circumference (AC):* transverse section of the abdomen at the level of the portal sinus and stomach, with ellipse placing at the outer surface.
- *Femur length (FL):* longest length of the ossified diaphysis.

- *The Uterine arteries:* after 20 weeks the abdominal route is the best choice. Transabdominally, the probe is placed longitudinally in the lower lateral quadrant of the abdomen, angled medially. Color flow mapping is useful to identify the uterine artery as it is seen crossing the external iliac artery.

3.3.2.1. Questionnaire: The questionnaire encompasses two main topics: maternal mental health (SCL90-r) and food frequency. The questions will be self-reported by the BiSC participants, via an online form created with Clinapsis. BiSC nurses will send a link to the participants that will direct them to the online questionnaire. Paper versions of the questionnaires will also be available. Partners of the participants will also receive a questionnaire to collect basic data on socioeconomics and lifestyle.

3.3.3 Week 32nd (3rd trimester) of pregnancy

The following tasks are conducted during the first visit and after having obtained the signed consent form from participants:

- Maternal anthropometric measures and blood pressure
- Maternal neurodevelopment: memory and attention
- Questionnaire
- Collection of biological samples: urine, blood, vaginal and rectal swabs, hair and nail
- Personal and home exposure assessment to air pollution, noise and other exposures
- Fetus evaluation: anthropometry, placental function, echocardiography and neurosonography

3.3.3.1. Maternal anthropometric measures and blood pressure: we will follow the same protocol as the one followed at the follow-up of the 12^{th} week of pregnancy (see section 3.3.1).

3.3.3.2. Maternal neurodevelopment (memory and attention): this assessment includes a test to assess working memory (N-back) and another to assess visual reasoning (PMA-R). The nurses/midwives will be in charge of explaining to the participants how the neuropsychological assessment tests work. The order of performance of the tests will be:

1) N-Back, for the evaluation of working memory.
2) PMA-R, to assess visual reasoning.

N-back is a paradigm for evaluating working memory widely used in neuroimaging research. The test consists of remembering a previous stimulus presented (in this case visually) as a function of the load of the n. That is, with a 0-back task we have to remember the stimulus just prior to the one presented, when it is 1-back we have to remember the penultimate stimulus and so on. In the task format that we have adapted for the study, it involves pressing one of the mouse buttons depending on the load of the n. The task will be divided into a stimulus (numbers) and 3 different n conditions or loads (0-back, 1-back, 2-back, 3-back). In the PMA-R the participant must look at a series of letters and choose which letter is missing. This test follows an abstract reasoning pattern and is based on the letters of the Spanish alphabet. The participant has 6 minutes to answer each exercise with different degrees of difficulty. If in one session you cannot figure out which letter is missing, you can move on to the next exercise by pressing the arrow. This test correlates with fluid intelligence and is similar to Raven's test of abstract reasoning.

3.3.3.3. Questionnaire: part of the questions will be administrated by face-to-face interviews by the environmental fieldworkers and by BiSC nurses, and the rest will be self-administrated as described below:

- Sociodemographic characteristics (self-administrated by the participants)
- Stress (self-administrated by the participants)
- Depression (administrated by BiSC nurses in the 3r trimester hospital visit)
- Environmental exposure (one part is self-administrated by the participants and other part is administrated through face-to-face interviews conducted by BiSC environmental fieldworkers)
- Lifestyle (one part is self-administrated by the participants and other part is administrated

through face-to-face interviews conducted by BiSC environmental fieldworkers)

- Home characteritzation (administrated by the BiSC environmental fieldworkers in the 3rd trimester home visit only if the participant has changed her address since the 1st trimester)

Questions will be asked both in Catalan and Spanish and will be filled in Clinapsis. The participants will fill the environmental exposure and lifestyle online questionnaires through the link provided by the nurses in the 1st trimester of pregnancy. Paper versions of the questionnaires are also available.

3.3.3.4. Biological samples (urine, blood, vaginal and rectal swabs, hair and nail): for blood and urine we will follow the protocol detailed in the follow-up of the 12th week of pregnancy (see section 3.3.1). Vaginal and rectal swabs will be collected to measure microbiota composition and diversity in the vagina and the gastrointestinal tract, respectively, in future analyses. Sampling will be conducted by gynecologists.

Hair sample collection will be done at the hospitals by the BiSC nurses during third trimester visit. Training of the nurses will be needed for this procedure. Figure 6 shows the area from which the hair samples should be obtained and stored. Hair will be used to measure biomarkers of stress, including cortisol and its metabolites.

Figure 6. Hair sampling example.

Nail sampling will be conducted by the mothers at home, and samples collected by fieldworkers during the home visit. A kit and instructions will be provided to the participants. Nails will be used to measure the chemical exposome in the future.

3.3.3.5. Personal and home exposure assessment to air pollution, noise and other exposures: we will follow the protocol detailed in the follow-up of the 12th week of pregnancy (see section 3.3.1).

3.3.3.6. Fetus evaluation: for fetal biometry we will follow the same protocol as the one followed at the follow-up of the 20th week of pregnancy (see section 3.3.2). In addition, we will conduct:

- *Fetoplacental hemodynamics:* which includes umbilical artery (UA), the middle cerebral artery (MCA), the cerebroplacental ratio (CPR), the ductus venosus (DV), the Uterine arteries (UtA). Prenatal Doppler ultrasound examinations will be performed in the absence of fetal movements. Pulse Doppler parameters will be performed automatically from three or more consecutive waveforms, with the angle of insonation as close to zero as possible.
- *Fetal cardiovascular assessment:* fetal cardiovascular remodeling will be assessed using different morphometric and functional parameters described below. During each exam, a movie clip of apical and lateral four chamber view, including at least three complete heart cycles without fetal movements, will be stored for off-line analysis. Morphometric parameters include: cardiac area, left and right sphericity, left and right atrial diameters and areas, fetal cardiac diameters (length and transverse diameter) and area, longitudinal cardiac diameter (LCD) of the heart, transverse diameter (TCD), left, right and septal wall thicknesses, and aortic/pulmonary outflows. Functional

parameters include valve E/A index, E wave deceleration, duration of A wave, aortic and pulmonary artery flows, TAPSE, SAPSE and MAPSE, the MPI, tissular Doppler measurements (optional).

- *Fetal neurological assessment:* fetal brain exam will be perform and standardize based on the ISUOG guidelines for fetal brain assessment which include the following parameters: Axial planes (transventricular plane, transthalamic plane or biparietal diameter plane:, transcerebellar plane), sagittal planes (midsagittal plane, parasagittal plane) and coronal planes (transcaudate plane, transcerebellar plane).

3.4. Follow-up at and after birth

Figure 7 illustrates the follow-up from birth until the age of 18 months.

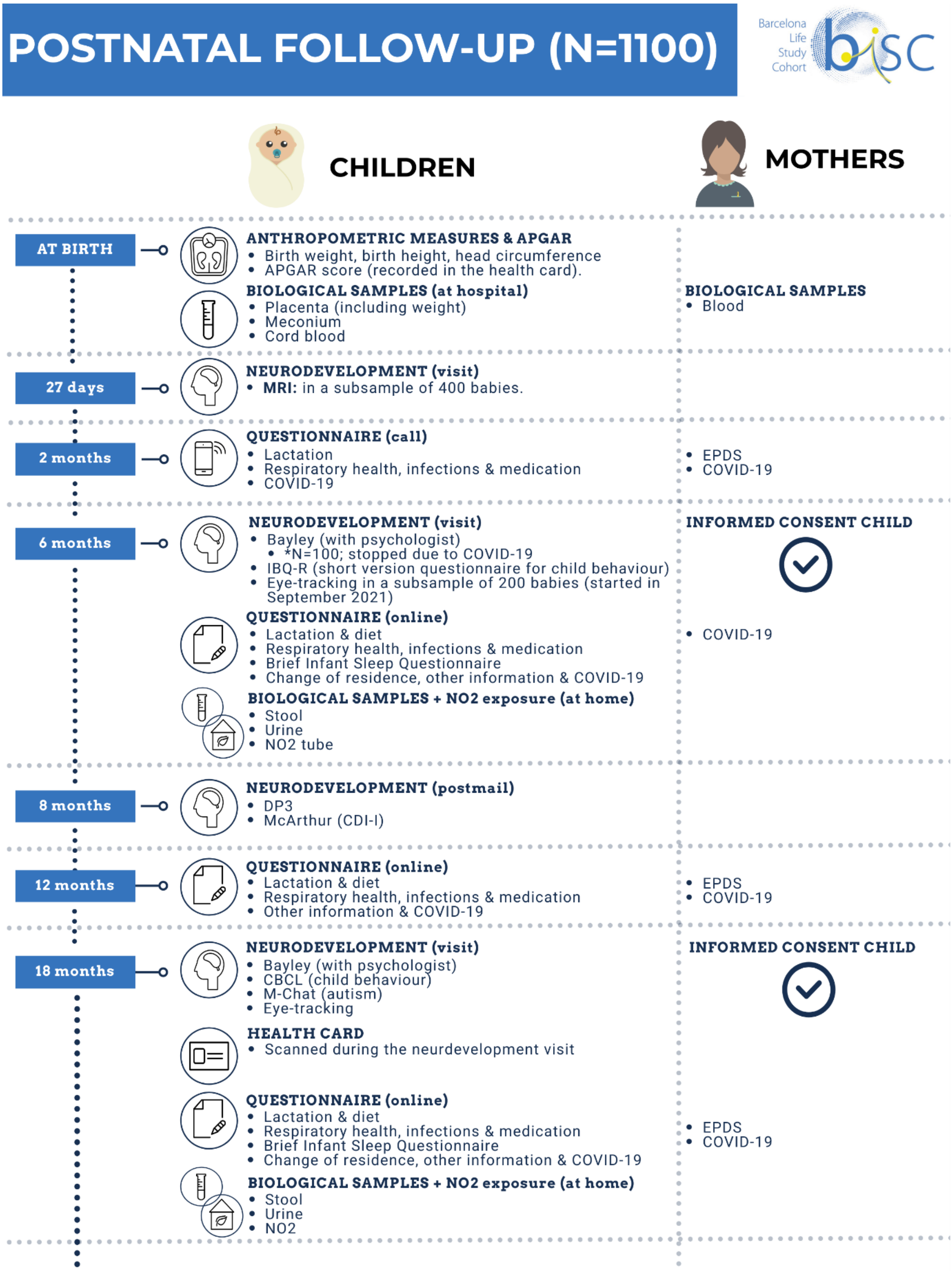
3.4.1 Follow-up at birth

The following tasks are conducted at birth:

- Obtaining the consent form from the parents for the participation of their baby in the BiSC study (see section 5 - Ethical consideration - and annexes in section 7 for details on the consent forms for the babies, including the specific consent form for the MRI).
- Anthropometric measures of the baby: birth weight & height and head circumference.
- APGAR score.
- Collection of maternal blood.
- Collection of placenta, cord blood and meconium of the baby.

3.4.1.1. Anthropometric measures of the baby & APGAR score: the complete somatometry should be done within the first 12 hours of life, and is possible, within the first 6 hours of life. It will be performed by qualified nursing staff from the Maternity ward, delivery room or Neonatal Unit. The information will be written in the official form of the hospital and in the medical card of the child.

Figure 7. BiSC postnatal follow-up.



The gynecologists will record the Apgar score just after birth. The score is commonly used as a rapid method to assess health's status of the newborn immediately after birth. It has been designed to evaluate signs of hemodynamic compromise such as cyanosis, hypoperfusion, bradycardia, hypotonia, respiratory depression or apnea. Some covariables such as gestational age, birth weight, maternal medications, drug use, anesthesia or congenital anomalies should be taken into account because they can influence the Apgar scor. Newborns can be classified in a) critically low, when the total apgar score is between 0 and 3; b) below normal, when the total score is between 4 and 6; and c) considered as normal, when the total score is 7 or higher.

3.4.1.2. Collection of maternal blood: blood should be collected only by trained personnel using aseptic methods. Sampling location should be an isolated, peaceful area (e.g., a separate room) with all the necessary equipment prepared beforehand. We will take measures to avoid hemolysis (lysis of red blood cells) when collecting blood samples, and to prevent backflow of tube additives from the tube into the individual's arm. The blood sample will be collected, if possible, before delivery and as close as possible to the time of delivery. In any case, time of blood extraction will be registered and time from extraction to delivery will be estimated. A total of 15 ml of blood will be obtained in different tubes and processed in the laboratory to separate different blood fractions (plasma, serum, whole blood, red cells, and buffy coat for DNA), as summarized in Figure 8. After being processed, samples will be stored at -80ºC until analysed in different laboratories to obtain data on genotyping, epigenetics, transcriptomics, metabolomics, proteomics and chemical analyses of the exposome, among others. Time from collection to storage will be recorded.

3.4.1.3. Collection of cord blood: cord blood samples will be obtained as indicated for mother samples, with the exception that blood in PAXgene tubes for transcriptomic analyses will also be obtained (Figure 9). Cord blood will be collected after delivery waiting at least 1 minute until the cord stops pulsing the blood to the fetus. Following the birth of the baby the cord will be double-clamped at 15-20 cm from the baby and with a distance of 2-5 cm between clamps. The needle will be inserted into the umbilical vein to collect 20 ml of cord blood. In uncomplicated deliveries, the cord blood will be collected prior to the delivery of the placenta. If not possible, it will be collected afterwards. If the cord is collapsed, then blood will be collected directly from the placenta blood vessels.

Figure 8. Overview of the protocol for maternal blood sampling and storage at birth.

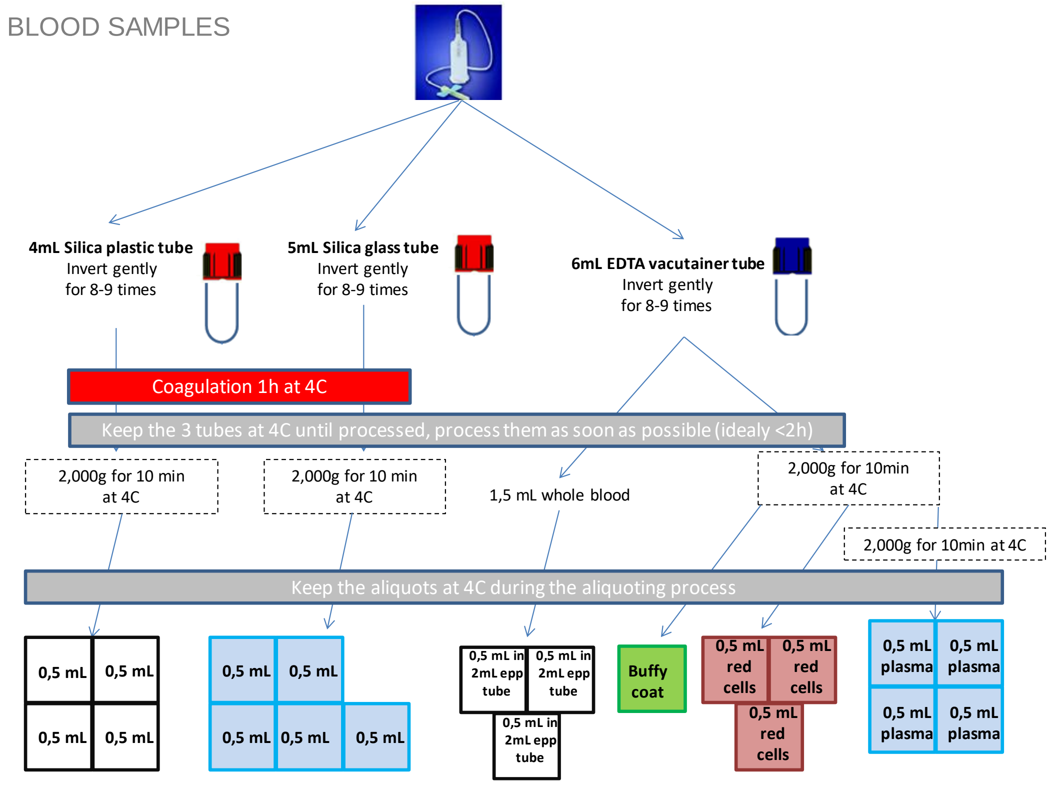
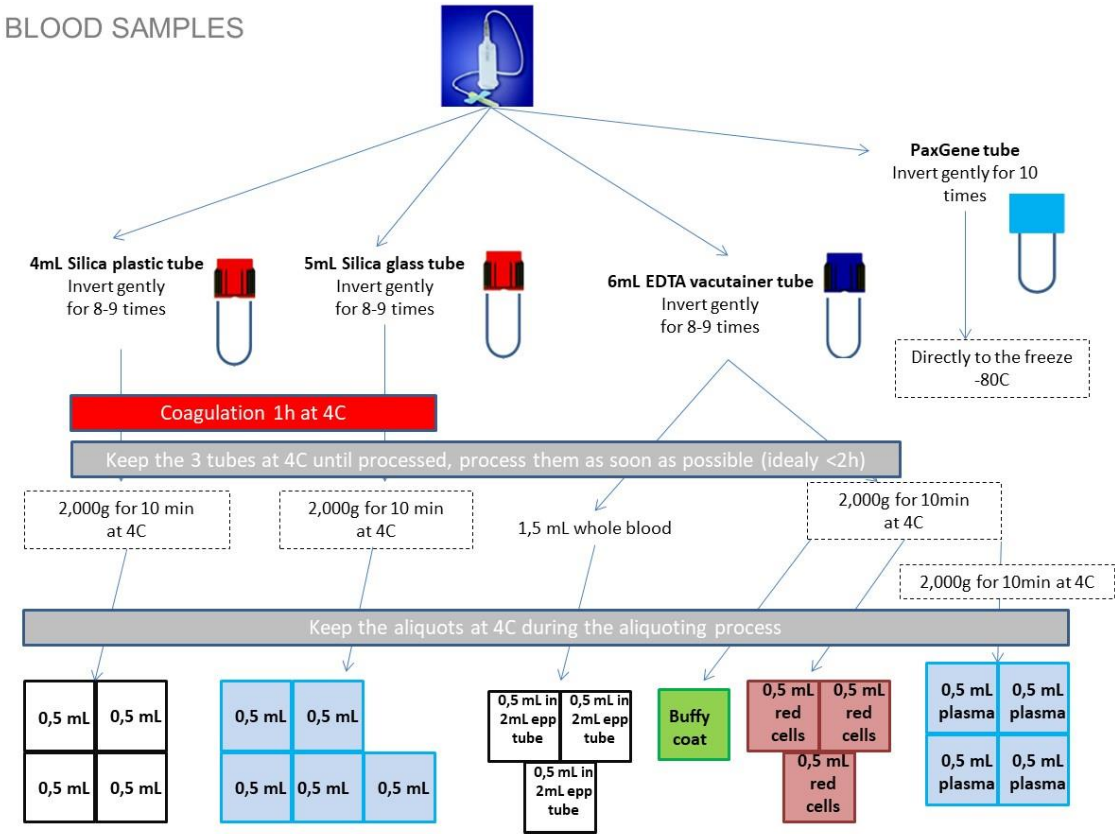


Figure 9. Overview of the protocol for cord blood sampling and storage at birth.



3.4.1.4. Collection of placenta and cord tissue: as soon as possible after delivery, the placenta and the remaining cord will be weighted and placenta and cord blood biopsis will be obtained following the instructions shown in Figure 10. Briefly, 2 biopies of 0.4 x 0.4 x 2.5 cm (full thickness of the placenta) will be collected in two different quadrants. The biopsies will be taken approximately at 4 cm from the cord insertion in an homogenious region (ie. without blood vessels). Each biospy will be cut in 2, one directly snap frozen in liquid nitrogen and then at -80ºC, and the other will be put in RNAlater for 1 week. Then, it will be cut in smaller fractions as indicated in Figure 11. The placenta samples will be used to analyze the chemical exposome and other exposures such as air pollution particles and also to assess the molecular responses to that exposome, including the epigenome, transcriptome, metabolome, and proteins, in different laboratories.

Figure 10. Collection of placenta and cord tissue biopsies.

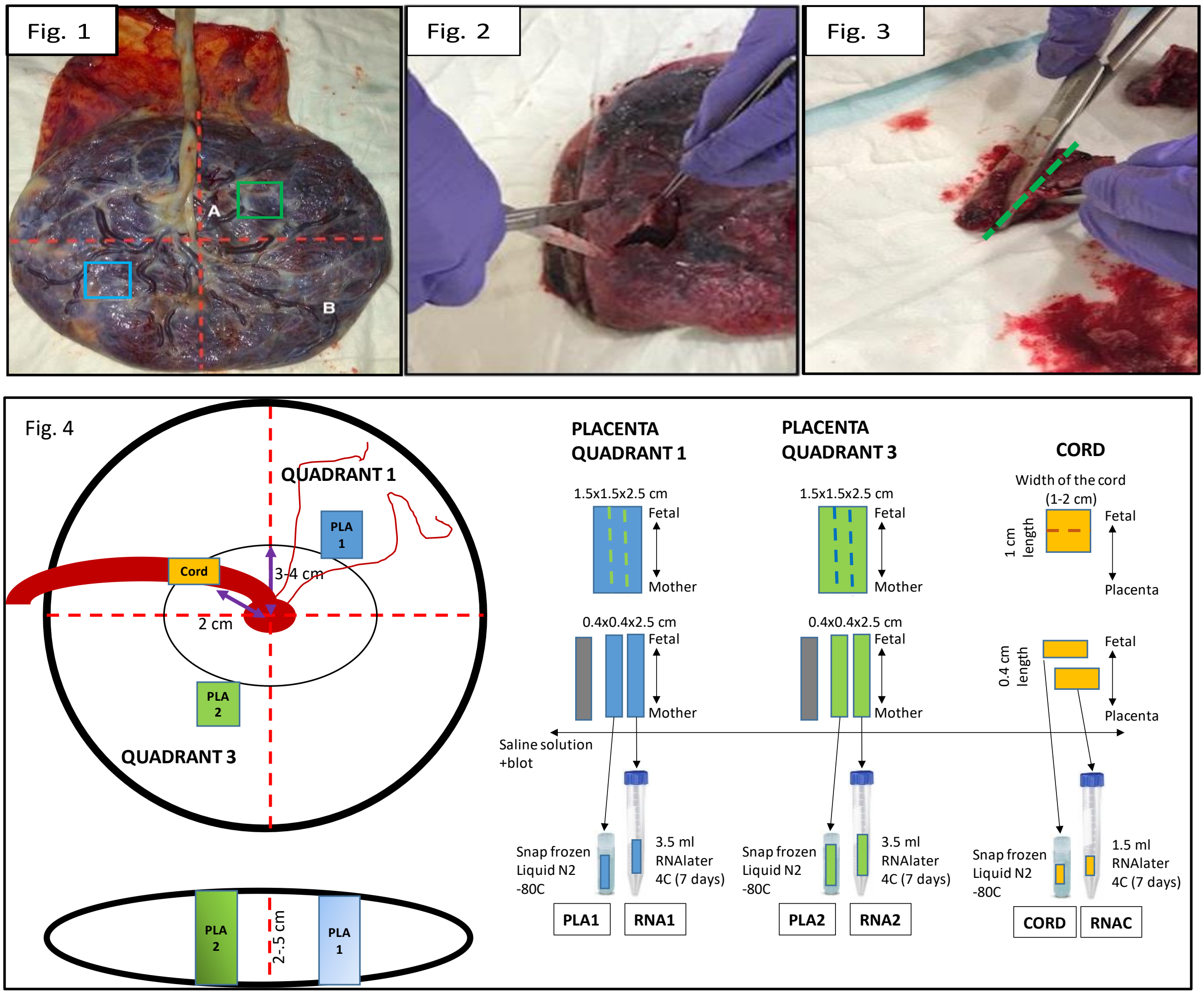
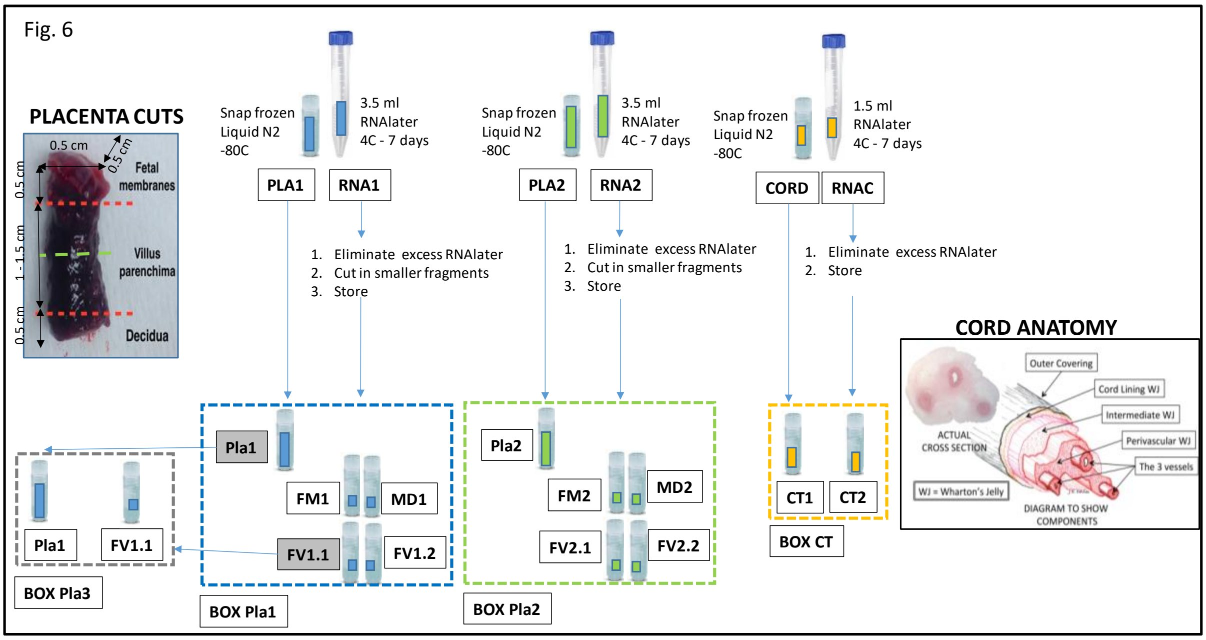


Figure 11. Processing of placenta and cord tissue biospies collected in RNAlater



3.4.1.5. Collection of meconium: the meconium is the first stool of a newborn infant. It is produced in utero and consists of materials such as epithelial cells, bile, mucous, and more. In most newborns, meconium is generally passed in the first day or so of life, has no odor, and appears as a very dark, tar-like substance. This helps distinguish meconium from the next phase of passage called transitional stool. Transitional stool will start to have an odor and present with a browner, greener, or more yellow color as the newborn starts digesting milk. Meconium samples are collected by the mothers during their hospital admission after birth. During the first routine diaper change, the sample will be collected. Hospital personnel will then collect the sample and store it at -20ºC until sent to the lab for long term storage at -80ºC. The sample will be used to explore the metagenome and the chemical exposome, among others.

3.4.2. Follow-up the age of 27 days (MRI)

The following tasks are conducted at the age of 27 days:

- Magnetic resonance imaging (MRI).

The proposal to collaborate in this part of the study will be presented to all participants who had previously participated in the assessment of household environmental exposure with at least 1 complete evaluation, except to the ones whose new-borns meet any of the exclusion criteria. The aim of the MRI-study and the procedure will be clearly explained by the BISC midwives in the 32-week visit.

The exclusion criteria for the MRI-study are:

- New-borns conditions:
  - Premature birth (<37 weeks)
  - Diagnosis of neurological disease, congenital disease or congenital malformation, and/or the presence of respiratory diseases or conditions that may affect both the results and the new-born safety during the procedure.
  - If the child has suffered any surgery since birth.
- Participants conditions:
  - No assessment of household environmental exposure has been performed (at least 1 visit is needed).
- MRI-study related conditions. The new-born will have to fulfil any of the conditions provided in the Annex 2 at birth, the doctor will fill in the checklist of MRI compatibility and will provide a medical certification in order to assess if there is any MRI-study related condition that may pose an exclusion criterion (Annex 2).

Fifteen days after birth, the BISC technician will call the participants and will ask their agreement with their participation in this part of the study. The BISC technician will respond to the questions that may arise. Also, the BISC technician will make an appointment to conduct the MRI to the closest day to the 27^{th} day since birth once the participants agree to participate. Ideally, the MRI test will be performed at the 27^{th} day since the date of birth, with a time frame of +/- 3 days (the test can not be conducted after the child is 30 days old). The MRI will take place at the facilities of Fundació Pasqual Maragall (FPM). At the day of the MRI, both the FPM and the BiSC (ISGlobal) informed consents will be given to participants to be signed before the MRI-study. While the study is in progress, the mother or the father (only one of them) will be allowed to stay in the room where the MRI-technicians are placed, which is separated from the MRI-room by a glass wall, being able to attend the procedure and watch the new-born until it finishes. Before entering the room, parents and a doctor need to sign the FPM consent form to confirm that there are conditions of the mother or the father that prevent them to be in the exploring room. Before starting, participants will be accommodated in the waiting room and will be asked to feed the new-borns until they fall sleep. No sedation will be used in order to perform the imaging acquisition. The test will be performed as soon as the baby has fallen asleep – with no pre-established order. If they do not fall asleep, another appointment will be made provided that it does not exceed the 27±3 days period stablished to perform de MRI-study.

The new-born will be placed in a MRI-adapted foam cradle that has been specifically designed for this study. It adjusts both to a new-born's body and the MRI-coil. The new-borns will be wrapped in blankets for protecting against the cold in the MRI-room (with an approximated temperature of 19ºC) and for limiting their movements throughout the exam. Only if necessary, an immobilizer will be used. Hearing protectors will also be used (cotton and earplugs). In order to assess the new-born safety during the procedure, the respiratory function will be measured with a paediatric pulse oximeter and a respiratory rate measure.

The parameters and the sequences and timings of the MRI are (Figure 12):

- T1-voxel: 1x1x1mm3.
- DWI: direction 24, b-value 1200 s/mm2, 2.2 isotropic resolution.
- 2.8 x 2.8 x 2.8 mm3 TR= 2s.

Figure 12. MRI parameters

Survey_NEONATS	FLAIR_NEONATS	T1_NEONATS	RS_NEONATS	rDWI_NEONATS	DWI_NEONATS	Total
x	x	x	x	x	x	**6**
9	43	120	8142	61	1525	**10420**
00:35	01:18	04:03	06:03	00:15	03:31	**0:15:45**

The aim is to obtain information on anatomical volumes of main structures (cortex, white matter, deep grey matter, ventricles and cerebellum), and dimensions of pre-selected pathways (visual, auditory and sensorymotor) and white matter tracts with known brain functions in neonates, as well as deterministic tractography [number of streamlines belonging to each pathway, fractional anisotropy, and integrity in *a priori* selected pathways of interest (motor, somatosensory, auditory, visual and limbic) and major white matter tracks (CC, inferior frontooccipital fasciculus, middle cerebellar peduncle)] and functional and structural connectivity (strength, global efficiency, local efficiency and clustering) from functional and diffusion MRI.

In total, the MRI- image acquisition procedure should not last more than 30 minutes; if the new-born wakes up during the imaging acquisition, a second and last attempt will be made to continue with the procedure. If they remain awake, the study will be stopped and hereby terminated.

After the study, a neuroradiologist hired by FPM will generate a neuro-radiological report within 15 days after the MRI. In case of brain anomalies or urgent findings we have established a protocol in order to inform the families and eventually guide proper actions.

3.4.3 Follow-up at age 2 months

The following tasks are conducted at the age of 2 months:

- Questionnaire

This very short follow-up includes a short questionnaire to assess postpartum depression and short questions about breastfeeding and the child's illness during the first two months of life. Information on COVID-19 infection is also asked. A second goal is to remember the mothers that they are part of the BiSC community and that a new follow-up will take place at the age of 6 months. The follow-up will be done by telephone, but all responses will be automatically recorded in the EU Survey system (https://ec.europa.eu/eusurvey/), specifically in the specific form https://ec.europa.eu/eusurvey/runner/BiSC_2m_followup_CAST" (see Annexes, section 7). The phone call takes less than 20 minutes in total.

3.4.4 Follow-up at age 6 months

The following tasks are conducted at the age of 6 months:

- Baby personal air pollution exposure assessment.
- Residential noise exposure assessment for participants with no information collected during pregnancy (same protocol as during pregnancy).
- Neuropsychological evaluation with Bayley Scale for Infant and Toddler Development, Third edition (BSID-III)]. *Note: this test was stopped due to COVID-19 pandemic (final*

N=100).

- Neuropsychological evaluation with eye-tracking.
- Questionnaire.
- Collection of biological samples: urine and stool.

3.4.4.1. Personal air pollution exposure assessment: NO_2 is a perfect indicator of all combustion sources, and it is feasible to assess infant's personal exposure to NO_2 because the tubes are light and do not need to be manipulated. Thus, passive NO_2 samplers attached to child for a one-week period will be used to assess personal exposure to NO_2. The tubes will be delivered to the mothers by mail and collected after one week by fieldworkers. Tubes will be stored at 4ºC until sent to a specialized lab for their analysis. We will perform temporal adjustment using a factor based on hybrid temporal geographical land use regression models to estimate pollutant levels during each month of life up to the date of neurodevelopment exam. Data on NO_2 from the samplers attached to the children will be combined with the home levels of NO_2 and particles derived from the mother to estimate post-natal exposure to $PM_{2.5}$.

3.4.4.2. Residential noise exposure assessment: we will follow the same protocol as the one followed during pregnancy (see section 3.3.1).

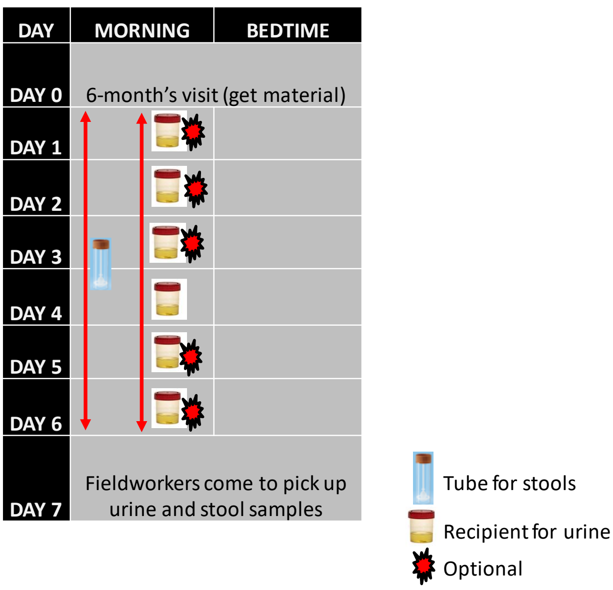
3.4.4.3. Neuropsychological evaluation with BSID-III: the Bayley's examinations will be performed by a single trained blinded observer (psychologist), who will invite all families to attend the 6 months' visit. She will evaluate 5 distinct scales of development: cognitive, language, motor competencies (fine and gross), and socio-emotional and adaptive behaviors. The Bayley III is a gold-standard measure with proven validity and reliability.

3.4.4.4. Neuropsychological evaluation with eye-tracking: two experiments are conducted: experiment 1 is Visual Recognition Memory (VPC) and experiment 2 is Object Occlusion. In the first experiment infants familiarize themselves with two identical stimuli and then on test show a new one, which infants should prefer if they memorized the others. A ball passes behind an occluder. We register the extent to which infants understand the permanence of an object, and when it passes behind something, they anticipate the appearance on the other side (i.e. predictive tracking).

3.4.4.5. Questionnaire: The follow-up includes a questionnaire to assess breastfeeding practices, diet of the child, child's illness since the previous follow-up, and other information such as pet ownership, daycare attendance, exposure to tobacco smoke or change of residential address and the characteristics of the new home. Information on COVID-19 infection is also asked. A second questionnaire collects information on the sleep quality of the child, infant child behavior (IBQ-R test). Participants are contacted by email and requested to fill in the two EU Survey system based questionnaires "https://ec.europa.eu/eusurvey/runner/BiSC_6m_followup_CAST_part1" (25 minutes) and "https://ec.europa.eu/eusurvey/runner/BiSC_6m_followup_CAST_part2" (10 minutes); see Annexes, section 7.

3.4.4.6. Biological samples (urine and stool): families will receive at home the kit and the instructions to collect themselves both urine and stool samples from their child. At home samples will be stored a -20ºC. After collection, a fieldworker will visit the families to collect the samples and freeze them at -80ºC after processing them. Regarding stool, only one sample will be collected in a sterile tube, while for urine mothers can choose between collecting one or six samples in a week (see Figure 13). The urine sample will be used to analyze the chemical exposome, among other factors. We aim to have 10% of the families volunteering for this intensive collection of urine samples in order to calculate inter-day variability of the chemicals and extrapolate measurement error the rest of samples. Stool sample will be used to assess the gut microbiome and also metabolites.

Figure 13. Scheme of child urine and stool collection at 6-month visit.



3.4.5 Follow-up at age 8 months

The following tasks are conducted at the age of 8 months:

- Neuropsychological evaluation with DP3 and McArthur.

At the age of 8 months of the child families will receive via post mail the two questionnaires: DP3 and McArthur. After filling them in, families will return the questionnaires to the neuropsychologist, who will calculate the scores and inform the families in case of incidental findings. DP3 allows to quickly assess the five main areas of child development (Cognition, Motor, Socio-emotional, Communication and Adaptive Behavior) and offers a global index of child development. The MacArthur Communicative Development Inventories (CDI) reflect the normal process of early language acquisition through a set of diverse manifestations: Prelinguistic Gestures, Prelinguistic Vocalizations, Vocabulary, and Grammar. MacArthur has become an essential instrument with important innovations in early communication and linguistic development and that is allowing progress in the early detection of language disorders.

3.4.6 Follow-up at age 12 months

The following tasks are conducted at the age of 12 months:

- Questionnaire

This follow-up includes a short questionnaire to assess postpartum depression, breastfeeding practices, diet and the child's illness since the last follow-up. Information on COVID-19 infection is also asked. A second goal is to remember the mothers that they are part of the BiSC community and that a new follow-up will take place at the age of 18 months. Participants are contacted by email and requested to fill in the EU Survey system based questionnaire "https://ec.europa.eu/eusurvey/runner/BiSC_12m_followup_CAST" (15 minutes; see Annexes, section 7).

3.4.7 Follow-up at age 18 months

The following tasks are conducted at the age of 18 months:

- Baby personal air pollution exposure assessment.

- Neuropsychological evaluation with BSID-III.
- Neuropsychological evaluation with CBCL and M-Chat.
- Neuropsychological evaluation with eye-tracking.
- Questionnaire.
- Collection of biological samples: urine and stool.
- Health card information.

3.4.7.1. Personal air pollution exposure assessment: we will follow the same protocol as the one followed at the age of 6 months (see section 3.4.4).

3.4.7.2. Neuropsychological evaluation with BSID-III: we will follow the same protocol as the one followed at the age of 6 months (see section 3.4.4).

3.4.7.3. Neuropsychological evaluation with CBCL and M-Chat: the neuropsychologist will contact the participating families to request them to fill in the questionnaire "https://ec.europa.eu/eusurvey/runner/BiSC_18m_neuro" (see Annexes, section 7), which includes the Child Behaviour Checklist (CBCL) and M-Chat (Cecklist for Autism in Toddlers). The CBCL is used to detect behavioural and emotional problems in children and adolescents. The CBCL is completed by parents. The M-Chat is a questionnaire to determine possible cases of children with autism in the routine evolutionary examination of 18 months. Answering both questionnaires takes less than 10 minutes.

3.4.7.4. Neuropsychological evaluation with eye-tracking: two experiments are conducted: experiment 1 is Visual Recognition Memory (VPC) and experiment 2 is Word-learning. In the first experiment infants familiarize with two identical stimuli and then on test show a new one, which infants should prefer if they memorized the others. The second experiment based on the Intermodal Preferential Looking Paradigm (IPLP), and it includes four test blocks so as to better capture infants' ability to learn two novel-word/novel object pairings.

3.4.7.5. Questionnaire: The follow-up includes a questionnaire to assess short questionnaire to assess postpartum depression, breastfeeding practices, diet of the child, child's illness since the previous follow-up, and other information such as use of screens, pet ownership, daycare attendance, sleep quality of the child, exposure to tobacco smoke or change of residential address and the characteristics of the new home. Information on COVID-19 infection is also asked. Participants are contacted by email and requested to fill in the EU Survey system based questionnaire "https://ec.europa.eu/eusurvey/runner/BiSC_18m_followup" (20 minutes); see Annexes, section 7.

3.4.7.6. Biological samples (urine and stool): we will follow the same protocol as the one followed at the age of 6 months (see section 3.4.4).

3.4.7.7. Health card information: at the visit with the neuropsychologist for the BSID-III, parents will provide the health card and the information will be scanned and afterwards digitalized using an EUSurvey form created for the purpose (https://ec.europa.eu/eusurvey/runner/BiSC_18m_carnet_salut).

3.5 Data management & analysis

Size and Power calculation: BiSC will recruit 1100 pregnant women. Based on our experience (response rate and attrition) in the INMA birth cohort[32], we estimate that it will be necessary to contact a total of n=2200 pregnant women. From the 1100 women recruited, we expect 95% live births (n~1045), and 95% of the new-borns to be followed-up at age 18 months (n>990). We expect to obtain a transvaginal neurosonography and Doppler scan for most of them. Based in our cohort studies and the observed association between residential exposure to NO_2 and the general Bayley's cognition scale, a study with 990 babies will have 95% power to detect the observed decrease of 1.98 points associated with a 10 µg/m3 increase in NO_2. This calculation is based on a mean and standard deviation of the outcome of 100±15, and of NO_2 of 48±1597, and a type I error rate of 0.05. Based on our MRI studies in healthy children[33], We expect that ~400 mothers will agree for their baby undergo a neonatal MRI. In turn, based on our neonatal MRI studies[20], we estimate that at least 300 MRI will be validly acquired. A study with 300 new-borns will have 95% power to detect a 5% decrease in the total area of the corpus callosum/cephalic index (mean 2.03, standard deviation: 0.44).

Data collection files: the data collection files (maternal address, telephone information, ID number) are kept in separate, password protected database on a secure server at ISGlobal and are only accessible to study staff with the appropriate training and clearance to access personal health information. Biological samples and medical record/questionnaire data are only labeled with this study ID and no other identifying information. Data linking subject names with study

IDs are kept in a separate, password-protected database on a secure server controlled by Dr. Sunyer and the BiSC data manager. Participants and staff are informed of the confidentiality of information and assured that the data will only be used for statistical purposes and group analyses in which individuals cannot be identified. Data will only be presented in tabular form, and no information on individual identification will be revealed in any published reports. No data beyond what has been consented will be obtained without authorization from the subject.

Data management and cleaning: BiSC data management team will be the responsible to clean the data (with the help of key investigators from each area) and store it in Comma Separated Values (csv) files, which can be linked via the unique BISC id. The repository data includes codebooks and Standard Operation Procedures.

Internal requests: All internal requests for data must be submitted in writing for documentation, and addressed to the BiSC Publication Committee (PC). The request will include: Title, Lead author(s) (junior and senior), Rationale and background, Research hypotheses/objectives, Analysis plan, including exposures, outcomes and main covariates considered, and statistical analysis protocol, Key references, Foreseen timeline and target submission date, and Foreseen co-authorship. The PC will review the proposal to verify that the proposal format has been followed and to determine if there is potential overlap with any other papers or abstracts, proposed or in progress. The PC will then work with the authors to reduce or remove overlap. Accepted manuscript proposals will be available on the BISC website intranet to help investigators determine available topics in advance. When the above requirements have been satisfied, the BiSC data management team will prepare a comma separated value (csv) file. The use of this text format will ensure compatibility with any statistical software. Internal researchers will have to destroy the data once the analysis has been completed. Authorship will follow BiSC regulation to ensure that appropriate credit is given to BiSC researchers and all public and private funding supporting BiSC will be acknowledged in the published work. Authors will be notified of the intent to publish and will be provided with an advance copy of any submitted manuscript that relies on the requested data.

External use: Data obtained as part of this project will be made available to external investigators to this proposal through a formal Data Transfer Agreement (DTA). In no case will explicit identifiers (e.g. contact information, medical record numbers) be included in analytic files for either internal use or sharing with external collaborators.

Data transfer: To ensure secure transfer of data and to facilitate access of authorized collaborators to these increasingly large data sets, ISGlobal has implemented a secure data transfer system (private cloud - Namek), which is mounted as a Virtual File System to the main ISGlobal servers, that guarantees the security and integrity of all data from Information System, and also complies with the General Data Protection Regulation (GDPR).

Data analyses: BiSC will estimate the effects of air pollutants, as well as other exposures, on birth outcomes and maternal and child health, including child brain development. We will also explore whether this effect is direct or indirect through the mediation of factors such as placental impairment, and will consider potentially more vulnerable subgroups based on sex or contextual variables such as noise, greenness or SES (i.e. interaction). To do so, we will combine classical epidemiological analysis with novel methods based on the counterfactual framework for causal inference when analysing mediation.

We will use general linear models, distributed lag models and b-splines to test for association between total maternal exposure to air pollution and dose during different time windows of pregnancy (weekly, monthly and trimester) and birth outcomes and pre and postnatal brain development, including fetal and neonatal imaging outcomes. A similar approach will be followed for other exposures. We will construct a conceptual model that accounts for effects and correlations between exposures at different time points by creating an exposure lag space. We will use inverse probability of attrition weighting (IPW) to control for potential bias by non-response when analyzing the subsample in the case of neonatal MRI or other neuropshychological tests that we will perform in subsamples (e.g. BSID-III at the age of 6 months). We will explore the most relevant window(s) of exposure to air pollution or other pollutants using distributed lag models.

Based on up-to-date published evidence, we will draw a direct acyclic graph (DAG) to decide which of the potential confounding variables selected *a-priori* should be included in the statistical models, including: contextual socioeconomic indicators at residential address and individual data for total personal exposure and dose; and temporal variables such as temperature and humidity for the spatial-temporal variables. To test the independent and interaction effects between the various exposures, after controlling for collinearity, we will apply penalized regression methods.

We will also conduct a formal mediation analysis to evaluate the role of placental function in mediating the association between environmental exposures during pregnancy and the outcomes of interest (e.g. birth outcomes and pre and postnatal brain development). Potential effect modifiers will be tested (e.g. maternal stress, physical activity, diet, and noise sensitivity, canopy measures).

4. COVID19 in BiSC

4.1 Objectives:

The COVID19 outbreak, which turned to be a pandemic, occurred during the recruitment of the pregnant women in the BiSC birth cohort. We therefore decided to include the effects of COVID19 in our study, including the impacts on the maternal stress and child behavior and the molecular and clinical implications in pregnant women and their children. The specific objectives are:

1) To describe the temporal evolution of psychological stress in pregnant women and women with a recent delivery during the different phases of the COVID-19 pandemic.
2) To identify the main predictors (i.e. health-related, economic insecurities, and confinement conditions) of the psychological stress, post-traumatic stress disorder and depression linked to the COVID-19 pandemic.
3) To evaluate the associations of the maternal psychological problems induced by the pandemic (e.g. confinement measures and economic insecurities) with the neurodevelopment of the child, as already assessed in the general BiSC protocol.
4) To understand the molecular and clinical implications in pregnant women and their children.

4.2 Methods:

All the procedures described below have been approved by the CEIm.

- ***Questionnaires:*** during the strict confinement between March to June 2020 in Spain, we contacted the 550 women that had been already recruited by BiSC. We followed them up through a total of 4 web-based questionnaires to characterize their evolving situation (economic, home and family conditions of confinement, and fears related to the pandemic) and also their mental health (perceived stress). After confinement, in late June 2020, we assessed the accumulated stress-related hormones in hair, and resent the questionnaire, also adding maternal mental health assessment for anxiety and depression. The last questionnaire will be resent several months later (6 to 12 months later depending on the state of the pandemic) to capture the long-term evolution in mental health of mothers and will include the assessment of stress, anxiety, depression and post-traumatic stress. We provide the initial questionnaire to show the type of questions asked to the participants (see Annexes, section 7).

- *Maternal and partner's stress and mental health:* besides the existing data already collected in BiSC on maternal health (e.g. EPDS), we will additionally assess mental health of women with the post-traumatic stress disorder (PTSD) (Symptom Severity Scale: in Spanish EGS-R) test. Partners' mental health will be assessed with the same questionnaires after confinement, except depression, which will be characterized with the Becks Depression Inventory (BDI-II), as the EPDS is for pregnancy.

- *Biological samples:* in June 2020, hair was collected to characterize the stress-related hormones in women (cortisol, 20-alpha-dihydrocortisol, 20-beta -dihydrocortisol, cortisone, 11-dehydrocorticosterone, and 20-alpha-dihydrocortisone, 20-beta-dihydrocortisone). In addition, we specifically requested pregnant women their consent to use their biological samples (e.g. blood, placenta, etc) in studies related to COVID19.

- *Child neurodevelopment:* we will use the neurodevelopment outcomes already assessed in BiSC at the age of 6 and 8 months.

- *Other variables:* questionnaires include additional questions regarding: COVID-19 infection and contacts, confinement situation (e.g. number of times they go out of home, home characteristics, couple dynamics and conflict resolution styles, domestic violence, change in diet, sedentarism, etc.), degree of concern related to health impacts derived from (potential) COVID-19 infection, and economic insecurity (of family and in general).

- *Data analysis:* we will follow the procedures described in section 3.5.

5. Ethical considerations

Ethical issues within the BiSC project are related to:

a) the recruitment of pregnant women and their offsprings
b) the acquisition of images via transvaginal neurosonography
c) the collection of biological samples (hair, placenta tissue) in women
d) personal air pollution monitoring and geolocalization during pregnancy and in the new borns
e) the administration of neurodevelopment tests to children (age 6 and 18 months)
f) the acquisition of brain MRI in new borns
g) the administration of several questionnaires to the women (SES, diet and other life-style habits, maternal IQ)
h) personal data collection and storage
i) genetic and epigenetic analysis

Research studies in Spain are regulated by both international and national legal and ethical rules. The Principal Investigator and the research team are aware and will conform to the International, European and National legislations in all the various aspects of the research as detailed below.

The candidate project is aware of further relevant guidance and codes, including:

- The Nuremberg Code (1946) addressing volunteer consent and proper acting;
- The Revised Declaration of Helsinki in its last version of 2013
- The convention for the protection of human rights and dignity of human being with regard to the application of biology and medicine called the "Convention on Human Rights and Biomedicine" (Council of Europe, 1997) and its additional protocol on biomedical research (2005)
- The Recommendation Rec (2006)4 of the Committee of Ministers to member states on research on biological materials of human origin (Council of Europe) are the main international guidelines for medical research.
- Convention of the Council of Europe on Human Rights and Biomedicine signed in Oviedo on 4 April 1997, and the Additional Protocol on the Prohibition of Cloning

Human Beings signed in Paris 12 January 1998;
- The Spanish Law on Biomedical Research (14/2007, of 3rd July) which regulates biomedical research in Spain
- The charter of Fundamental rights of the EU Directive 95/46/EC of the European Parliament and of the Council of 24 October 1995 on the protection of individuals with regard to processing of personal data and on the free movement of such data;
- UN Convention on the Rights of the Child (1990);
- The Royal Decree that establishes the basic requirements for the authorisation and functioning of biobanks with biomedical research purpose and for the processing of human samples and regulating the functioning and organisation of the National Register of Biobanks for Biomedical Research (1716/2011, of 18th November).

We have reviewed the guidance available on the web (http://ec.europa.eu/research/participants/docs/h2020-funding-guide/cross-cutting-issues/ethics_en.htm; http://ec.europa.eu/research/swafs/index.cfm?pg=policy&lib=ethics).

ISGlobal- Campus Mar is bond to the PS-Mar Ethics Committee (Clinical Research Ethics Committee of the Municipal Health Care Service, created and accredited for the first time on November 11th, 1993 by the General Direction of Health Resources of the Department of Health of the Government of Catalonia, in accordance with the Order of 26 October 1992). The PS-Mar CEIC evaluates all research protocols in humans conducted by ISGlobal-Campus Mar researchers. According to Spanish regulations, our local Ethic Committee will follow the implementation of the study by giving its approval to every protocol (including Participant Information Sheet and Consent Form) that will be developed through the study. All ISGlobal researchers are self-regulated by the Code of Good Scientific Practice (http://www.prbb.org/system/uploads/attachment/file/3/en/eng_a4.pdf).

General procedures included in the research protocol to safeguard the privacy of study subjects:
- Written consent will be obtained from all the participants in the study to use their personal data.
- All material obtained in the framework of the project (questionnaires, lab samples, MRI, etc) will be identified through a code, the name and/or other personal data that

could allow the identification of the participant will never be indicated. This unique identifier will link all basic data required for the study. The master key file linking the centre's study numbers with personal identifiers will be maintained in a password protected file with limited access.

- All files containing personal data will be stored in encrypted and password-locked files. Access to these files will be limited to authorized project personnel;
- Only researchers linked to the project will have access to personal data.
- Personal data will not be transferred, except in the cases considered by law.
- Reported study results will pertain to analyses of aggregate data. No individual's name will be associated with any published or unpublished report of this study.
- All project personnel will be trained in the importance of confidentiality of individual records and required to sign a confidentiality agreement.

We provide detailed information regarding:

- Inform consents: the BiSC protocol includes 7 informed consents (see Annexes for details). Four of them are provided to the participants during pregnancy, when women and their partners are invited to participate. The other three informed consents are provided to the participants at birth, and are necessary for the participation of the baby in the study.

<u>Enrollment of women and their partners:</u> prior to the enrolment into the study, the BISC nurse shall provide the potential participant with detailed information on the study aims and objectives and the tasks that participants are expected to accomplish. Moreover, the BISC nurse shall highlight the volunteer nature of the participation and the ability of the participant to leave the study whenever she wishes together with assurances that not accepting to participate or leaving the study by no means would affect the healthcare that she would receive. The potential participant will be then given as much time that she needs to properly study the informed consent sheet in a peaceful environment without perceiving any need to rush or any pressure to accept. The BISC nurse should be available to the potential participant to answer the queries that might arise when studying the informed consent sheet. See annexes, section 7, for details on the consent forms for the mothers and their partners, including specific informed consents for genetic and molecular studies.

<u>Enrollment of the baby:</u> prior to the enrolment of the baby into the study, the BISC fieldworker shall provide the mothers of the child and their partners with detailed information on the study

aims and objectives and the tasks that participants are expected to accomplish from the BISC fieldworker. Moreover, the BISC fieldworker shall highlight the volunteer nature of the participation and the ability of the participant and her baby to leave the study whenever she and her partner wishes, together with assurances that not accepting to participate or leaving the study by no means would affect the healthcare that she would receive. The potential participant will be then given as much time that she needs to properly study the informed consent sheet in a peaceful environment without perceiving any need to rush or any pressure to accept. The BISC fieldworker should be available to the potential participant to answer the queries that might arise when studying the informed consent sheet (see annexes, section 7, for details on the consent forms for the babies, including the specific consent form for the MRI). See annexes, section 7, for details on the consent forms for the babies, including the specific consent form for the MRI and genetic and molecular studies. In all cases one of the parents or a legal tutor will have to sign the consent form.

- Recruitment and informed consent procedures, including details on the procedures used to ensure that there is no coercion on participants

The recruitment of pregnant women will be done in collaboration with the three Hospitals involved in the study (see above). Women will be recruited at their first prenatal visit. During the visit at the hospital and prior to any procedure, participants will be informed by BiSC midwife or nurse (well informed about the study and specifically trained in research ethics) about the study procedures, duration and their right to withdraw the study at any point without any consequence. If the woman agrees to participate, the consent form will be signed and a copy will be given to her.

- Research with pregnant women: procedures to ensure welfare

All investigators involved in the study will be appropriately trained and well qualified to conduct research with humans and to perform all study procedures.

The protocol will include details about which measures will be taken to ensure welfare of participants in all study procedures. These are summarized below:

<u>Transvaginal neurosonography</u>

Transvaginal neurosonography is a routinely exam in obstetrics used to detect brain malformations. It will be performed using a two-dimensional transabdominal and transvaginal approach at week 32. All scans will be performed by the same experienced examiner using the same equipment (Voluson W10 Expert scanner, equipped with a 5–9 MHz transvaginal transducer). Each neurosonography will last 20 to 30 min. Ultrasonographic exams do not have secondary effects. We will follow the criteria established by the International Society of Ultrasound in Obstetrics (Sonographic examination of the fetal central nervous system: guidelines for performing the 'basic examination' and the 'fetal neurosonogram'. UOG 2007; 29: 109)116.

Questionnaire (SES, diet…)

- Questionnaires will be administered via in-person interviews, by phone or by email (see details above).
- The participant will be located in a quiet room or at home, when she has the right time and conditions to answer the questionnaire.
- The interviewer will ensure the questionnaire is well understood and that the volunteer feels comfortable before and during the questionnaire administration.

MRI testing in the newborns

Image acquisition MRI examinations will be performed at Fundació Pasqual Maragall (FPM) following the protocol described above. To avoid movement artefacts, term-neonates will be scanned while spontaneously asleep after feeding. Particular care will be taken to protect neonatal welfare during the MRI acquisition; to minimize noise using customized headphones and covering the magnet tunnel with special noise protection foam; and to achieve sufficient immobilization and handling of the infant's head within a neonatal coil, without compromising optimal signal-to-noise ratio: a dedicated 32-channel receive array coil and positioning device to facilitate careful image acquisition, monitoring and immobilization of the child, and management of acoustic noise. MRI will be acquired using a whole body magnetic resonance scanner. An expert neuroradiologist will visually inspect all acquired structural T1 and T2 weighted images to exclude brain abnormalities or apparent artefacts, and tests will be excluded accordingly (around 15% due to white matter (WM) lesions, awakening or excessive movement during acquisition).

An expert neurologist will review all the scans routinely; incidental findings that may have clinical significance will be communicated following the established protocol (see above). This option entails a clinical read of all research scans, not just those identified as presenting a possible incidental finding. We will follow the general recommendations on disclosing incidental findings proposed by Anastasova et al (2013) Communication of results and disclosure of incidental findings in longitudinal paediatric research, and the The Management of Incidental Findings Detected During Research Imaging (2011) report of the The Royal College of Radiologists (RCR) and the Scottish Imaging Network: A Platform for Scientific Excellence (SINAPSE).

Neurodevelopment testing in children

Neurodevelopmental outcomes will be assessed using the BSID-III, and by measuring eye tracking at ages 6, and 18 months using the Tobii system. The Bayley's examinations will be performed by a single trained blinded observer (psychologist), and will evaluate 5 distinct scales of development: cognitive, language, motor competencies (fine and gross), and socio-emotional and adaptive behaviours. The BSID-III is a gold-standard measure with proven validity and reliability conducted by a neuropsychologist observing the responses of the baby to an ordered set of games. To complement this direct observation method, which is sensitive to the observer bias, we will use an 'objective' procedure, namely eye tracking. This will be measured using a remote eye-tracker, which uses a video camera to evaluate the distance and speed of eye movements, and where on the screen the infant is looking in response to viewing faces. Eye tracking is also seen as a game by babies and parents and has not any invasive or disturbing manoeuvre. Other neuropsychological tests will also be implemented; these tests will be self-responded by the parents: IBQ-R (2 months), DP3 and McArthur-CDI (8 months), M-Chat and CBCL (18 months).

Ethics advisor

We have appointed Prof Magí Farré as the ethics advisor for the BiSC project. Prof Farré is going to be external advisor for the whole BiSC study and has reviewed and approved the present document. Magí Farré (MD, PhD) is Professor of Pharmacology at Universitat Autònoma de Barcelona, Spain. He holds an MD degree with specialization in Clinical Pharmacology and a PhD in Pharmacology in Universitat Autònoma de Barcelona. He also has a postgraduate Diploma in Bioethics and Quality of Life (Universitat de Barcelona) and a Master in Medical Ethics (Organización Médica Colegial). During the last 25 years, Magi

Farré has been member of the Human Research Ethics Committee of the Parc de Salut MAR (CEIC-PSMAR, Barcelona) and have acted as its Chairman for the last 10 years. In 2015, Magí Farré joined Hospital Universitari Germans Trias i Pujol in Badalona, where he is Head of the Clinical Pharmacology Unit and Chairman of the Human Research Ethics Committee of the Hospital (CEIC-HUGTiP, Badalona). He has previous experience as Ethics advisor for seven European founded projects.

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7. Annexes

The following annexes are attached:

- General consent form for the mothers (Mothers_General Consent form_BISC_v7 2021 01 04.pdf)
- Genetic consent form for the mothers (Mothers_GeneticConsent form_BISC_v3_2021 03 17.pdf)
- General consent form for the partners (Partners_GeneralConsent form_BISC_v2 2018 12 19.pdf)
- Genetic consent form for the fathers (Partners_GeneticConsent form_BISC_v4_2021 03 17.pdf)
- General consent form for the babies (Babies_GeneralConsent form_BISC_v7 2021 01 04.pdf)
- Genetic consent form for the babies (Babies_GeneticConsent form_BISC_v3 2021 03 17.pdf)
- MRI consent form for the babies (Annex_3_Babies_MRI_Consent form_BISC_v5 2018 09 26.pdf)
- Questionnaire of the 2 months follow-up (BiSC_2m_followup_CAST_25_01_2021_ES.pdf)
- Questionnaires of the 6 months follow-up (BiSC_6m_followup_CAST_part1_08_04_2021_ES.pdf and BiSC_6m_followup_CAST_part2_08_04_2021_ES.pdf)
- Questionnaire of the 12 months follow-up (BiSC_12m_followup_CAST_08_04_2021_ES.pdf)
- Questionnaires of the 18 months follow-up (BiSC_18m_followup_08_04_2021_ES.pdf and BiSC_18m_neuro_08_04_2021_ES.pdf)
- COVID initial questionnaire (BISC_covid19_inicial_03_03_2020_ES.pdf)