The INTERBIO-21st Study

The Functional Classification of Abnormal Fetal and Neonatal Growth Phenotypes

Study Protocol

The INTERBIO-21st Consortium

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Credits

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This protocol should be referenced as:
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(www.interbio21.org.uk)

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List of abbreviations used

AGA	Appropriate for Gestational Age
BPD	Biparietal Diameter
BMI	Body Mass Index
CRL	Crown Rump Length
CI	Confidence Interval
DDT	Dichloro-Diphenyl-Trichloroethane
DSS	Demographic Surveillance System
ENID	Early Nutrition and Immune Development
FGLS	Fetal Growth Longitudinal Study
GWAS	Genome Wide Association Studies
HC	Head Circumference
IQTL	Imprinted Quantitative Trait Loci
IUGR	Intrauterine Growth Restriction
KDH	Kilifi District Hospital
LBW	Low Birth Weight
LMP	Last Menstrual Period
MeDIP-Chip	Methylated-Cytosine DNA Immunoprecipitation-Microarray Chip
MRC	Medical Research Council
MMN	Multiple Micronutrient
NICU	Neonatal Intensive Care Unit
NCSS	Newborn Cross-sectional Study
PPFS	Preterm Postnatal Follow-up Study
PCR	Polymerase Chain Reaction
RCT	Randomised Control Trial
RR	Risk Ratio
RT-PCR	Real Time PCR
SMRU	Shoklo Malaria Research Unit.
SGA	Small for Gestational Age
SNP	Single Nucleotide Polymorphism

INTERBIO-21st	The INTERBIO-21st Study: The Functional Classification of Abnormal Fetal and Neonatal Growth Phenotypes
INTERGROWTH-21st	The INTERGROWTH-21st Project: The International Fetal and Newborn Growth Consortium for the 21st Century

SUMMARY

The INTERBIO-21st Study aims to evaluate newborn phenotypes so as to understand better the relationship between the causes of IUGR/SGA and preterm birth syndromes. It is based upon our hypothesis, presented in the initial INTERGROWTH-21st Project, that phenotypic subgroups other than those defined by birth weight and gestational age alone are needed to determine a newborn's nutritional status and assess the effectiveness of interventions to prevent and/or treat the effects of an adverse intrauterine environment. In effect, therefore, we are aiming to produce a more "functional" description of these syndromes.

The redefinition of newborn subgroups will arise from evaluating a combination of factors in pregnancies with normal and abnormal outcomes. These factors include maternal health; fetal growth patterns; growth patterns of fetal organs; newborn body composition and physiological function; micronutrient levels and data from epigenetic experiments. We will initially characterise normal genetic variability and normal variability across the epigenome in uncomplicated pregnancies, and compare these data to the variability observed in a sample of high-risk pregnancies. In a series of case-control studies, we will evaluate the effects of adverse environmental and nutritional factors (and other biomarkers), which possibly interact with genetic factors and the epigenome, on the sub-groups of IUGR/SGA and preterm birth.

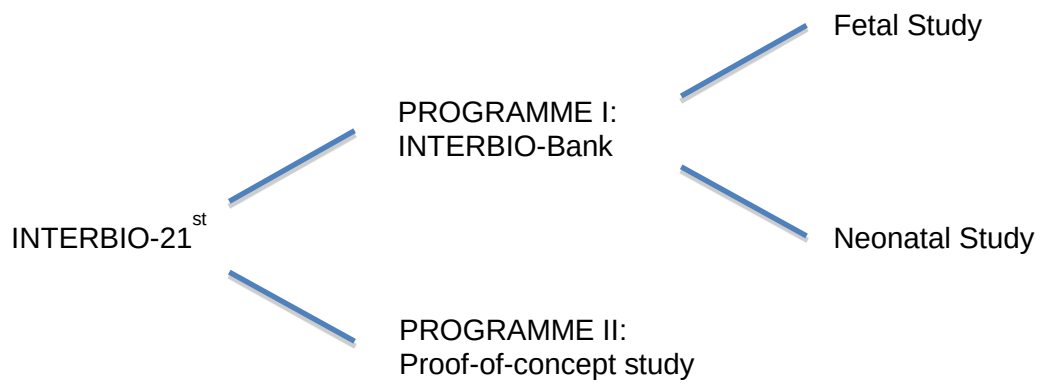
The rigorous clinical and laboratory-based characterization of newborn phenotypes and their different aetiologies in relation to morbidities, especially those that are common in resource-poor settings, should lead to better clinical management of pregnancies and newborn complications. This will contribute to the selection of more effective preventive interventions and screening strategies by improving their specificity.

Specifically, we shall:

PROGRAMME I: Create a unique biobank (INTERBIO-Bank) of maternal blood, maternal faeces and cord blood/placental samples from at least six populations with different risk profiles, including women at high risk for preterm delivery and IUGR/SGA because of malnutrition and/or infection. We shall follow a longitudinal and cross-sectional study design in two sub-studies. These samples will be used primarily to explore risk factors and biomarkers for the subgroups of IUGR/SGA and preterm delivery.

PROGRAMME II: Conduct, in the first of a series of experiments, a hypothesis-testing, proof-of-concept study comparing DNA methylation patterns and micronutrient status in term AGA and IUGR/SGA newborns drawn from the INTERBIO-Bank.

Figure 1: INTERBIO-21st Study Flow Diagram



BACKGROUND

The INTERBIO-21st Study builds upon the International Fetal and Newborn Growth Consortium for the 21st Century (INTERGROWTH-21st), a unique, population-based project that is being conducted in eight different geographical locations in Brazil, China, India, Italy, Kenya, Oman, the UK and US. (www.intergrowth21.org.uk).

The primary objective of INTERGROWTH-21st is to develop new "prescriptive" standards, conceptually similar to the WHO Child Growth Standards, describing optimal fetal and preterm neonatal growth and newborn nutritional status, and to relate these to neonatal health risk.

This objective is being achieved by implementing three studies involving detailed and highly standardised recording of maternal characteristics and anthropometry, pregnancy complications, exposure to pollutants, fetal growth, neonatal anthropometry and perinatal outcomes:

1. Fetal Growth Longitudinal Study (FGLS): ultrasound and clinical assessment of fetal growth every five weeks throughout pregnancy from <14 weeks, with accurate early pregnancy dating, in eight populations with optimal health, in defined geographical areas with low environmental risks. It will produce ultrasound and clinical Fetal Growth Standards.

2. Preterm Postnatal Follow-up Study (PPFS): follow-up of infants from the FGLS cohort born prematurely with regular anthropometry and nutritional evaluation to describe their postnatal growth pattern up to 2 years. It will produce Preterm Postnatal Growth Standards.

All newborns from the complete cohort (FGLS and PPFS) will be seen at 1 and 2 years to evaluate health, nutrition and development.

3. Newborn Cross-Sectional Study (NCSS): anthropometric measures, neonatal morbidity and mortality, and pregnancy complications assessed in all newborns at each of the study centres over a 12 month period, i.e. all deliveries are being captured over 12 months from the same areas. It will produce Newborn Birth Weight for Gestational Age Standards.

The secondary objectives are:

a) **Clinical:** to develop a prediction model, based on multiple 2-dimensional (2D) ultrasound measurements, for estimating gestational age during mid-late pregnancy for use in populations of pregnant women without access to early/frequent antenatal care;

b) **Epidemiological:** to study in this multi-ethnic, population-based sample the determinants of LBW and its components (preterm delivery, impaired fetal growth and their subgroups) under current healthcare conditions, and

c) **Biological:** to acquire additional 3-dimensional (3D) images to create an anatomical and growth databank of individual fetal organs as a unique source of biological information for future research.

The study populations from these geographically defined areas have no socio-economic constraints on growth; low morbidity and perinatal mortality, and adequate nutritional status. To be included, women must be non-smokers, with a normal pregnancy history, and without health problems likely to influence fetal growth or indicate a risk for pregnancy-related pathological conditions.

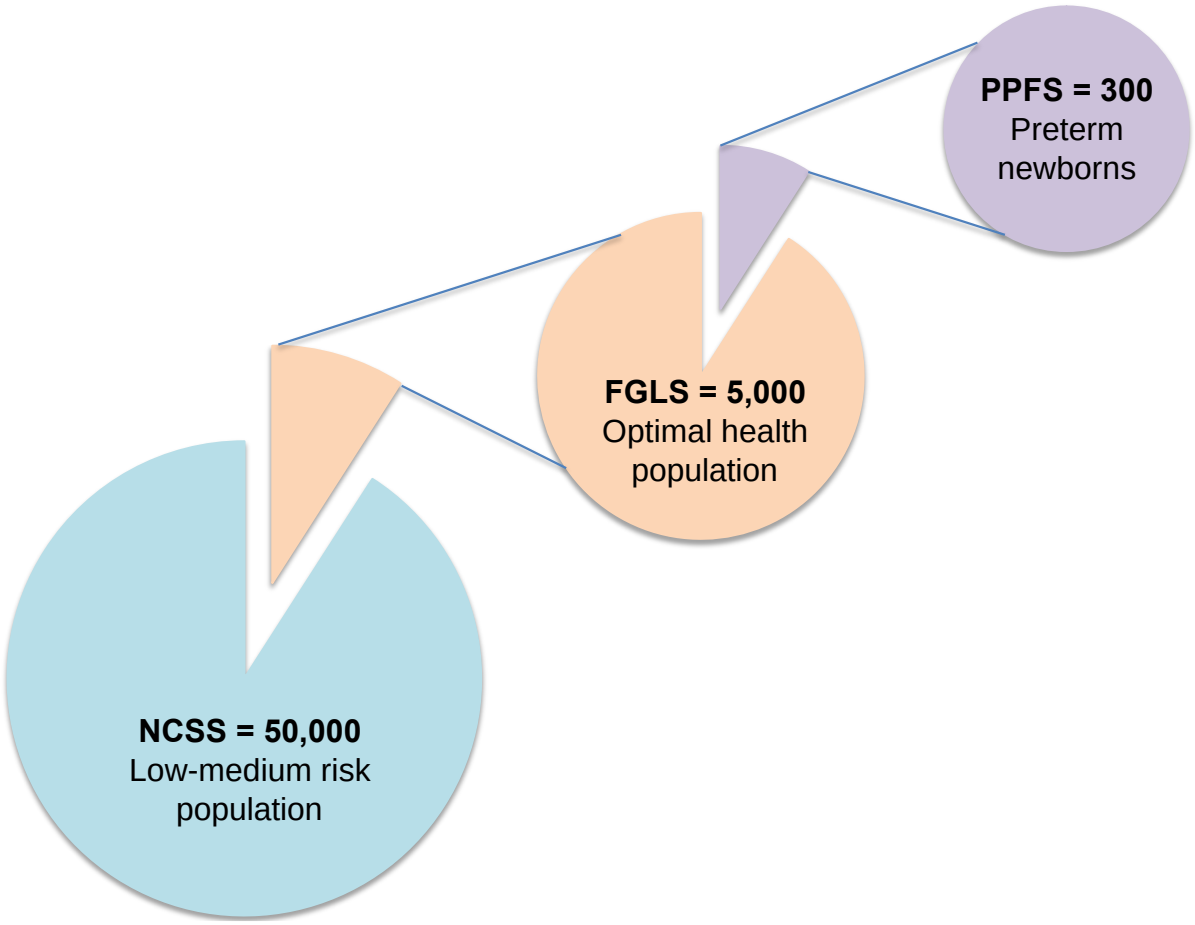
In **FGLS**, women are screened $<14^{+0}$ weeks at their first antenatal visit and followed-up with standard clinical and 2D ultrasound examinations every five weeks, i.e. up to six times during pregnancy. In **PPFS**, preterm infants ($> 26^{+0}$ but $< 38^{+0}$ weeks) born from this sample are being followed-up during their first 8 months of life with the same protocol and set of anthropometric measures used in the WHO Child Growth Study. Postnatal growth is being evaluated from both delivery and conception for comparison with the corresponding *in utero* measurements. All infants from FGLS and PPFS will also be seen at 1 and 2 years to evaluate health, nutrition and development.

In **NCSS**, all newborns at the study centres, born during a fixed 12 month period, have anthropometric measurements taken immediately after birth. Only babies born to women who meet the same inclusion criteria used in FGLS are being selected to construct the newborn standards. Birth weight and gestational age will also be related to neonatal morbidity and mortality outcomes to construct risk-related newborn weight for gestational age standards.

Standard quality control measures are being used, including adaptation of the ultrasound machines to ensure that blinded measurements are taken; a unique system of random evaluation and repetition of

ultrasound measurements (from stored images) to monitor validity and reliability, and continuous real time assessment of all data collected. Anthropometric measures of all neonates are being monitored and standardised centrally. All data are entered and managed in an on-line system specifically developed for the study, including a means of transferring blinded data directly from the ultrasound equipment to the database. This allows initiation of data analysis soon after data collection is completed.

Figure 2: Three INTERGROWTH-21st cohorts



PROGRAMME I. INTERBIO-Bank

Create a biobank of maternal blood and cord blood/placental samples

Background

The aim is to establish a biobank (INTERBIO-Bank) of maternal blood, maternal faeces and cord blood/placental samples from healthy and complicated pregnancies to allow nutritional, epigenetic and other biomarker studies to be performed.

Collecting a heterogeneous group of cases will allow us to explore the wide range of aetiological factors (genetic, metabolic, vascular, autoimmune, infectious etc.) contributing to the development of complicated pregnancies that may present in the same way phenotypically (e.g. low gestational age), as well as the interactions between risk factors and outcomes. Ultimately, we aim to integrate all the pregnancy-related, clinical and biomarker data to improve the phenotypic characterisation of newborns, so as to facilitate the development of targeted interventions and screening strategies in pregnancy and early infant life. [a]

The pathways leading to pregnancy complications, e.g. preterm delivery, IUGR and SGA syndromes, are almost certainly controlled by multiple molecular, genetic, epigenetic and biochemical mechanisms. What is less clear is the relative contributions from risk factors such as infections, nutritional status and other environmental exposures (e.g. pesticides, aflatoxins), especially in resource-poor settings.

We hypothesise that:

- There is more than one preterm delivery phenotype associated with inter-related pathways, i.e. the heterogeneous causes have different functional effects on the fetus/newborn.
- Similarly, the IUGR/SGA phenotype has several intrauterine growth patterns, multiple causes (e.g. small maternal stature, poor maternal nutrition, infection, prematurity and utero-placental insufficiency), and neonatal and infant outcomes.
- Hence, it is inappropriate to manage SGA and preterm newborns as single clinical entities, as usually occurs, based on the potentially false assumption that, *irrespective of the cause*, the adverse effects on the fetus and the clinical manifestations in the newborn are uniform;
- These phenotypes will best be characterised by integrating measures of maternal health, fetal growth patterns, better estimation of gestational age and metabolic function, with biomarker data;

More rigorous clinical and laboratory-based characterisation of such phenotypic subgroups and their different aetiologies should lead to better clinical management of newborn complications and the development of more effective preventive interventions and screening strategies by improving their specificity. This is important because a lack of specificity of interventions tested in previous RCTs, particularly those to prevent preterm delivery, could have resulted in interventions that are actually effective in some phenotypic subgroups, being abandoned because they failed to show an overall protective effect.

A good example is the finding that calcium supplementation in low-risk women with low-calcium diets, significantly reduces the risk of pre-eclampsia (RR 0.48; 95% CI 0.33-0.69) but its impact on preterm birth (RR 0.81; 95% CI 0.64-1.03) borders on significance [1]. However, when the analysis was restricted to the four small RCTs including women at high risk of pre-eclampsia (n=568), there was a large and significant decrease in preterm birth (RR 0.45, 95% CI 0.24 to 0.83) [2]. Hence, it is possible that the magnitude of the effect of supplementation varies because the predominant preterm birth subgroups are different.

Similarly, although malaria infection clearly affects birth weight and gestational duration in epidemiological studies, a Cochrane systematic review of anti-malarial interventions in pregnancy showed that - among women in their 1st or 2nd pregnancies - treatment reduced anaemia, parasitaemia, placental malaria, perinatal deaths and low birth weight (RR 0.57; 95% CI 0.46-0.72), but had no effect on preterm births in the only trial assessing this outcome [2 3]. Thus, anti-malarial interventions may be effective in preventing only a subgroup of preterm births that is not seen when small trials use overall preterm rate as the primary outcome. Lastly, despite the considerable epidemiological evidence that gynaecological infections and bacterial vaginosis are associated with preterm birth, the results of several RCTs of

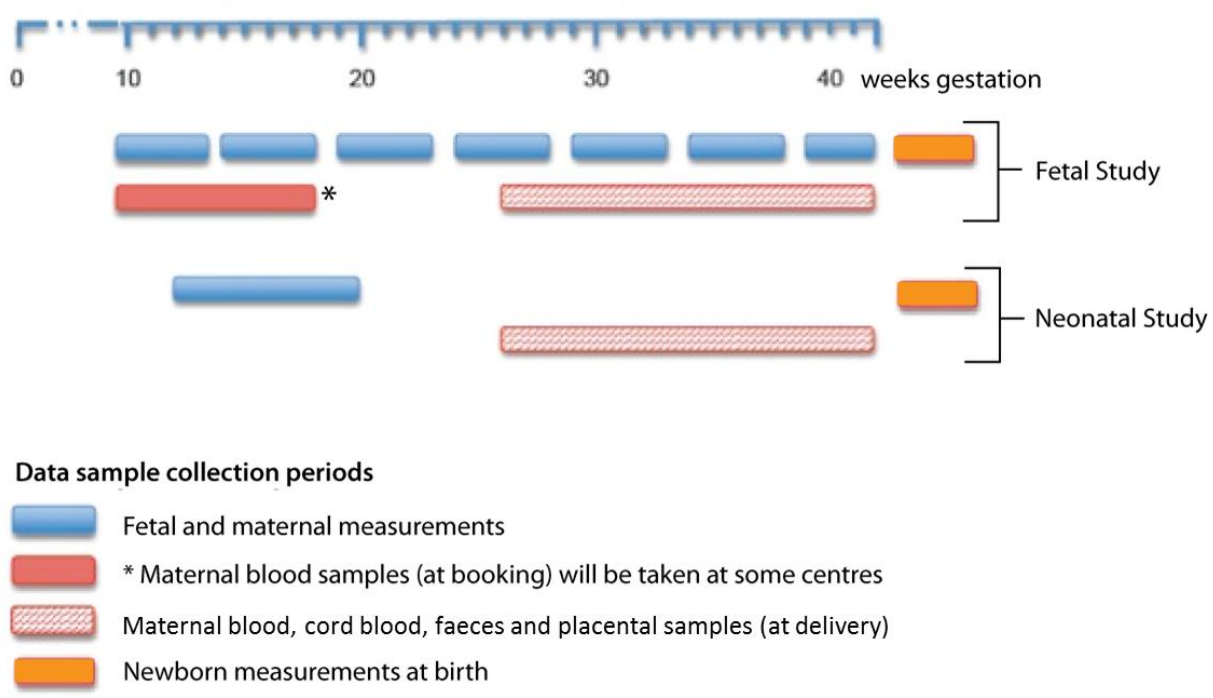
[a] (Kramer MS, Victora CG Humana (2000); Barros FC, BMC Pregnancy and Childbirth (2010)

antibiotic treatment of such infections have generally been disappointing [4 5]. However, it is possible that such treatments are still effective in reducing certain subgroups of preterm birth.

In addition, interventions that are phenotype-specific may, in the long-term, prevent the adverse metabolic and cardiovascular consequences of fetal malnutrition in adulthood. This general approach is of special relevance to resource-poor settings where targeting more homogeneous pregnancy and newborn sub-groups could considerably enhance the effectiveness of available resources.

The very thorough and highly standardised characterisation of antenatal events, using the same protocols in all the pregnancies will make this, to the best of our knowledge, the most comprehensive biobank in the world for nutritional, epigenetic and other biomarker studies in pregnancy.

Figure 3: INTERBIO 21st Fetal and Neonatal Studies: data and sample collection periods



The biobank will be used for studies such as: genetics (SNP genotyping); epigenetics (DNA methylation, histone modification, imprinting, miRNA); expression analyses (mRNA and protein); micronutrient assays; immunohistochemistry; biomarker discovery and validation relating to outcomes such as preterm birth. Anonymised samples will also be made available to other biobanks via a process governed by the Biobank Management Group.

INTERBIO-Bank study design

We aim to collect and store maternal blood, maternal faeces and cord blood/placental samples (see Figure 3) to create a biobank from the following populations:

- 1) "Fetal Study" pregnancies in three centres currently in the INTERGROWTH-21st Project (Nagpur, India; Nairobi, Kenya; Oxford, UK), supplemented by high-risk pregnancies in centres in resource-poor settings, monitored using the same protocol: **INTERBIO-21st Fetal Study**
- 2) "Neonatal Study" pregnancies in the same three centres (Nagpur, India; Nairobi, Kenya; Oxford, UK), supplemented by high-risk pregnancies in centres in resource-poor settings, monitored using the same protocol: **INTERBIO-21st Neonatal Study**

The INTERBIO-21st Fetal Study will provide detailed phenotypic information based on fetal growth patterns and biological samples to investigate maternal/fetal nutritional status and maternal/placental/fetal biomarkers in pregnancies with optimal outcomes, as well as those complicated by a range of factors, including HIV, malaria, malnutrition and anaemia in resource-poor settings. In the field of DNA methylation in particular, this will be an important first step in describing normal variability in fetal/placental methylomes and how methylation signatures relate to both healthy and adverse clinical outcomes.

The INTERBIO-21st Neonatal Study will provide detailed newborn phenotypic information (including accurate gestational age at birth and neonatal morbidity) and biological samples for case-control studies of maternal/fetal nutritional and maternal/placental/fetal biomarkers in healthy pregnancies, as well as those complicated by a range of factors, including HIV, malaria, malnutrition and anaemia in resource-poor settings.

1) INTERBIO-21st Fetal Study: Collect and store maternal blood, maternal faeces and cord blood/placental samples from pregnancies in three INTERGROWTH-21st centres (n=500 per centre), supplemented by samples from high-risk populations monitored using the same protocols in centres in resource-poor settings (n=500 per centre).

In all centres, we plan to collect and store maternal blood, maternal faeces and cord blood/placental samples at delivery (in addition to the pregnancy and fetal growth data) from a total of 2,500 pregnancies (500 per centre). For details of blood, faecal and tissue sample collection see the INTERBIO-21st Operations Manual.

Detailed information will also be acquired about gestational age and fetal growth patterns starting at <14 weeks' gestation. This is of great relevance because of the recent evidence that fetal growth discrepancies, which can be detected by ultrasound as early as the 1st trimester, are associated with increased risks of preterm birth, low birth weight, and SGA at birth [6].

Therefore, the three INTERGROWTH-21st centres will each start collecting blood, faeces and placental samples from women already enrolled in the study; they will continue enrolling women until the target number of 500 for the INTERBIO-21st Fetal Study is reached.

For example, if one of the three INTERGROWTH-21stcentres still has 200 women yet to deliver within the FGLS sample, they should enrol these 200 women into the INTERBIO-21st Fetal Study and recruit an extra 300 to reach the total of 500 women required. The additional 300 women should be selected from the entire population of women attending for antenatal care from <14 weeks' gestation, irrespective of their risk profile for adverse pregnancy/neonatal outcomes.

The data collection forms will include a code to clearly identify women from FGLS and women from the overall population so that the final analysis can be carried out independently for each group of women.

The INTERBIO-21st Fetal Study will monitor fetal and newborn growth using the same protocol as the FGLS component of INTERGROWTH-21st: http://www.medscinet.net/intergrowth/protocol.aspx

Inclusion criteria for INTERBIO-Bank

INTERGROWTH-21st centres that have already completed FGLS

1. Women from the entire population of women attending for antenatal care from <14 weeks' gestation, irrespective of their risk profile for adverse pregnancy/neonatal outcomes, should be recruited for INTERBIO-bank. However to participate, women must be at least 18 years old and their pregnancy must have been conceived naturally. Women who have a BMI over 35 must be excluded from the study as their weight will be a barrier to accurate ultrasound scans. All other women are eligible.

New INTERBIO-21st centres

1. Women from the entire population of women attending for antenatal care from <14 weeks' gestation, irrespective of their risk profile for adverse pregnancy/neonatal outcomes, should be recruited for INTERBIO-bank. However to participate, women must be at least 18 years old and their pregnancy must have been conceived naturally. Women who have a BMI over 35 must be excluded from the study as their weight will be a barrier to accurate ultrasound scans. All other women are eligible.

Estimation of gestational age at study entry

Gestational age at study entry will be estimated by ultrasound measurement of CRL <14 weeks. When LMP is available this should also be recorded. This estimation of gestational age by CRL takes into consideration that in a large proportion of very high risk pregnancies the LMP may not be known.

Fetal growth monitoring

After the first scan between 9^{+0} and 14^{+0} weeks, we will perform scans at ~5 weekly (±1 week) intervals. After the dating scan, 6 further visits (for fetal biometry) will be scheduled at ~5 weekly (± 1 week) intervals (i.e. 14-18, 19-23, 24-28, 29-33, 34-38 and 39-42 weeks). Seven measurements will be taken at each visit from 14^{+0} weeks onwards: Biparietal Diameter (BPD); Occipito-Frontal Diameter (OFD); Head Circumference (HC); Transverse abdominal diameter (TAD); Anterio-posterior abdominal diameter (APAD); Abdominal Circumference (AC) and Femur Length (FL). At each visit, the measurements will be obtained 3 times from 3 separately generated ultrasound images in a "blinded" fashion, and submitted electronically (with the associated images) to the Project Coordinating Unit. All the study centres will use equipment with similar characteristics. The staff will be appropriately trained following standardised procedures according to the corresponding FGLS Protocol and Ultrasound Operations Manual.

Pregnancy follow-up

Women in the study will receive standardised antenatal care (with some local variations) based on the recommended WHO package, part of which involves screening for conditions that emerge during pregnancy. All women recruited will be followed throughout pregnancy from the time of the first visit, irrespective of the pregnancy outcome.

Severe perinatal morbidity and mortality outcomes

We have decided to use an un-weighted composite outcome including at least one of the following conditions: stillbirth, neonatal death until hospital discharge of the newborn, newborn stay in Neonatal Intensive Care Unit (NICU) for ≥7 days or other severe neonatal complications. We believe this is a good proxy for adverse perinatal outcomes across countries. We have used it as a primary neonatal outcome in recent publications and it has been well accepted. Its only disadvantage is that it risks excluding, from the total number of early neonatal deaths, some cases amongst healthy, mostly term babies delivered vaginally who, after hospital discharge at 48 hours, develop severe complications or death up to 7 days post-natally without returning to the same hospital. However, missing these isolated cases is preferable to performing thousands of unnecessary home visits.

2) INTERBIO-21st Neonatal Study: Collect and store maternal blood, maternal faeces and cord blood/placental samples at birth from pregnancies in three INTERGROWTH-21st centres (200 newborns at $<38^{+0}$ weeks' gestation plus 200 controls, and 200 IUGR/SGA plus 200 controls in each centre), supplemented by samples from high-risk pregnancies in resource-poor settings. For details of sample collection see INTERBIO-21st Operations Manual.

NCSS pregnancies in INTERGROWTH-21st are ideal, population-based cohorts for nutritional, epigenetic and other biomarker studies to study the causes of pregnancy complications and how they influence growth and development, principally for the reasons outlined in Box 1.

Box 1: Some unique characteristics of studies conducted using NCSS protocols

Geographically diverse populations

Large, population-based, sample size with severe morbidity and mortality outcomes

Early pregnancy dating by ultrasound provided by small number of standardised operators

Standardised methodology for maternal, newborn and infant follow-up anthropometric measures

Maternal morbidities during pregnancy captured prospectively

Environmental characterisation of the populations and individual participants

However, we recognise the need to enrich the collection of complicated pregnancies from populations with other risk factors that are especially relevant to the needs of developing countries. Therefore, we will supplement sample collection in the three INTERGROWTH-21st centres by also collecting samples from pregnancies from the general population in resource-poor settings where there is a high risk of fetal growth impairment and preterm delivery because of infection, malnutrition, poor socio-economic status and past adverse pregnancy outcomes. This strategy will increase the generation of cases from a relatively small population given the higher incidence of the conditions.

In these centres, we will collect and store samples from 800 pregnancies per centre:

- Maternal blood, maternal faeces, cord blood and placental samples will be collected from pregnancies (cases) that have delivered at $<38^{+0}$ weeks gestation (n=200 per centre) or have resulted in IUGR/SGA newborns (n=200 per centre). Newborns that were born at $<38^{+0}$ weeks gestation and were IUGR/SGA will be included in both sets of cases as the case-control analysis will be carried out separately for each outcome.
- We will also collect the same samples from term AGA newborns (controls), i.e. non-IUGR, normal birth weight newborns at term, as a reference group (n=400 per centre, i.e. one control for each case).

All cases and controls are required to have had, reported in their medical records, an estimation of gestational age by ultrasound measurement of either CRL <14 weeks or HC <24 weeks. When LMP is available this should also be recorded. If the LMP is not available it should be recorded as such and ultrasound estimations will be used.

Because of the different populations in the centres selected, all analyses in this case-control strategy will be stratified by centre, and will only be pooled if there is no statistical evidence of heterogeneity**.**

Anthropometric measurements

All babies, i.e. all cases and controls, born during the study period will have weight, length and head circumference taken within 24 hours of delivery:

Standardised, electronic, digital, newborn weighing scales with a precision of 10g will be used and their calibration status will be checked twice a week; they will be replaced if they are faulty and cannot be repaired. We shall also provide all clinics with standardised infantometers for length (precision 0.1 cm) and tape measures for head circumference (precision 0.1 cm); these will be similarly calibrated and

maintained. All anthropometrists will be trained centrally and monitored during the study following standard procedures by the Anthropometric Standardization Unit; they in turn will train the nurses/midwives in how to apply the study's measurement protocol.

For a small subgroup, the following additional anthropometric measurements will be taken: arm circumference; thigh circumference; abdominal circumference and skinfold thickness, as well as neonatal body composition using air displacement plethysmography (PEA POD) in some centres.

Follow-up

All newborns during the study period, including those on NICU or special care, will be followed on a daily basis until hospital discharge to document severe morbidity and detect neonatal death. We will make strenuous efforts to coordinate and promote evidence-based care for the neonates born $<38^{+0}$ weeks gestation using materials developed as part of our best practice programme, by liaising with the lead neonatologist in each NICU before and during the study. We recognise that differences in practice will persist despite our best efforts, especially in resource-poor settings. However, we believe this is unavoidable in a very pragmatic study such as this, which is trying to reflect what happens on a daily basis in clinical practice. Furthermore, we will similarly make strenuous efforts to standardise the main protocols for feeding practices in each NICU before the study starts. During the routine site-visits by members of the Study Coordinating Unit and the Anthropometric Team we will monitor the implementation of the protocols.

Severe perinatal morbidity and mortality outcomes

We have decided to use an un-weighted composite outcome including at least one of the following conditions: stillbirth, neonatal death until hospital discharge of the newborn, newborn stay in NICU for ≥7 days or other severe neonatal complications. We have used such an outcome recently [7] [8]; it requires limited standardization of clinical diagnoses across hospitals and is well accepted as a marker in large, international, population-based studies of newborns that are severely ill.[b] It could be argued, however, that intrapartum stillbirth may not be related to fetal growth and should not be included in this index. We believe this is a valid point but as it will not be possible to separate those intrapartum deaths that are related to IUGR from those that are unrelated, we suggest keeping the index as it is. We believe this is a good proxy for adverse perinatal outcomes across countries.

On-line data management and statistical analysis

All clinical data will be entered into an on-line data management system specifically developed for the study. It includes a method for direct transfer of blinded data from the ultrasound machines to the database. This on-line system has the practical benefit of allowing on-going quality control, correction of errors or missing values and the initiation of data analysis soon after data collection is completed. It will be used for data management and monitoring all sub-studies, including patient recruitment and follow-up, and is based on the INTERGROWTH-21st Electronic Data Management System. The system permits all participants' data to be incorporated into the data files via the Internet as soon as they are available. Included within the system is a review process to ensure that all data are complete.

All sample related data will be entered separately into a data management system specifically developed for the study. The system allows samples to be tracked from the time of collection through processing, storage in the participating centres, and transport to a centralised facility. Each participant will have a unique identifier number, which will be used to link the clinical and sample databases. The number will also be used to barcode individual samples and aliquots. Quality control for this aspect of the study will be monitored by a team from GAPPS.

These systems will provide the Data Management Unit with a detailed daily record of patient enrolment and data entry, at both individual and institutional levels to monitor progress against the milestones listed

[b] Others have also used these composite indices of neonatal morbidity (Hannah ME, Hannah WJ Kewson SA et al (2000); Wapner RJ, Sorokin Y, Thom EA (2006); Joseph KS, Fahey J, Platt R (2009)).

in the protocol. Corresponding actions, such as telephone calls, web conferences and site visits will take place within a week of detecting a problem in a centre to ensure that appropriate corrective measures are taken.

Selection of Cases and Controls

All live and stillborn infants in the study hospitals during the data collection period, whether or not they survive until hospital discharge, will be screened. However, multiple births and post-term births (>42 weeks^{+0}), will not be included.

Each newborn infant will fall into one of the four groups below:

Set	Infants born <38 weeks' gestation	Infants born IUGR/SGA	Description	Number of births at study site	Number to be included in the case-control study
A	Yes	No	Non-IUGR/SGA infants born <38^{+0} weeks	A	A (all)
B	No	Yes	IUGR/SGA infants born ≥38^{+0} weeks	B	B (all)
C	Yes	Yes	IUGR/SGA infants born <38^{+0} weeks	C	C (all)
D	No	No	Non-IUGR/SGA infants born ≥38^{+0} weeks	D	Sample = A+B+C

All mothers admitted for delivery (spontaneous or induced labour, or elective C-section) will be screened to check if they had gestational age estimated by CRL at <14 weeks or HC at <24 weeks. If not, they are not eligible for the study. If a mother had one or both of these two measurements, the screening form will be completed to collect the information required to classify her infant as: a) <38^{+0} weeks or ≥38^{+0} weeks, and b) IUGR/SGA or non-IUGR/SGA (based on the charts provided).

Operational definition of cases and controls in the maternity wards

To simplify the identification of cases and controls during screening, the following procedures will be used (see instructions in Appendix II):

First, gestational age will be assessed using CRL or HC. **Cases, born at <38^{+0} weeks, will be live or stillborn infants with gestational age assessed by an early ultrasound (either CRL at <14 weeks or HC at <24 weeks)**, regardless of whether or not they presented with IUGR/SGA at any time during pregnancy or at birth. These infants correspond to groups A and C in the table above.

Second, BW for gestational age will be assessed for infants born ≥38^{+0} weeks. **Cases, IUGR/SGA, will be live or stillbirths whose BW for gestational age is below the 10th centile of the INTERGROWTH-21st neonatal standard as defined on the form.** These infants correspond to group B in the table above. In the data analysis phase, infants from group C (IUGR/SGA infants born <38^{+0} weeks) will be added to those in group B so as to include all IUGR/SGA infants regardless of their gestational age at birth.

Third, the screening form will also identify **potential controls, that is, non-IUGR/SGA infants who were <u>not</u> born <38^{+0} weeks** (group D in the table above). The first potential control born after each case (either a case born <38^{+0} weeks or an IUGR/SGA case) in the same hospital[c] will be enrolled in the study as a control. After enrolling a case, a control must be recruited. If two cases are born in succession, the

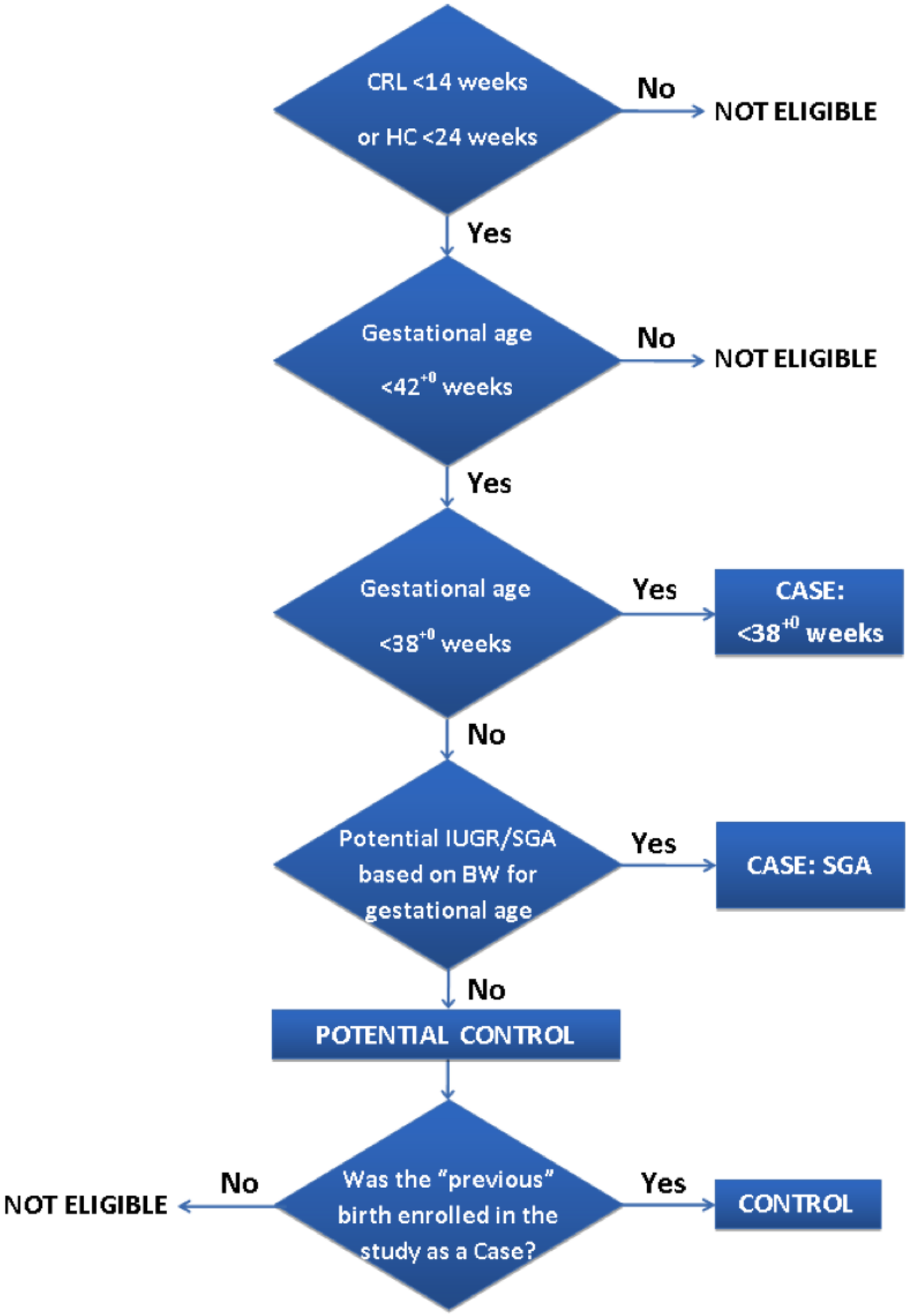
[c] If there is more than one hospital at a given study site, and if presumed risk factors vary by hospital (e.g. one primarily attracts mothers of low socioeconomic status, and another attracts high income mothers), it may be necessary to weight the analyses to reproduce a control group that is representative of the study population; ignoring such differences may lead to overmatching.

second case cannot be recruited and instead screening for a control continues. Once a case-control pair have been recruited and processed, sites then screen for another case.

At each site, 200 cases born $<38^{+0}$ weeks and 200 IUGR/SGA cases will be recruited, along with 400 controls. If a site collects 200 cases born $<38^{+0}$ weeks before it has collected 200 IUGR/SGA cases, it will stop recruiting cases born $<38^{+0}$ weeks and their corresponding controls, and will continue recruiting IUGR/SGA cases until 200 (and their controls) have been recruited - and vice-versa, if the quota of 200 IUGR/SGA cases is collected before 200 cases born $<38^{+0}$ weeks are enrolled.

Note that the only criteria for matching cases and controls are: a) hospital of birth and b) approximate date of birth (usually same day, sometimes the next day if there are no controls on the same day).

Figure 4: Neonatal Study Eligibility Flow Diagram



Definitions of cases and controls for the data analyses

Cases born <38^{+0} weeks' gestation for the data analyses will include all births at <38^{+0} weeks whether or not they present with IUGR/SGA (groups A and C).

IUGR/SGA cases for the data analyses will include the operational definition of IUGR/SGA cases (group B) plus those cases born <38^{+0} weeks who are also IUGR/SGA (group C); the latter were collected as a sub-set of cases born <38^{+0} weeks.

Infants in group C (IUGR/SGA infants born <38^{+0} weeks) will be included in both groups of cases, as the case-control analyses will be carried out separately for each outcome.

The table below provides the definition of controls for the analyses.

Controls for cases born <38^{+0} weeks will be a sample of live and stillborn infants born ≥38^{+0} weeks. In the statistical analyses, a proportion of term IUGR/SGA (xB) cases will be added to the operational controls (group D).

Controls for IUGR/SGA cases will be a sample of live and stillborn infants who are not IUGR/SGA at birth. In the analyses, they will include all operational controls (group D) plus a proportion of infants born <38^{+0} weeks who are not IUGR/SGA at birth(xA).

Table 2. Case-control comparisons in the data analyses.

Comparison	Cases	Controls	Comments
Infants born <38^{+0} weeks case-control study	A + C	D + xB	To reproduce the control population, set B (IUGR/SGA only) will be down-weighted by a factor x which is equal to the sampling fraction for set D, that is the proportion of all infants in the control pool who were included in the detailed study (cases).
IUGR/SGA case-control study	B + C	D + xA	As above, for set A (infants born <38^{+0} weeks only).

Subgroup analyses will include cases born <38^{+0} weeks, stratified according to: a) gestational age groups (the exact groupings will be decided based on the number of births each week of gestational age, so that there will be at least 100 cases in each sub-group) or b) by preterm phenotype, using the newly proposed INTERGROWTH-21st classification system. For IUGR/SGA, subgroup analyses will include stratification by: a) IUGR/SGA severity (<3rd, 3-5th, 6th-9th centile groups) and b) gestational age.

Appendix II provides more detailed information on different strategies for selecting controls for case-control studies than we considered when planning the study, but some of these proposed strategies were not practical. As proposed above, INTERBIO-21st will adopt a traditional case-non-case design, and odds ratios will be used to estimate relative risks. This is based on the assumption that cases will be relatively rare, i.e. <10% of the overall number of births.

We estimate that the overall birth rate for infants born <38^{+0} weeks will be <10% and the overall IUGR/SGA rate will be <10-15%. However, by collecting data on all four sets (A, B, C and D), it will also be possible, with appropriate statistical weights in the analyses, to carry out case-base analyses using Poisson regression with robust variance, if the outcomes end up being more common (>10%).

Sample processing

The sample collection, processing and storage procedures will be performed in a standardised manner based on protocols described in detail in the INTERBIO-21st Operations Manual that has been developed with the assistance of the GAPPS team, and researchers at the Universities of Oxford [9] and Cambridge [10], and Johns Hopkins Bloomberg School of Public Health.

In brief, maternal and cord blood samples will be collected to store whole blood, plasma and the buffy coat for a wide range of purposes, including DNA extraction for genetic and epigenetic studies and micronutrient assays. Two placental biopsies will be taken for immunohistochemistry and DNA extraction and in RNA later for expression studies (if the sample is obtained <30 mins after delivery). In addition, we intend to collect and store samples for a number of future, as yet unspecified, biomarker assays relating to preterm delivery and fetal growth.

It is vitally important to ensure that samples are collected in a standardised way with adequate monitoring of quality control, principally because sample quality, quantity and handling can greatly influence the results of microarray and sequencing experiments [11].

The primary reason (aside from quality control) for ensuring that samples are collected, stored and processed in a uniform manner is to facilitate the anticipated interchange of data, in the future, with other biobanks. Standardising phenotypic definitions, sample collection methods and analyses fosters trans-national collaboration and networking [12]. We shall therefore also seek advice from groups such as the Public Population Project in Genomics (http://www.p3g.org), which promotes international harmonization and collaboration in population genomics and biobanking by sharing research tools and expertise.

Faecal samples: We wish to collect a faecal sample from mothers, oportunistically at the time of delivery, for metabiomic studies. Although it has been suggested that we should also collect stool samples from infants every 6 months, we feel that this is a rather large-scale undertaking that is beyond our remit.

We certainly appreciate the importance of looking for maternal intestinal co-infections and microbiota. In fact, we published on this subject in 1989: in a prospective study of 14,914 pregnant Guatemalan women, the incidence of IUGR increased with the number of parasitic species detected [13].

Sample size

This is a great challenge in any field-study of this magnitude and even more difficult when exploring risk factors with relatively unknown degrees of association and prevalence in the population. The key issue is to reach a balance between logistical demands, including the need to maintain data quality in these populations, and power calculations especially for the planned epigenetic studies. Having said that, our co-investigators, Krina Zondervan and Cecilia Lindgren in the Wellcome Trust Centre for Human Genetics, Oxford, have considerable experience of conducting candidate gene and genome-wide association studies (GWAS) in related fields and the lessons learned over the last 15 years will be pertinent to the proposed studies.

To illustrate the point, Cardon & Zondervan reviewed how the complex interplay between genotype, phenotype, environmental factors and sample size affects the ability to detect disease susceptibility variants in population-based association studies [14]. They concluded that thousands of cases and controls are required to detect common variants with small effect sizes in such studies.

Three examples demonstrate the need to study large numbers to identify genes influencing quantitative traits involved in metabolic function, such as birth weight. Nearly 120,000 individuals were genotyped to identify three loci influencing anthropometric measures (waist circumference and waist-hip-ratio) of central obesity and fat distribution in a recently published meta-analysis of 16 GWAS, followed by large-scale replication testing [15]. Using similar methodology (GWAS meta-analysis followed by replication), over 90,000 individuals were genotyped to confirm that two loci are associated with BMI and to identify six additional loci [16] and, more recently, nearly 40,000 European individuals were genotyped in identifying variants in *ADCY5* and near *CCNI* associated with fetal growth and birth weight [17].

For the nested case-control studies, we are collecting samples from 2000 controls; and 2,000 cases from pregnancies with adverse outcomes, e.g. delivery at $<38^{+0}$ weeks' gestation, term IUGR/SGA. In addition we have the potential to include 400 cases and 2100 controls from the FGLS population in the analysis, taking into consideration the possibility of selection bia in the selection of FGLS population controls.

It is very unlikely that fewer than these numbers will be needed to study the effects of adverse intra-uterine effects on epigenetic profiles, especially as there is emerging evidence from genome-wide epigenetic studies in animals that imprinted quantitative trait loci (iQTL) affect body weight and growth [18] and adult body composition [19] in much more complex and diverse patterns than previously assumed.

Selection of study centres

We aim to use the same rigorous processes to select the new sites for this extension as originally adopted in the selection of the current INTERGROWTH-21st centres. However, in this case, the selection criteria will inevitability involve finding a balance between obvious opportunities (e.g. having access to a malnourished pregnant population with a high prevalence of malaria/HIV) and the risks of working in a research naïve environment with limited existing access to antenatal care.

The criteria the INTERBIO-21st Steering Committee will use to select the centres will include factors such as: 1) existing research infrastructure and capacity; 2) existing maternity services, including antenatal ultrasound; 3) support of local health authorities; 4) previous experience in collecting biological samples; 5) geographical location to retain global coverage; 6) prevalence of key exposure variables, i.e. risk factors; 7) costs; 8) leveraged funding from other donors, and 9) need ideally for all samples in the proof-of-concept study to be analysed in a centralised facility.

Staged introduction of sample collection at likely study sites

Phase I

Shoklo Malaria Research Unit, Mae Sot, Thailand

KEMRI-Coast Centre for Geographical Medicine & Research, Kilifi, Kenya

John Radcliffe Hospital, Oxford, UK

Phase II (start late 2011-early 2012)

Ketkar Nursing Home, Nagpur, India

MRC Laboratories Keneba, The Gambia

The Aga Khan University Hospital, Nairobi, Kenya

Aga Khan University Medical Centre, Karachi, Pakistan

PROGRAMME II: Proof-of-concept study

Background

Understanding the gene-environmental interactions underlying the plasticity of the epigenome at certain times from fetal life to infancy will be crucial to developing interventions, particularly in pregnancy, that might correct or at least prevent the long-term, adverse consequences [20]. We believe that the key to doing so effectively is to recognise that phenotypes other than birth weight and gestational age alone are needed to determine the nutritional status of the newborn and assess the effectiveness of interventions.

The redefinition of newborn phenotypes will arise from evaluating a combination of factors in pregnancies with normal and abnormal outcomes. These include maternal health; fetal growth patterns measured using 2D ultrasound; growth patterns of individual fetal organs measured using 3D ultrasound; newborn body composition and physiological function; micronutrient levels and data from epigenetic experiments, which will initially characterise normal variability across the epigenome in uncomplicated pregnancy and then, in carefully designed nested case-control studies, evaluate the effects of adverse environmental and nutritional factors on the epigenome (and other biomarkers) in a pool of complicated and uncomplicated pregnancies.

General Objectives

The aim is to conduct a hypothesis-testing, proof-of-concept study comparing 500 normal birth weight and 500 term IUGR/SGA newborns (using both cord blood and placental samples) taken from the samples collected in the context of both the INTERBIO-21st Fetal and Newborn Studies. This will be the first in a series of experiments utilizing samples collected for the INTERBIO-Bank.

We aim to assess DNA methylation patterns in ~100 imprinted genes previously implicated in fetal growth. Our hypothesis is that maternal micronutrient deficiency, particularly of folate and other methyl donor factors, results in impaired fetal growth, development and pregnancy outcomes, through altered DNA methylation.

We will therefore correlate these methylation patterns with pregnancy (clinical outcomes, fetal growth), nutritional (micronutrient assays), and neonatal (growth, development and body composition) data, which will allow us to:

1. Study the effects of environmental and nutritional factors on the epigenome;
2. Develop new phenotypic definitions of LBW and other adverse pregnancy outcomes

If validated, the results could inform knowledge-based actions to address underlying problems, such as poor nutrition and infection, leading to improved outcomes. The data will, in addition, serve to define normal variability in the epigenome and inform the design of future epigenome-wide studies, once the cost has fallen, as inevitably it will with technological advances.

In the long-term, we would also wish to correlate these epigenetic findings with single nucleotide polymorphism (SNP) genotyping data from a GWAS given the increasing evidence that epigenetic regulation is influenced by genetic factors and the recently published data implicating variants in *ADCY5* and near CCNI with fetal growth and birthweight [17].

Specific Objectives

We plan to study the methylation profiles of the ~100 imprinted genes that have to date been implicated in fetal growth, although the final list of candidate genes will be taken from our own systematic search of the literature, as well as existing databases, such as http://www.geneimprint.com and http://igc.otago.ac.nz.

Where possible, we will analyse cord blood and placental tissue separately to compare the methylation profiles of both tissues. The underlying rationale is as follows:

1. There is increasing evidence that placental function and gene expression respond to, and are marked by, environmental insults. The placenta can therefore serve as a 'record of *in utero* exposure and pathology' [21]. Effects on the fetus almost certainly occur downstream of these events and so comparing the epigenetic profiles of both tissues in individual pregnancies may help to differentiate the various causes of IUGR/SGA and preterm delivery.
2. Alterations in DNA methylation in humans appear to be tissue-specific:
    a. Katari et al. (2009) have reported significantly different DNA methylation levels at specific CpG sites between cord blood and placenta [22].
    b. Guo et al. (2008) have described similar findings in two imprinting clusters: the H19 promoter is unmethylated and IGF2 DMR2 hypomethylated in placenta. However, in cord blood, these two regions maintain the differential methylation status seen in most other tissues [23].
    c. Yuen et al. (2009) have observed DNA methylation of the promoter in *TUSC3* and *WNT2* in placental, and not the associated fetal, tissues; within individual placentas, methylation was confined to trophoblastic chorionic villi, and not amnion, chorion, cord or decidua [24].

Study design

For this proof-of-concept study, we will randomly select 500 term IUGR/SGA cases from the INTERBIO-Bank. The 500 normal birth weight controls will be taken either from the population at least risk within the INTERBIO-21st Fetal Study or from the total with normal outcomes from the entire study population, and matched with the cases. A final decision will be made by the INTERBIO-21st Steering Committee.

Methods

We have given considerable thought to the best technological platform for assessing methylation profiles and we have consulted widely with leading experts in the scientific community and industry. There are a large number of different platforms available and many more being developed; in general, there is an inverse relationship between the cost of analysis and the resolution/coverage of the genomic region being studied. At this stage, however, we have decided to use Methylated-Cytosine DNA Immunoprecipitation-Microarray Chip (MeDIP-Chip) followed by bisulfite-(BS) PCR and high throughput sequencing for validation of differentially methylated loci [11].

The approach is well described in a recently published proof-of-concept study assessing whether 'DNA methylation in a subset of genomic loci may connect end-stage cardiomyopathy with different etiologies' [25]. In brief, these authors performed a preliminary analysis using MeDIP-Chip (Nimblegen, WI, US); validated differential methylation loci by BS-PCR and high throughput sequencing; identified three angiogenesis-related genetic loci that were differentially methylated with the BATMAN algorithm [26], and using quantitative RT-PCR, found that the expression of these genes differed significantly between cardiomyopathy hearts and normal controls.

However, we are aware that the samples may not be analysed for at least another two years by which time the technology is likely to have changed considerably, costs will have fallen and genome-wide profiling in large numbers of samples will be affordable. We are therefore in preliminary discussions with a number of companies, including Nanopore (Oxford, UK), http://www.nanoporetech.com, and Pacific Biosciences (Menlo Park, CA, US), http://www.pacificbiosciences.com, who may soon be able to offer high-throughput, single molecule sequencing [27]. Whichever platform is used, however, the intention ideally is to analyse all samples in a centralised facility; in fact, this applies to all the experiments proposed in the proof-of-concept study.

Specific experiments

Placenta v. cord blood methylation profiles: To the best of our knowledge, no study has compared the methylation profiles of the ~100 imprinted genes in placental tissue and cord blood. The outcomes of these comparisons will potentially shed light on the regulatory mechanisms and epigenetic profiles of adverse and healthy pregnancy outcomes.

Cases v. controls methylation profiles: The results of the placenta v. cord blood studies will help to determine which sample sets are compared in trying to identify the methylation profiles associated with adverse pregnancy and newborn outcomes. Comparisons will also be made between ethnic sub-groups. All the above experiments will be performed in duplicate with adequate quality control measures,

Sample pooling for methylation profiles: Pooling samples of 'healthy' controls to act as a reference standard for epigenetic studies has been proposed in the literature [11]. Given that FGLS provides an ideal opportunity to use samples from newborns whose intra-uterine growth has been optimal, we plan to explore this possibility with FGLS samples drawn from the three INTERGROWTH-21st centres. This might involve pooling samples collected both within and across these centres, although the experiments would need to be performed in India if samples are collected there.

Placental expression analyses: We will follow the same experimental design outlined in the Movassagh et al. (2010) study [26]. Quantitative real-time PCR will be performed for target genes, selected from the methylation studies, using validated Taqman Gene Expression Assay primers (Applied Biosystems, Foster City, CA) normalised against house-keeping gene data. In the long-term, we also plan to characterise global expression patterns in placental tissue using the new Illumina HT-12 v4 expression chip, for comparison between sub-groups and methylation profiles, as well as between normal and adverse pregnancy and newborn outcomes.

Nutritional status

To supplement the epigenetic studies above, we will also assess the nutritional status of the 500 cases and 500 controls selected for the proof-of-concept study, by measuring:

- Micronutrients in maternal blood at booking and cord blood at delivery
- Putative markers of methyl donation, e.g. S-adenosylmethionine (SAM) to S-adenosyl-homocysteine (SAH) ratio
- Neonatal body composition

The rationale for adding these measures is that they should facilitate the interpretation of the epigenetic data and the characterization of specific sub-phenotypes, in particular IUGR and SGA.

Micronutrient assays: As with the epigenetic studies, there are a large number of technological platforms available to assay micronutrients and some controversy regarding the most appropriate ones to measure. The assessment of micronutrients in mother's blood is made even more complex by physiological alterations such as haemodilution and the hyperlipidaemic state of pregnancy [28 29].

We will therefore seek guidance from the Biomarker Group consisting of experts in the field before finalizing the list of analytes and the methods to use. We will also draw heavily on the expertise of our collaborators at SMRU, Thailand, and MRC Gambia, who have considerable experience of assessing nutritional status in their populations. At present, based on unpublished data from their studies and our reading of the literature, the following analytes have been proposed as candidates to measure:

- Retinol Binding Protein (RBP)/Vitamin A
- Iodine (maternal) and TSH (newborn)
- Ferritin and Soluble Transferrin Receptor (sTfR) – markers of Fe deficiency
- Zinc protoporphyrin (ZnPP)
- Folate, thiamine, choline and zinc
- DDT
- Aflotoxin-albumin
- Vitamin D
- CRP and αGP

In Thailand, we will also measure Dichloro-Diphenyl-Trichloroethane (DDT) metabolite levels as DDT was used as an insecticide for malaria control in Northern Thailand until it was replaced by Deltamethrin in

2000. However, high serum DDT residues, which affect serum retinol levels and probably thiamine as well, are still detected in pregnant women living in the Mae La camp [30]. In The Gambia, aflatoxin-albumin adducts will be assayed as the group has previously demonstrated that *in utero* exposure to aflatoxins (a common environmental exposure derived from consumption of poorly-stored groundnuts) is associated with significantly impaired post-natal infant growth [31]. We also plan to ask the local investigators to identify other possible chemical exposures to measure. Final decisions about which exposures to measure and where the samples will be analysed will be made by the Biomarker Group; however, we will ideally use centralised facilities.

Neonatal body composition: As part of our Wellcome Trust/EPSRC funded research program, we are already starting to measure neonatal body composition in: a) term normal birth weight, b) preterm and c) term IUGR/SGA infants enrolled in the UK component of FGLS and PPFS. To do so, we are using an infant-sized, air-displacement plethysmograph (PEA POD Infant Body Composition System, Life Measurement, Concord, CA, US). The study is being conducted so as to correlate fetal growth patterns with better measures than birth weight alone, i.e. the relative contributions of body fat, lean tissues and bone, all of which are key indicators of the adequacy of intra-uterine nutrition.

The PEA POD system compares well with the 4-compartment reference model, which is considered the best choice for assessing body composition in humans. In contrast, however, it is easy to perform; takes only a few minutes to complete; infant movement during the measurement is not a significant problem; the measurements can be repeated as frequently as needed, and the results are immediately available [32].

The system is becoming recognised as an established method to assess neonatal body composition in developed countries [33-35], and it has been suggested that it may offer important insights into which fetal growth parameters most closely reflect the generalised nutritional state of neonates and infants [35]. However, there are no published data about its use in resource-poor settings as, to the best of our knowledge, the system has been installed in only one site in such a setting, as part of a collaboration between Jimma University, Ethiopia, and the Department of Human Nutrition, University of Copenhagen.

We now propose installing PEA POD systems in four of the centres in resource-poor settings to give a much more detailed assessment of nutritional status and growth than birth weight and gestational age alone.

Sample size for epigenetic studies

As discussed on page 16, it is extremely difficult to provide reliable power calculations at the moment for epigenetic studies: the field is too new and very few relevant studies have been conducted, especially in humans, to enable power calculations to be performed. It is also unclear at present to what extent it will be necessary to map DNA methylation at high resolution across the entire genome [36], which will inevitably influence the epigenotyping strategy and choice of platform, e.g. bisulfite sequencing or array-based technology. However, having said that, the sample size chosen matches that in the NIH National Standard for Normal Fetal Growth Study and we feel comfortable that it provides a reasonable compromise between cost, expediency and logistical demands.

The estimated samples sizes required to detect the effects of methylation status on adverse pregnancy outcomes are inevitably based on a range of assumptions, since the spectrum of methylation changes and their corresponding effect sizes are unknown. Table 1 shows the sample sizes required to detect differential methylation in cases vs. controls. The following assumptions are made:

1. Methylation status is either on/off, and so the proportion of cases vs. controls with methylated status is analysed.
2. Methylation proportion among controls of 0.2, with proportion in cases varying from 0.3-0.5, corresponds to an odds ratios (OR) of the effect of methylation status on outcome from 1.7-4.0.
3. A significance threshold α of 5.0×10^{-4} (Bonferroni-corrected threshold for 100 candidate imprinted genes) vs. 5.0×10^{-7} (commonly applied genome-wide significance threshold in GWA studies [37]).
4. Power of 80% vs. 90%
5. Case: control ratio either 1:1 or 1:3

Table 1. Sample sizes* to detect differential methylation status between cases and controls

				Sample size for candidate gene study (α=5.0x10^{-4})		Sample size for genome-wide study (α=5.0x10^{-7})	
	methylation proportion among controls	methylation proportion among cases	OR (PAF)**	Ca:Co 1:1	Ca:Co 1:3	Ca:Co 1:1	Ca:Co 1:3
Power=80%	0.2	0.3	1.71 (0.12)	719	459	1313	913
		0.35	2.15 (0.19)	342	215	623	388
		0.4	2.67 (0.25)	204	127	370	260
		0.45	3.27 (0.32)	137	85	248	151
		0.5	4.00 (0.38)	99	61	179	125
Power=90%	0.2	0.3	1.71 (0.12)	847	559	1512	966
		0.35	2.15 (0.19)	412	263	716	451
		0.4	2.67 (0.25)	245	155	425	265
		0.45	3.27 (0.32)	164	103	284	175
		0.5	4.00 (0.38)	118	74	204	126

* Sample size for cases is given. Calculations include a continuity correction allowing for normal approximation of the binomial distribution.
** OR = odds ratio; PAF = population attributable fraction

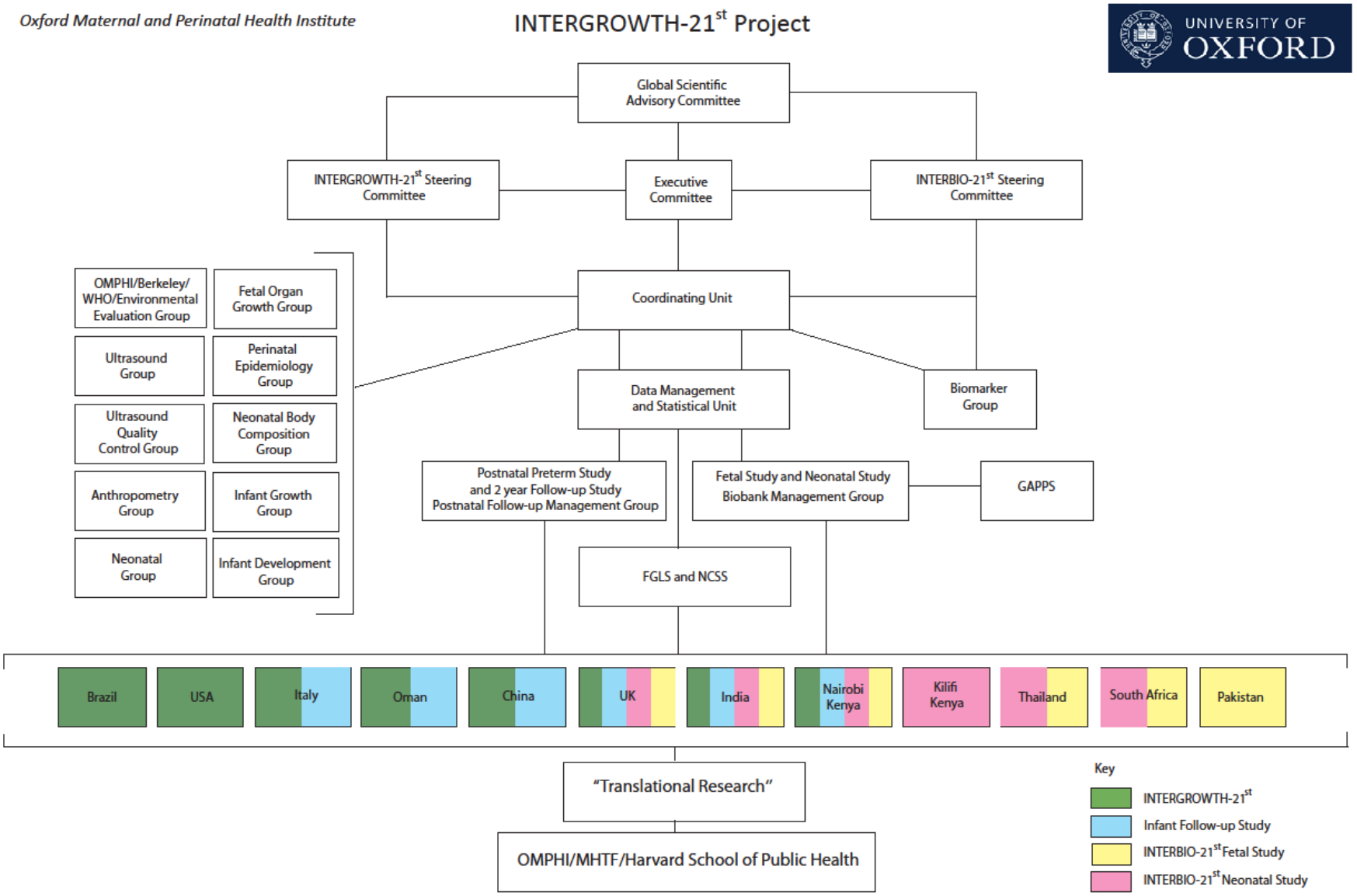
Table 1 demonstrates the approximate power of the proposed experiments with 500 cases and 500 controls. However, for future experiments, based on conservative estimates (OR=2.2 and PAF=0.2), we will have considerable power to detect differences even for 90% power, given that we could have a 1:3 case: control ratio (i.e. 1,000 infants born at <38^{+0} weeks' gestation or 1,000 term IUGR/SGA newborns and at least 3,000 term, non-IUGR/SGA controls). Nevertheless, it is worth emphasizing that these are approximate calculations and that, in a study of this magnitude and complexity, logistical and budgetary considerations must inevitably play an important role in the selection of the sample size.

Data quality: Standardization of the research staff, who will be responsible for obtaining the neonatal body composition data, represents a challenge. However, we will employ the same quality control measures that are now being used in FGLS and PPFS for the ultrasound and anthropometric data to ensure that the quality of the data is maintained.

Publications and Authorship

This is to be discussed at the first INTERBIO steering committee meeting.

Appendix I: Figure 5: INTERBIO-21st Governance



Appendix II:

We are aiming to collect:

- **200 Cases <38^{+0} weeks, including ALL babies delivered at <36 weeks and 200 Corresponding controls**
- **200 Small for Gestational Age Cases, including ALL babies delivered with a birthweight <P3 and 200 corresponding controls**

Instructions for recruiting Cases and Controls for the Neonatal Study

Each day an INTERBIO-21st midwife/researcher will screen all women on the delivery suite and those scheduled for a Caesarean section using the tablet computer provided. The tablet is programmed with an algorithm incorporated into a simple, user friendly application which, when completed, will select the correct proportion of cases and controls by gestational age and birthweight. This method removes the need for the user to make a decision and keeps the user blind to who is recruited as a case or a control. The proportion of cases and controls eligible for the study by gestational age is as follows:

Gestational age or birthweight for gestational age percentile	% to be recruited	Case/Control
GA <36 weeks (up to and including 35+6)	100%	Preterm case
GA 36+0 to 36+6	50%	Preterm case
GA 37+0 to 37+6	5%	Preterm case
BW/GA <P3	100%	SGA case
BW/GA P3-P9.9	50%	SGA case
GA 38+0 to 41+6 weeks and BW/GA >=P10	Will vary according to the number of cases recruited	Potential controls, which are to be sampled immediately after each case.

P3:

Weeks	≥36^{+0}≤36^{+6}	≥37^{+0}≤37^{+6}	≥38^{+0}≤38^{+6}	≥39^{+0}≤39^{+6}	≥40^{+0}≤40^{+6}	≥41^{+0}≤41^{+6}
Cut-off value	2000g	2200g	2300g	2450g	2600g	2700g

P10:

Weeks	≥36^{+0}≤36^{+6}	≥37^{+0}≤37^{+6}	≥38^{+0}≤38^{+6}	≥39^{+0}≤39^{+6}	≥40^{+0}≤40^{+6}	≥41^{+0}≤41^{+6}
Cut-off value	2300g	2450g	2600g	2750g	2900g	3000g

The midwife/researcher using the tablet will approach and screen all women. For an eligible woman they will ensure that consent has been acquired, then recruit the woman and collect biological samples. Some descriptive information, including age, parity and schooling will be collected on all women that are screened using the tablet, whether they are enrolled into the study or not. The midwife/researcher should aim to recruit as many women as possible each day given the circumstances on the delivery suite and the capacity of the laboratory. The numbers of cases and controls recruited each day will be site specific.

Appendix III:

Technical note on selection of controls by Prof Cesar Victora

Selection of appropriate controls in case-control studies is one of the most complex issues in epidemiological design, and also one in which recent progress has obliterated pre-existing ideas, in particular the notion that controls had to be "healthy" or "normal"[d]. There are currently two key concerns in the selection of controls. First, controls should represent the population from which the cases were selected. This will ensure internal validity of the study by avoiding selection bias. It is not required that controls should be healthy in all respects, because in the population where the cases came from there will be unhealthy subjects (for example, controls born at $<38^{+0}$ weeks' gestation may be IUGR). Second, control selection should be driven by the epidemiological measure of effect that one wishes to estimate. In etiological research the most appropriate measure of effect is the incidence density ratio (IDR), or rate ratio, which is equal to the ratio between the incidence rates in the exposed and unexposed groups. Nevertheless, it is not always possible to estimate the IDR directly in case-control designs, and feasibility considerations may lead to other approaches for selecting controls.

There are three main types of case-control studies, which differ according to the type of controls. If the outcome being studied is relatively rare (say, 10% or less), then the three types of controls produce similar results (see attached spreadsheet, "INTERBIO control selection.xls"). Nevertheless, delivery rates at <38 weeks' gestation could be above 10% in some study sites.

Birth study on infants born at <38 weeks' gestation

Below are three potential methods for selecting controls for births $<38^{+0}$ weeks' gestation.

Case-concurrent design

Data from the fetal study allow adopting the case-concurrent method. If information on exposure (for example questionnaire-based exposure variables) is available for all mothers in the fetal growth study, there is no need to do a case-control analysis, because one will already have data on the whole cohort of pregnancies. The data can be analysed with standard cohort analyses (e.g. Cox regression) where the denominator is fetus-weeks-at-risk. If obtaining information on exposure for the whole cohort is too expensive (e.g. GWAS, single SNPs or some biomarkers) then one can do nested case-control analyses.[e] In this design, whenever a birth at <38 weeks' gestation occurs, the next woman attending for antenatal care or ultrasound examination, with the same gestational age, would be selected as a matched control. This design has the advantage of estimating IDR directly, whether or not the outcome (birth at $<38^{+0}$ weeks' gestation) is common. The main disadvantage is that the study cases and controls would be restricted to women who comply with the entrance criteria for the fetal growth study, and who attend antenatal care frequently. This approach may leave out many of the high risk women who would not comply with these strict criteria, and as a consequence the study may miss out on important risk factors.

Case-non-case design

Non-case controls in the study will include infants born $\geq 38^{+0}$ weeks' gestation, regardless of whether or not they present IUGR. Both cases and controls will be selected in the neonatal study. Because there are many more potential controls than cases, controls will be sampled to improve the efficiency of the study, and to avoid carrying out expensive tests on all non-cases. A detailed discussion of the approach to selecting such controls is available in the body of this protocol. The case-non-case design is easy to explain to a wider audience than the other two designs discussed here, and it will provide an estimate of the odds ratios associated with specific exposures, which is a good estimate of the IDR when rates of delivery $<38^{+0}$ weeks' gestation is relatively low, but will overestimate the IDR if the delivery rate $<38^{+0}$ weeks is high (see attached spreadsheet, "INTERBIO control selection.xls"). Logistic regression is the method of choice for analyzing case-non-case designs.

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[d] *Olsen, J., Cesar, V., Ebrahim, S., Pearce, N. The idea of the healthy control is sick. Available at: http://www.ieaweb.org/index.php?option=com_content&view=article&id=62:the-idea-of-the-healthy-control-is-sick&catid=22:rapid-response&Itemid=54 [accessed 25/07/2011]*

[e] *For exposures that will also be collected in the neonatal study, it will be possible to carry out separate analyses in the fetal and neonatal studies, and compare their results; if results are similar, the validity of the findings will be enhanced.*

Case-base design

In this design, controls are sampled from all pregnant women, including those who delivered <38^{+0} weeks' gestation. The latter women will therefore be included as both cases (all women with delivery at <38^{+0} weeks' gestaition) and controls (a sample of these women, using the same sampling fraction as that of women with a delivery age >38^{+0} weeks). The case-base design estimates the prevalence ratio – it is important to remember that prevalence is obtained by dividing subjects with a given characteristic (for example, birth at <38^{+0} weeks' gestation) by the whole population, which includes all births. This justifies the inclusion of women with deliveries at <38^{+0} weeks' gestation in the control group as well. Prevalence ratios obtained from a case-base design tend to overestimate the IDR for births at <38^{+0} weeks' gestation (see attached spreadsheet, "INTERBIO control selection.xls"), particularly when the rate of delivery at <38^{+0} weeks' gestation is high. By collecting data on the four subgroups of births (A, B, C and D) as described in the body of this protocol, it is possible to use weighting to reproduce a case-base analysis. Analyses of case-base designs may be carried out using Poisson regression with robust variance.

IUGR study

Below are three potential methods for selecting controls for IUGR births. The same principles discussed above for the design for births at <38^{+0} weeks' gestation, also apply here, with a few modifications.
Unlike births at <38^{+0} weeks' gestation - which would be defined equally in the fetal and neonatal studies – IUGR would have different definitions, as discussed below.

Case-concurrent design

In the fetal study with serial ultrasound measurements, it may be possible to identify the approximate time at which a fetus became growth-restricted, and use a case-concurrent design. Controls would be fetuses who are not growth-restricted at the time their corresponding cases start faltering. This design is a theoretical possibility, but in practice it may be hard to pinpoint the exact gestational age at which faltering started, and it would also be necessary to decide how to handle fetuses with temporary faltering followed by catch-up growth, and whose weight for gestational age goes back to the normal range. Therefore, although such studies are possible in theory, they are unlikely to be feasible. In addition, as mentioned above in the context of the case-concurrent design for deliveries at <38^{+0} weeks' gestation, the sample of women with frequent measurements in the fetal study is likely biases, and may exclude high-risk pregnancies which are less likely to attend antenatal care frequently.

Case-non-case design

Both for case-non-case and for case-base designs, the cases would include IUGR infants at birth, defined as BW/GA <10th centile. In the case-non-case design, controls would be a sample of all infants who do not present IUGR at birth. The measure of effect would be the odds ratio, which overestimates the IDR and the prevalence ratio when IUGR prevalence exceeds 10% (see attached spreadsheet, "INTERBIO control selection.xls"). Such high rates are common in some parts of the world such as South Asia and Central America.

Case-base design

IUGR at birth is a point prevalence measure, more specifically the proportion of all babies who are born with low weight for their gestational age. For example, 12% of all newborns in a population may present IUGR - note that the denominator of the prevalence measure includes births with and without IUGR. The case-base design directly estimates the prevalence ratio, because the control group includes a sample of all births, regardless of their gestational age at delivery or IUGR status. It may be argued that for IUGR the prevalence ratio is a better measure than the IDR, in particular given how hard it is to define the precise incidence and timing of IUGR onset (as discussed above under the case-concurrent design).

Conclusions

By collecting data on the four subgroups of births (A, B, C and D) as described in the body of this protocol, it is possible to carry out both case-non-case and case-base analyses in the neonatal study.
We are proposing for logistical reasons that the neonatal study controls should be initially selected from non-cases and that the primary analyses should entail case-non-case comparisons. However, it is equally possible to analyse the data with a case-base comparison, by using statistical weighting to correct for the over-sampling of infants born <38^{+0} weeks' gestation and those who are IUGR, and therefore reproducing the whole population of births.

If information on exposures (e.g. through a questionnaire) are available for all births in the neonatal study, then it is possible to carry out a direct analysis of prevalence ratios, without the need for sampling controls. On the other hand, for exposures that are expensive to measure (e.g. lab tests) then sampling controls is a necessity.

Appendix IV:

Definition of intrauterine growth restriction in field studies

A specific limitation of the anthropometric definition of IUGR is the fact that some small babies are biologically small, yet healthy. This could theoretically be overcome by incorporating putative biological or physical markers associated with IUGR/SGA to improve the definition, and in doing so, potentially separate those newborns that are biologically small (yet healthy) from those that are true IUGR/SGA. However, this may be a less relevant issue for the high-risk and undernourished populations we are planning to study where the proportion of the total IUGR/SGA population, that is composed of "healthy" small IUGR/SGA newborns is tiny, compared to a healthy well-nourished western population where biologically "small" babies can represent an important proportion of the IUGR population.
Furthermore, we believe there is not enough evidence presently that such markers can differentiate IUGR/SGA sub-groups sufficiently to justify their incorporation in the planned field studies, especially as these are taking place predominantly in developing countries. For example, it has been suggested that first trimester Doppler ultrasound has a role in distinguishing some of the etiologies of IUGR/SGA. In our opinion, even if these findings are eventually confirmed in a large-scale RCT and shown to be associated with a reduction in perinatal mortality, it would simply not be practical to introduce these additional measurements in the study sites we are planning to use and on the scale of our prospective data collection. Other biological markers that have been proposed as a way of characterizing IUGR/SGA better are still being assessed and, therefore, one of the aims of the new study is to contribute to the evaluation of such markers in IUGR/SGA.

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