Scottish Advanced Fetal Research Study

SAFeR

Full Title: Normal development of the human fetus and the influences and mechanisms by which that development occurs and is perturbed.

Sponsor: University of Aberdeen

Funders: Medical Research Council,
Society for Endocrinology,
NHS Grampian

Chief Investigator: Prof. Paul Fowler

Study Management Group

Chief Investigator: Professor Paul Fowler BSc Hons, PhD, FRSB
Institute of Medical Sciences,
School of Medicine, medical Sciences & Nutrition, Foresterhill
Aberdeen AB25 2ZD
Scotland
United Kingdom
Telephone no: 01224 437528
E-mail: p.a.fowler@abdn.ac.uk

Sponsor:
The Sponsor for this study is the University of Aberdeen/NHS Grampian.

For Further information the sponsor contact is:

Ms Patricia Burns
Research Governance Management
Foresterhill House Annexe
Foresterhill
Aberdeen
AB25 2ZBTelephone no: 01224 551123
Fax no: 01224 550559
E-mail: patriciaburns@abdn.ac.uk

Funder:

MRC
Polaris House
North Star Avenue
Swindon
SN2 1ET

Society for Endocrinology
22 Apex Court
Woodlands
Bristol
BS32 4JT

NHS Grampian
Research & Development
Foresterhill House Annexe
Aberdeen
AB25 2ZB

This protocol describes the "**Normal development of the human fetus and the influences and mechanisms by which that development"** study and provides information about procedures for entering participants. Every care was taken in its drafting but corrections or amendments may be necessary. These will be circulated to investigators in the study. Problems relating to this study should be directed, in the first instance, to the Chief Investigator. This protocol will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (2nd edition). It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

Table of Contents

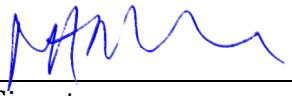
List of Abbreviations:

SAFeR:	Scottish Advanced Fetal Research study
ToP	Termination of Pregnancy
PoC	Products of Conception
SOPs	Standard Operating Procedures

PROTOCOL APPROVAL

Signatures

By signing this document I am confirming that I have read, understood and approve the protocol for the above study.

Prof Paul A. Fowler		05/112015
Chief Investigator	Signature	Date
Dr Alex Douglas		05/11/2015
Individual Responsible for Statistical Review	Signature	Date

1.0. Introduction

1.1 Overview

In-utero exposure to drugs and chemicals through maternal smoking, alcohol use, drug abuse, prescription medicines and occupational/lifestyle exposures is widespread. Such exposures can alter fetal development and programming, leading to the effects becoming "locked in" from birth and causing long-term adverse consequences for the individual. These include costly and widespread conditions such as obesity, hypertension, metabolic syndrome and infertility (Cupul-Uicab et al. 2012, Hackshaw et al. 2011, Reynolds et al. 2011, Snijder et al. 2011, Snijder et al. 2012)*. The weight of evidence linking these conditions to fetal recreational drug or environmental chemical exposures, including cigarettes, alcohol, air pollution, food contact materials, is overwhelming. What is lacking is an understanding of how fetal drug exposure translates to adult ill-health and this is due, largely, to our inability to study the problem directly in affected human fetuses.*

1.2 Drug use during pregnancy

While the overall incidence of smoking in the UK has fallen over the last 20 years, significant numbers of women continue to smoke: typically around 30% of women aged <30 years. In Aberdeen, for example, 31% of women are smokers when first pregnant and typically fewer than 4% of pregnant women stops smoking during pregnancy (Tappin et al. 2010). In the UK, roughly 80% of young women drink (40% heavily) and up to 20% use recreational drugs, with smokers more likely to also drink. Women who use drugs tend to stop taking ecstasy when they become pregnant but 50% continue to use cannabis (Moore et al. 2010). Questionnaire sampling suggests that alcohol use also declines when women become pregnant but in the UK more than 20% continue to drink during pregnancy (6% heavily) (Mehta et al. 2009). Unfortunately, fetal alcohol exposure is difficult to measure reliably (Joya et al. 2012), especially below levels inducing fetal alcohol syndrome. Thus national exposure levels may be higher than assumed and exposure for the individual neonate is generally unknown. It should be noted that second hand (side-stream) cigarette smoke exposure is also associated with a significant increase in congenital defects and post-natal health deficits (Meeker and Benedict 2013).

1.3 Social deprivation

Social deprivation is a significant risk factor for childhood health (Weightman et al. 2012) and some of this may come from programming during fetal life (Reynolds et al. 2013). Furthermore, as we have seen in our studies of the human fetal liver exposed to maternal cigarette smoking (Drake et al. 2015, Filis et al. 2015, O'Shaughnessy et al. 2011), there is also sexual dimorphism in the growth response to maternal stress (Giesbrecht et al. 2015). The sex of the fetus is important in terms of the placenta (Muralimanoharan et al. 2015) and this also holds true for the effects of another adverse maternal lifestyle factor, obesity. Furthermore, adverse lifestyle choices such as drinking and smoking are more prevalent amongst the most deprived women (e.g. (Raisanen et al. 2014)). This means that the fetus and placenta will be subjected to multiple adverse environmental factors in some women. Distinguishing between those fetuses and placentas that are affected significantly by such multiple "hits" and those who are not is a significant health challenge.

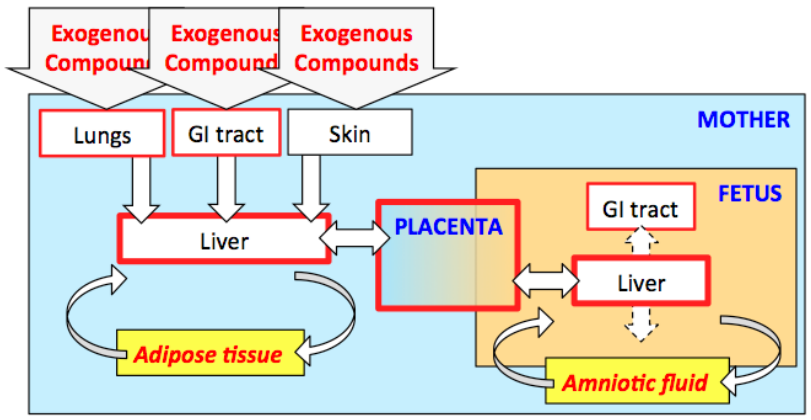
1.4 The placenta is at risk from adverse maternal lifestyle factors

Both maternal smoking (Kawashima et al. 2014, Shinjo et al. 2014) and, to a lesser extent, alcohol use (Lui et al. 2014, Wang et al. 2014) are implicated in reduced placental and fetal development, although the effects become more evident later in pregnancy (Anblagan et al. 2013). The placenta has been studied as a potential matrix with which to detect adverse exposures using biomarkers, such as environmental heavy metal or polycyclic aromatic hydrocarbons. Furthermore, there are emerging studies showing that specific biomarkers in the placenta can highlight specific risks (e.g. Arrebola et al. 2015). However, the use of the placenta

as a matrix with which to monitor diseases in a more advanced clinical perspective has been hampered by lack of focused experimental and epidemiological data. In addition, sex differences in placental responses to adverse conditions (Brown et al. 2014) mean that the sex of the new-born will be an important criteria in biomarker searches.

1.5 The human lifestyle and the fetus are very different from the rodent

Maternal intake of chemicals, including pollutants and drugs, occurs via multiple routes of exposure (**Fig.1**), with maternal biotransformation (metabolism, modification, conjugation, sulphation) in the gastro-intestinal tract, liver and placenta. The fetus will be exposed to xenochemicals (e.g. nicotine metabolite cotinine (Fowler et al. 2008) through the feto-maternal interface at the placenta. Fetal blood from the placenta travels through the umbilical vein and supplies 70% of the blood to the fetal liver. The fetal liver is, therefore, exposed to high concentrations of maternally-derived xenochemicals/drug metabolites. Our own data shows that maternal smoking increased total polycyclic aromatic hydrocarbons (PAHs, a significant health-damaging component of air pollution) in the female human liver 5.7-fold (P=0.011) (Fowler et al. 2014). Unlike the rodent, the human 2nd trimester fetal liver expresses many xenochemical sensing and metabolising transcripts (O'Shaughnessy et al. 2011), which will act to protect the fetus from exposure. We demonstrated that expression of these transcripts change in responses to chemicals in cigarette smoke (O'Shaughnessy et al. 2011), probably due to increased levels of compounds activating the xenosensors AHR, PXR and CAR. We have also carried out a pilot proteomic study (Filis et al. 2015) of a closely matched subset of fetal livers previously published (O'Shaughnessy et al. 2011, O'Shaughnessy et al. 2013). Proteins involved in cellular homeostasis (males) and gastrointestinal diseases (females) were dysregulated differently between the sexes if the mother smoked. Maternal smoking, therefore, causes sex-specific changes in the human fetal liver proteome, affecting diverse pathway and potentially laying the foundations for disease later in the offspring's life.



KEY: red outline = major biotransforming organs; GI = gastrointestinal tract
Chemicals are primarily lost via maternal exhalation, urination and defecation.

Fetal urination and drinking passes chemicals back and forth between fetus and amniotic fluid, which acts as a chemical reservoir.

Fig.1: How exogenous chemicals reach the fetus. Maternal routes of ingestion/absorption pass via maternal liver and the placenta to the fetus where further biotransformation occurs.

One of the effects of fetal xenochemical exposure is to alter fetal liver function, and this is likely to have knock-on effects to other aspects of fetal growth. We have shown that fetal liver function is sexually dimorphic, probably through androgen receptor expression (O'Shaughnessy et al. 2013), thus playing a role in the differential growth of male and female fetuses. Furthermore, the human fetal liver expresses an extensive range of steroidogenic and steroid metabolising enzymes enabling a significant modulation of fetal circulating steroids (O'Shaughnessy et al. 2013). This makes the fetal liver capable of altering steroid hormone signalling from the placenta, the mother and other fetal organs (e.g. adrenal glands) and suggests that it is a key endocrine organ during human fetal development. Overall, therefore, the fetal liver is likely to play a dual critical role in human pregnancy, protecting the fetus from xenotoxicant exposure by oxidation/reduction/conjugation reactions and by regulating steroid hormone exposure.

Liver development is an on-going complex interaction of factors likely to have significant knock-on effects on fetal development in general. However, the fetal liver is also a target for these xenotoxicants, with consequences for its function. Our own data (Drake et al. 2015) shows that maternal smoking disturbs sex differences in the IGF system, significantly reducing hepatic *IGF2* in male fetuses, one way in which growth may be dysregulated in-utero, thus programming the fetus towards IUGR and adulthood metabolic syndrome. We also found that maternal smoking altered DNA methylation at a number of genes susceptible to the in-utero environment, including the imprinted gene *IGF2* and glucocorticoid receptor (*NR3C1*). Reduced male fetal hepatic *DNTM1* expression demonstrates a sex-specific way by which maternal smoking alters CpG residue methylation.

1.6 Importance of fetal liver development for adult health (currently funded by the Medical Research Council)

There is strong evidence to suggest that dysregulaton of human fetal liver development has consequences for adult health. One link is through impaired fetal growth, which can lead to fetal programming of metabolic syndrome in adulthood (Latini et al. 2004). A down-stream consequence of increasing metabolic syndrome levels is increased non-alcoholic fatty liver disease, which affects 17-30% of the population in Western countries (Kneeman et al. 2012) and which also has roots in disturbed fetal growth. Programming of metabolic syndrome may also be partially affected by alterations in steroid enzyme activity (Nyirenda et al. 2009) and there is strong evidence for greater risk of fetal programming of dysregulated hepatic lipid metabolism in males (Choi et al. 2007). The link between fetal environment (including nutritional variation), the liver and adult disease is poorly understood (e.g. (Morrison et al. 2010)) and further research into the factors leading from dysregulated fetal liver development to disturbed adult metabolic processes is required.

1.7 Normal human fetal development remains poorly understood

We have previously (approved by the NHS Grampian Research Ethics Committees (REC 04/S0802/21)) reported adverse effects of maternal cigarette smoking on the fetal testis (Fowler et al. 2008, Fowler et al. 2009), ovary (Anderson et al. 2014, Fowler et al. 2014), urogenital tract (Fowler et al. 2011b) and liver (Drake et al. 2015, Filis et al. 2015, O'Shaughnessy et al. 2011, O'Shaughnessy et al. 2013). However, there are many aspects of human fetal development that remain very poorly understood, in part because researchers have relied on studies of rodents and other animals and only a limited number of studies have used either miscarried fetuses (therefore of suspect normality) or electively terminated fetuses from normal pregnancies. We have also contributed significantly to understanding of normal fetal development, and sex differences, in terms of the gonads, urogenital tract and the liver (Drake et al. 2015, Filis et al. 2015, Fowler et al. 2009, Fowler et al. 2009, Fowler et al. 2011a, Fowler et al. 2011b, Fowler et al. 2014, O'Shaughnessy et al. 2007, O'Shaughnessy et al. 2011, O'Shaughnessy et al. 2011, O'Shaughnessy et al. 2013). It is very important to continue this process of characterising the processes of normal human fetal development in order to better understand how that development is controlled, how it can be disturbed (e.g. by maternal lifestyle or pollution) and how this can be detected. Furthermore, health-risks posed by chemicals and medicines (e.g. plastics like bisphenol A, pain-killers), to both the mother and the fetus (e.g. paracetamol (Snijder et al. 2012, van den Driesche et al. 2015)) requires better fundamental understanding of how the fetus and its placenta develop and how that process is regulated. A current example is a study we are preparing for publication: we have found that the human male fetus has at least 2,000 times higher levels of oestrogens circulating in its blood than an adult man. This will likely have significant implications for the risk posed to the developing male fetus by environmental oestrogens (e.g. chemicals from the pill found in water), which are considered a major cause for concern in the environmental toxicology field. Out other on-going studies include research on the fetal endocrine systems focussing on the fetal adrenal and thyroid glands, both in terms of normal development and the effects of maternal smoking.

1.8 Summary

We, and others, have shown that human fetal development, which lays the foundations of adult health and function (fetal programming), is quite different from the rodent and frequently exhibits surprising aspects. It has become evident that the close interconnectivity of the developing fetal organs and also the placenta, means that a much more holistic approach to research aiming to understand human fetal development and the challenges posed to programming for a health adulthood is critical. To that end we are requesting a carefully considered expansion in the numbers and ages range (from 11-21 to 7-20 weeks of gestation) of fetuses we can study and in the range of organs, body fluids and maternal information collected. Specific project areas are highlighted in the associated SAFER project REC application.

2.0 Study Objectives

2.1 Objectives

Our overarching objective is to intensively and systematically study the human fetus during a normal pregnancy and pregnancies where aspects of maternal lifestyle and environment will challenge the fetus. We aim to provide fundamental information to better understand the mechanisms involved and to detect and treat or ameliorate adverse effects during pregnancy (such as maternal smoking/drinking, deprivation, exposure to pollution). In the long term findings from this research will be important for future studies aimed at enabling better health in later life.

2.1.1 Primary Objective

To provide fundamental information to better understand the mechanisms involved and to detect and treat or ameliorate adverse effects during pregnancy.

2.1.2 Secondary Objectives

The secondary objectives of this study are al aimed at better understanding of normal processes during development and how maternal lifestyle and environment can affect it. Our research will involve a wide range of laboratory, cell and tissue culture and xenografting as well as microscopic techniques to study chemicals, hormones, proteins, genes and DNA in the fetal tissues. We have developed sophisticated approaches to minimise how much tissue we require to carry our as many different measurements as possible in the same sample. It should be noted that the proposed studies are collaborative with different research groups since no single research group could carry out all the studies in isolation. Furthermore, the proposed studies will inform back to animal and cell studies, enabling more accurate interpretation of their own findings in light of mechanisms and specific conditions actually at play in the human fetus.

(1) Human fetal endocrine system

We aim to investigate how the different hormone systems in the fetus develop and are affected by adverse maternal factors such as cigarette smoking and obesity. These include the testis, ovary, adrenal gland, thyroid gland, kidney and the hypothalamus, pineal gland and pituitary gland in the brain. In addition, we also aim to study the placenta, including its endocrine function, and how it is affected by maternal environment, since it is a key organ of pregnancy. Changes in programming that establishes the endocrine system can have lifelong consequences for health and wellbeing.

(2) Human fetal reproductive and urogenital system

We aim to investigate how the ovary, testis, urogenital tract (including prostate gland, breast buds and uterus), genitalia and kidney develop in the human fetus. There is considerable evidence that maternal smoking and over the counter medicine use can have negative impacts on this system and we will investigate when such impacts begin and what mechanisms are

involved. In addition, common cancers, such as testis cancer, may have fetal origins and certainly events in fetal life may predispose the resulting adult to develop cancers. In the case of testis, ovary, breast buds and prostate gland, an additional aim is to determine whether and how events between 7 and 20 weeks of gestation increase the likelihood of cancers in these organs in adulthood.

(3) Mechanistic studies, Epilepsy and other brain disorders

We aim to investigate development of the fetal central nervous system, including the brain. The first study planned is to investigate changes in genes and the structure of DNA in the brain related to epilepsy and other neurodevelopmental disorders linked with maternal cigarette smoking during pregnancy. A second aim is to use the smoke-exposed fetuses to better understand the role vitamin A plays in normal fetal development, including development of the central nervous system. Similar concerns are expressed in the literature with respect to maternal alcohol consumption and by accessing data from the FAST alcohol questionnaire that is already collected at clinic, we will be able to address this issue systematically with respect to all our studies, including fetal brain development.

(4) Metabolism and liver disease

We aim to continue our studies (funded by the Medical Research Council) of the human fetal liver and other organs important for metabolism, including pancreas and fat cells and cells that will become fat cells (precursor cells). Programming of metabolism during life in the womb is important for adult health and better understanding of the development of the metabolic systems and how they are perturbed by factors such as maternal smoking and deprivation is an important basis of developing strategies for wellbeing post-natally. A critical disease of growing importance in these studies is non-alcoholic fatty liver disease, which is increasing in incidence, especially in obese individuals. Given that the human fetal liver is active and responding to maternal environment, some of the basis of this disease may be established in fetal life. In addition, the development of thyroid and adrenal glands is also essential in establishing normal metabolic processes. Cutting edge techniques, such as large-scale metabolomic and lipidomic studies, including analysis of a wide range of dietary metabolites, will also be used.

(5) Heart and cardiovascular system

The development of the heart and the major artery, the aorta, between 7 and 20 weeks of gestation will be characterised. The effects of adverse in-utero environment of the developmental processes will then be investigated.

(6) Immune system development

The thymus is critical and large organ in fetal development that is important to establish the immune system. The spleen manufactures antibodies and, in fetal life, red blood cells (as does the fetal liver) and recycles red blood cells. Both organs have important role in maintaining the immune system. We aim to investigate whether adverse maternal lifestyle and/or environment can alter thymus and spleen development and function.

(7) Bone, cartilage and joints

Very little is known about normal bone and cartilage development in the human fetus. Importantly, it is not even know when the cells involved in bone, cartilage and joint development begin to alter their behaviour into a more “adult” format. The reason this is important is that in musculoskeletal disease in the elderly, especially osteoarthritis, early stages of disease is usually characterised by renewed tissue growth – i.e. a return to a more “fetal” pattern of cell behaviour. The role of cell programming during fetal life and better understanding of the correct development and maintenance of bone, cartilage and joints, and how programming is affected by the fetal environment, is therefore one of our aims.

(8) The development of the placenta between 7 and 20 weeks of gestation

We know a great deal about the term placenta because it is so easy to obtain and to relate directly to the accompanying new-born and its parents. However, the placenta is a dynamically developing organ that is vital for normal pregnancy and that is affected by maternal lifestyle (e.g. placental growth is reduced if the mother continues to smoke while pregnant). We aim therefore to better understand how the placenta develops during this window in gestation and how it is affected by maternal environment.

(9) Nerve repair

Spinal cord injury is a massive health problem globally. Recently it has been shown that human fetal central nervous system cells or embryonic nerve stem cells have the potential to assist in novel treatment strategies to repair spinal cord injury. This final secondary objective is focussed on a proof of concept study to determine whether culturing severed nerves in the presence of human fetal nerve cells assists in correct and functional nerve regrowth and re-joining across the induced gap. Any further studies, if the proof of concept aim is realised in a satisfactory manner would require a separate ethic application.

(10) Maternal emotional health and wellbeing effects on the fetus

Maternal stress is a well-known negative influence on fetal development. Our aim is to perform a pilot study relating indices of fetal development, such as morphology, endocrine status, to maternal records of emotional health and wellbeing and abuse. The principal outcome will be an indication as to whether maternal stress is affecting fetal development at a general level between 7 and 20 weeks of gestation. Placental biomarkers identified in other studies listed above will also be related to measures of maternal stress in order to establish whether there may be placental biomarkers of early fetal response to maternal stress.

(11) Fetal lung

While it is well known that the adverse effects of maternal smoking include increased incidences of respiratory problems, such as asthma. However, there are also data suggesting that the offspring exposed to cigarette smoke may be more likely to develop lung cancer. Our aim is to determine whether maternal smoking disturbs fetal lung development early in pregnancy and if this includes signs of increased likelihood of cancer development, such as polycyclic aromatic hydrocarbon-DNA adducts in the fetal lungs.

(12) Quantifying human fetal exposure to pollutants, drugs, pharmaceuticals and other chemicals

It is simple to measure chemicals in the blood or urine of the mother, or in amniotic fluid collected during amniocentesis. However, amniocentesis is not performed for research purposes because of not inconsiderable risk to the fetus. This means that we have to reply on animal studies of such chemicals getting to the fetus but this is rather inadequate due to major species differences. Therefore we aim to measure chemicals and drugs of potential health concern (such as bisphenol A which is in plastics and over the counter drugs taken by mothers which may affect the fetus, such as paracetamol) in the fetal tissues, including blood and urine where available. This is the only way to accurately assess how much of these compounds actually get into the fetus itself. This is important since the fetus is considered much more vulnerable than the adult to toxic chemicals. In addition, we aim to also measure these compounds in the placentas (and cord blood where available) from the same fetuses because the placenta is readily available at term and we aim to investigate in the placenta biomarkers of fetal exposure to adverse compounds (e.g. alcohol) that are detected in the second trimester fetal liver.

(13) Non-genetic programming of the fetus for adult life

Epigenetic changes are changes that involve marks being put onto DNA that affects how genes are activated. Therefore, an epigenetic change can change, sometimes greatly, the link between the amount of a gene present in cell and its effect. This linkage can be quite large with a gene being practically silenced in terms of its effect, even though there may be plenty of the gene present in the cell. Conditions within the womb, such a maternal smoking, can have epigenetic effects. We aim therefore to better understand how the environment experience by the fetus can affect development and programming of adult health and disease by epigenetic mechanisms.

2.2 Outcomes

2.2.1 Primary Outcomes

The provision of an extensive dataset on the human fetus during normal pregnancy and pregnancies where aspects of maternal lifestyle and environment will challenge the fetus, informing on future heath and function.

2.2.2 Secondary Outcomes

The headline secondary objectives of the study, applying to a wide range of body systems and organs, are:

(1) The identification of key stages and regulators required for the normal development of the human fetus and its placenta.

(2) The quantification of maternal lifestyle factors, including body burdens of toxicant chemicals (e.g. from smoking, from air pollution, from food contact material) and determination of their relationship with adverse fetal and placental outcomes.

(3) Mechanistic insights into how fetal human development is controlled and how this control can be disturbed by maternal factors.

(4) Establishment of the risks of adverse in-utero environment for future generations. This is epigenetic and focuses on ways in which fetal event, without any changes in genes themselves, can mis-programme the development of future generations.

3.0 Study Design

3.1 Study Description

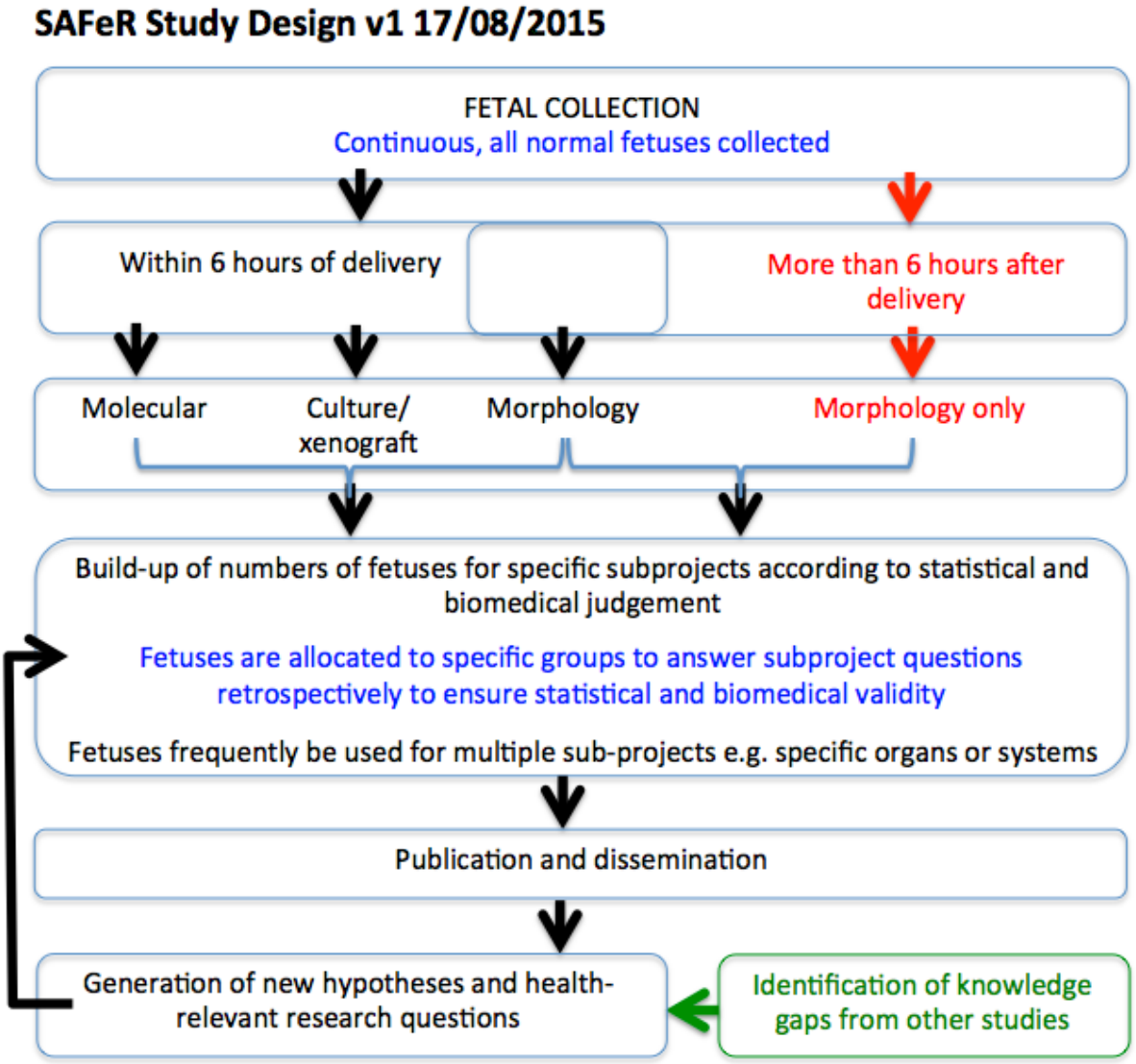
Normally, for a study, a specific question is addressed by prospectively recruiting a carefully considered, statistically validated, number of patients of specific types. Effects or differences are then measured as detailed in the ethics application. For our study this approach is simply not possible. There are several reasons for this, most importantly:

(1) It is impossible to predict the categories of pregnancies that become available, including maternal characteristics, sex and age of the fetus, normality of the fetus and whether the mother will withdraw from the study.
(2) It takes months and even years to collect a statistically valid number of specific fetuses suitable to answer secondary research questions.
(3) It is evident that there are very clear sex differences during development and for the majority of studies it is essential to have matching numbers of male and female fetuses.
(4) In our experience of the last 10 years of such studies, new data very frequently leads to unexpected new secondary questions.

For these reasons, the only viable strategy is to collect every fetus that becomes available with written, informed, consent and store the tissues and data collected in ways appropriate for a

range of different research methods to be used. We have consulted with the Grampian Biorepository with respect to this application. Thereafter, once appropriate numbers of fetuses best matching a specific sub-project become available, the sub-project can begin. Essentially this is a type of retrospective design but this is the only scientifically and statistically valid manner to proceed under the circumstances (see 3.2 Study Design Flowchart below).

3.2 Study Design Flowchart



4.0 Study Population

4.1 Number of Participants

Based on analysis of recent figures, approximately 800 and 1,400 fetuses ≥7 weeks of gestation are terminated medically in Aberdeen and Glasgow respectively. Of these some 5%10% are expected to consent. At a highly conservative estimate, over 10 years, this would be 1,200 fetuses Aberdeen and Glasgow combined should be able to achieve this target. For reasons laid out here and in the full ethics application, we cannot have a priori finalisation of sample size. Therefore, we request approval to aim to collect 1,200 fetuses, enabling sufficient numbers to perform appropriately powered sub-studies.

4.2 Inclusion criteria

- Women at 7-20 weeks of gestation (critical stage of fetal development).
- Women aged 16 years and older, deemed capable of making a rational decision.
- Absence of fetal anomaly at ultrasound scan (only normal fetuses are required).
- Women who are fluent English speakers. This is in order to ensure the woman understands that fetal tissues will be collected.

4.3 Exclusion criteria

- Women exhibiting considerable emotional distress.
- Fetal anomalies identified at ultrasound scan (it is essential to ensure fetal normality).

5.0 Patient selection and enrolment

NB: see SAFeR Consent to Disposal flowchart (page 19) in conjunction with sections 5 and 6 below

5.1 Identifying participants

Women seeking a termination will attend clinic to receive their first medication around 36-48 hours prior to ward admission as part of standard care. The study will be explained to potential participants during this appointment. Patients will in no way be persuaded, induced or coerced into participating in this study. The researchers and laboratory staff play not part what so ever in participant identification. The **SAFeR Study Information Sheet** will be offered to women who fit the inclusion criteria by members of the direct clinical team. The women will be able to take this information sheet home with them and will have at least 24 hours prior to ward admission for reflection. Where a woman is approached, this is recorded in her clerking sheets.

The following data are routinely collected by clinical staff and recorded on the clinical record of each woman attending:

1. Age
2. Weight
3. Height
4. LMP
5. Previous pregnancies or ToPs
6. Medication use (both over-the-counter and prescribed)
7. Recreational drug use, including cigarette and ecigarette use
8. FAST alcohol score (validated questionnaire)
9. Postcode*
10. Intimate partner abuse (short list of questions)
11. Emotional health and wellbeing (short list of questions)
12. Normality and stage of the pregnancy based on ultrasound scan

note the postcode will never be supplied to any researcher involved in the study

These data are noted on the Clerking Sheet for each woman, which is routinely passed on to the nurses if the woman is progressing to termination of pregnancy (ToP). The women are not asked any additional questions for the SAFeR study.

5.2 Consenting participants

This is performed by a member of the direct clinical team when patients are admitted to the appropriate ward. The clinical staff both in the clinics and in wards will independently assess

the women according to inclusion and exclusion criteria. Members of the clinical team will be GCP trained and will have experience of this vulnerable patient group.

In the ward, women will be asked if they have read the **SAFeR Study Information Sheet** (provided at clinic at least 24 hours previously) and whether they are willing to take part in the study. If they consent, they will be asked to sign the **SAFeR Consent Form**. The nurses will remain with the women throughout the day and will be available to answer questions at any time. **The participants are able to sign the consent form and change their mind at any stage, right up to the point where the products of conception are removed from the ward**. Once the consent form is signed, the nurses will notify the laboratory staff by telephone. The laboratory staff will then open a new entry in the electronic **SAFeR MONITORING LOG**, which is held in a secure and restricted, password-protected, filespace on a University of Aberdeen server (therefore continuously backed-up) and begin recording the entire process.

5.2.1 Post-consent stages in the ward

The ToP proceeds in exactly the same manner as it would if the women did not consent. The nurses also check that all approved data from the clerking sheets is recorded in the **Maternal Datasheet**. The clerking sheets remain confidential and are **never** seen by the research team. Once the products of conceptions (fetus and placenta) are delivered by the mother there are two possible scenarios:

CONSENT IS WITHDRAWN:
In this case the ward proceeds with the disposal of the products of conception to the mortuary according to current legislation. The nurses notify the laboratory to enable logging of the withdrawal of consent. The process is then at an end.

CONSENT IS MAINTAINED:
In this case the nurses clamp the umbilical cord, place the products of conception in the appropriate sealed opaque container and telephone the laboratory staff. They also enter the SAFER number and date (only) on the **Fetal Datasheet**. No maternal identifiers are recorded in either the **Fetal** or **Maternal Datasheet**. When the products of conception are collected by the laboratory staff, the nurses record this in their logbook. This acts as an independent cross-check for whether the fetus was removed from the ward. Once the products of conception have been removed from the ward, the nurses also note in the TOPS coding sheet that the pregnancy has entered the **SAFeR** study. This then enables the Data Management Team (under Dr Katie Wilde), University of Aberdeen, who host the Grampian TOPS database, to record this fact and to send Professor Fowler monthly reports on the 5% category of the SIMD score that each woman (whose products of conception have been taken to the laboratory) falls within. This enables the relative deprivation status of the mother to be used in the research project in a manner that will have absolutely no impact what so ever on her absolute anonymity (e.g. avoids the need for postcode data to be used by the researchers).

Before leaving the ward the laboratory staff check that they have all relevant documentation (copy of **CONSENT FORM** and **SAFeR MATERNAL** and **FETAL DATASHEETS** and the **NHS MORTUARY FORM**S). The laboratory staff then transport the products of conception to the laboratory. On the way they stop at the relevant **NHS Grampian Biorepository office** to pass on the **SAFeR CONSENT FORM** and thus register the PoC and the SAFeR study number with the Biorepository. The consent forms are available at the Biorepository for NHS audit purposes. The laboratory staff then continue to the laboratory. In the event that the Biorepository is shut (i.e. after hours working), the **SAFeR Consent Form** will be taken to the Biorepository on the earliest available working day.

5.2.2 Post-consent stages in the laboratory

Laboratory staff collects the products of conception from the ward as soon as possible, after being notified by the nurses, and transport them back to the laboratory in the sealed opaque container carried in a sealable opaque cold box (i.e. 3 layers of containment of the PoC). On the way to the laboratory (working hours) or as soon as possible on the following working day the **CONSENT FORM** must be lodged with the Biorepository. Upon arrival in the laboratory, remaining available documentation from the ward (**SAFeR MATERNAL** and **FETAL DATASHEETS** and the **NHS MORTUARY FORMS**) must be re-checked and any irregularities not observed in the ward queried with the nurses and logged. There are two possible scenarios that affect exactly how the products of conception are handled in the laboratory:

PRODUCTS OF CONCEPTION DELIVERED WITHIN 8 HOURS OF ToP TREATMENT ONSET:
In this case the samples are collected and processed in a manner suitable for molecular biology, cell/tissue culture and histology/morphology, requiring rapid processing of samples for molecular biology to liquid nitrogen and then storage at -80°C and/or processing to culture conditions and processing for histology/morphology

PRODUCTS OF CONCEPTION DELIVERED MORE THAN 8 HOURS OF ToP TREATMENT ONSET:
In this case the samples are collected and processed in a manner suitable for histology/morphology or other measurements less affected by length of time after fetal death, such as measurement of stable pollutants. Processing is less urgent and if necessary can occur the following day. Products of conceptions that are not processed within 48 hours of delivery must be disposed of immediately.

SAMPLE PROCESSING:
A detailed protocol for dissection (**SAFeR Tissue Collection Protocol**) is used by laboratory staff to guide the processing of the products of conception. The processing is conducted as rapidly as possible in an isolated laboratory using a class 2 cabinet to ensure laboratory staff safety. The laboratory has opaque windows and is allocated to Prof Fowler's research group uniquely. Any staff present, who is not involved in the SAFeR study, will leave the laboratory immediately, prior to the transport bag being opened. This ensures that the process is not observed and is conducted with as much respect and sensitivity for the fetus as possible.

Mr Paul Brown, Consultant Paediatric Pathologist, NHS Grampian is a named collaborator and is available to inspect fetuses should the laboratory staff have cause for concern with respect to normality. He is also able to provide additional training and advice to the laboratory staff harvesting the tissue samples.

During dissection and processing the **SAFeR Fetal Datasheet** must be completed fully. Once all the samples have been processed, the person undertaking the processing must update the **SAFeR Monitoring Log**.

DISPOSAL OF PRODUCTS OF CONCEPTION:
Once all sample processing is completed the **SAFeR Mortuary Receipt Form** must be completed and the fetus and the **NHS Mortuary Forms** must be taken to the mortuary the next working day for disposal according to current legislation. The **NHS Mortuary Forms** are retained by the mortuary. The fetuses must be disposed of according to the Scottish CMO guidance 2012 update (SGHD/CMO(2012)7) on "SOHHD/DGM (1992)4: Sensitive disposal of fetuses and fetal tissues following termination of pregnancy" with a minimum standard of disposal being disposal at a crematorium. At this point the laboratory staff have no access to any patient identifiers and no record of these will be retained by the laboratory staff. Any enquiries requiring identifiers must thereafter be conducted via the Biorepository.

The **SAFeR Mortuary Receipt Form** must be signed by the ARI mortuary staff and retained for the **SAFeR Site File**. The **SAFeR Monitoring Log** has a specific entry to record this.

5.3 Participant withdrawal

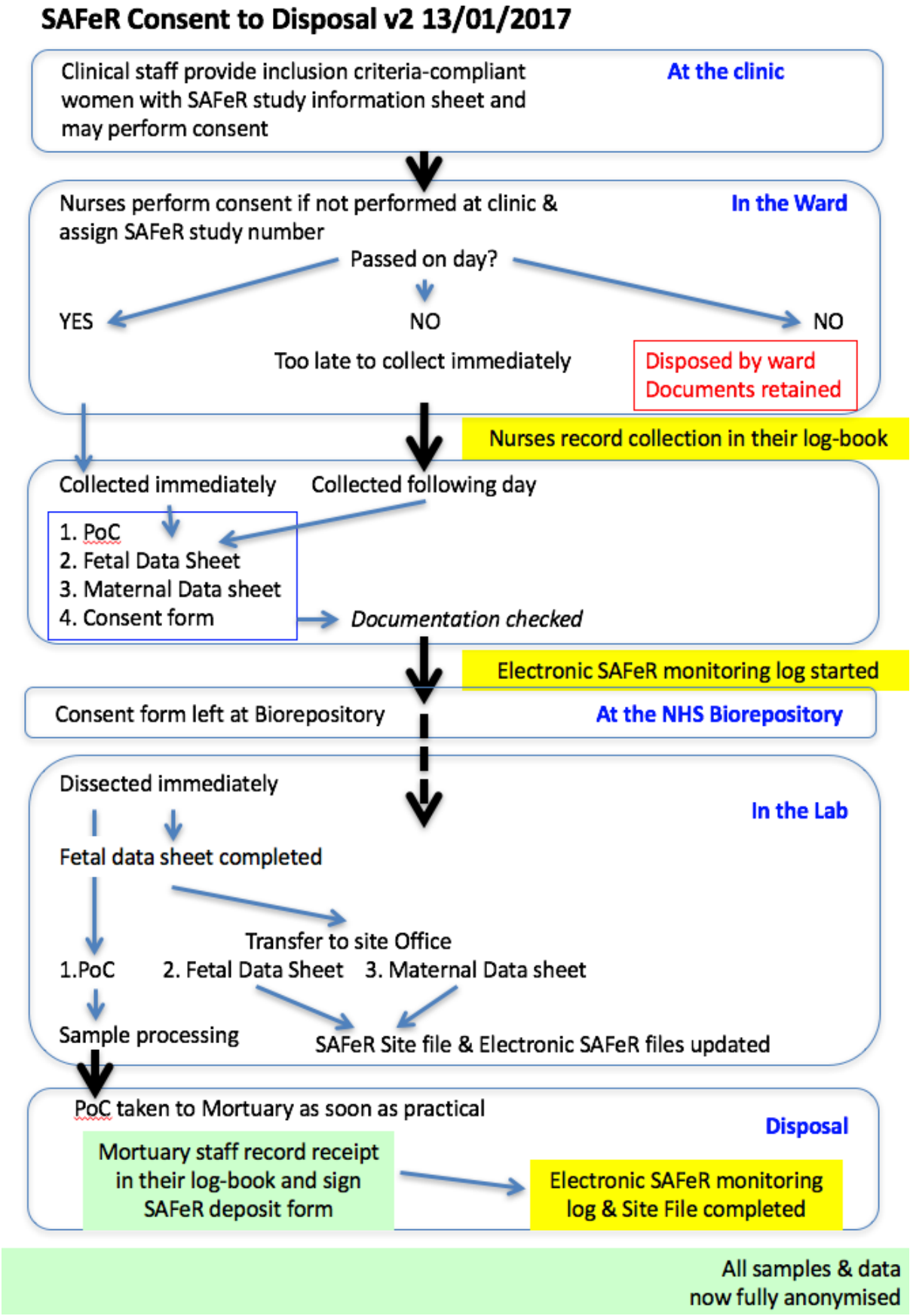
In this case, the ward proceeds with the disposal of the products of conception to the mortuary according to current legislation. The nurses notify the laboratory to enable logging of the withdrawal of consent. The process is then at an end. Once the products of conception leave the ward, the woman is no longer able to withdraw,

6. Data Collection and Management

The PI then maintains the **SAFeR Site File,** cross-checks and closes the **SAFeR Monitoring Log** for that fetus and enters fetal and maternal data into the **SAFeR database**. This is the research database and contains no identification information other than the unique study (SAFeR) number assigned to the products of conception by the nurses when the mother was consented. Electronic copies of the completed **SAFeR Maternal** and **FETAL datasheets** are then emailed to the Biorepository

The **SAFeR database** and the **SAFeR Monitoring Log** are located in a secure folder with highly restricted, password-protected access, backed-up University server. New staff joining the team will complete the Good Clinical Practice (GCP) course before taking part in the research or having access to these two files. Data will be stored in accordance with the University of Aberdeen Data Protection Policy and the Data Protection Act (1998). The research described here will comply with University of Aberdeen SOPs.

Site-specific Note: each site has its own SAFeR Monitoring log, but the SAFeR database is located in a secure folder with highly restricted, password-protected access, backed-up University of Aberdeen server. Named team members at each site will have access to this database only with prof Fowler's specific approval. These individuals will be recorded and counter-signed in the Aberdeen Site file


SAFeR Consent to Disposal v2 13/01/2017
Clinical staff provide inclusion criteria-compliant women with SAFeR study information sheet and may perform consent
At the clinic
Nurses perform consent if not performed at clinic & assign SAFeR study number
In the Ward
Passed on day?
YES
NO
NO
Too late to collect immediately
Disposed by ward
Documents retained
Nurses record collection in their log-book
Collected immediately
Collected following day
1. PoC
2. Fetal Data Sheet
3. Maternal Data sheet
4. Consent form
Documentation checked
Electronic SAFeR monitoring log started
Consent form left at Biorepository
At the NHS Biorepository
Dissected immediately
In the Lab
Fetal data sheet completed
Transfer to site Office
1.PoC
2. Fetal Data Sheet
3. Maternal Data sheet
Sample processing
SAFeR Site file & Electronic SAFeR files updated
PoC taken to Mortuary as soon as practical
Disposal
Mortuary staff record receipt in their log-book and sign SAFeR deposit form
Electronic SAFeR monitoring log & Site File completed
All samples & data now fully anonymised

7. Statistics and Data Analysis

7.1 Proposed Analysis

The SAFeR study core personnel include Dr Alex Douglas, a highly experienced bio-medical statistician and data modeller. This is necessary because of the complexity of some of the data and statistical analyses to be performed.

The SAFeR project (like its successful predecessor, the FEGO project) has an unusual design. This is simply because of the nature of the patients recruited (women undergoing elective terminations of pregnancy) and the samples obtained (products of conception). In addition, there is a steady incidence of withdrawals of consent once the patient see the products of conception. We are not able to have any information on the fetus prior to recruitment and successful return of the samples to the laboratory. This means that it is simply not possible to prospectively adjust numbers of fetuses falling into four principal groups (i) male, (ii) female, (iii) non-exposed, (iv) stage of gestation (7-20 weeks). There is certainly no prior planning possible with respect to which fetuses will have high or low levels of different exposures or different maternal lifestyle factors. This means that recruitment and collection of samples has to be conducted without specific target numbers until the point sufficient numbers of fetuses appropriate for specific sub-studies are available. Therefore the studies themselves have to be conducted retrospectively. Building up an extensive collection of fetuses also gives us the flexibility the flexibility to collaborate in a range of projects to answer study questions by bringing in world-leading expertise. Again, these could not be arranged prospectively.

Examples of power analyses (accepted as suitable by the MRC when funding our study) are as follows (but each sub-study will require its own power analysis):

FOR RNA-SEQ: The power of and RNA-seq experimental design to detect differential gene expression was assessed using the Scotty software package. Since there are no RNA-seq pilot data on the human fetal liver available, the power analysis was based on a publicly available human liver RNA-seq dataset, which enabled us to compare two treatment groups. Although our dataset will be more extensive, this allowed a preliminary power analysis to be performed. A total of 60 fetuses would have 85% power to detect a 2-fold change difference in gene expression assuming a sequencing depth of 30 M reads, a type I error rate of 1 % and equal number of fetuses in each group.

FOR qPCR: To establish the likely power of the study we examined a representative outcome based on existing data from the 2nd trimester human fetus, using our most constrained population size. Based on the liver expression of GSTP1 (expressed relative to house-keeping genes), a study of 60 fetuses would have 80% power to detect differences in gene expression of 700 units between fetuses from mothers who used drugs compared with those who do not, assuming a common standard deviation of 951 units, a type 1 error rate of 5% and equal numbers of fetuses in each group.

The statistical approach depends upon the specific sub-project, its study design, its hypotheses, questions and objectives, the numbers of suitable fetuses available and the specific methodologies to be used. Examples of statistical analytical methods (accepted as suitable by the MRC when funding our study) are as follows (but each sub-study will require its own tailored statistical analytical approach):

The RNA-seq data will be quality controlled using FastQC, quality filtering applied and the data aligned to the current reference human genome using BWA on the University of Aberdeen Maxwell computer cluster. Thereafter, HTSeq will be used in conjunction with current annotation to determine the number of reads aligning to each gene, feeding into the expression

analysis. Following quantification of reads aligning to each gene, genes with altered transcript numbers will be identified using an empirical Bayes moderated generalised linear modelling approach as implemented in the Bioconductor package edgeR. This approach permits the efficient estimation of gene-specific biological variation and will allow us to identify gene transcripts that are significantly differentially expressed according to fetal sex, level of maternal drug exposure and gestation period. Type I error rates (false discovery rates, FDR) will be addressed: (i) non-specific filtering of genes with low numbers of reads across all treatment groups will be performed prior to statistical modelling to reduce the number of transcripts tested and therefore reduce the potential FDR. (ii) P-values associated with the differential expression analysis of each transcript will be adjusted using the Benjamini & Hochberg algorithm to control the FDR at <0.05. Co-expression of these significant transcripts will be further investigated by clustering transcripts based on expression profiles across treatment groups using hierarchical clustering techniques.

8. Study Management

The CI will have the overall responsibility of the study, assisted by the Glasgow site specific PI. Any queries will be resolved by the CI or a delegated member of the study team. A study-specific Delegation Log will be prepared for each site, detailing the responsibilities of each member of staff working on the study.

9. Inspection of Records

The CI, PIs and all institutions involved in the study will permit study-related monitoring, audits, and REC review. The CI agrees to allow the Sponsor direct access to all study records and source documentation. Due to the nature of this study, the consent process and tissue storage will be monitored frequently by NHS monitors who work closely with Research Governance.

10. Good Clinical Practice

10.1 Ethical Conduct of the Study

The study will be conducted in accordance with the principles of good clinical practice (GCP). In addition to Sponsorship approval, a favourable ethical opinion will be obtained from the appropriate REC and appropriate NHS R&D approval will be obtained prior to commencement of the study.

10.2 Confidentiality

All laboratory specimens, evaluation forms, reports, and other records will be identified in a manner designed to maintain participant confidentiality. All records will be kept in a secure storage area with limited access to study staff only. Clinical information will not be released without the written permission of the participant, except as necessary for monitoring and auditing by the Sponsor or its designee. The CI and study staff involved with this study will not disclose or use for any purpose other than performance of the study, any data, record, or other unpublished, confidential information disclosed to those individuals for the purpose of the study. Prior written agreement from the Sponsor or its designee will be obtained for the disclosure of any said confidential information to other parties.

10.3 Data protection

The CI and study staff involved with this study will comply with the requirements of the Data Protection Act 1998 with regard to the collection, storage, processing and disclosure of personal information and will uphold the Act's core principles. The CI and study staff will also adhere, if appropriate, to the current version of the NHS Scotland Code of Practice on Protecting Patient Confidentiality. Access to collated participant data will be restricted to the CI and appropriate

study staff. Computers used to collate the data will have limited access measures via user names and passwords. Published results will not contain any personal data that could allow identification of individual participants.

10.4 Insurance and Indemnity

The University of Aberdeen and Grampian Health Board are Co-Sponsoring the study.
Insurance: The University of Aberdeen will obtain and hold a policy of Public Liability Insurance for legal liabilities arising from the study.
Indemnity: The Sponsor does not provide study participants with indemnity in relation to participation in the Study but has insurance for legal liability as described above.

11. Study Conduct Responsibilities

11.1 Protocol Amendments, Deviations and Breaches

The CI will seek approval for any amendments to the Protocol or other study documents from the Sponsor, REC and NHS R&D Office(s). Amendments to the protocol or other study docs will not be implemented without these approvals. In the event that a CI needs to deviate from the protocol, the nature of and reasons for the deviation will be recorded in the CRF, documented and submitted to the Sponsor. If this necessitates a subsequent protocol amendment, this will be submitted to the Sponsor for approval and then to the appropriate REC and lead NHS R&D Office for review and approval. In the event that a serious breach of GCP is suspected, this will be reported to the Sponsor immediately using the form "Breach Report Form".

11.2 End of Study

The end of study is defined as last termination date. The Sponsor, CI and/or the TSC have the right at any time to terminate the study for clinical or administrative reasons. The end of the study will be reported to the Sponsor and REC within 90 days, or 15 days if the study is terminated prematurely. The CI will ensure that any appropriate follow up is arranged for all participants. A summary report of the study will be provided to the Sponsor and REC within 1 year of the end of the study.

12. Reporting, publications and notifications of results

12.1 Authorship Policy

Ownership of the data arising from this study resides with the study team and their respective employers. On completion of the study, the study data will be analysed and tabulated, and a clinical study report will be prepared.

12.2 Publication

The clinical study report will be used for publication and presentation at scientific meetings. Investigators have the right to publish orally or in writing the results of the study. Summaries of results will also be made available to Investigators for dissemination within their scientific and clinical areas (where appropriate and according to their discretion).

13. Bibliography

Anblagan D, Jones NW, Costigan C, Parker AJ, Allcock K, Aleong R, Coyne LH, Deshpande R, Raine-Fenning N, Bugg G *et al*. Maternal smoking during pregnancy and fetal organ growth: a magnetic resonance imaging study. *PLoS One* 2013:**8**:e67223.

Anderson RA, McIlwain L, Coutts S, Kinnell HL, **Fowler** PA and Childs AJ. Activation of the aryl hydrocarbon receptor by a component of cigarette smoke reduces germ cell proliferation in the human fetal ovary. *Mol Hum Reprod* 2014:**20**:42-48.

Arrebola JP, Molina-Molina JM, Fernandez MF, Saenz JM, Amaya E, Indiveri P, Hill EM, Scholze M, Orton F, Kortenkamp A *et al*. A novel biomarker for anti-androgenic activity in placenta reveals risks of urogenital malformations. *Reproduction* 2015:**149**:605-613.

Brown ZA, Schalekamp-Timmermans S, Tiemeier HW, Hofman A, Jaddoe VW and Steegers EA. Fetal sex specific differences in human placentation: a prospective cohort study. *Placenta* 2014:**35**:359-364.

Choi GY, Tosh DN, Garg A, Mansano R, Ross MG and Desai M. Gender-specific programmed hepatic lipid dysregulation in intrauterine growth-restricted offspring. *Am J Obstet Gynecol* 2007:**196**:477.e1-477.e7.

Cupul-Uicab LA, Skjaerven R, Haug K, Melve KK, Engel SM and Longnecker MP. In Utero Exposure to Maternal Tobacco Smoke and Subsequent Obesity, Hypertension, and Gestational Diabetes Among Women in the MoBa Cohort. *Environ Health Perspect* 2012:**120**:355-360.

Drake AJ, O'Shaughnessy PJ, Bhattacharya S, Monteiro A, Kerrigan D, Goetz S, Raab A, Rhind SM, Sinclair KD, Meharg AA, **Fowler PA**. In utero exposure to cigarette chemicals induces sex-specific disruption of one-carbon metabolism and DNA methylation in the human fetal liver. *BMC Med* 2015:**13**:18-014-0251-x.

Filis P, Nagrath N, Fraser M, Hay DC, Iredale JP, O'Shaughnessy P and **Fowler** PA. Maternal smoking dysregulates protein expression in second trimester human fetal livers in a sex-specific manner. *J Clin Endocrinol Metab* 2015:jc20143941.

Fowler PA, Anderson RA, Saunders PT, Kinnell H, Mason JI, Evans DB, Bhattacharya S, Flannigan S, Franks S, Monteiro A *et al*. Development of Steroid Signaling Pathways during Primordial Follicle Formation in the Human Fetal Ovary. *J Clin Endocrinol Metab* 2011a:**96**:1754-1762.

Fowler PA, Bhattacharya S, Flannigan S, Drake AJ and O'Shaughnessy PJ. Maternal Cigarette Smoking and Effects on Androgen Action in Male Offspring: Unexpected Effects on Second-Trimester Anogenital Distance. *J Clin Endocrinol Metab* 2011b:**96**:E1502-1506.

Fowler PA, Bhattacharya S, Gromoll J, Monteiro A and O'Shaughnessy PJ. Maternal Smoking and Developmental Changes in Luteinizing Hormone (LH) and the LH Receptor in the Fetal Testis. *J Clin Endocrinol Metab* 2009:**94**:4688-4695.

Fowler PA, Cassie S, Rhind SM, Brewer MJ, Collinson JM, Lea RG, Baker PJ, Bhattacharya S and O'Shaughnessy PJ. Maternal Smoking during Pregnancy Specifically Reduces Human Fetal Desert Hedgehog Gene Expression during Testis Development. *J Clin Endocrinol Metab* 2008:**93**:619-26.

Fowler PA, Childs AJ, Courant F, MacKenzie A, Rhind SM, Antignac JP, Le Bizec B, Filis P, Evans F, Flannigan S *et al*. In utero exposure to cigarette smoke dysregulates human fetal ovarian developmental signalling. *Hum Reprod* 2014:**29**:1471-1489.

Fowler PA, Flannigan S, Mathers A, Gillanders K, Lea RG, Wood MJ, Maheshwari A, Bhattacharya S, Collie-Duguid ES, Baker PJ *et al*. Gene expression analysis of human fetal ovarian primordial follicle formation. *J Clin Endocrinol Metab* 2009:**94**:1427-1435.

Giesbrecht GF, Campbell T, Letourneau N and APrON Study Team. Sexually dimorphic adaptations in basal maternal stress physiology during pregnancy and implications for fetal development. *Psychoneuroendocrinology* 2015:**56**:168-178.

Hackshaw A, Rodeck C and Boniface S. Maternal smoking in pregnancy and birth defects: a systematic review based on 173 687 malformed cases and 11.7 million controls. *Hum Reprod Update* 2011:**17**:589-604.

Joya X, Friguls B, Ortigosa S, Papaseit E, Martinez SE, Manich A, Garcia-Algar O, Pacifici R, Vall O and Pichini S. Determination of maternal-fetal biomarkers of prenatal exposure to ethanol: A review. *J Pharm Biomed Anal* 2012:**69**:209-222.

Kawashima A, Koide K, Ventura W, Hori K, Takenaka S, Maruyama D, Matsuoka R, Ichizuka K and Sekizawa A. Effects of maternal smoking on the placental expression of genes related to angiogenesis and apoptosis during the first trimester. *PLoS One* 2014:**9**:e106140.

Kneeman JM, Misdraji J and Corey KE. Secondary causes of nonalcoholic fatty liver disease. *Therap Adv Gastroenterol* 2012:**5**:199-207.

Latini G, De Mitri B, Del Vecchio A, Chitano G, De Felice C and Zetterstrom R. Foetal growth of kidneys, liver and spleen in intrauterine growth restriction: "programming" causing "metabolic syndrome" in adult age. *Acta Paediatr* 2004:**93**:1635-1639.

Lui S, Jones RL, Robinson NJ, Greenwood SL, Aplin JD and Tower CL. Detrimental effects of ethanol and its metabolite acetaldehyde, on first trimester human placental cell turnover and function. *PLoS One* 2014:**9**:e87328.

Meeker JD and Benedict MD. Infertility, Pregnancy Loss and Adverse Birth Outcomes in Relation to Maternal Secondhand Tobacco Smoke Exposure. *Curr Womens Health Rev* 2013:**9**:41-49.

Mehta C, Masson G, Iqbal Z, O'Mahony F and Khalid R. Prevalence of excessive alcohol consumption in pregnancy. *Public Health* 2009:**123**:630-631.

Moore DG, Turner JD, Parrott AC, Goodwin JE, Fulton SE, Min MO, Fox HC, Braddick FM, Axelsson EL, Lynch S *et al*. During pregnancy, recreational drug-using women stop taking ecstasy (3,4-methylenedioxy-N-methylamphetamine) and reduce alcohol consumption, but continue to smoke tobacco and cannabis: initial findings from the Development and Infancy Study. *J Psychopharmacol* 2010:**24**:1403-1410.

Morrison JL, Duffield JA, Muhlhausler BS, Gentili S and McMillen IC. Fetal growth restriction, catch-up growth and the early origins of insulin resistance and visceral obesity. *Pediatr Nephrol* 2010:**25**:669-677.

Muralimanoharan S, Guo C, Myatt L and Maloyan A. Sexual dimorphism in miR-210 expression and mitochondrial dysfunction in the placenta with maternal obesity. *Int J Obes (Lond)* 2015:**39**:1274-1281.

Nyirenda MJ, Carter R, Tang JI, de Vries A, Schlumbohm C, Hillier SG, Streit F, Oellerich M, Armstrong VW, Fuchs E *et al*. Prenatal programming of metabolic syndrome in the common marmoset is associated with increased expression of 11beta-hydroxysteroid dehydrogenase type 1. *Diabetes* 2009:**58**:2873-2879.

O'Shaughnessy PJ, Baker PJ, Monteiro A, Cassie S, Bhattacharya S and **Fowler** PA. Developmental Changes in Human Fetal Testicular Cell Numbers and Messenger Ribonucleic Acid Levels during the Second Trimester. *J Clin Endocrinol Metab* 2007:**92**:4792-801.

O'Shaughnessy PJ, Monteiro A, Bhattacharya S and **Fowler** PA. Maternal Smoking and Fetal Sex Significantly Affect Metabolic Enzyme Expression in the Human Fetal Liver. *J Clin Endocrinol Metab* 2011:96:2851-2860.

O'Shaughnessy PJ, Monteiro A, Bhattacharya S, Fraser MJ and **Fowler** PA. Steroidogenic enzyme expression in the human fetal liver and potential role in the endocrinology of pregnancy. *Mol Hum Reprod* 2013:**19**:177-187.

O'Shaughnessy PJ, Monteiro A and **Fowler** PA. Identification of stable endogenous reference genes for real-time PCR in the human fetal gonad using an external standard technique. *Mol Hum Reprod* 2011:**17**:620-625.

Raisanen S, Kramer MR, Gissler M, Saari J, Hakulinen-Viitanen T and Heinonen S. Smoking during pregnancy was up to 70% more common in the most deprived municipalities - a multilevel analysis of all singleton births during 2005-2010 in Finland. *Prev Med* 2014:**67**:6-11.

Reynolds JN, Weinberg J, Clarren S, Beaulieu C, Rasmussen C, Kobor M, Dube MP and Goldowitz D. Fetal alcohol spectrum disorders: gene-environment interactions, predictive biomarkers, and the relationship between structural alterations in the brain and functional outcomes. *Semin Pediatr Neurol* 2011:**18**:49-55.

Reynolds RM, Labad J, Buss C, Ghaemmaghami P and Raikkonen K. Transmitting biological effects of stress in utero: implications for mother and offspring. *Psychoneuroendocrinology* 2013:**38**:1843-1849.

Shinjo A, Ventura W, Koide K, Hori K, Yotsumoto J, Matsuoka R, Ichizuka K and Sekizawa A. Maternal smoking and placental expression of a panel of genes related to angiogenesis and oxidative stress in early pregnancy. *Fetal Diagn Ther* 2014:**35**:289-295.

Snijder CA, Brouwers MM, Jaddoe VW, Hofman A, Roeleveld N and Burdorf A. Occupational exposure to endocrine disruptors and time to pregnancy among couples in a large birth cohort study: the Generation R Study. *Fertil Steril* 2011:**95**:2067-2072.

Snijder CA, Kortenkamp A, Steegers EA, Jaddoe VW, Hofman A, Hass U and Burdorf A. Intrauterine exposure to mild analgesics during pregnancy and the occurrence of cryptorchidism and hypospadia in the offspring: the Generation R Study. *Hum Reprod* 2012:**27**:1191-1201.

Tappin DM, MacAskill S, Bauld L, Eadie D, Shipton D and Galbraith L. Smoking prevalence and smoking cessation services for pregnant women in Scotland. *Subst Abuse Treat Prev Policy* 2010:**5**:1.

van den Driesche S, Macdonald J, Anderson RA, Johnston ZC, Chetty T, Smith LB, McKinnell C, Dean A, Homer NZ, Jorgensen A *et al*. Prolonged exposure to acetaminophen reduces testosterone production by the human fetal testis in a xenograft model. *Sci Transl Med* 2015:**7**:288ra80.

Wang N, Tikellis G, Sun C, Pezic A, Wang L, Wells JC, Cochrane J, Ponsonby AL and Dwyer T. The effect of maternal prenatal smoking and alcohol consumption on the placenta-to-birth weight ratio. *Placenta* 2014:**35**:437-441.

Weightman AL, Morgan HE, Shepherd MA, Kitcher H, Roberts C and Dunstan FD. Social inequality and infant health in the UK: systematic review and meta-analyses. *BMJ Open* 2012:**2**:10.1136/bmjopen-2012-000964. Print 2012.