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Complete List of Authors:	Rostom, Hussam; University of Oxford Medical Sciences Division, Nuffield Department of Women's & Reproductive Health Meng, Xin; University of Oxford Medical Sciences Division, Nuffield Department of Women's & Reproductive Health Price, Helen; University of Oxford Medical Sciences Division, Nuffield Department of Women's & Reproductive Health Fry, Alexandria; University of Oxford Medical Sciences Division, Nuffield Department of Women's & Reproductive Health Elajnaf, Taha; University of Oxford Medical Sciences Division, Nuffield Department of Women's & Reproductive Health Humphrey, Robert; Oxford University Hospitals NHS Foundation Trust, Clinical Biochemistry Guha, Nishan; Oxford University Hospitals NHS Foundation Trust, Clinical Biochemistry James, Tim; Oxford University Hospitals NHS Foundation Trust, Clinical Biochemistry Kennedy, Stephen; University of Oxford Medical Sciences Division, Nuffield Department of Women's & Reproductive Health Hannan, Fadil; University of Oxford Medical Sciences Division, Nuffield Department of Women's & Reproductive Health
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Title: Protocol for an observational study investigating hormones triggering the onset of sustained lactation: the INSIGHT Study

Hussam Rostom[1], Xin Meng[1], Helen Price[1], Alexandria Fry[1], Taha Elajnaf[1], Robert Humphrey[2], Nishan Guha[2], Tim James[2], Stephen H. Kennedy[1], Fadil M. Hannan[1*]

[1]Larsson-Rosenquist Foundation Oxford Centre for the Endocrinology of Human Lactation, Nuffield Department of Women's & Reproductive Health, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

[2]Department of Clinical Biochemistry Level 4, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

*Corresponding author:

Name: Prof Fadil M. Hannan

Affiliation: Larsson-Rosenquist Foundation Oxford Centre for the Endocrinology of Human Lactation, Nuffield Department of Women's & Reproductive Health, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom.

E-mail address: fadil.hannan@wrh.ox.ac.uk.

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ABSTRACT

Introduction: Lactation is a hormonally controlled process that promotes infant growth and neurodevelopment and reduces the long-term maternal risk of diabetes, cardiovascular disease and breast cancer. Hormones, such as prolactin and progesterone, mediate mammary development during pregnancy and are critical for initiating copious milk secretion with 24-72 hours postpartum. However, the hormone concentrations mediating lactation onset are ill-defined.

Methods and analysis: The primary objective of the Investigating hormones triggering the onset of sustained lactation (INSIGHT) study is to establish reference intervals for the circulating hormone concentrations initiating postpartum milk secretion. The study will also assess how maternal factors such as parity, pregnancy co-morbidities, and complications during labour and delivery, which are known to delay lactation, may affect hormone concentrations. This single centre observational study will recruit up to 1068 pregnant women over a 3-year period. A baseline blood sample will be obtained at 36 weeks' gestation. Participants will be monitored during postpartum days 1-4. Lactation onset will be reported using a validated breast fullness scale. Blood samples will be collected before and after a breastfeed on up to two occasions per day during postpartum days 1-4. Colostrum, milk and spot urine samples will be obtained on a single occasion. Serum hormone reference intervals will be calculated as mean ± 1.96SD, with 90% confidence intervals determined for the upper and lower reference limits. Differences in hormone values between healthy breastfeeding women and those at risk of delayed onset of lactation will be assessed by repeated measures two-way ANOVA or a mixed linear model. Correlations between serum hormone concentrations and milk composition and volume will provide insights into the endocrine regulation of milk synthesis.

Ethics and dissemination: The findings will be published in high-ranking journals and presented at national and international conferences.

Trial registration number: ISRCTN12667795

Strengths and limitations of this study

- The INSIGHT Study comprises biological and comprehensive phenotype data.
- Metabolites associated with the onset of lactation will be evaluated in serum, urine and milk samples.
- Participants will benefit from breastfeeding support provided by the study investigators.
- A pilot study is being undertaken to evaluate study feasibility as it may be difficult to collect multiple samples during the early postpartum period.

Key words (5-8): Secretory activation, hormones, lactogenesis, delayed onset of lactation, breastfeeding.

INTRODUCTION

Lactation is tightly regulated by metabolic and reproductive hormones.[1] It plays a key role in infant health and neurodevelopment, and decreases infant mortality and morbidity from infections during the first year of life.[2 3] Longer-term benefits of breastfeeding for the offspring include a reduced risk of obesity and type 2 diabetes, and higher performance in intelligence tests.[3-5] Lactation also confers benefits on breastfeeding women, and is associated with decreases in the risk of maternal diseases such as type 2 diabetes, cardiovascular diseases, and breast and ovarian cancer.[6-9]

Hormones influence the mammary gland from the onset of puberty until the end of pregnancy to induce formation of a branched epithelial milk-secreting organ. From puberty-onwards, oestrogen and the growth hormone/insulin-like growth factor (IGF-1) axis promote elongation and branching of the mammary ductal structure.[10 11] During pregnancy, prolactin, progesterone, oestradiol, insulin, and glucocorticoid hormones such as cortisol stimulate the formation of milk-producing lobuloalveolar structures at the end of mammary ducts (Table 1).[12-15]

Table 1. Major reproductive and metabolic hormones influencing mammary gland function in pregnancy and at the onset of lactation

Hormone	Role in pregnancy	Role at the onset of lactation	References
Reproductive			
Prolactin	Lobuloalveolar formation Milk synthesis	Tight junction formation Milk synthesis	14 17 18 24 25
Progesterone	Mammary ductal branching Lobuloalveolar formation	Withdrawal of progesterone initiates tight junction formation and increases milk synthesis	14 26 27
Oestradiol	Mammary ductal branching Lobuloalveolar formation	Not known	12
Metabolic			
Cortisol	Lobuloalveolar formation Milk synthesis	Tight junction formation	15 24
Insulin	Lobuloalveolar formation Milk synthesis	Milk synthesis	13 20 28

Prolactin is the principal hormone promoting milk synthesis, and circulating concentrations progressively increase throughout pregnancy and are correlated with the rate of lactogenesis, as assessed by urinary lactose excretion.[16] Prolactin signals via the STAT5 transcription factor to increase expression of mammary-specific genes mediating the production of lactose, milk proteins and lipids.[17] [18] These lactogenic effects of prolactin are augmented by thyroid hormones, insulin and glucocorticoids, which enhance STAT5 activation and/or the transcriptional activation of milk synthesis genes in mammary cells.[19-21]

Hormones also trigger the postpartum onset of copious milk secretion, known as secretory activation, which typically occurs between 24-72 hours following child-birth (Table 1).[1] Secretory activation is associated with an increase in milk volume, which leads to the maternal perception of milk 'coming in', characterised by breast fullness and swelling.[22] [23] Secretory activation is mediated by a marked decrease in the circulating concentration of progesterone following delivery of the placenta, and requires a high circulating prolactin concentration in the presence of insulin and cortisol.[1] At the onset of lactation these hormonal changes increase milk synthesis and induce the formation of tight junction complexes between mammary cells, which establishes an osmotic gradient that stimulates water movement into the alveolar lumen (Table 1).[24-28]

Secretory activation is critical for the establishment of lactation, and an absence or delay in onset of lactation is a major cause of inadequate and/or early cessation of breastfeeding.[29] [30] Although the key hormones involved in secretory activation have been described, their circulating concentrations required for initiating lactation in the early postpartum period are not well defined.[31] Furthermore, alterations in circulating metabolites associated with secretory activation remain to be fully characterised.

METHODS AND ANALYSIS

Objectives

Investigating hormones triggering the onset of sustained lactation (INSIGHT) is a single-centre observational study aiming to: 1) characterise the circulating hormone concentrations required for initiating postpartum milk secretion, and 2) assess how maternal factors such as parity, pregnancy co-

morbidities, and complications during labour and delivery, which are known to delay lactation (Table 2), may affect hormone concentrations.

Table 2. Examples of causes of delayed onset of lactation and association with endocrine disturbances

Cause	Effect on lactation	Associated endocrine abnormalities	References
Maternal factors/co-morbidities			
Pituitary insufficiency e.g. Sheehan's syndrome	Absent or delayed onset	↓ multiple pituitary hormones	42
Polycystic ovary syndrome	Delayed onset and/or lactation insufficiency due to lack of prenatal breast enlargement	↑androgens, obesity-related endocrine abnormalities (see-below)	43 44
Obesity	Delayed onset	↓ mammary responsiveness to prolactin	47 48
Primiparity	Delayed onset	Possible ↓ mammary responsiveness to pregnancy-related hormones	45 46
Labour and delivery factors			
Medications e.g. epidural analgesia, i.v. oxytocin	Delayed onset	↓oxytocin	49 50
Caesarean section	Delayed onset	↓oxytocin, ↓prolactin	30 51

Primary objective

Establishment of reference intervals for hormones initiating postpartum onset of lactation.

Secondary objectives

- Measure longitudinal changes in circulating lactation hormones and metabolites during the first 4 days postpartum.
- Evaluate effects of maternal co-morbidities, medications, mode of delivery and pregnancy complications on lactation hormone concentrations.
- Assess whether lactation hormone concentrations correlate with the timing of the onset of milk secretion.
- Evaluate the relationship between lactation hormones and breast milk volume.
- Characterise breast milk hormone composition.

Outcomes

Primary outcome

Reference intervals will be established for lactation hormones during the early postpartum period before and after a breastfeed during the morning and afternoon, which will account for the effect of diurnal variation and infant suckling on hormone concentrations.

Secondary outcomes

Serial blood sampling during the early postpartum period will allow assessment of longitudinal changes in the concentrations of hormones during secretory activation. Correlation of hormone concentrations with maternal co-morbidities, and complications during pregnancy and delivery, will help elucidate how these factors cause delayed onset of lactation. Furthermore, correlation of hormone concentrations with milk composition and volume will provide insights into the endocrine regulation of milk synthesis.

Study design

Pilot study phase

To assess whether sample collection will be feasible during the early postpartum period, an initial pilot study involving up to 100 women will be performed. Postpartum samples will be collected during a single study visit on postpartum day 4, either at the participant's home or in hospital. These samples will be used for an initial analysis of lactation hormone concentrations, and the data included in the final analysis.

Main study phase

To assess changes in lactation hormones during the early postpartum period, blood sampling will be performed at around 36 weeks' gestation, as a baseline measure, and also before and after a breastfeed during the first 4 days after birth. Participants can provide blood samples on a single postpartum day, or opt for serial sample collection during postpartum days 1-4. Participants will record their breast fullness to determine the timing of lactation onset, as previously described [30]. Demographic and clinical characteristics such as participant age, co-morbidities, medications and complications occurring during pregnancy and delivery, will also be recorded. Infant birth weight will be recorded, and the infant weighed before and after a breastfeed to estimate milk volume. Up to 10 mL of milk will be obtained

for biochemical analysis and a spot urine sample will be collected to assess lactation hormone metabolites. The study protocol is shown in figure 1.

Participants

Over a 3-year period, the study will recruit up to 1000 pregnant women attending maternity units within Oxfordshire, UK, who either have no risk factors for delayed onset of lactation (n~600), or one or more risk factors such as primiparity or obesity (n~400) (Table 2).

Inclusion criteria

- Pregnant women, aged ≥18 years.
- Intention to fully or partially breastfeed.
- Willing and able to give informed consent for participation in the study.

Exclusion criteria

Participants may not enter or continue in the study if ANY of the following apply:

- Preterm birth (<37 weeks' gestation).
- Severe maternal illness, including postpartum depression or psychosis.
- Severe neonatal illness including major congenital abnormalities and infants who are only expected to live for a short period of time.
- Prolonged separation of infant from mother, e.g. due to admission to the neonatal unit.
- Maternal or neonatal COVID-19 test positivity
- Mother or infant infected with blood borne viruses such as HIV.
- Resides outside of Oxfordshire.
- Safeguarding issues that may impede the safety of research staff carrying out home visits.
- Current participation in another research study involving investigational medicinal products.

Participant enrolment

Screening and recruitment

Potential participants will be screened by assessing their eligibility and intention to breastfeed. Introductory study information will be provided during antenatal clinic or ultrasound scan visits. Women can also identify themselves as eligible from advertising materials such as posters, social media advertising, and the advertisement on the Larsson-Rosenquist Foundation Oxford Centre for the Endocrinology of Human Lactation (https://www.ocehl.ox.ac.uk/insight) website.

Informed consent

A trained member of the research team will obtain written informed consent. Written and verbal information will be provided to the participant detailing the exact nature of the study and any risks involved in taking part. Potential participants will be given the opportunity to discuss the study with the investigator, their general practitioner (GP) or other independent parties to decide whether to participate. It will be clearly stated that the participant is free to withdraw from the study at any time and for any reason without prejudice to her or her infant's future care, without affecting her rights, and with no obligation to give the reason for withdrawal.

Informed consent will be obtained electronically (e-consent) on a tablet device. If this is unavailable, then written informed consent will be obtained. The participant will be provided with a copy of the completed consent form and participant information sheet. The e-consent form will be stored on the trial specific database held on the University of Oxford High Compliance Server, and a copy kept in the medical records. The participant must complete the e-consent form or hardcopy version of the Informed Consent form before any study procedures are performed.

Study schedule

Baseline assessment

The participant's co-morbid conditions, medications and pregnancy complications will be recorded, and a blood sample collected.

Subsequent visits

Admission in labour or for Caesarean section: The research team will check that participants are ≥37 weeks' gestation, as defined by the first trimester dating ultrasound scan.

Day 0-1 postpartum: Continued consent will be confirmed with the participant. Delivery or Caesarean section complications, and use of medications (e.g. syntocinon, analgesia), will be recorded. Blood sampling will commence within 8 hours of birth and samples obtained pre- and post-infant feeding. Self-reporting of breast fullness will also commence.

Day 1-4 postpartum: The research midwife or research assistant will visit the participant in hospital, or at home. Blood samples will be taken pre- and post-breastfeed. Breastfeeding support will be provided, if requested by the participant. A colostrum sample of ~1ml and urine sample of ~20ml will be obtained on a single occasion.

Day 4 postpartum: A milk sample (~10 ml) will be obtained. Milk volume will be recorded by weighing the infant pre- and post-feed. Daily recordings of breast fullness will be collected on the final study visit.

Study procedures

Sample collection and handling

Around 10 mL of blood will be collected per sampling time-point, and a maximum of four blood samples (two from a morning and two from an afternoon breastfeeding session) will be obtained per participant over a 24-hour period. Thus, a maximum of 16 blood samples will be obtained from each participant over a 4-day period. Colostrum and milk will be collected using an electric or manual breast pump, or by hand expression into a sterile container. On the day of collection, blood and milk samples will be centrifuged and aliquoted centrally. Samples will either be analysed freshly or frozen at -80°C for later analysis.

Biochemical measurements

Lactation hormones, such as prolactin, progesterone, oestradiol, cortisol and insulin (Table 1), will be measured with validated immunoassay methods. Metabolites will be assayed in blood, milk and urine

samples using techniques including mass spectrometry. The biochemical and cellular composition of colostrum and breast milk will be assessed by flow cytometry, immunoassay and mass spectrometry.

Any residual sample will be stored long-term for use in future ethically approved studies in accordance with University of Oxford guidelines and the Human Tissue Authority's Code of Practice. At the end of the study, with consent, samples may also be transferred to an ethically approved Research Tissue Bank for long-term storage. However, if consent has not been received then the samples will be destroyed according to local protocol.

Recording breast fullness and factors influencing the onset of lactation

Participants will record their perception of lactation onset on a daily basis from birth until postpartum day 4 using a validated breast fullness numerical scale of 1-5, anchored with 1=no change, 3=noticeably fuller, and 5=uncomfortably fuller, as described.[30] The onset of lactation will be indicated by the time when breast fullness first reached scale point 3.[30] The following factors will also be recorded at each study visit: frequency and duration of mother-infant skin-to-skin contact; frequency and duration of breastfeeds, and use of formula milk or other feeds.

Infant weighing

The volume of the participant's breast milk expressed during a feed will be estimated by weighing her infant immediately before and after feeding using a calibrated portable electronic scale, as described.[32]

Withdrawal or discontinuation of participants

A participant may choose to withdraw from the study at any time, or choose to stop blood sampling at any time and remain on study follow-up. Participants who withdraw from the study can permit data and samples obtained up until the point of withdrawal to be retained for analysis. In addition, the Chief Investigator may discontinue a participant from the study at any time. Reasons include ineligibility (either during the study or retrospectively if overlooked during screening and the study), significant protocol deviation, significant non-compliance with study requirements, or as a clinical decision.

If the participant withdraws from the study before donating any samples then no data will be used. Withdrawn participants will be replaced. If the participant is withdrawn due to an adverse event, the Chief Investigator will arrange for referral to her GP or an appropriate health care professional involved in her care. In the event of a serious adverse event, the participant will be withdrawn from the study. The reason for withdrawal will be recorded in the case report form.

Statistics and analysis

Primary outcome analysis

The reference interval (RI), which represents the middle 95% of measurements for a lactation hormone in a population of breastfeeding women with no risk factors for delayed onset of lactation (Table 2), will be derived according to the Clinical and Laboratory Standards (CLSI) and International Federation of Clinical Chemistry (IFCC) recommendations by two methods.[33 34] Data will be log-transformed to achieve a parametric distribution. The mean and standard deviation (SD) will be derived, and the RI calculated as mean ± 1.96SD, with 90% confidence intervals (CIs) determined for the upper and lower reference limits. If hormone values cannot be normalised by logarithmic transformation, then a non-parametric percentile method will be used. This involves ordering measurements from smallest to largest, with the RI determined as being between the 2.5th and 97.5th centile of the ordered observations. For a sample size of 120, the 2.5th and 97.5th centiles represent the 3rd and 118th observations, respectively.

As samples will be taken pre- and post-feed, and at different times during the day, these RIs will consider the effect of diurnal variation and infant suckling on hormone concentrations.

Secondary outcome analysis

Differences in hormone values between participants with no risk factors for delayed onset of lactation and those with ≥1 risk factor will be assessed by repeated measures two-way ANOVA or a mixed linear model. The log-transformed hormone value will be used as the outcome variable, and the two nominal variables will be 1) absence or presence of risk factors for delayed lactation, and 2) the timing of the sample (pre- or post-breastfeeding).

Hormone values from a subset of participants willing to provide serial blood samples over a 4 day period will be used to assess longitudinal hormone changes. A mixed linear model, which incorporates factors such as breastfeeding status and sampling pre- and post-feeds, will be used to analyse the change in lactation hormone values over the study period whilst accounting for any missing data.

To determine whether lactation hormone concentrations are associated with breast milk volume or the timing of onset of lactation, bivariate correlations will be assessed using the Pearson or Spearman Rank correlation tests. Adjustment for potential confounding variables will be undertaken using a multivariate linear regression model.

Sample size determination for primary outcome

Around 120 participants with no risk factors for delayed onset of lactation are required to establish an RI with acceptable 90% confidence intervals for the upper and lower reference limits.[34] This study involves establishing RIs at a single time-point in the third trimester and for up to 16 different time-points over a 4-day period. Each participant is expected to provide blood samples for an average of four time-points, and therefore 480 participants will likely be required to provide samples for all 16 time-points. A total of N=1920 postpartum blood samples (120 blood samples at each of 16 time-points) are required for the primary outcome. The pilot study is also expected to contribute around 100 samples from 50 participants (two samples per participant) to the primary outcome. Therefore, 455 participants providing an average of 4 samples each (total of 1820 postpartum samples) will be recruited to the main study. Thus, N=505 participants (50 from pilot study and 455 from main study) will be recruited in total for the primary outcome analysis.

Sample size determination for secondary outcomes

A major secondary outcome is alteration in lactation hormones for participants at risk of delayed onset of lactation compared to participants with no risk factors. Prolactin is critical for secretory activation, and sample size has been determined using published prolactin values for breastfeeding women.[35] The effect size (d) for a 10% change in serum prolactin is 0.57, given that a 10% change is 18.3 µg/L and

pooled SD is 32 µg/L.[35] G*Power statistical software was used to calculate sample size with this effect size and the following parameters: a two tailed t test for difference between two independent means, alpha error probability of 0.05, power of 0.95 and a 1:1 allocation ratio. This calculation indicates that a sample size of 81 participants with ≥1 risk factor for delayed onset of lactation is required at each time-point for secondary outcome analysis. Assuming each participant provides samples for an average of four time-points, then 324 participants are required to provide samples for all 16 time points. A total of N=1296 postpartum blood samples (81 blood samples at each of 16 time-points) are required for the secondary outcomes. The pilot study is also expected to contribute ~100 samples from 50 participants (two samples per participant) to the secondary outcome. Therefore, 299 participants providing an average of 4 samples each (total of 1196 postpartum samples) will be recruited to the main study. Thus, N=349 participants (50 from pilot study and 299 from main study) will be recruited in total for the secondary outcome analysis.

Accounting for participant withdrawal

In total, 505 participants with no risk factors for delayed onset of lactation, and 349 participants with ≥1 risk factor are required for the primary and secondary outcome analyses. Assuming a 20% drop out rate, then 632 participants with no risk factors and 436 participants with ≥1 risk factor for delayed onset of lactation (1068 participants in total) will need to be recruited.

Decision points and end of study

The protocol will be evaluated in a pilot study involving ~100 participants. Depending on the results, a decision will be made whether to limit recruitment to certain participant groups, such as multiparous women with no breastfeeding issues who are more willing to participate in the study and provide multiple blood samples. In addition, if sample collection from participants at home proves difficult, then the protocol will be amended to collect blood samples whilst participants are in hospital following child birth, and only collect limited samples during home study visits.

The end of the study is the point at which all data have been collected and analysed, and the database is locked. If it is not possible to collect sufficient blood samples to establish reference intervals at any of the time-points, then the study will be stopped.

Procedure for accounting for missing, unused and spurious data

If there are insufficient (i.e. N<120) samples to calculate the RIs at each time-point using log-normal distribution or the centile method, then a bootstrap calculation method will be used to derive RIs. Bootstrapping involves random re-sampling of the original data observations to generate replacement values and establishment of a 'pseudosample' from which RIs can be derived.[36] This method requires normally distributed data obtained from a minimum of 40 samples.[37] The derivation of hormone RIs can also be affected by values lying above or below the assay detection limit. For very high values, sample dilution will be undertaken prior to biochemical analysis.

Data management

Data will be recorded using an electronic case report form (eCRF), and then transferred to the study specific database. This holds the unique participant study numbers, sample numbers, screening log, e-consent forms, as well as clinical and laboratory data. Any hard copies of the CRFs and consent forms will be kept in a locked filing cabinet in the key-coded locked office of the Lead Research Midwife. The consent form will be retained to meet the UK Human Tissue Authority traceability requirements, and also as the basis for retention of details and approach for any future ethically approved studies.

Quality assurance procedures

The study may be monitored or audited in accordance with the current approved protocol, Good Clinical Practice, relevant regulations and standard operating procedures.

Patient and public involvement

Healthcare professionals such as community midwifes reviewed the study protocol and participant-facing information. In addition, breastfeeding women on the hospital postnatal ward, and in the community, reviewed the study advertising and participant-facing information, and provided feedback on the appeal and comprehensibility of this information.

ETHICS AND DISSEMINATION

Study conduct

The study will be conducted in accordance with the principles of the Declaration of Helsinki and with Good Clinical Practice. The Chief Investigator will submit an annual progress report to the Research Ethics Committee, Health Research Authority (HRA) host organisation, Sponsor and funder. An end-of-study notification and final report will be submitted to the same parties. The findings will be published in high-ranking journals in the field and presented at national and international conferences.

Participant confidentiality

The study will comply with the General Data Protection Regulation (GDPR) and Data Protection Act 2018, which require data to be de-identified as soon as it is practical to do so. Participants will be identified only by a participant study number on any electronic database.

Other ethical considerations

Incidental findings are not expected from the sample analysis. Hormone values are likely to vary substantially within a healthy mother during the early postpartum period, and such alterations will not require referral for clinical investigations or management.

DISCUSSION

This protocol has been established to investigate hormone concentrations triggering secretory activation, and ascertain whether alterations in lactation hormones are associated with a delay in the onset of copious milk secretion. Delayed onset of lactation, defined as occurring >72 hours postpartum, affects between 17-40% of breastfeeding women, and is reported to cause excessive neonatal weight loss during the first postpartum week, and lead to increased use of formula supplementation and decreased duration of breastfeeding.[29 30 38-41] A range of maternal factors, which potentially contribute to endocrine dysregulation, may lead to delayed onset of lactation (Table 2). For example, endocrine disorders due to pituitary or ovarian abnormalities have been reported to delay lactation onset or cause lactation failure (Table 2).[42-44] Furthermore, parity influences the timing of secretory activation, with primiparous mothers experiencing a significantly increased duration of time from parturition until onset of lactation compared to multiparous mothers.[45] Such effects may have an endocrine basis, as rodent studies have shown an effect of parity on mammary hormone responsiveness. Thus, mammary glands from parous mice exhibit a more rapid expansion of ductal structures and earlier onset of milk protein synthesis when exposed to pregnancy-associated hormones, compared to the mammary glands of hormone-treated nulliparous mice.[46] In addition, maternal obesity, occurring either at the pre-pregnant stage or at the time of delivery, is reported to delay onset of lactation,[47] and a mouse study has revealed that obesity-induced lactation insufficiency is associated with mammary gland resistance to prolactin.[48] Medications administered during labour may also influence lactation hormones and cause delayed onset of lactation.[49 50] The administration of infused oxytocin with epidural analgesia to women in spontaneous labour is reported to decrease postpartum plasma concentrations of oxytocin, which is required for stimulating milk let-down during a breastfeed.[50] Moreover, Caesarean section is an independent risk factor for delayed onset of lactation. Delivery by emergency Caesarean section decreases the pulsatility of oxytocin secretion and impairs prolactin secretion during a breastfeed in the early postpartum period.[30 51]

The characterisation of hormone and metabolite concentrations at the physiological onset of lactation, as well as in women at risk of delayed onset of lactation, will improve understanding of the

factors involved in the initiation of successful lactation. The study also has the potential to facilitate development of hormonal biomarkers that can predict those likely to suffer from low-volume or delayed breast milk production, and help identify individuals who may benefit from galactagogue therapy to stimulate lactation. In addition, the study will provide biological insights into how lactation hormones influence the volume and composition of breast milk.

FOOTNOTES

Contributors

HR, XM, HP, TE, SHK and FMH wrote the protocol. HP and AF are involved with consenting participants and collecting samples. HR, XM, RH, TE, NG and TJ are involved in sample processing and analysis. SHK and FMH conceived the study.

Funding

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Competing interests

None declared

Patient consent for publication

Not required

Access to data

Study investigators have full access to data

Authorship eligibility criteria

Individuals making substantive contribution to the design, conduct, interpretation, and reporting of this study will be granted authorship on the final study report.

Ethics approval

Approval for this study had been granted by the East of England - Cambridgeshire and Hertfordshire Research Ethics Committee (REC No. 20/EE/0172), by the Health Research Authority (HRA), and by the Oxford University Hospitals NHS Foundation Trust. It was originally approved in October 2020, with amendments approved in May 2021

Study sponsor

University of Oxford Research Goverance, Ethics & Assurance Team,

Boundary Brook House, Churchill Drive, Headington, Oxford, OX3 7GB

License statement

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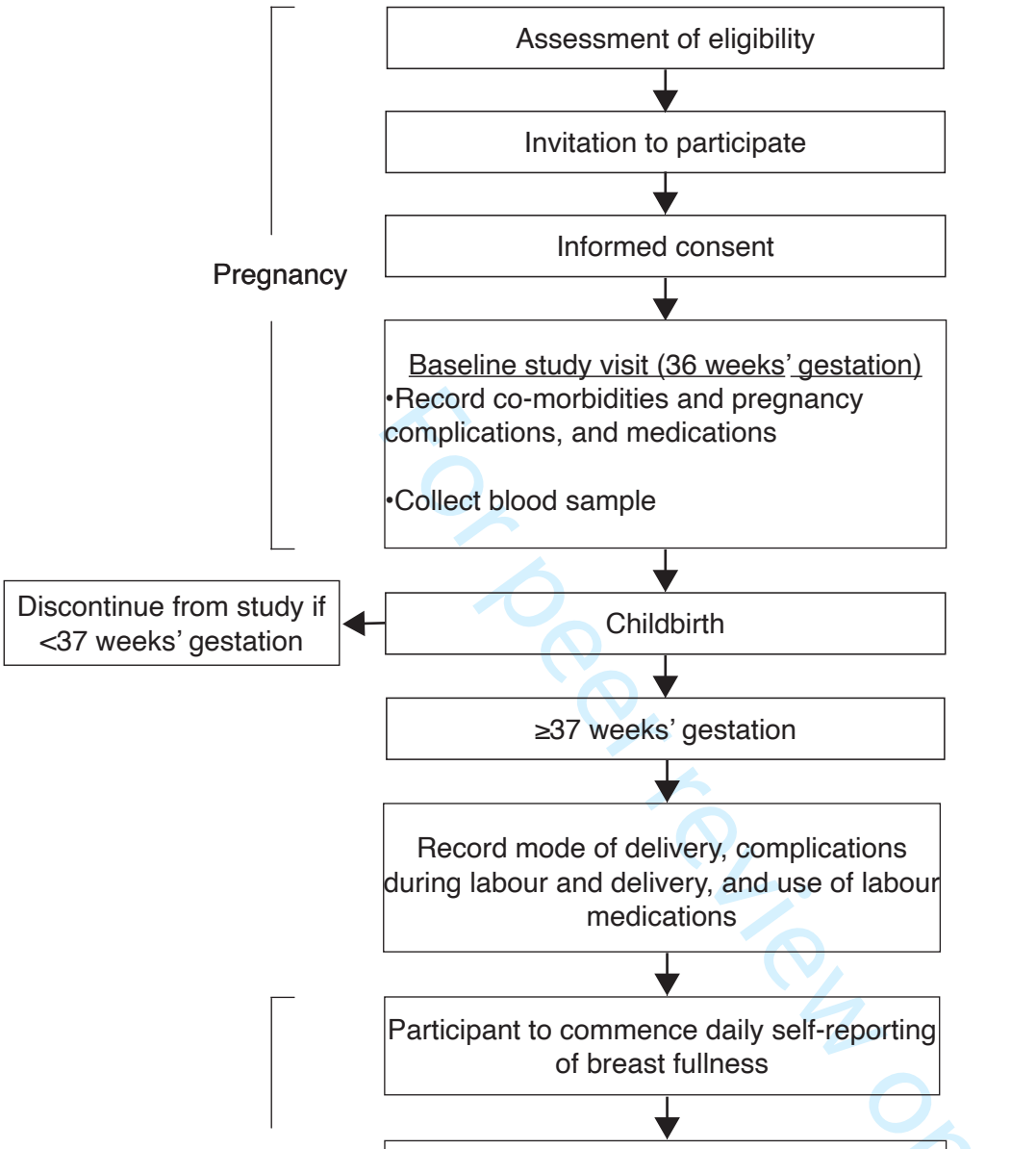
FIGURE LEGENDS

Figure 1. Flowchart of INSIGHT study procedures. *Procedures undertaken only on a single occasion.



SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Page 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 2
	2b	All items from the World Health Organization Trial Registration Data Set	Page 2
Protocol version	3	Date and version identifier	Version 2.1, dated 5th August 2021
Funding	4	Sources and types of financial, material, and other support	Page 17
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Pages 1 and 17
	5b	Name and contact information for the trial sponsor	Page 18
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	No role in any of these activities
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Not applicable

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Pages 4 & 5
	6b	Explanation for choice of comparators	Not applicable for this observational study
Objectives	7	Specific objectives or hypotheses	Page 5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Page 5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Pages 7-8
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Page 7
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Not applicable
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	Not applicable
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Not applicable
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Page 7
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Page 6

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	See Figure 1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Pages 12-13
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	Not applicable
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Not applicable
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Not applicable
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Not applicable
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Not applicable
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Page 14

	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	Not applicable
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Page 14
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Pages 11-12
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Not applicable
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Pages 13-14
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	An external DMC is not required for this low-risk observational single centre study
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	Pages 13-14
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	Page 11
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Page 14
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 18

Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	This will be communicated by the chief investigator
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 8
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Not applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Page 15
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 17
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Page 17
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Page 11
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Page 15
	31b	Authorship eligibility guidelines and any intended use of professional writers	Page 17
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	Not applicable
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Not applicable

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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Protocol for an observational study investigating hormones triggering the onset of sustained lactation: the INSIGHT Study

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Title: Protocol for an observational study investigating hormones triggering the onset of sustained lactation: the INSIGHT Study

Hussam Rostom[1], Xin Meng[1], Helen Price[1], Alexandria Fry[1], Taha Elajnaf[1], Robert Humphrey[2], Nishan Guha[2], Tim James[2], Stephen H. Kennedy[1], Fadil M. Hannan[1*]

[1]Larsson-Rosenquist Foundation Oxford Centre for the Endocrinology of Human Lactation, Nuffield Department of Women's & Reproductive Health, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

[2]Department of Clinical Biochemistry Level 4, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

*Corresponding author:

Name: Prof Fadil M. Hannan

Affiliation: Larsson-Rosenquist Foundation Oxford Centre for the Endocrinology of Human Lactation, Nuffield Department of Women's & Reproductive Health, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom.

E-mail address: fadil.hannan@wrh.ox.ac.uk.

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ABSTRACT

Introduction: Lactation is a hormonally controlled process that promotes infant growth and neurodevelopment and reduces the long-term maternal risk of diabetes, cardiovascular disease and breast cancer. Hormones, such as prolactin and progesterone, mediate mammary development during pregnancy and are critical for initiating copious milk secretion with 24-72 hours postpartum. However, the hormone concentrations mediating lactation onset are ill-defined.

Methods and analysis: The primary objective of the Investigating hormones triggering the onset of sustained lactation (INSIGHT) study is to establish reference intervals for the circulating hormone concentrations initiating postpartum milk secretion. The study will also assess how maternal factors such as parity, pregnancy co-morbidities, and complications during labour and delivery, which are known to delay lactation, may affect hormone concentrations. This single centre observational study will recruit up to 1068 pregnant women over a 3-year period. A baseline blood sample will be obtained at 36 weeks' gestation. Participants will be monitored during postpartum days 1-4. Lactation onset will be reported using a validated breast fullness scale. Blood samples will be collected before and after a breastfeed on up to two occasions per day during postpartum days 1-4. Colostrum, milk and spot urine samples will be obtained on a single occasion. Serum hormone reference intervals will be calculated as mean ± 1.96SD, with 90% confidence intervals determined for the upper and lower reference limits. Differences in hormone values between healthy breastfeeding women and those at risk of delayed onset of lactation will be assessed by repeated measures two-way ANOVA or a mixed linear model. Correlations between serum hormone concentrations and milk composition and volume will provide insights into the endocrine regulation of milk synthesis.

Ethics and dissemination: Approval for this study had been granted by the East of England - Cambridgeshire and Hertfordshire Research Ethics Committee (REC No. 20/EE/0172), by the Health Research Authority (HRA), and by the Oxford University Hospitals NHS Foundation Trust. The findings will be published in high-ranking journals and presented at national and international conferences.

Trial registration number: ISRCTN12667795

Strengths and limitations of this study

- The INSIGHT Study comprises biological and comprehensive phenotype data.
- Metabolites associated with the onset of lactation will be evaluated in serum, urine and milk samples.
- Participants will benefit from breastfeeding support provided by the study investigators.
- Risk of limited participant recruitment and retention during late pregnancy and the early postpartum period.

Key words (5-8): Secretory activation, hormones, lactogenesis, delayed onset of lactation, breastfeeding.

INTRODUCTION

Lactation is tightly regulated by metabolic and reproductive hormones.[1] It plays a key role in infant health and neurodevelopment, and decreases infant mortality and morbidity from infections during the first year of life.[2 3] Longer-term benefits of breastfeeding for the offspring include a reduced risk of obesity and type 2 diabetes, and higher performance in intelligence tests.[3-5] Lactation also confers benefits on breastfeeding women, and is associated with decreases in the risk of maternal diseases such as type 2 diabetes, cardiovascular diseases, and breast and ovarian cancer.[6-9]

Hormones influence the mammary gland from the onset of puberty until the end of pregnancy to induce formation of a branched epithelial milk-secreting organ. From puberty-onwards, oestrogen and the growth hormone/insulin-like growth factor (IGF-1) axis promote elongation and branching of the mammary ductal structure.[10 11] During pregnancy, prolactin, progesterone, oestradiol, insulin, growth hormone/IGF-1 and glucocorticoid hormones such as cortisol stimulate the formation of milk-producing lobuloalveolar structures at the end of mammary ducts (Table 1).[12-17]

Table 1. Major reproductive and metabolic hormones influencing mammary gland function in pregnancy and at the onset of lactation

Hormone	Role in pregnancy	Role at the onset of lactation	References
Reproductive			
Prolactin	Lobuloalveolar formation Milk synthesis	Tight junction formation Milk synthesis	16 18-21
Progesterone	Mammary ductal branching Lobuloalveolar formation	Withdrawal of progesterone initiates tight junction formation and increases milk synthesis	16 22 23
Oestradiol	Mammary ductal branching Lobuloalveolar formation	Not known	13
Metabolic			
Cortisol	Lobuloalveolar formation Milk synthesis	Tight junction formation	17 18
Insulin	Lobuloalveolar formation Milk synthesis	Milk synthesis	15 24 25
Growth hormone/IGF-1	Lobuloalveolar formation	Not known	12 14

Prolactin is the principal hormone promoting milk synthesis, and circulating concentrations progressively increase throughout pregnancy and are correlated with the rate of lactogenesis, as assessed by urinary lactose excretion.[26] Prolactin signals via the STAT5 transcription factor to increase expression of mammary-specific genes mediating the production of lactose, milk proteins and lipids.[20 21] These lactogenic effects of prolactin are augmented by thyroid hormones, insulin and glucocorticoids, which enhance STAT5 activation and/or the transcriptional activation of milk synthesis genes in mammary cells.[24 27 28]

Hormones also trigger the postpartum onset of copious milk secretion, known as secretory activation, which typically occurs between 24-72 hours following child-birth (Table 1).[1] Secretory activation is associated with an increase in milk volume, which leads to the maternal perception of milk 'coming in', characterised by breast fullness and swelling.[29 30] Secretory activation is mediated by a marked decrease in the circulating concentration of progesterone following delivery of the placenta, and requires a high circulating prolactin concentration in the presence of insulin and cortisol.[1] At the onset of lactation these hormonal changes increase milk synthesis and induce the formation of tight junction complexes between mammary cells, which establishes an osmotic gradient that stimulates water movement into the alveolar lumen (Table 1).[18 19 22 23 25]

Secretory activation is critical for the establishment of lactation, and an absence or delay in onset of lactation is a major cause of inadequate and/or early cessation of breastfeeding.[31 32] Although the key hormones involved in secretory activation have been described, their circulating concentrations required for initiating lactation in the early postpartum period are not well defined.[33] Furthermore, alterations in circulating metabolites associated with secretory activation remain to be fully characterised.

METHODS AND ANALYSIS

Objectives

Investigating hormones triggering the onset of sustained lactation (INSIGHT) is a single-centre observational study aiming to: 1) characterise the circulating hormone concentrations required for initiating postpartum milk secretion, and 2) assess how maternal factors such as parity, pregnancy co-

morbidities, and complications during labour and delivery, which are known to delay lactation (Table 2), may affect hormone concentrations.

Table 2. Examples of causes of delayed onset of lactation and association with endocrine disturbances

Cause	Effect on lactation	Associated endocrine abnormalities	References
Maternal factors/co-morbidities			
Pituitary insufficiency e.g. Sheehan's syndrome	Absent or delayed onset	↓ multiple pituitary hormones	34
Polycystic ovary syndrome	Delayed onset and/or lactation insufficiency due to lack of prenatal breast enlargement	↑androgens, obesity-related endocrine abnormalities (see-below)	35 36
Obesity	Delayed onset	↓ mammary responsiveness to prolactin	37 38
Primiparity	Delayed onset	Possible ↓ mammary responsiveness to pregnancy-related hormones	39 40
Labour and delivery factors			
Medications e.g. epidural analgesia, i.v. oxytocin	Delayed onset	↓oxytocin	41 42
Caesarean section	Delayed onset	↓oxytocin, ↓prolactin	32 43

Primary objective

Establishment of reference intervals for hormones initiating postpartum onset of lactation.

Secondary objectives

- Measure longitudinal changes in circulating lactation hormones and metabolites during the first 4 days postpartum.
- Evaluate effects of maternal co-morbidities, medications, mode of delivery and pregnancy complications on lactation hormone concentrations.
- Assess whether lactation hormone concentrations correlate with the timing of the onset of milk secretion.
- Evaluate the relationship between lactation hormones and breast milk volume.
- Characterise breast milk hormone composition.

Outcomes

Primary outcome

Reference intervals will be established for lactation hormones during the early postpartum period before and after a breastfeed during the morning and afternoon, which will account for the effect of diurnal variation and infant suckling on hormone concentrations.

Secondary outcomes

Serial blood sampling during the early postpartum period will allow assessment of longitudinal changes in the concentrations of hormones during secretory activation. Correlation of hormone concentrations with maternal co-morbidities, and complications during pregnancy and delivery, will help elucidate how these factors cause delayed onset of lactation. Furthermore, correlation of hormone concentrations with milk composition and volume will provide insights into the endocrine regulation of milk synthesis.

Study design

Pilot study phase

To assess whether sample collection will be feasible during the early postpartum period, an initial pilot study involving up to 100 women will be performed. Postpartum samples will be collected during a single study visit on postpartum day 4, either at the participant's home or in hospital. These samples will be used for an initial analysis of lactation hormone concentrations, and the data included in the final analysis.

Main study phase

To assess changes in lactation hormones during the early postpartum period, blood sampling will be performed at around 36 weeks' gestation, as a baseline measure, and also before and after a breastfeed during the first 4 days after birth. Participants can provide blood samples on a single postpartum day, or opt for serial sample collection during postpartum days 1-4. Participants will record their breast fullness to determine the timing of lactation onset, as previously described [32]. Demographic and clinical characteristics such as participant age, co-morbidities, medications and complications occurring during pregnancy and delivery, will also be recorded. Infant birth weight will be recorded, and the infant weighed before and after a breastfeed to estimate milk volume. Up to 10 mL of milk will be obtained

for biochemical analysis and a spot urine sample will be collected to assess lactation hormone metabolites. The study protocol is shown in figure 1.

Participants

Over a 3-year period, the study will recruit up to 1000 pregnant women attending maternity units within Oxfordshire, UK, who either have no risk factors for delayed onset of lactation (n~600), or one or more risk factors such as primiparity or obesity (n~400) (Table 2).

Inclusion criteria

- Pregnant women, aged ≥18 years.
- Intention to fully or partially breastfeed.
- Willing and able to give informed consent for participation in the study.

Exclusion criteria

Participants may not enter or continue in the study if ANY of the following apply:

- Preterm birth (<37 weeks' gestation).
- Severe maternal illness, including postpartum depression or psychosis.
- Severe neonatal illness including major congenital abnormalities and infants who are only expected to live for a short period of time.
- Prolonged separation of infant from mother, e.g. due to admission to the neonatal unit.
- Maternal or neonatal COVID-19 test positivity
- Mother or infant infected with blood borne viruses such as HIV.
- Resides outside of Oxfordshire.
- Safeguarding issues that may impede the safety of research staff carrying out home visits.
- Current participation in another research study involving investigational medicinal products.

Participant enrolment

Screening and recruitment

Potential participants will be screened by assessing their eligibility and intention to breastfeed. Introductory study information will be provided during antenatal clinic or ultrasound scan visits. Women can also identify themselves as eligible from advertising materials such as posters, social media advertising, and the advertisement on the Larsson-Rosenquist Foundation Oxford Centre for the Endocrinology of Human Lactation (https://www.ocehl.ox.ac.uk/insight) website.

Informed consent

A trained member of the research team will obtain written informed consent. Written and verbal information will be provided to the participant detailing the exact nature of the study and any risks involved in taking part. Potential participants will be given the opportunity to discuss the study with the investigator, their general practitioner (GP) or other independent parties to decide whether to participate. It will be clearly stated that the participant is free to withdraw from the study at any time and for any reason without prejudice to her or her infant's future care, without affecting her rights, and with no obligation to give the reason for withdrawal.

Informed consent will be obtained electronically (e-consent) on a tablet device. If this is unavailable, then written informed consent will be obtained. The participant will be provided with a copy of the completed consent form and participant information sheet. The e-consent form will be stored on the trial specific database held on the University of Oxford High Compliance Server, and a copy kept in the medical records. The participant must complete the e-consent form or hardcopy version of the Informed Consent form before any study procedures are performed.

Study schedule

Baseline assessment

The participant's co-morbid conditions, medications and pregnancy complications will be recorded, and a blood sample collected.

Subsequent visits

Admission in labour or for Caesarean section: The research team will check that participants are ≥37 weeks' gestation, as defined by the first trimester dating ultrasound scan.

Day 0-1 postpartum: Continued consent will be confirmed with the participant. Delivery or Caesarean section complications, and use of medications (e.g. syntocinon, analgesia), will be recorded. Blood sampling will commence within 24 hours of birth and samples obtained pre- and post-infant feeding. Self-reporting of breast fullness will also commence.

Day 1-4 postpartum: The research midwife or research assistant will visit the participant in hospital, or at home. Blood samples will be taken pre- and post-breastfeed. Breastfeeding support will be provided, if requested by the participant. A colostrum sample of ~1ml and urine sample of ~20ml will be obtained on a single occasion.

Day 4 postpartum: A milk sample (~10 ml) will be obtained. Milk volume will be recorded by weighing the infant pre- and post-feed. Daily recordings of breast fullness will be collected on the final study visit.

Study procedures

Sample collection and handling

Around 10 mL of blood will be collected per sampling time-point, and a maximum of four blood samples (two from a morning and two from an afternoon breastfeeding session) will be obtained per participant over a 24-hour period. Thus, a maximum of 16 blood samples will be obtained from each participant over a 4-day period. Blood sampling will be undertaken at similar times during successive study visits to minimise the effect of circadian rhythm on hormone concentrations.

Colostrum and milk will be collected using an electric or manual breast pump, or by hand expression into a sterile container. On the day of collection, blood samples will be centrifuged and aliquoted centrally. Milk samples will be divided into skimmed milk (centrifuged) and whole milk aliquots (uncentrifuged). Samples will either be analysed freshly or frozen at -80°C for later analysis.

Biochemical measurements

Lactation hormones, such as prolactin, progesterone, oestradiol, cortisol, insulin, growth hormone and IGF-1 (Table 1), will be measured with validated immunoassay methods. Milk components and metabolites with potential as biomarkers for secretory activation will be assayed in blood, milk and urine samples using techniques including mass spectrometry. These molecules include: carbohydrates such as lactose, glucose, sialic acid, and oligosaccharides; amino acids such as alanine, aspartate and glutamate; lipids such as free fatty acids and triacylglycerols; and intermediary metabolites involved in lipid synthesis such as citrate and oxaloacetate.[44] The biochemical and cellular composition of colostrum and breast milk will be assessed by flow cytometry, immunoassay and mass spectrometry.

Any residual sample will be stored long-term for use in future ethically approved studies in accordance with University of Oxford guidelines and the Human Tissue Authority's Code of Practice. At the end of the study, with consent, samples may also be transferred to an ethically approved Research Tissue Bank for long-term storage. However, if consent has not been received then the samples will be destroyed according to local protocol.

Recording breast fullness and factors influencing the onset of lactation

Participants will record their perception of lactation onset on a daily basis from birth until postpartum day 4 using a validated breast fullness numerical scale of 1-5, anchored with 1=no change, 3=noticeably fuller, and 5=uncomfortably fuller, as described.[32] The onset of lactation will be indicated by the time when breast fullness first reached scale point 3.[32] The following factors will also be recorded at each study visit: frequency and duration of mother-infant skin-to-skin contact; frequency and duration of breastfeeds, and use of formula milk or other feeds.

Infant weighing

The volume of the participant's breast milk expressed during a feed will be estimated by weighing her infant immediately before and after feeding using a calibrated portable electronic scale, as described.[45]

Withdrawal or discontinuation of participants

A participant may choose to withdraw from the study at any time, or choose to stop blood sampling at any time and remain on study follow-up. Participants who withdraw from the study can permit data and samples obtained up until the point of withdrawal to be retained for analysis. In addition, the Chief Investigator may discontinue a participant from the study at any time. Reasons include ineligibility (either during the study or retrospectively if overlooked during screening and the study), significant protocol deviation, significant non-compliance with study requirements, or as a clinical decision.

If the participant withdraws from the study before donating any samples then no data will be used. Withdrawn participants will be replaced. If the participant is withdrawn due to an adverse event, the Chief Investigator will arrange for referral to her GP or an appropriate health care professional involved in her care. In the event of a serious adverse event, the participant will be withdrawn from the study. The reason for withdrawal will be recorded in the case report form.

Statistics and analysis

Primary outcome analysis

The reference interval (RI), which represents the middle 95% of measurements for a lactation hormone in a population of breastfeeding women with no risk factors for delayed onset of lactation (Table 2), will be derived according to the Clinical and Laboratory Standards (CLSI) and International Federation of Clinical Chemistry (IFCC) recommendations by two methods.[46 47] Data will be log-transformed to achieve a parametric distribution. The mean and standard deviation (SD) will be derived, and the RI calculated as mean ± 1.96SD, with 90% confidence intervals (CIs) determined for the upper and lower reference limits. If hormone values cannot be normalised by logarithmic transformation, then a non-parametric percentile method will be used. This involves ordering measurements from smallest to largest, with the RI determined as being between the 2.5th and 97.5th centile of the ordered observations. For a sample size of 120, the 2.5th and 97.5th centiles represent the 3rd and 118th observations, respectively.

As samples will be taken pre- and post-feed, and at different times during the day, these RIs will consider the effect of diurnal variation and infant suckling on hormone concentrations.

Secondary outcome analysis

Differences in hormone values between participants with no risk factors for delayed onset of lactation and those with ≥1 risk factor will be assessed by repeated measures two-way ANOVA or a mixed linear model. The log-transformed hormone value will be used as the outcome variable, and the two nominal variables will be 1) absence or presence of risk factors for delayed lactation, and 2) the timing of the sample (pre- or post-breastfeeding).

Hormone values from a subset of participants willing to provide serial blood samples over a 4 day period will be used to assess longitudinal hormone changes. A mixed linear model, which incorporates factors such as breastfeeding status and sampling pre- and post-feeds, will be used to analyse the temporal change in lactation hormone values over the study period whilst accounting for any missing data.

To determine whether lactation hormone concentrations are associated with breast milk volume or the timing of onset of lactation, bivariate correlations will be assessed using the Pearson or Spearman Rank correlation tests. Adjustment for potential confounding variables will be undertaken using a multivariate linear regression model.

Sample size determination for primary outcome

Around 120 participants with no risk factors for delayed onset of lactation are required to establish an RI with acceptable 90% confidence intervals for the upper and lower reference limits.[47] This study involves establishing RIs at a single time-point in the third trimester and for up to 16 different time-points over a 4-day period. Each participant is expected to provide blood samples for an average of four time-points, and therefore 480 participants will likely be required to provide samples for all 16 time-points. A total of N=1920 postpartum blood samples (120 blood samples at each of 16 time-points) are required for the primary outcome. The pilot study is also expected to contribute around 100 samples from 50 participants (two samples per participant) to the primary outcome. Therefore, 455 participants providing an average of 4 samples each (total of 1820 postpartum samples) will be recruited to the main

study. Thus, N=505 participants (50 from pilot study and 455 from main study) will be recruited in total for the primary outcome analysis.

Sample size determination for secondary outcomes

A major secondary outcome is alteration in lactation hormones for participants at risk of delayed onset of lactation compared to participants with no risk factors. Prolactin is critical for secretory activation, and sample size has been determined using published prolactin values for breastfeeding women.[48] The effect size (d) for a 10% change in serum prolactin is 0.57, given that a 10% change is 18.3 µg/L and pooled SD is 32 µg/L.[48] G*Power statistical software was used to calculate sample size with this effect size and the following parameters: a two tailed t test for difference between two independent means, alpha error probability of 0.05, power of 0.95 and a 1:1 allocation ratio. This calculation indicates that a sample size of 81 participants with ≥1 risk factor for delayed onset of lactation is required at each time-point for secondary outcome analysis. Assuming each participant provides samples for an average of four time-points, then 324 participants are required to provide samples for all 16 time points. A total of N=1296 postpartum blood samples (81 blood samples at each of 16 time-points) are required for the secondary outcomes. The pilot study is also expected to contribute ~100 samples from 50 participants (two samples per participant) to the secondary outcome. Therefore, 299 participants providing an average of 4 samples each (total of 1196 postpartum samples) will be recruited to the main study. Thus, N=349 participants (50 from pilot study and 299 from main study) will be recruited in total for the secondary outcome analysis.

Accounting for participant withdrawal

In total, 505 participants with no risk factors for delayed onset of lactation, and 349 participants with ≥1 risk factor are required for the primary and secondary outcome analyses. Assuming a 20% drop out rate, then 632 participants with no risk factors and 436 participants with ≥1 risk factor for delayed onset of lactation (1068 participants in total) will need to be recruited.

Decision points and end of study

The protocol will be evaluated in a pilot study involving ~100 participants. Depending on the results, a decision will be made whether to limit recruitment to certain participant groups, such as multiparous women with no breastfeeding issues who are more willing to participate in the study and provide multiple blood samples. In addition, if sample collection from participants at home proves difficult, then the protocol will be amended to collect blood samples whilst participants are in hospital following child birth, and only collect limited samples during home study visits.

The end of the study is the point at which all data have been collected and analysed, and the database is locked. If it is not possible to collect sufficient blood samples to establish reference intervals at any of the time-points, then the study will be stopped.

Procedure for accounting for missing, unused and spurious data

If there are insufficient (i.e. N<120) samples to calculate the RIs at each time-point using log-normal distribution or the centile method, then a bootstrap calculation method will be used to derive RIs. Bootstrapping involves random re-sampling of the original data observations to generate replacement values and establishment of a 'pseudosample' from which RIs can be derived.[49] This method requires normally distributed data obtained from a minimum of 40 samples.[50] The derivation of hormone RIs can also be affected by values lying above or below the assay detection limit. For very high values, sample dilution will be undertaken prior to biochemical analysis.

Data management

Data will be recorded using an electronic case report form (eCRF), and then transferred to the study specific database. This holds the unique participant study numbers, sample numbers, screening log, e-consent forms, as well as clinical and laboratory data. Any hard copies of the CRFs and consent forms will be kept in a locked filing cabinet in the key-coded locked office of the Lead Research Midwife. The consent form will be retained to meet the UK Human Tissue Authority traceability requirements, and also as the basis for retention of details and approach for any future ethically approved studies.

Quality assurance procedures

The study may be monitored or audited in accordance with the current approved protocol, Good Clinical Practice, relevant regulations and standard operating procedures.

Patient and public involvement

Healthcare professionals such as community midwifes reviewed the study protocol and participant-facing information. In addition, breastfeeding women on the hospital postnatal ward, and in the community, reviewed the study advertising and participant-facing information, and provided feedback on the appeal and comprehensibility of this information.

ETHICS AND DISSEMINATION

Study conduct

The study will be conducted in accordance with the principles of the Declaration of Helsinki and with Good Clinical Practice. The Chief Investigator will submit an annual progress report to the Research Ethics Committee, Health Research Authority (HRA) host organisation, Sponsor and funder. An end-of-study notification and final report will be submitted to the same parties. The findings will be published in high-ranking journals in the field and presented at national and international conferences.

Participant confidentiality

The study will comply with the General Data Protection Regulation (GDPR) and Data Protection Act 2018, which require data to be de-identified as soon as it is practical to do so. Participants will be identified only by a participant study number on any electronic database.

Other ethical considerations

Incidental findings are not expected from the sample analysis. Hormone values are likely to vary substantially within a healthy mother during the early postpartum period, and such alterations will not require referral for clinical investigations or management.

DISCUSSION

This protocol has been established to investigate hormone concentrations triggering secretory activation, and ascertain whether alterations in lactation hormones are associated with a delay in the onset of copious milk secretion. Delayed onset of lactation, defined as occurring >72 hours postpartum, affects between 17-40% of breastfeeding women, and is reported to cause excessive neonatal weight loss during the first postpartum week, and lead to increased use of formula supplementation and decreased duration of breastfeeding.[31 32 51-54] A range of maternal factors, which potentially contribute to endocrine dysregulation, may lead to delayed onset of lactation (Table 2). For example, endocrine disorders due to pituitary or ovarian abnormalities have been reported to delay lactation onset or cause lactation failure (Table 2).[34-36] Furthermore, parity influences the timing of secretory activation, with primiparous mothers experiencing a significantly increased duration of time from parturition until onset of lactation compared to multiparous mothers.[40] Such effects may have an endocrine basis, as rodent studies have shown an effect of parity on mammary hormone responsiveness. Thus, mammary glands from parous mice exhibit a more rapid expansion of ductal structures and earlier onset of milk protein synthesis when exposed to pregnancy-associated hormones, compared to the mammary glands of hormone-treated nulliparous mice.[39] In addition, maternal obesity, occurring either at the pre-pregnant stage or at the time of delivery, is reported to delay onset of lactation,[38] and a mouse study has revealed that obesity-induced lactation insufficiency is associated with mammary gland resistance to prolactin.[37] Medications administered during labour may also influence lactation hormones and cause delayed onset of lactation.[41 42] The administration of infused oxytocin with epidural analgesia to women in spontaneous labour is reported to decrease postpartum plasma concentrations of oxytocin, which is required for stimulating milk let-down during a breastfeed.[42] Moreover, Caesarean section is an independent risk factor for delayed onset of lactation. Delivery by emergency Caesarean section decreases the pulsatility of oxytocin secretion and impairs prolactin secretion during a breastfeed in the early postpartum period.[32 43]

The characterisation of hormone and metabolite concentrations at the physiological onset of lactation, as well as in women at risk of delayed onset of lactation, will improve understanding of the

factors involved in the initiation of successful lactation. The study also has the potential to facilitate development of hormonal biomarkers that can predict those likely to suffer from low-volume or delayed breast milk production, and help identify individuals who may benefit from galactagogue therapy to stimulate lactation. In addition, the study will provide biological insights into how lactation hormones influence the volume and composition of breast milk.

FOOTNOTES

Contributors

HR, XM, HP, TE, SHK and FMH wrote the protocol. HP and AF are involved with consenting participants and collecting samples. HR, XM, RH, TE, NG and TJ are involved in sample processing and analysis. SHK and FMH conceived the study.

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Competing interests

None declared

Patient consent for publication

Not required

Access to data

Study investigators have full access to data

Authorship eligibility criteria

Individuals making substantive contribution to the design, conduct, interpretation, and reporting of this study will be granted authorship on the final study report.

Ethics approval

Approval for this study had been granted by the East of England - Cambridgeshire and Hertfordshire Research Ethics Committee (REC No. 20/EE/0172), by the Health Research Authority (HRA), and by the Oxford University Hospitals NHS Foundation Trust. It was originally approved in October 2020, with amendments approved in May 2021.

Study sponsor

University of Oxford Research Goverance, Ethics & Assurance Team,

Boundary Brook House, Churchill Drive, Headington, Oxford, OX3 7GB

License statement

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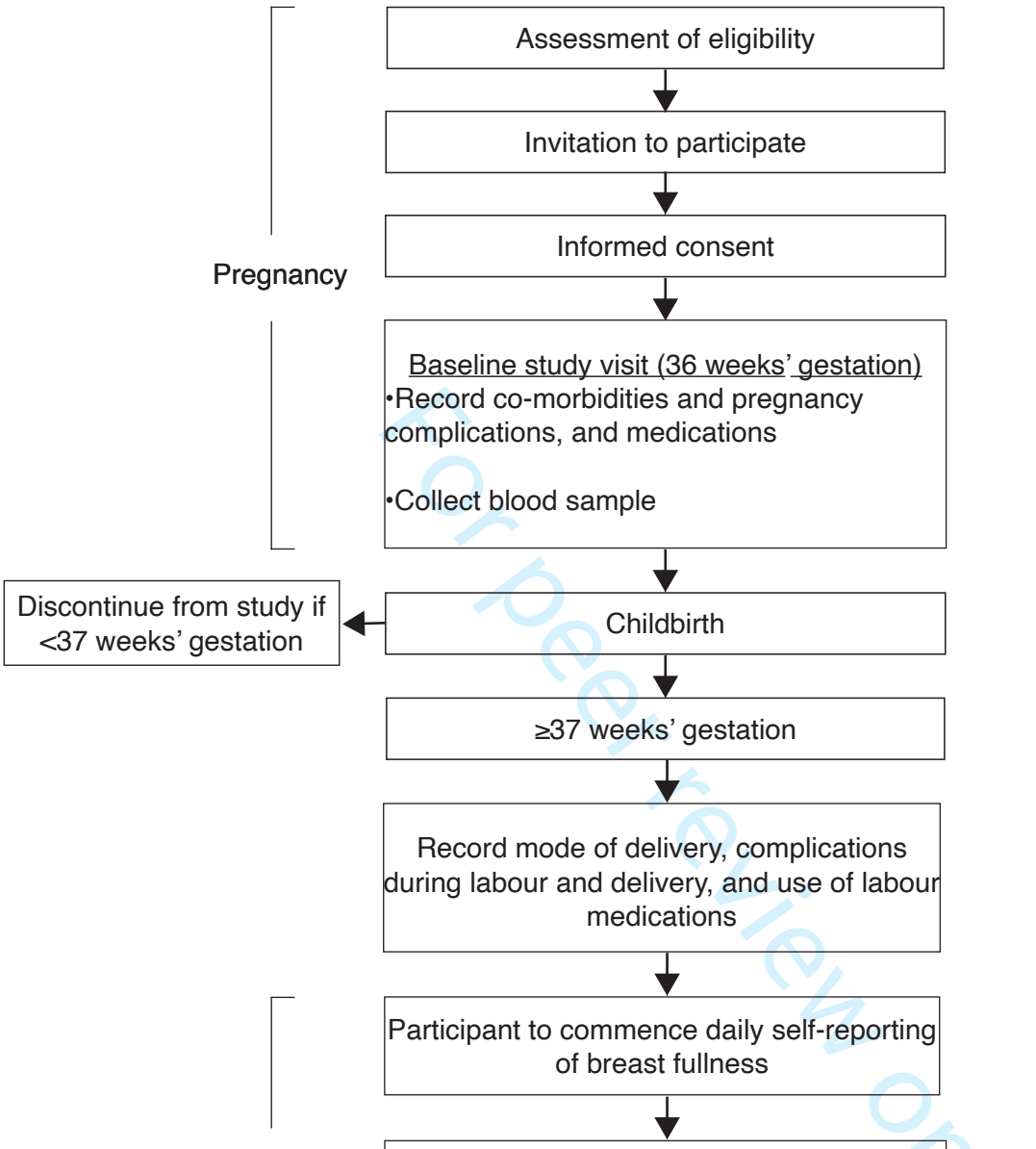
FIGURE LEGENDS

Figure 1. Flowchart of INSIGHT study procedures. *Procedures undertaken only on a single occasion.



SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Page 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 2
	2b	All items from the World Health Organization Trial Registration Data Set	Page 2
Protocol version	3	Date and version identifier	Version 2.1, dated 5th August 2021
Funding	4	Sources and types of financial, material, and other support	Page 17
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Pages 1 and 17
	5b	Name and contact information for the trial sponsor	Page 18
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	No role in any of these activities
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Not applicable

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Pages 4 & 5
	6b	Explanation for choice of comparators	Not applicable for this observational study
Objectives	7	Specific objectives or hypotheses	Page 5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Page 5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Pages 7-8
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Page 7
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Not applicable
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	Not applicable
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Not applicable
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Page 7
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Page 6

Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	See Figure 1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Pages 12-13
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 8
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	Not applicable
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Not applicable
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Not applicable
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Not applicable
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Not applicable
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Page 14

	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	Not applicable
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Page 14
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Pages 11-12
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Not applicable
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Pages 13-14
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	An external DMC is not required for this low-risk observational single centre study
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	Pages 13-14
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	Page 11
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Page 14
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 18

Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	This will be communicated by the chief investigator
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 8
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Not applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Page 15
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 17
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Page 17
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Page 11
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Page 15
	31b	Authorship eligibility guidelines and any intended use of professional writers	Page 17
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	Not applicable
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Not applicable

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.