BMJ Open

Serum hepcidin and iron status parameters in pregnant women and the association with adverse maternal and foetal outcome: a study protocol

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2019-032280
Article Type:	Protocol
Date Submitted by the Author:	11-Jun-2019
Complete List of Authors:	Amstad Bencaiova, Gabriela; University Hospital Basel, Department of Obstetrics; Vogt, Deborah; University of Basel, Clinical Trial Unit Hoesli, Irene; University Hospital, University Basel, Obstetrics
Keywords:	hepcidin, pregnancy, BMI, pregnancy outcome

Serum hepcidin and iron status parameters in pregnant women and the association with adverse maternal and foetal outcome: a study protocol

G Amstad Bencaiova, D R Vogt, I Hoesli

Department of Obstetrics and Antenatal Care, University Hospital Basel, University Basel, Switzerland

Clinical Trial Unit, Department of Clinical Research, University Hospital Basel and University of Basel

Corresponding author: Gabriela Amstad, MD

Department of Obstetrics and Antenatal Care,

University Hospital of Basel

Spitalstrasse 21

CH-4031 Basel

Switzerland

Email: gabriela.amstad@usb.ch

Phone +41 61 556 59 22

Fax +41 61 265 9198

Abstract

Introduction: Hepcidin production is normally upregulated by iron stores, and in obesity has been shown to be overexpressed and correlated with low iron status. The increased hepcidin may restrain the iron release from the cells by affecting the expression of ferroportin, which probably associates with the development of diabetes complication. Firstly, we investigate the difference of serum hepcidin and iron parameters between obese and non-obese pregnant women; secondly, we examine the correlation between serum hepcidin and adverse maternal and neonatal outcomes in pregnant women.

Methods and analysis: This is a mono-centre, observational study with a study (obese) and a control group (non-obese women). 188 singleton pregnancies will be recruited in the first trimester. Thereof, we expect 75 with a BMI $\geq$ 30 kg/m^2 and 113 with a BMI 18.5 -- 30 kg/m^2. Serum hepcidin, iron and haematological parameters will be measured at 11-14, 24-28, 32-36 weeks of gestation and at time of delivery. Blood pressure, weight, BMI and smoking status will be examined at all visits. We will assess the composite endpoints adverse maternal outcome (including pre-eclampsia, gestational hypertension, gestational diabetes, haemorrhage, placenta abruption), and adverse neonatal outcome (preterm birth, intrauterine growth restriction, preterm premature rupture of membranes, Apgar score <7 at 5 minutes, still birth, neonatal death).

Recruitment has started in April 2019.

Ethics and dissemination: This study received ethical approval from the ethics committee in Basel. It was registered under http://www.ClinicalTrials.gov (NCT03792464) on 7 January 2019.

Trial registration number: NCT03792464.

Strengths and limitations of this study

This is a prospective study to examine the difference of serum hepcidin and iron parameters between obese and non-obese pregnant women.

For the first time to our knowledge, a prospective study will be performed in order to verify the association between hepcidin and pregnancy outcome.

At the time of planning this study, to our knowledge, no data on hepcidin levels of obese pregnant women in the first trimester were available. Hepcidin levels in our study might thus deviate from our assumptions for sample size estimation. Therefore, we will perform a blinded sample size re-estimation.

Introduction

Obesity is the most common problem in obstetrics that affects both the mother and her offspring.[1] Obesity causes short term and long term problems for the mother, such as increasing her risk of gestational diabetes (GDM) and pre-eclampsia during pregnancy and further increases the risk of developing the metabolic syndrome in later life. The offspring have an increased risk of obstetric morbidity and mortality and, consistent with the developmental origins of health and disease, a long term risk of childhood obesity and metabolic dysfunction.[1]

Obesity is also associated with an increased risk of iron deficiency, attributable to adiposity-related inflammatory mediators on iron regulatory pathways.[2] The pro-inflammatory cytokine interleukin-6 (IL-6), frequently elevated in obesity, has been shown to induce expression of hepcidin[2], a negative regulator of intestinal iron absorption and macrophage iron efflux. Normal foetal growth and development is dependent upon maternal iron sufficiency during pregnancy. Pregnant women have an increased requirement of iron to support foetoplacental development, expansion of maternal red blood cell mass, and to compensate for intrapartum blood loss; to meet this need, absorption of dietary iron is enhanced concomitant with increased utilization of existing iron stores. During a healthy pregnancy, hepcidin is reduced, enabling iron transfer to the fetus.[3-6] Obesity in

pregnancy may lead to hepcidin excess and decreased iron transfer to the fetus.[7-9] Low iron stores are reported to be more common in obese pregnant women.[10] The correlation between serum ferritin and hepcidin has been shown in previous studies.[3,5,8,11,12] Although in other studies, no correlation between serum hepcidin and iron status has been found.[4,6,13] To strengthen the link between the poor iron status of obesity and hepcidin, there is the interesting observation that hepcidin is expressed not only in the liver but also in adipose tissue and that mRNA expression is increased in adipose tissue of obese pregnant patients.[7] The increased hepcidin may restrain the iron release from the cells by affecting the expression of ferroportin, which probably associates with the development of diabetes complication.[14] Iron content appears to be increased in the subcutaneous and visceral adipose tissue of obese patients, and negatively correlated with adiponectin expression, which could be contributing to insulin resistance and the metabolic complications of obesity.[15]

There are few studies examining the influence of maternal obesity on maternal iron status, with some indicating that iron status may be compromised [(7-9,16)], and others reporting no impact.[17] However dietary or iron supplementation was not assessed and might be responsible for divergent results. There is no study evaluating the difference of serum hepcidin and ferritin between obese and non-obese pregnant women longitudinally during pregnancy as well as the association between these parameters and adverse pregnancy outcome.

Methods and analysis

Primary objective

The primary endpoint is serum hepcidin level (ng/ml) measured at 11-14 weeks of gestation.

Secondary objectives

1. To compare iron status parameters, haemogram and CRP measured in the first trimester between obese and non-obese pregnant women.

2. To describe the course of serum hepcidin, iron status parameters, haemogram, CRP, weight and BMI during pregnancy and to compare them between obese and non-obese pregnant women.

3. To describe the correlations between serum hepcidin, iron status parameters, haemogram, CRP, weight and BMI in each trimester and at delivery.

4. To investigate whether adverse pregnancy outcome is associated with serum hepcidin and iron status in the first trimester and during total pregnancy.

5. To investigate whether adverse neonatal outcome is associated with serum hepcidin and iron status in the first trimester and during total pregnancy.

Study design

This is a mono-centre, observational study with a study (obese) and a control group (non-obese women). This study protocol was developed on the basis of

Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT; see online SPIRIT checklist for further details).

Study settings

The study will be conducted at the University Hospital of Basel, Department of Obstetrics and Antenatal Care with 2800 deliveries/year. The study has started in April 2019. We estimate to recruit all patients within 18 months. A total of N=188 healthy pregnant women (expected 75=40% with BMI ≥ 30 kg/m2) will be recruited in the first trimester in our outpatients' department. Considering a drop-out rate of 10% we will have a total of 169 evaluable women.

Eligible criteria

Inclusion criteria

- Age ≥ 18 years
- Singleton pregnancy
- BMI ≥ 18.5 kg/m^2
- Gestational age at recruitment: 11-14 of gestational weeks
- Written informed consent.

Exclusion criteria

- Foetal genetic, chromosomal or intervention-requiring morphological abnormalities
- Chronic disease of heart, liver, kidney, cardiovascular system, gastrointestinal tract, neurologic, autoimmune, haematological disorders and psychiatric disorders or known infection like hepatitis or HIV
- The inability to read and/or understand the participant's information sheet.

Assessment of primary outcome

The serum hepcidin will be investigated at 11-14, 24-28, 32-36 weeks of gestation and at labour. Blood samples are collected by venepuncture. The measurement of serum hepcidin will be conducted at the University Hospital of Basel, Department of Biomedicine. Serum hepcidin will be measured with an ELISA. No additional visit is necessary besides the standard routine antenatal care visits.

Assessment of secondary outcomes

Serum iron parameters (serum ferritin and soluble transferrin receptors) will be measured at 11-14, 24-28, 32-36 weeks of gestation and at labour. Iron parameters will be determined using routine laboratory methodology. Blood samples will be collected by venepuncture. All blood measurements will be

conducted at the University Hospital of Basel, Department of Laboratory Medicine and Department of Biomedicine.

The following maternal outcome will be investigated: pre-eclampsia defined according to the «Report of the American College of Obstetricians and Gynecologists` Task Force on Hypertension in pregnancy»[18], pregnancy induced hypertension[18], infection in pregnancy (urinary tract infection, vaginal infection, chorioamnionitis etc.), anaemia and iron deficiency in pregnancy according to the World Health Organization (WHO) with ferritin cut-off of 15 µg/l[19], cholestasis of pregnancy, gestational diabetes mellitus according to the results of an oral 75g glucose test, mode of delivery, abnormal placentation, placental abruption, thromboembolism in pregnancy and puerperium, peripartum and postpartum haemorrhage, estimated blood loss, anaemia in postpartum, puerperal infection or sepsis, transfusion requirement. Pre-eclampsia, gestational hypertension, GDM, cholestasis of pregnancy, peripartal haemorrhage (increased blood loss peripartal or significant haemoglobin- difference between before and after delivery > 30 g/l), infections, anaemia and placenta abruption are defined as adverse maternal outcome.

The following neonatal outcome should be investigated: gestational age at birth, birth weight, Apgar score, pH levels, preterm delivery < 37 weeks of gestation, preterm premature rupture of membranes (PPROM), macrosomia with birth weight above 95th percentile, intrauterine growth restriction (IUGR) with birth weight below 5th percentile, low birth weight (LBW) with birth weight below

2500 g, still birth defined according to WHO[20], neonatal death defined according to UNICEF and WHO[21], admissions to the neonatal unit care (NICU). Preterm birth, IUGR, PPROM, macrosomia, Apgar score < 7 at 5, still birth and neonatal death are defined as adverse neonatal outcome

Participant time line and study procedures

All healthy pregnant patients with regular care at our outpatients' department are counselled and asked at six –10 weeks of gestation to participate. At 10+0 to 13+6 weeks of gestation, all women have to undergo a first trimester ultrasound scan which is standard care. The ultrasound scan is used to confirm gestational age, diagnose any major foetal abnormalities. All pregnant women that meet in-/exclusion criteria will be recruited in the first trimester in the framework of regular pregnancy visit through physicians, midwifes and study coordinators. Serum hepcidin, iron and haematological parameters will be measured at 11-14, 24-28, 32-36 weeks of gestation and at labour. The blood pressure, weight, weight gain, body mass index (BMI) and smoking status will be examined at all visits as a standard of care. Time and event schedule provides an overview about the schedule of observations and assessments (Table 1).

Haematological parameters

This is a routine examination on the visit 1, 2, 3 and 4 haemoglobin (Hb), red blood cell count (RBC), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), percentage of microcytic erythrocytes (MRC), hypochromic red blood cells (HRC) and reticulocyte haemoglobin content (CHr) are measured using a haematology analyser. Mean corpuscular haemoglobin is automatically calculated from Hb and RBC.

Serum ferritin, CRP, hepcidin and soluble transferrin receptors

There are additional examinations on the visit 1, 2, 3 and 4. Ferritin is assessed by chemiluminescence immunoassay and CRP is assessed by immunoturbidimetry. Soluble transferrin receptors and serum hepcidin are measured with an ELISA.

Recording of medications

All medications being continued by a patient on enrolment and all medications given in addition must be documented on the Case report form (CRF) and in the patient`s medical records.

Sample size

Sample size is estimated so that a difference between obese and non-obese pregnant women regarding the primary endpoint- serum hepcidin level in the first trimester- can be shown with 80% power, at a significance level $\alpha = 5\%$.

Sample size estimation is based on the following assumptions:

• The proportion of obese women is expected to be 40%.

• We assume hepcidin levels to be normally distributed with equal variance for obese and non-obese women.

• Based on results reported in Dao et al.[9] for obese women in the second trimester, and assuming these are similar in the first trimester, we assume a mean hepcidin level of 13.5 ng/ml in the first trimester and a standard deviation of $\sigma = 9.0$ ng/ml. Dao et al.[9] report a mean difference of 8.0 ng/ml between obese and non-obese women, while Garcia-Valdes et al.[7] report a mean difference of approximatively 5.0 ng/ml. Here, we aim to show a clinically relevant difference of 4.0 ng/ml, thus we assume $\mu = 17.5$ ng/ml in non-obese women.

Sample size was calculated using a re-sampling procedure. Each sample size (ni=1,...,71 =20, ..., 300) was evaluated first determining the number of obese women (expected 40%, c.f. above) by sampling 999 times from a binomial distribution with n = ni and probability p = 0.4. Then, 999 times ni observations, were sampled from the above described distributions for non-obese women and obese women, respectively. The difference in each sample between obese and

non-obese women was then tested using a two- samples t-test. The null hypothesis was rejected when the difference was statistically significant ($p < 0.05$). Sample size was set to ensure a significant result in at least 80 % of the cases (power: $1 - \beta = 0.8$), at a significance level $\alpha = 5$ %.

Supposing to show a hepcidin difference of 4.0 ng/ml, a total of 188 women should be recruited in order to have a total of 169 evaluable women, considering a drop-out rate of 10%. A sample size re-estimation is planned as soon as hepcidin levels of the first trimester of 80% of the initially estimated number of women (i.e. 136 out of 169) will be available (c.f. Interim Analysis).

Data collection

The study data recorded in the CRF will be transferred to a corresponding electronic CRF (e-CRF) by a designated person. The principal investigator will be responsible for assuring that the data entered into the e-CRF is complete, accurate, and that the entry and updates are performed in timely manner. All information recorded in the e-CRFs will be traceable to the source documents in the patient's file and in the date source files.

The e-CRF will be implemented by the Data management group at the Clinical Trial Unit Basel using the electronic data capture (EDC) software SecuTrial. The EDC system runs on a server maintained by the IT-department of the University Hospital Basel.

Internal data management will be conducted. The data-review and data-handling documents, to be developed during the initiation phase of the study, will include specifications for consistency and plausibility checks on data and will also include data handling rules for obvious data errors.

In compliance with the International Council on Harmonization and GCP guidelines the investigator/institution will maintain all source documents that support the data collected from each patient, and all documents as specified in Essential Documents for the Conduct of a study and as specified by the applicable regulatory requirements. The investigator/institution will take measures to prevent accidental or premature destruction of these documents. Essential documents must be retained until at least 10 years after the last approval. If the responsible investigator retires, relocates, or for other reasons withdraws from the responsibility of keeping the study records, custody must be transferred to a person who will accept the responsibility.

Withdrawal and discontinuation

Patient participation may be terminated prior to completing the Visit 4 for any of the following reason:

- The patient withdraws her informed consent for whatever reason.
- Non cooperative patient, lack of compliance.

- Occurrence of a transient disease, which might distort the results of this study or represents a contraindication.

Premature termination of the study must be agreed upon by the principal investigator and must be documented. Reasons for premature termination include: inadequate recruitment of patients, a protocol violation was identified or developed during the study, failure of responsible investigator to comply with the protocol or GCP guidelines.

Planned analysis

The statistical analysis will be conducted by the Clinical Trial Unit Basel. Detailed methodology for summaries and statistical analyses of the data collected in this study will be documented in a statistical analysis plan. The statistical analysis plan will be finalized before database closure and will be under version control at the Clinical Trial Unit Basel. The Full Analysis Set will consist of all included women for whom serum hepcidin level in the first trimester (11-14 weeks of gestation) is available. Demographics and baseline characteristics will be reported for obese and non-obese woman separately and taken together.

Primary analysis

The primary objective is to compare the primary endpoint between obese and non-obese pregnant women. If data are approximatively normally distributed, a two-sample t-test will be applied. Otherwise, data transformation (e.g. log), the use of a generalized linear model (GLM) with a Poisson error distribution, or the use of a non-parametric Wilcoxon two-sample rank test will be considered.

Secondary analyses

Secondary endpoints measured in the first trimester (secondary objective 1) will be compared between obese and non-obese pregnant women as described for the primary endpoint. The course of the secondary endpoints during pregnancy (secondary objective 2) will be compared between obese and non-obese pregnant women using a random slope / random intercept linear mixed effects models for each endpoint. The model will include the respective secondary endpoint as dependent variable, study group (obese vs. non-obese), gestation week (continuous) and the interaction between study group and gestation week as fixed effects, and woman (patient ID) as random effect with random slope and random intercept. In case of an obvious non-linear time course, time will be included as categorical variable (time point: first, second and third trimester and delivery) instead, and a random intercept model will be applied (thus no random slope).

The correlations between secondary endpoints measured during the course of pregnancy (secondary objective 3) will be described by means of scatterplots and Spearman's rank correlation coefficient for each trimester and at delivery separately. Adverse maternal outcome and adverse neonatal outcome will be analysed for an association with the listed secondary endpoints in the first trimester using logistic regression (secondary objectives 4 and 5). As a first step, univariate models will be fit. Each model will include the respective binary endpoint as dependent variable, and one of the secondary endpoints measured in the first trimester as explanatory variable. In order to test for a combined effect of the secondary endpoints, in a second step, multivariable logistic regression models will be fit. A forward model selection approach will be applied, based on Akaike's information criterion (AIC). Starting with the "null model" - the simplest model, including no explanatory variable - single predictor variables (i.e. secondary endpoints) will repeatedly be added if and as far as AIC can be decreased. This will result in a final model that includes the set of variables that (together) best describe the outcome. (A forward model selection approach is chosen, due to the relatively large number of predictors of interest in comparison to the expected number of events.) For each statistical model, odds ratios will be presented for each explanatory variable with 95% confidence intervals and p-values. Further, the frequency of each composite will be given for obese and non-obese women.

Interim analysis

The estimation of the variance (or the distribution in general) of hepcidin levels is crucial for determining the sample size of the study, but hepcidin levels observed in the study may deviate considerably from our assumptions. We will therefore re-estimate the sample size as soon as hepcidin levels of the first trimester of 80 % of the initially estimated number of women (i.e. 136 out of 169) will be available. We will re-estimate the variance σ2 obese and σ2 non–obese in a blinded manner – only the hepcidin levels of the third trimester will be available to the statistician performing the interim analysis. The overall variance σ2 will be estimated (one-sample variance estimator), assuming σ2 = σ2 obese = σ2 non–obese. Since no hypothesis test is performed, no p-value adjustment to control type I error is needed. Using the re-estimated standard deviation, the sample size N' will be re-estimated as before. If there should be clear evidence against a normal distribution of hepcidin levels (based on visual inspection of quantile-quantile plots), a t-test based on transformed data or a non-parametric Wilcoxon two-sample rank test will be used. The sample size will be increased in order to include N' evaluable patients whenever N' > N, preserving a power of 80 %. A sample size reduction or early stopping of the study will not be considered.

Deviations from the original statistical plan

If substantial deviations of the analysis as outlined in these sections are needed for whatever reason, the protocol will be amended. All deviations of the analysis from the protocol or from the detailed analysis plan will be listed and justified in a separate section of the final statistical report.

Handling of missing data

Presumably, there may be reasons for drop outs confounded with the measurements. For example, some composites of adverse maternal and neonatal outcome will lead to a preterm end of pregnancy, which will result in missing measurements after the first trimester. These data will be missing not at random. It is important to keep these drop outs in the analysis set regarding secondary objectives 4 and 5. In case there will be many such drop-outs (i.e. missing not at random), inverse probability of censoring weights (IPCW) will be considered, and a sensitivity analysis with a complete case analysis set will be performed.

Missing data and drop-outs will be handled using the method most appropriate, based on an examination of missing values before data base closure. Details will be specified in the statistical report and analysis plan. The number of missing values in each endpoint and the number of drop-outs will be summarized for obese and non-obese women.

Confidentiality and Coding

Project data will be handled with uttermost discretion and is only accessible to authorized personnel who require the data to fulfil their duties within the scope of the research project. On the CRFs and other project specific documents, participants are only identified by a unique participant number. Biological material in this project is not identified by participant name but by a unique participant number.

Biological material and health-related personal data will be coded. The coding key will be located by the investigator and the members of her research team. Access to this key will only have the investigator and the members of her research team. Biological material is appropriately stored in a restricted area only accessible to authorized personnel. Data generation, transmission, storage and analysis of health related personal data and the storage of biological samples within this project will follow strictly the current Swiss legal requirement for data protection and will be performed according to the HRO Art. 5. Health related personal data captured during this project and biological samples from participants are strictly confidential and disclosure to third parties is prohibited; coding will safeguard participants` confidentiality. Only the investigator and members of her research team will have access to project plan, dataset, statistical code etc. during and after the research project.

Retention and destruction of study data and biological material

The investigator/institution will take measures to prevent accidental or premature destruction of these documents. Essential documents must be retained until at least 10 years after the last approval. After measurement, biological material will be destroyed at the in University Hospital of Basel, Department of Laboratory Medicine.

Data sharing

After publication of the study individual anonymous participant data including variable keys will be available upon request by the corresponding author. Researchers may request data to repeat the analyses or use the data for secondary analyses (e.g. systematic review and meta-analysis).

Discussion

Obesity is a low-grade chronic inflammatory condition linked to the overexpression of hepcidin and secondarily to iron homeostasis. Increased hepcidin in obese pregnant women without gestational diabetes mellitus was shown in previous studies.[7, 9, 22, 23] Obesity- related inflammation may induce hepcidin biosynthesis with a consequent reduction of iron supply.[7, 24] Anelli et al. showed lower haemoglobin concentration in obese pregnant women.[22] Interestingly, anaemia was not related to the increase in hepcidin, while this was positively associated with maternal BMI.[22]

Few studies have evaluated the influence of obesity on hepcidin and iron status during pregnancy, but their results are inconclusive. Whereas increased hepcidin in obese pregnant women was shown in previous studies, [7, 9, 22, 23] it has not been confirmed in another studies.[25, 26] Due to previous data, we expect to observe an increase in the serum hepcidin in obese pregnant women, consequentially increased soluble transferrin receptors as a marker for iron deficiency in cells and a lower level of haemoglobin because of iron deficiency.

There is unclear which obese pregnant women develop during pregnancy complications such as gestational diabetes, pregnancy induced hypertension, pre-eclampsia, intrahepatic cholestasis, intrauterine growth restriction etc. Increased serum hepcidin could indicate the increased risk of adverse maternal and neonatal outcome. Chen et al showed that increased hepcidin restrains the

iron release from the cells by affecting the expression of ferroportin, which probably associates with the development of diabetes complication.[14] High ferritin in the first trimester was also associated with GDM.[27, 28]

The role of hepcidin in pre-eclamptic pregnant women is unclear. Hepcidin levels in the first half of pregnancy were found significantly higher in women who subsequently developed pre-eclampsia compared to mothers having a physiological pregnancy until term.[30] Reason could be due to an inflammatory condition that characterizes the pre-eclamptic syndrome long before the symptoms onset. The identification of women at elevated risk of preeclampsia may be sufficiently early to allow the prophylactic use of low-dose aspirin, which has been demonstrated to reduce the prevalence of preeclampsia when started before 16 weeks` gestation. For the first time to our knowledge, a prospective study will be performed in order to verify the association between hepcidin in obese women and pregnancy outcome such intrahepatic cholestasis of pregnancy, haemorrhage, intrauterine growth restriction etc.

In conclusion, we will contribute new information regarding the effects of obesity on iron and hepcidin levels. Our findings will improve the understanding of hepcidin and iron metabolism in obese pregnant women and provide direction for maternal and foetal complications. We expect that the usefulness of hepcidin in the early detection of women with an increased risk of developing maternal and foetal complications could be confirmed by our prospective cohort study. This may conduce to establish new promising biomarkers and possible life care

intervention, aimed at reducing the obesity-related risks in pregnancy. Our findings could be of clinical and public health importance since serum hepcidin as a biological marker for pregnancy risk can improve the prenatal care and optimize peripartal and postpartal management in the future.

Ethics and dissemination

This study received ethical approval from the ethics committee in Basel (Project-ID: 2017-02322). It was registered under http://www.ClinicalTrials.gov (NCT03792464) on 7 January 2019. All members of the research team are aware of the guidelines for good clinical practice for obtaining consent. The principal investigator must ensure that all ethical and legal requirements have been met before the first patient is enrolled in the study. This protocol is to be followed exactly. To alter the protocol, amendments must be written, receive approval from the appropriate personnel, and receive Ethics Committee approval prior to implementation. Administrative changes (not affecting the patient benefit/risk ratio) may be made without the need for a formal amendment.

Before each patient is admitted to the study, signed informed consent will be obtained from the patient according to the regulatory and legal requirements. This consent form must be dated and retained by the investigator as part of the study records. The informed consent should be in accordance with the current revision of the Declaration of Helsinki, current International Conference on Harmonization (ICH) and Good Clinical Practice (GCP) guidelines.

The physicians and study coordinators must explain to potential participants the aims, methods, reasonably anticipated benefits of this study. Patients will be informed that they are free not to participate in the study and that they may withdraw consent to participate at any time. They will be told that competent

authorities may examine their records and authorized persons but that personal information will be treated as strictly confidential and will not be publicly available. Patients must be given the opportunity to ask questions. After this explanation and before entry into the study, consent should be appropriately recorded by means of the patient`s dated signature.

If a protocol amendment is required, the informed consent form may need to be revised to reflect the changes to the protocol. If the consent form is revised, it must be approved and signed by all patients subsequently enrolled in the study.

References

1. Catalano PM, Shankar K. Obesity and pregnancy: mechanisms of short term and long term adverse consequences for mother and child. *BMJ* 2017 Feb 8; 356:j1. doi: 10.1136/bmj.j1. Review.
2. Flynn AC, Begum S, White SL, et al. Relationships between maternal obesity and maternal and neonatal iron status. *Nutrients* 2018 Jul 30; 10(8). pii: E1000. doi: 10.3390/nu10081000.
3. van Santen S, Kroot JJ, Zijderveld G, et al. The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. *Clin Chem Lab Med* 2013; 51(7):1395-401.
4. Hedengran KK, Nelson D, Andersen MR. Hepcidin levels are low during pregnancy and increase around delivery in women without iron deficiency - a prospective cohort study. *J Matern Fetal Neonatal Med* 2016; 29(9):1506-8.
5. Kulik-Rechberger B, Kosciesza A, Szponar E, Domosud J. Hepcidin and iron status in pregnant women and full-term newborns in first days of life. *Ginekol Pol* 2016; 87(4):288-92.
6. Simavli S, Derbent AU, Uysal S, Turhan NÖ. Hepcidin, iron status, and inflammation variables among healthy pregnant women in the Turkish population. *J Matern Fetal Neonatal Med* 2014; 27(1):75-9.

7. Garcia-Valdes L, Campoy C, Hayes H, et al. The impact of maternal obesity on iron status, placental transferrin receptor expression and hepcidin expression in human pregnancy. *Int J Obes (Lond)* 2015; 39(4):571-8.
8. Nazif HK, El-Shaheed AA, El-Shamy KA, et al. Study of serum hepcidin as a potential mediator of the disrupted iron metabolism in obese adolescents. *Int J Health Sci (Qassim)* 2015; 9(2):172-8.
9. Dao MC, Sen S, Iyer C, et al. Obesity during pregnancy and fetal iron status: is Hepcidin the link? *J Perinatol* 2013; 33:177-81.
10. Bekri S, Gual P, Anty R, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. *Gastroenterology* 2006; 131:788-796.
11. Schulze KJ, Christian P, Ruczinski I, et al. Hepcidin and iron status among pregnant women in Bangladesh. *Asia Pac J Clin Nutr* 2008; 17(3):451-56.
12. Finkenstedt A, Wildschwendter A, Brasse-Lagnel CG, et al. Hepcidin is correlated to soluble hemojuvelin but not to increased GDF15 during pregnancy. *Blood Cells Mol Dis* 2012; 48:233-37.
13. Chelchowska M, Swiatek E, Ambroszkiewicz J, et al. [Concentrations of pro-hepcidin in serum of pregnant women and in umbilical cord blood]. *Ginekol Pol* 2008; 79(11):754-7.

14. Chen Y, Yin HQ, Liu HL, et al. Hepcidin and iron metabolism in non-diabetic obese and type 2 diabetic rats. *J Huazhong Univ Sci Technolog Med Sci* 2015; 35:851-857.

15. Pihan-Le Bars F, Bonnet F, Loréal O, et al. Indicators of iron status are correlated with adiponectin expression in adipose tissue of patients with morbid obesity. *Diabetes Metab* 2016; 42:105-11.

16. Cepeda-Lopez AC, Melse-Boonstra A, Zimmermann MB, Herter-Aeberli I. In overweight and obese women, dietary iron absorption is reduced and the enhancement of iron absorption by ascorbic acid is one-half that in normal-weight women. *Am J Clin Nutr* 2015; 102:1389-97.

17. Yanoff LB, Mentie CM, Denkinger B, et al. Inflammation and iron deficiency in the hypoferremia of obesity. *Int J Obes (Lond)* 2007; 31:1412-1419.

18. The American College of Obstetricians and Gynecologists. Hypertension in Pregnancy. *Task Force on Hypertension in pregnancy* 2013.

19. World Health Organization. Iron Deficiency Anaemia. Assessment, Prevention, and Control. *A guide for programme managers* 2001.

20. World Health Organization. Neonatal and perinatal mortality country, regional and global estimates. *Country, Regional and Global Estimates* 2006.

21. UNICEF, WHO, The World Bank. Levels and trends in child mortality. *Report* 2014.

22. Anelli GM, Cardellicchio M, Novielli C, et al. Mitochondrial content and hepcidin are increased in obese pregnant mothers. *J Matern Fetal Neonatal Med* 2018; 18:2388-2395.

23. Cepeda-López AC, Aeberli I, Melse Boonstra A, et al. Does obesity increase risk for iron deficiency? A review of the literature and the potential mechanisms. *Int J Vitam Nutr Res* 2010; 80:263-70.

24. Rehu M, Punnonen K, Ostland V, et al. Maternal serum hepcidin is low at term and independent of cord blood iron status. *Eur J Haematol* 2010; 85(4):345-52.

25. Cao C, Pressman EK, Cooper EM, et al. Pre-pregnancy body mass index and gestational weight gain have no negative impact on maternal or neonatal iron status. *Reprod Sci* 2016; 23:613-22.

26. Jones AD, Zhao G, Jiang Y., et al. Maternal obesity during pregnancy is negatively associated with maternal and neonatal iron status. *Eur J Clin Nutr* 2016; 70(8):918-24.

27. Rawal S, Hinkle SN, Bao W, et al. A longitudinal study of iron status during pregnancy and the risk of gestational diabetes: finding from a prospective, multiracial cohort. *Diabetologia* 2017; 60(2):249-57.

28. Bowers KA, Olsen SF, Bao W, et al. Plasma concentrations of ferritin in early pregnancy are associated with risk of gestational diabetes mellitus in women in the Danish National Birth Cohort. *J Nutr* 2016; 146(9):1756-61.

29. Cardaropoli S, Todros T, Nuzzo AM, et al. Maternal serum levels and placental expression of hepcidin in preeclampsia. *Pregnancy Hypertens.* 2018; 11:47-53.

Contributors GAB is the principal investigator who designed the study and drafted the article. DV is in charge with the Statistical analysis. IH reviewed and edited the manuscript. All authors read and approved the final manuscript.

Funding This study is financial supported by the «Gottfried und Julia Bangerter-Rhyner Stiftung» und "Freiwillige akademische Gesellschaft."

Competing interests None.

Patient consent Obtained.

Ethics approval The study was approved by the Ethics Committee of Basel (Protocol ID: 2017-02322).

Table 1. **Time and Event Schedule**

Visits	**Enrolment**	**1**	**2**	**3**	**4**
Gestational weeks	**6-10**	**11-14**	**24-28**	**32-36**	**Delivery**
Screen for eligibility Inclusion/exclusion criteria	X				
Written informed consent		X			
Demographic data		X			
Maternal age		X			
Gestational age		X	X	X	X
Gravidity		X			
Parity		X			
Blood pressure		X	X	X	X
Smoking status		X	X	X	X
Weight (kg)		X	X	X	X
Weight gain (kg)			X	X	X
BMI (kg/m^2)		X	X	X	X
Medication		X	X	X	X
Blood examination:					
Haemogram		X	X	X	X
CRP		X	X	X	X
Serum hepcidin		X	X	X	X
Serum ferritin		X	X	X	X
Transferrin receptors		X	X	X	X
Maternal and neonatal outcome					X

BMJ Open

Serum hepcidin and iron status parameters in pregnant women and the association with adverse maternal and foetal outcome: a study protocol for a prospective cohort study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2019-032280.R1
Article Type:	Protocol
Date Submitted by the Author:	13-Aug-2019
Complete List of Authors:	Amstad Bencaiova, Gabriela; University Hospital Basel, Department of Obstetrics; Vogt, Deborah; University of Basel, Clinical Trial Unit Hoesli, Irene; University Hospital, University Basel, Obstetrics
Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Diabetes and endocrinology, Haematology (incl blood transfusion), Nutrition and metabolism
Keywords:	hepcidin, pregnancy, BMI, pregnancy outcome

Serum hepcidin and iron status parameters in pregnant women and the association with adverse maternal and foetal outcome: a study protocol for a prospective cohort study

G Amstad Bencaiova, D R Vogt, I Hoesli

Department of Obstetrics and Antenatal Care, University Hospital Basel, University Basel, Switzerland

Clinical Trial Unit, Department of Clinical Research, University Hospital Basel and University of Basel

Corresponding author: Gabriela Amstad Bencaiova, MD

Department of Obstetrics and Antenatal Care,

University Hospital of Basel

Spitalstrasse 21

CH-4031 Basel

Switzerland

Email: gabriela.amstad@usb.ch

Phone +41 61 556 59 22

Fax +41 61 265 9198

Abstract

Introduction: Hepcidin production is normally upregulated by iron stores, and in obesity has been shown to be overexpressed and correlated with low iron status. The increased hepcidin may restrain the iron release from the cells by affecting the expression of ferroportin, which probably associates with the development of diabetes complication. Firstly, we investigate the difference of serum hepcidin and iron parameters between obese and non-obese pregnant women; secondly, we examine the correlation between serum hepcidin and adverse maternal and neonatal outcomes in pregnant women.

Methods and analysis: This is a mono-centre, prospective cohort study with a study (obese) and a control group (non-obese women). 188 singleton pregnancies will be recruited in the first trimester. Thereof, we expect 75 with a BMI ≥ 30 kg/m^2 and 113 with a BMI 18.5 -- 30 kg/m^2.

Serum hepcidin, iron and haematological parameters will be measured at 11-14, 24-28, 32-36 weeks of gestation and at time of delivery. Blood pressure, weight, BMI and smoking status will be examined at all visits. We will assess the composite endpoints adverse maternal outcome (including pre-eclampsia, gestational hypertension, gestational diabetes, haemorrhage, placenta abruption), and adverse neonatal outcome (preterm birth, intrauterine growth restriction, preterm premature rupture of membranes, Apgar score <7 at 5 minutes, still birth, neonatal death). Recruitment has started in April 2019.

Ethics and dissemination: This study received ethical approval from the ethics committee in Basel. The results of the study will be published in a peer-reviewed journal, and presented at national scientific conferences. It

was registered under http://www.ClinicalTrials.gov (NCT03792464) on 7 January 2019.

Trial registration number: NCT03792464.

Strengths and limitations of this study

For the first time to our knowledge, a prospective study will be performed in order to verify the association between hepcidin and pregnancy outcome.

At the time of planning this study, no data on hepcidin levels of obese pregnant women in the first trimester were available.

A blinded sample size re-estimation could be performed provided hepcidin levels deviate from our assumptions for sample size estimation.

An important limitation of the study may be the size of the study population for the analysis of secondary outcomes since we only powered for our primary outcome.

Our findings could generate the first signs of the potential association between hepcidin and adverse pregnancy outcomes and initiate other powered studies.

Introduction

Obesity is the most common problem in obstetrics that affects both the mother and her offspring.[1] Obesity causes short term and long term problems for the mother, such as increasing her risk of gestational diabetes (GDM) and pre-eclampsia during pregnancy and further increases the risk of developing the metabolic syndrome in later life. The offspring have an increased risk of obstetric morbidity and mortality and, consistent with the developmental origins of health and disease, a long term risk of childhood obesity and metabolic dysfunction.[1]

Obesity is also associated with an increased risk of iron deficiency, attributable to adiposity-related inflammatory mediators on iron regulatory pathways.[2] The pro-inflammatory cytokine interleukin-6 (IL-6), frequently elevated in obesity, has been shown to induce expression of hepcidin[2], a

negative regulator of intestinal iron absorption and macrophage iron efflux. Normal foetal growth and development is dependent upon maternal iron sufficiency during pregnancy. Pregnant women have an increased requirement of iron to support foetoplacental development, expansion of maternal red blood cell mass, and to compensate for intrapartum blood loss; to meet this need, absorption of dietary iron is enhanced concomitant with increased utilization of existing iron stores. During a healthy pregnancy, hepcidin is reduced, enabling iron transfer to the fetus.[3-6] Obesity in pregnancy may lead to hepcidin excess and decreased iron transfer to the fetus.[7-9] Low iron stores are reported to be more common in obese pregnant women.[10] The correlation between serum ferritin and hepcidin has been shown in previous studies.[3,5,8,11,12] Although in other studies, no correlation between serum hepcidin and iron status has been found.[4,6,13] To strengthen the link between the poor iron status of obesity and hepcidin, there is the interesting observation that hepcidin is

expressed not only in the liver but also in adipose tissue and that mRNA expression is increased in adipose tissue of obese pregnant patients.[7] The increased hepcidin may restrain the iron release from the cells by affecting the expression of ferroportin, which probably associates with the development of diabetes complication.[14] Iron content appears to be increased in the subcutaneous and visceral adipose tissue of obese patients, and negatively correlated with adiponectin expression, which could be contributing to insulin resistance and the metabolic complications of obesity.[15]

There are few studies examining the influence of maternal obesity on maternal iron status, with some indicating that iron status may be compromised [7-9,16], and others reporting no impact.[17] However dietary or iron supplementation was not assessed and might be responsible for divergent results. There is no study evaluating the difference of serum

hepcidin and ferritin between obese and non-obese pregnant women longitudinally during pregnancy as well as the association between these parameters and adverse pregnancy outcome.

Methods and analysis

Primary objective

The primary objective is serum hepcidin level (ng/ml) measured at 11-14 weeks of gestation.

Secondary objectives

1. To compare iron status parameters, haemogram and CRP measured in the first trimester between obese and non-obese pregnant women.

2. To describe the course of serum hepcidin, iron status parameters, haemogram, CRP, weight and BMI during pregnancy and to compare them between obese and non-obese pregnant women.

3. To describe the correlations between serum hepcidin, iron status parameters, haemogram, CRP, weight and BMI in each trimester and at delivery.

4. To investigate whether adverse pregnancy outcome is associated with serum hepcidin and iron status in the first trimester and during total pregnancy.

5. To investigate whether adverse neonatal outcome is associated with serum hepcidin and iron status in the first trimester and during total pregnancy.

Study design

This is a mono-centre, prospective cohort study with a study (obese) and a control group (non-obese women). This study protocol was developed on the basis of Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT; see online SPIRIT checklist for further details).

Study settings

The study will be conducted at the University Hospital of Basel, Department of Obstetrics and Antenatal Care with 2800 deliveries/year. The study has started in April 2019. We estimate to recruit all patients within 18 months. A total of N=188 healthy pregnant women (expected 75=40% with BMI ≥ 30 kg/m2) will be recruited in the first trimester in our outpatients' department. Considering a drop-out rate of 10% we will have a total of 169 evaluable women.

Eligible criteria

Inclusion criteria

- Age ≥ 18 years
- Singleton pregnancy
- BMI ≥ 18.5 kg/m^2

- Gestational age at recruitment: 11-14 of gestational weeks
- Written informed consent.

Exclusion criteria

- Foetal genetic, chromosomal or intervention-requiring morphological abnormalities
- Chronic disease of heart, liver, kidney, cardiovascular system, gastrointestinal tract, neurologic, autoimmune, haematological disorders and psychiatric disorders or known infection like hepatitis or HIV
- The inability to read and/or understand the participant's information sheet.

Assessment of primary outcome

The serum hepcidin will be investigated at 11-14, 24-28, 32-36 weeks of gestation and at labour. Blood samples are collected by venepuncture. The measurement of serum hepcidin will be conducted at the University Hospital of Basel, Department of Biomedicine. Serum hepcidin will be measured with an ELISA. No additional visit is necessary besides the standard routine antenatal care visits.

Assessment of secondary outcomes

Serum iron parameters (serum ferritin and soluble transferrin receptors) will be measured at 11-14, 24-28, 32-36 weeks of gestation and at labour. Iron parameters will be determined using routine laboratory methodology. Blood samples will be collected by venepuncture. All blood measurements will be conducted at the University Hospital of Basel, Department of Laboratory Medicine and Department of Biomedicine.

The following maternal outcome will be investigated: pre-eclampsia defined according to the «Report of the American College of Obstetricians and Gynecologists` Task Force on Hypertension in pregnancy»[18], pregnancy induced hypertension[18], infection in pregnancy (urinary tract infection, vaginal infection, chorioamnionitis etc.), anaemia and iron deficiency in pregnancy according to the World Health Organization (WHO) with ferritin cut-off of 15 µg/l[19], cholestasis of pregnancy, gestational diabetes mellitus according to the results of an oral 75g glucose test, mode of delivery, abnormal placentation, placental abruption, thromboembolism in pregnancy and puerperium, peripartum and postpartum haemorrhage, estimated blood loss, anaemia in postpartum, puerperal infection or sepsis, transfusion requirement. Pre-eclampsia, gestational hypertension, GDM, cholestasis of pregnancy, peripartal haemorrhage (increased blood loss peripartal or significant haemoglobin- difference between before and after delivery > 30 g/l),

infections, anaemia and placenta abruption are defined as adverse maternal outcome.

The following neonatal outcome should be investigated: gestational age at birth, birth weight, Apgar score, pH levels, preterm delivery < 37 weeks of gestation, preterm premature rupture of membranes (PPROM), macrosomia with birth weight above 95th percentile, intrauterine growth restriction (IUGR) with birth weight below 5th percentile, low birth weight (LBW) with birth weight below 2500 g, still birth defined according to WHO[20], neonatal death defined according to UNICEF and WHO[21], admissions to the neonatal unit care (NICU). Preterm birth, IUGR, PPROM, macrosomia, Apgar score < 7 at 5, still birth and neonatal death are defined as adverse neonatal outcome.

Participant time line and study procedures

All healthy pregnant patients with regular care at our outpatients' department are counselled and asked at six –10 weeks of gestation to participate. At 10+0 to 13+6 weeks of gestation, all women have to undergo a first trimester ultrasound scan which is standard care. The ultrasound scan is used to confirm gestational

age, diagnose any major foetal abnormalities. All pregnant women that meet in- /exclusion criteria will be recruited in the first trimester in the framework of regular pregnancy visit through physicians, midwifes and study coordinators. Serum hepcidin, iron and haematological parameters will be measured at 11-14, 24-28, 32-36 weeks of gestation and at labour. The blood pressure, weight, weight gain, body mass index (BMI) and smoking status will be examined at all visits as a standard of care. Time and event schedule provides an overview about the schedule of observations and assessments (Table 1).

Table 1. **Time and Event Schedule**

Visits	**Enrolment**	**1**	**2**	**3**	**4**
Gestational weeks	**6-10**	**11-14**	**24-28**	**32-36**	**Delivery**
Screen for eligibility Inclusion/exclusion criteria	X				
Written informed consent		X			
Demographic data		X			
Maternal age		X			
Gestational age		X	X	X	X
Gravidity		X			
Parity		X			
Blood pressure		X	X	X	X
Smoking status		X	X	X	X
Weight (kg)		X	X	X	X
Weight gain (kg)			X	X	X
BMI (kg/m^2)		X	X	X	X

Medication		X	X	X	X
Blood examination:					
Haemogram		X	X	X	X
CRP		X	X	X	X
Serum hepcidin		X	X	X	X
Serum ferritin		X	X	X	X
Transferrin receptors		X	X	X	X
Maternal and neonatal outcome					X

Haematological parameters

This is a routine examination on the visit 1, 2, 3 and 4 haemoglobin (Hb), red blood cell count (RBC), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), percentage of microcytic erythrocytes (MRC), hypochromic red blood cells (HRC) and reticulocyte haemoglobin content (CHr) are measured using a haematology analyser. Mean corpuscular haemoglobin is automatically calculated from Hb and RBC.

Serum ferritin, CRP, hepcidin and soluble transferrin receptors

There are additional examinations on the visit 1, 2, 3 and 4. Ferritin is assessed by chemiluminescence immunoassay and CRP is assessed by immunoturbidimetry. Soluble transferrin receptors and serum hepcidin are measured with an ELISA.

Recording of medications

All medications being continued by a patient on enrolment and all medications given in addition must be documented on the Case report form (CRF) and in the patient`s medical records.

Sample size

Sample size is estimated so that a difference between obese and non-obese pregnant women regarding the primary endpoint- serum hepcidin level in the first trimester- can be shown with 80% power, at a significance level $\alpha = 5\%$.

Sample size estimation is based on the following assumptions:

• The proportion of obese women is expected to be 40%.

• We assume hepcidin levels to be normally distributed with equal variance for obese and non-obese women.

• Based on results reported in Dao et al.[9] for obese women in the second trimester, and assuming these are similar in the first trimester, we assume a mean hepcidin level of 13.5 ng/ml in the first trimester and a standard deviation of $\sigma = 9.0$ ng/ml. Dao et al.[9] report a mean difference of 8.0 ng/ml between obese and non-obese women, while Garcia-Valdes et al.[7] report a mean difference of approximatively 5.0 ng/ml. Here, we aim to show a

difference of 4.0 ng/ml, thus we assume μ = 17.5 ng/ml in non-obese women. A clinical significance of the difference of 4.0 ng/ml will be monitored examining the correlation between serum hepcidin and adverse pregnancy outcomes.

Sample size was calculated using a re-sampling procedure. Each sample size (ni=1,...,71 =20, ..., 300) was evaluated first determining the number of obese women (expected 40%, c.f. above) by sampling 999 times from a binomial distribution with n = ni and probability p = 0.4. Then, 999 times ni observations, were sampled from the above described distributions for non-obese women and obese women, respectively. The difference in each sample between obese and non-obese women was then tested using a two- samples t-test. The null hypothesis was rejected when the difference was statistically significant ($p < 0.05$). Sample size was set to ensure a

significant result in at least 80 % of the cases (power: 1 – β = 0.8), at a significance level α = 5 %.

Supposing to show a hepcidin difference of 4.0 ng/ml, a total of 188 women should be recruited in order to have a total of 169 evaluable women, considering a drop-out rate of 10%. A sample size re-estimation is planned as soon as hepcidin levels of the first trimester of 80% of the initially estimated number of women (i.e. 136 out of 169) will be available (c.f. Interim Analysis). P-values resulting from secondary analyses will not be interpreted as confirmative but will be used to identify hypotheses worth of further investigation.

Data collection

The study data recorded in the CRF will be transferred to a corresponding electronic CRF (e-CRF) by a designated person. The data will be extracted

from clinical records by study nurses and study coordinators. The principal investigator will be responsible for assuring that the data entered into the e-CRF is complete, accurate, and that the entry and updates are performed in timely manner. All information recorded in the e-CRFs will be traceable to the source documents in the patient's file and in the date source files.

The e-CRF will be implemented by the Data management group at the Clinical Trial Unit Basel using the electronic data capture (EDC) software SecuTrial. The EDC system runs on a server maintained by the IT-department of the University Hospital Basel.

Internal data management will be conducted. The data-review and data-handling documents, to be developed during the initiation phase of the study, will include specifications for consistency and plausibility checks on data and will also include data handling rules for obvious data errors.

In compliance with the International Council on Harmonization and GCP guidelines the investigator/institution will maintain all source documents that support the data collected from each patient, and all documents as specified in Essential Documents for the Conduct of a study and as specified by the applicable regulatory requirements. The investigator/institution will take measures to prevent accidental or premature destruction of these documents. Essential documents must be retained until at least 10 years after the last approval. If the responsible investigator retires, relocates, or for other reasons withdraws from the responsibility of keeping the study records, custody must be transferred to a person who will accept the responsibility.

Withdrawal and discontinuation

Patient participation may be terminated prior to completing the Visit 4 for any of the following reason:

- The patient withdraws her informed consent for whatever reason.
- Non cooperative patient, lack of compliance.
- Occurrence of a transient disease, which might distort the results of this study or represents a contraindication.

Premature termination of the study must be agreed upon by the principal investigator and must be documented. Reasons for premature termination include: inadequate recruitment of patients, a protocol violation was identified or developed during the study, failure of responsible investigator to comply with the protocol or GCP guidelines.

Planned analysis

The statistical analysis will be conducted by the Clinical Trial Unit Basel. Detailed methodology for summaries and statistical analyses of the data collected in this study will be documented in a statistical analysis plan. The statistical analysis plan will be finalized before database closure and will be under version control at the Clinical Trial Unit Basel. The Full Analysis Set will consist of all included women for whom serum hepcidin level in the first trimester (11-14 weeks of gestation) is available. Demographics and baseline characteristics will be reported for obese and non-obese woman separately and taken together.

Primary analysis

The primary objective is to compare the primary endpoint between obese and non-obese pregnant women. If data are approximatively normally distributed, a two-sample t-test will be applied. Otherwise, data

transformation (e.g. log), the use of a generalized linear model (GLM) with a Poisson error distribution, or the use of a non-parametric Wilcoxon two-sample rank test will be considered.

Secondary analyses

Secondary endpoints measured in the first trimester (secondary objective 1) will be compared between obese and non-obese pregnant women as described for the primary endpoint. The course of the secondary endpoints during pregnancy (secondary objective 2) will be compared between obese and non-obese pregnant women using a random slope / random intercept linear mixed effects models for each endpoint. The model will include the respective secondary endpoint as dependent variable, study group (obese vs. non-obese), gestation week (continuous) and the interaction between study group and gestation week as fixed effects, and

woman (patient ID) as random effect with random slope and random intercept. In case of an obvious non-linear time course, time will be included as categorical variable (time point: first, second and third trimester and delivery) instead, and a random intercept model will be applied (thus no random slope).

The correlations between secondary endpoints measured during the course of pregnancy (secondary objective 3) will be described by means of scatterplots and Spearman's rank correlation coefficient for each trimester and at delivery separately. Adverse maternal outcome and adverse neonatal outcome will be analysed for an association with the listed secondary endpoints in the first trimester using logistic regression (secondary objectives 4 and 5). As a first step, univariate models will be fit. Each model will include the respective binary endpoint as dependent variable, and one of the secondary endpoints measured in the first

trimester as explanatory variable. In order to test for a combined effect of the secondary endpoints, in a second step, multivariable logistic regression models will be fit. A forward model selection approach will be applied, based on Akaike's information criterion (AIC). Starting with the "null model" - the simplest model, including no explanatory variable - single predictor variables (i.e. secondary endpoints) will repeatedly be added if and as far as AIC can be decreased. This will result in a final model that includes the set of variables that (together) best describe the outcome. (A forward model selection approach is chosen, due to the relatively large number of predictors of interest in comparison to the expected number of events.) For each statistical model, odds ratios will be presented for each explanatory variable with 95% confidence intervals and p-values. Further, the frequency of each composite will be given for obese and non-obese women.

Interim analysis

The estimation of the variance (or the distribution in general) of hepcidin levels is crucial for determining the sample size of the study, but hepcidin levels observed in the study may deviate considerably from our assumptions. We will therefore re-estimate the sample size as soon as hepcidin levels of the first trimester of 80 % of the initially estimated number of women (i.e. 136 out of 169) will be available. We will re-estimate the variance σ2 obese and σ2 non–obese in a blinded manner – only the hepcidin levels of the third trimester will be available to the statistician performing the interim analysis. The overall variance σ2 will be estimated (one-sample variance estimator), assuming σ2 = σ2 obese = σ2 non–obese. Since no hypothesis test is performed, no p-value adjustment to control type I error is needed. Using the re-estimated standard deviation,

the sample size N' will be re-estimated as before. If there should be clear evidence against a normal distribution of hepcidin levels (based on visual inspection of quantile-quantile plots), a t-test based on transformed data or a non-parametric Wilcoxon two-sample rank test will be used. The sample size will be increased in order to include N' evaluable patients whenever N' > N, preserving a power of 80 %. A sample size reduction or early stopping of the study will not be considered.

Deviations from the original statistical plan

If substantial deviations of the analysis as outlined in these sections are needed for whatever reason, the protocol will be amended. All deviations of the analysis from the protocol or from the detailed analysis plan will be listed and justified in a separate section of the final statistical report.

Handling of missing data

Presumably, there may be reasons for drop outs confounded with the measurements. For example, some composites of adverse maternal and neonatal outcome will lead to a preterm end of pregnancy, which will result in missing measurements after the first trimester. These data will be missing not at random. It is important to keep these drop outs in the analysis set regarding secondary objectives 4 and 5. In case there will be many such drop-outs (i.e. missing not at random), inverse probability of censoring weights (IPCW) will be considered, and a sensitivity analysis with a complete case analysis set will be performed.

Missing data and drop-outs will be handled using the method most appropriate, based on an examination of missing values before data base closure. Details will be specified in the statistical report and analysis plan.

The number of missing values in each endpoint and the number of drop-outs will be summarized for obese and non-obese women.

Confidentiality and Coding

Project data will be handled with uttermost discretion and is only accessible to authorized personnel who require the data to fulfil their duties within the scope of the research project. On the CRFs and other project specific documents, participants are only identified by a unique participant number. Biological material in this project is not identified by participant name but by a unique participant number.

Biological material and health-related personal data will be coded. The coding key will be located by the investigator and the members of her research team. Access to this key will only have the investigator and the members of her research team. Biological material is appropriately stored

in a restricted area only accessible to authorized personnel. Data generation, transmission, storage and analysis of health related personal data and the storage of biological samples within this project will follow strictly the current Swiss legal requirement for data protection and will be performed according to the HRO Art. 5. Health related personal data captured during this project and biological samples from participants are strictly confidential and disclosure to third parties is prohibited; coding will safeguard participants` confidentiality. Only the investigator and members of her research team will have access to project plan, dataset, statistical code etc. during and after the research project.

Retention and destruction of study data and biological material

The investigator/institution will take measures to prevent accidental or premature destruction of these documents. Essential documents must be

retained until at least 10 years after the last approval. After measurement, biological material will be destroyed at the in University Hospital of Basel, Department of Laboratory Medicine.

Data sharing

After publication of the study individual anonymous participant data including variable keys will be available upon request by the corresponding author. Researchers may request data to repeat the analyses or use the data for secondary analyses (e.g. systematic review and meta-analysis).

Patient and public involvement

Patients and the public were neither involved in developing the hypothesis, the specific aims or the research question, nor were they involved in developing plan for design or implementation of the study.

Discussion

Obesity is a low-grade chronic inflammatory condition linked to the overexpression of hepcidin and secondarily to iron homeostasis. Increased hepcidin in obese pregnant women without gestational diabetes mellitus was shown in previous studies.[7, 9, 22, 23] Obesity- related inflammation may induce hepcidin biosynthesis with a consequent reduction of iron supply.[7, 24] Anelli et al. showed lower haemoglobin concentration in obese pregnant women.[22] Interestingly, anaemia was not related to the increase in hepcidin, while this was positively associated with maternal BMI.[22]

Few studies have evaluated the influence of obesity on hepcidin and iron status during pregnancy, but their results are inconclusive. Whereas increased hepcidin in obese pregnant women was shown in previous studies, [7, 9, 22, 23] it has not been confirmed in another studies.[25, 26] Due to

previous data, we expect to observe an increase in the serum hepcidin in obese pregnant women, consequentially increased soluble transferrin receptors as a marker for iron deficiency in cells and a lower level of haemoglobin because of iron deficiency.

It is unclear which obese pregnant women develop during pregnancy complications such as gestational diabetes, pregnancy induced hypertension, pre-eclampsia, intrahepatic cholestasis, intrauterine growth restriction etc. Increased serum hepcidin could indicate the increased risk of adverse maternal and neonatal outcome. Chen et al showed that increased hepcidin restrains the iron release from the cells by affecting the expression of ferroportin, which probably associates with the development of diabetes complication.[14] High ferritin in the first trimester was also associated with GDM.[27, 28]

The role of hepcidin in pre-eclamptic pregnant women is unclear. Hepcidin levels in the first half of pregnancy were found significantly higher in women who subsequently developed pre-eclampsia compared to mothers having a physiological pregnancy until term.[29] Reason could be due to an inflammatory condition that characterizes the pre-eclamptic syndrome long before the symptoms onset. The identification of women at elevated risk of pre-eclampsia may be sufficiently early to allow the prophylactic use of low-dose aspirin, which has been demonstrated to reduce the prevalence of pre-eclampsia when started before 16 weeks` gestation.

For the first time to our knowledge, a prospective study will be performed in order to verify the potential association between hepcidin in obese women and pregnancy outcome such intrahepatic cholestasis of pregnancy, haemorrhage, intrauterine growth restriction etc. However, an important limitation of this study may be the size of the study population.

We only powered for our primary outcome, not for secondary analyses. On the other hand, our findings could generate the first signs of the potential association between hepcidin and adverse pregnancy outcomes. In conclusion, we will contribute new information regarding the effects of obesity on iron and hepcidin levels. Our findings will improve the understanding of hepcidin and iron metabolism in obese pregnant women and provide direction for maternal and foetal complications. Our results could initiate planning and conducting of powered studies to verify the potential association between hepcidin and adverse pregnancy outcomes. Acknowledgement of this hypothesis may conduce to establish new promising biomarkers and possible life care intervention, aimed at reducing the obesity-related risks in pregnancy.

Ethics and dissemination

This study received ethical approval from the ethics committee in Basel (Project-ID: 2017-02322). It was registered under http://www.ClinicalTrials.gov (NCT03792464) on 7 January 2019. All members of the research team are aware of the guidelines for good clinical practice for obtaining consent. The principal investigator must ensure that all ethical and legal requirements have been met before the first patient is enrolled in the study. This protocol is to be followed exactly. To alter the protocol, amendments must be written, receive approval from the appropriate personnel, and receive Ethics Committee approval prior to implementation. Administrative changes (not affecting the patient benefit/risk ratio) may be made without the need for a formal amendment. Before each patient is admitted to the study, signed informed consent will be obtained from the patient according to the regulatory and legal

requirements. This consent form must be dated and retained by the investigator as part of the study records. The informed consent should be in accordance with the current revision of the Declaration of Helsinki, current International Conference on Harmonization (ICH) and Good Clinical Practice (GCP) guidelines.

The physicians and study coordinators must explain to potential participants the aims, methods, reasonably anticipated benefits of this study. Patients will be informed that they are free not to participate in the study and that they may withdraw consent to participate at any time. They will be told that competent authorities may examine their records and authorized persons but that personal information will be treated as strictly confidential and will not be publicly available. Patients must be given the opportunity to ask questions. After this explanation and before entry into

the study, consent should be appropriately recorded by means of the patient`s dated signature.

If a protocol amendment is required, the informed consent form may need to be revised to reflect the changes to the protocol. If the consent form is revised, it must be approved and signed by all patients subsequently enrolled in the study.

The findings of this study will be published in a peer-reviewed journal, and presented at national scientific conferences, to disseminate the results to academic and health professional audiences, and made available to participants and to a wider public on our website at the time of publication.

References

1. Catalano PM, Shankar K. Obesity and pregnancy: mechanisms of short term and long term adverse consequences for mother and child. *BMJ* 2017 Feb 8; 356:j1. doi: 10.1136/bmj.j1. Review.
2. Flynn AC, Begum S, White SL, et al. Relationships between maternal obesity and maternal and neonatal iron status. *Nutrients* 2018 Jul 30; 10(8). pii: E1000. doi: 10.3390/nu10081000.
3. van Santen S, Kroot JJ, Zijderveld G, et al. The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. *Clin Chem Lab Med* 2013; 51(7):1395-401.
4. Hedengran KK, Nelson D, Andersen MR. Hepcidin levels are low during pregnancy and increase around delivery in women without iron deficiency - a prospective cohort study. *J Matern Fetal Neonatal Med* 2016; 29(9):1506-8.

5. Kulik-Rechberger B, Kosciesza A, Szponar E, Domosud J. Hepcidin and iron status in pregnant women and full-term newborns in first days of life. *Ginekol Pol* 2016; 87(4):288-92.

6. Simavli S, Derbent AU, Uysal S, Turhan NÖ. Hepcidin, iron status, and inflammation variables among healthy pregnant women in the Turkish population. *J Matern Fetal Neonatal Med* 2014; 27(1):75-9.

7. Garcia-Valdes L, Campoy C, Hayes H, et al. The impact of maternal obesity on iron status, placental transferrin receptor expression and hepcidin expression in human pregnancy. *Int J Obes (Lond)* 2015; 39(4):571-8.

8. Nazif HK, El-Shaheed AA, El-Shamy KA, et al. Study of serum hepcidin as a potential mediator of the disrupted iron metabolism in obese adolescents. *Int J Health Sci (Qassim)* 2015; 9(2):172-8.

9. Dao MC, Sen S, Iyer C, et al. Obesity during pregnancy and fetal iron status: is Hepcidin the link? *J Perinatol* 2013; 33:177-81.

10. Bekri S, Gual P, Anty R, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. *Gastroenterology* 2006; 131:788-796.

11. Schulze KJ, Christian P, Ruczinski I, et al. Hepcidin and iron status among pregnant women in Bangladesh. *Asia Pac J Clin Nutr* 2008; 17(3):451-56.

12. Finkenstedt A, Wildschwendter A, Brasse-Lagnel CG, et al. Hepcidin is correlated to soluble hemojuvelin but not to increased GDF15 during pregnancy. *Blood Cells Mol Dis* 2012; 48:233-37.

13. Chelchowska M, Swiatek E, Ambroszkiewicz J, et al. [Concentrations of pro-hepcidin in serum of pregnant women and in umbilical cord blood]. *Ginekol Pol* 2008; 79(11):754-7.

14. Chen Y, Yin HQ, Liu HL, et al. Hepcidin and iron metabolism in non-diabetic obese and type 2 diabetic rats. *J Huazhong Univ Sci Technolog Med Sci* 2015; 35:851-857.

15. Pihan-Le Bars F, Bonnet F, Loréal O, et al. Indicators of iron status are correlated with adiponectin expression in adipose tissue of patients with morbid obesity. *Diabetes Metab* 2016; 42:105-11.

16. Cepeda-Lopez AC, Melse-Boonstra A, Zimmermann MB, Herter-Aeberli I. In overweight and obese women, dietary iron absorption is reduced and the enhancement of iron absorption by ascorbic acid is one-half that in normal-weight women. *Am J Clin Nutr* 2015; 102:1389-97.

17. Yanoff LB, Mentie CM, Denkinger B, et al. Inflammation and iron deficiency in the hypoferremia of obesity. *Int J Obes (Lond)* 2007; 31:1412-1419.

18. The American College of Obstetricians and Gynecologists. Hypertension in Pregnancy. *Task Force on Hypertension in pregnancy* 2013.

19. World Health Organization. Iron Deficiency Anaemia. Assessment, Prevention, and Control. *A guide for programme managers* 2001.

20. World Health Organization. Neonatal and perinatal mortality country, regional and global estimates. *Country, Regional and Global Estimates* 2006.

21. UNICEF, WHO, The World Bank. Levels and trends in child mortality. *Report* 2014.

22. Anelli GM, Cardellicchio M, Novielli C, et al. Mitochondrial content and hepcidin are increased in obese pregnant mothers. *J Matern Fetal Neonatal Med* 2018; 18:2388-2395.

23. Cepeda-López AC, Aeberli I, Melse Boonstra A, et al. Does obesity increase risk for iron deficiency? A review of the literature and the potential mechanisms. *Int J Vitam Nutr Res* 2010; 80:263-70.

24. Rehu M, Punnonen K, Ostland V, et al. Maternal serum hepcidin is low at term and independent of cord blood iron status. *Eur J Haematol* 2010; 85(4):345-52.

25. Cao C, Pressman EK, Cooper EM, et al. Pre-pregnancy body mass index and gestational weight gain have no negative impact on maternal or neonatal iron status. *Reprod Sci* 2016; 23:613-22.

26. Jones AD, Zhao G, Jiang Y., et al. Maternal obesity during pregnancy is negatively associated with maternal and neonatal iron status. *Eur J Clin Nutr* 2016; 70(8):918-24.

27. Rawal S, Hinkle SN, Bao W, et al. A longitudinal study of iron status during pregnancy and the risk of gestational diabetes: finding from a prospective, multiracial cohort. *Diabetologia* 2017; 60(2):249-57.

28. Bowers KA, Olsen SF, Bao W, et al. Plasma concentrations of ferritin in early pregnancy are associated with risk of gestational

diabetes mellitus in women in the Danish National Birth Cohort. *J Nutr* 2016; 146(9):1756-61.

29. Cardaropoli S, Todros T, Nuzzo AM, et al. Maternal serum levels and placental expression of hepcidin in pre-eclampsia. *Pregnancy Hypertens.* 2018; 11:47-53.

Contributors GAB is the principal investigator who designed the study and drafted the article. DV is in charge with the Statistical analysis. IH reviewed and edited the manuscript. All authors read and approved the final manuscript.

Funding This study is financial supported by the «Gottfried und Julia Bangerter-Rhyner Stiftung» und “Freiwillige akademische Gesellschaft."

Competing interests None.

Patient consent Obtained.

Ethics approval The study was approved by the Ethics Committee of Basel (Protocol ID: 2017-02322).

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1, 3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5, 6
Objectives	3	State specific objectives, including any prespecified hypotheses	7
Methods			
Study design	4	Present key elements of study design early in the paper	7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	8, 9
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	9, 10, 11
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*11, 12, 13*
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	13
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	17-19
		(*b*) Describe any methods used to examine subgroups and interactions	18
		(*c*) Explain how missing data were addressed	20
		(*d*) If applicable, explain how loss to follow-up was addressed	20
		(*e*) Describe any sensitivity analyses	19, 20
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	It is a study protocol
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	24
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	25
Generalisability	21	Discuss the generalisability (external validity) of the study results	26
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	34

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.