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Endometrial factors in the implantation failure spectrum: protocol of a MUltidisciplinary observational cohort study in women with Repeated Implantation failure and recurrent Miscarriage (MURIM Study)

Linda Stevens Brentjens [ID] ,[1,2] Denise Habets,[1,2,3] Janneke Den Hartog,[1,2] Salwan Al-Nasiry,[1,2] Lotte Wieten,[2,3] Servaas Morré,[2,4] Aafke Van Montfoort,[1,2] Andrea Romano,[1,2] Ron van Golde[1,2]

ABSTRACT

Introduction Women with repeated implantation failure (RIF) and unexplained recurrent miscarriage (RM) are proposed to be at opposite ends of the implantation spectrum, with RM representing an overly receptive endometrium (implantation of genetically aberrant or poor-quality embryos) versus RIF representing an overly selective endometrium (no implantation even with good quality embryos). In both cases, often no explanation for reproductive failure can be found and although promising add-on treatments have been introduced, therapeutic options are frequently limited to supportive care. Both RM and RIF are multifactorial and research indicates that the interplay between steroidogenesis, uterine natural killer (uNK) cells and the microbiome determine the capacity of the endometrium to be a biosensor for invading embryos. Our objective is to elucidate whether there is a difference in endometrial receptivity parameters (ie, steroid metabolism, uNK cells and the microbiome) between women aged 18–38 years with reproductive failure (RIF and RM), and fertile controls.

Methods and analysis Single-centre, observational cohort study. Endometrial biopsies, vaginal swabs and peripheral blood will be collected during the window of implantation and menstrual blood in the subsequent menstruation. The study parameters are the steroid profile (steroid levels and mRNA levels, protein expression and activity of steroid enzymes) in endometrial tissue and peripheral blood, as well as the activating or inhibitory phenotype of uNK cells based on receptor expression in menstrual blood and endometrial tissue and determination of the vaginal and endometrial microbiome using the inter spacer bacterial profiling technique.

Ethics and dissemination The protocol is approved by the local medical ethical review committee at the Maastricht University Medical Centre. Findings from this study will be shared with the academic and medical community and the patient organisations to optimise and individualise medical care of patients with implantation failure and miscarriages.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This study will provide novel, important information for patients diagnosed with implantation failure and miscarriages, and for clinicians to consider new therapeutic approaches.
⇒ Embryo implantation will be studied in the most extensive way throughout the whole implantation spectrum, comparing data from patients with repeated implantation failure (RIF) to data from patients with recurrent miscarriage (RM) and fertile controls.
⇒ Endometrial intracrinology, the microbiome and reproductive immunology will be assessed to determine their contribution to endometrial receptivity and selectivity.
⇒ The absence of data on chromosomal abnormalities in the previously transferred embryos in these patients might be a limitation as this is thought to be a contributing factor in patients with RIF and RM.

INTRODUCTION

Reproductive failure is a condition in which couples experience involuntary childlessness despite their effort for conceiving. Recurrent failure to conceive can either imply a condition in which embryos repeatedly fail to implant (non-receptive or overly selective) or a condition in which embryos repeatedly implant, but pregnancies end in miscarriage (non-selective or overly receptive). Both outcomes represent an enormous emotional burden for couples with the desire of having children.[1] Although several factors involved in

these clinical entities have been identified, in most cases the underlying causes and mechanisms are unknown and treatment options are therefore scarce.

Repeated implantation failure (RIF) is defined by failure of implantation after three transfers of high-quality embryos or after placement of 10 or more embryos in multiple transfers regardless of the quality in in vitro fertilisation (IVF/ICSI) cycles.[2] When excluding factors known to have a negative impact on embryo implantation such as maternal age (over 38 years of age) and poor ovarian response, the remaining incidence of RIF is around 4% of IVF couples.[3] Recurrent miscarriage (RM), defined as the loss of two or more pregnancies before 20 weeks of gestation, affects 1% of couples trying to conceive.[4] Factors that contribute to the risk of RM are advanced maternal age, previous miscarriages, obesity (body mass index (BMI)>30 kg/m^2), antiphospholipid syndrome, parental chromosomal anomalies, uterine malformations, diabetes mellitus, thyroid disease and both inherited and acquired thrombophilia. However, over 50% of RM cases remain unexplained. In these women, no therapeutic options other than supportive care are available until and in a subsequent pregnancy.[4]

Reproductive failure may have certain common features that are important to control reproductive functions. Hormonal regulation by steroids, mainly ovarian oestrogen and progesterone, influences gene expression in endometrial cells and the balance between steroids is fundamental for successful decidualisation, implantation and pregnancy maintenance. Alterations in oestrogen and progesterone levels and their receptors during the luteal phase change endometrial receptivity.[5] In addition, the endometrium is able to modify and fine-tune blood-derived steroids intracellularly to create the optimal endocrine milieu for its functions. Local steroid regulation, known as intracrinology, is controlled by steroidogenic enzymes that activate or deactivate oestrogen and other circulating steroids.[6 7]

As has recently been extensively reviewed by Brosens et al, the endometrium provides an implantation checkpoint which should be passed, to limit the risk of a maternal investment in failing pregnancy.[8] A complex interplay between steroidogenesis, uterine natural killer (uNK) cells and the microbiome capacitates this biosensor role of the endometrium. Rising progesterone levels during the luteal phase lead to a dramatic increase in uNK cell numbers, causing uterine NK cells to become the predominant type of leucocyte in the uterus during implantation.[9] In this phase, oestrogen primed decidual cells undergo inflammatory stress creating progesterone resistant senescent cells vulnerable for breakdown (and subsequent menstruation). However, co-operation of progesterone-dependent decidual cells and uNK cells enable transformation of the stroma towards a decidual matrix receptive for implantation.

Furthermore, oestrogen generation regulates immune cell function during endometrial remodelling towards a receptive endometrium, where immune cells have to create a tolerant immunological environment for the semiallogenic embryo invading the maternal tissue. However, immune cells are not only regulated by steroids such as progesterone and oestrogen. The identification of bacterial RNA (16S rRNA) in the endometrium has fuelled the notion that the uterus is nonsterile, contrary to what has previously been thought.[10] These microbial species, known as the microbiome, constitute an additional player in the complex uterine environment leading towards successful implantation. It is suggested that the vaginal microbiome reflects an equilibrium in immunotolerance and nutritional environment of the endometrium and thereby is a 'proxy' for endometrial receptivity.[11] Recently, it was found that determining the vaginal microbiota composition enables stratification of the chance of becoming pregnant prior to the start of an IVF/ICSI treatment with high specificity. The validity of the model was shown in an external validation cohort, in which none of the women with an unfavourable profile became pregnant in the subsequent cycle.[12]

In the present study, we hypothesise that in patients with RIF and RM, the role of the endometrium as a biosensor in the implantation checkpoint is altered, and that steroid regulation, uNK cell functioning and the microbiome are aberrantly regulated when compared with controls. To the best of our knowledge, this is the first time that the complex interplay between the systemic and the endometrial endocrine milieus, the uNK cells and the microbiome composition are studied in the full reproductive failure spectrum of patients with RIF and RM to ultimately bring therapeutic approaches closer to the patient.

METHODS AND ANALYSIS
Study objective and design
The aim of this single-centre observational cohort study is to explore the differences in endometrial receptivity parameters between women with reproductive failure (RIF and RM, cases) and fertile controls. Women referred to the department of Obstetrics and Gynaecology of the Maastricht University Medical Centre+ (MUMC+) for RIF or RM treatment will be included as study groups. Women referred for IVF or ICSI treatment because of a severe male factor (Semen Volume x Concentration x Motility (VCM) <1), bilateral tubal factor or preimplantation genetic testing (PGT) without concurrent subfertility will serve as a control group. After informing possible participants, they sign an informed consent form to confirm that they are willing to participate in the study.

Endometrial biopsies, vaginal swabs and peripheral blood will be collected during the window of implantation in a natural cycle (figure 1). Menstrual blood will be self-collected at home during the subsequent menstruation. All women will have a follow-up period of 12 months after inclusion in which pregnancy results will be monitored.
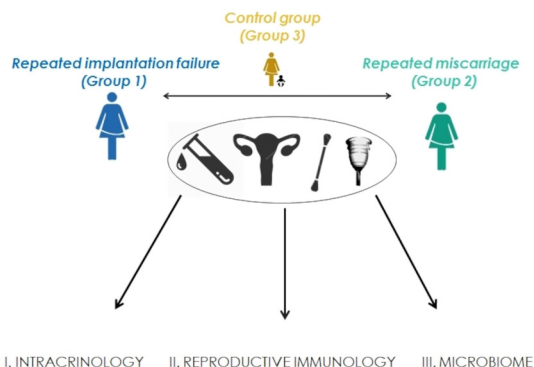
Figure 1 Study design. A single blood draw, endometrium biopsy and vaginal swab will be performed in three groups of patients (repeated implantation failure, repeated miscarriage and a control group of healthy parous women or women with presumed fertility). Menstrual blood will be collected in the subsequent menstruation.

Primary objectives

The primary objective of this study is to elucidate whether there is a difference in endometrial receptivity parameters in women with reproductive failure (RIF and RM), and fertile controls. The three endometrial receptivity parameters that are studied are:

1. The steroid profile: 17β-estradiol, oestrone, progesterone, pregnenolone, 17OH-pregnenolone, 17OH-progesterone, dehydroepiandrosterone, androstenedione, testosterone, androsterone, dihydrotestosterone, 21OH-progesterone, 11-deoxycortisol, cortisol, cortisone, aldosterone and corticosterone. The steroid profile will be determined in endometrial tissue and peripheral blood by using liquid chromatography mass spectrometry (LC-MS).[13 14] In addition, mRNA levels, protein expression and the activity of enzymes involved in local steroid metabolism of endometrial tissue will be determined by RNA-sequencing, immunohistochemistry and high-performance liquid chromatography (HPLC).
2. The activating or inhibitory phenotype of NK cells based on receptor expression in menstrual blood and endometrial tissue.
3. The vaginal and endometrial microbiome using the inter spacer bacterial profiling technique.

Secondary objectives

Baseline parameters that will be collected by a questionnaire are age, length, weight, ethnicity, alcohol, drugs, nicotine use, use of vitamins, diet specifics, use of antibiotics, medication, illness, menstrual cycle data, previous conceptions, duration of infertility, type of infertility, own birth weight and own gestational age. In the 12-month follow-up period, data on clinical pregnancy rate, implantation rate and live birth rate will be collected.

Sample size

This is a pilot study that compares the steroid profile, immune cell population and microbial composition in three study groups (RIF, RM and controls). The novelty of this study and its explorative design makes it difficult if not impossible to define what would be a clinically relevant difference to detect. Therefore, we aim to include as many patients as possible during a fixed period of time, namely between April 2019 and December 2022. Based on data of previous years, we expect approximately 150–200 inclusions in total.

Study population and recruitment

Patients with RIF will be recruited at the outpatient clinic at the Centre for Reproductive Medicine of the MUMC+, in which approximately 550 couples per year undergo either IVF, ICSI or PGT. They can also be recruited at our RIF clinic after referral by other Dutch fertility centres. Due to the previously observed willingness of patients to contribute to scientific studies on implantation failure, it is expected that the majority of eligible patients is willing to participate. Previous studies on endometrial biopsies have shown that the procedure is safe and might increase pregnancy chances (SCRaTCH Study), which could further contribute to willingness of patients.[15] Inclusion criteria are female age between 18–38 years, with primary or secondary infertility, written informed consent and RIF defined as: failure of implantation after three transfers of high-quality embryo's or after placement of 10 or more embryos in multiple transfers irrespective of embryo quality. Exclusion criteria are clinically relevant intrauterine pathologies, BMI>35 kg/m^2, untreated endocrine abnormalities and PGT treatment.

Patients with RM will be recruited at the outpatient clinic of Obstetrics and Gynaecology of the MUMC+, in which approximately 75 couples per year receive specialised investigations and care for RM. It is expected that the majority of eligible patients is willing to participate, due to the previously observed willingness of patients to contribute to other scientific studies. Females aged 18–38 years old with unexplained RMs, defined as two or more miscarriages not caused by abnormal parental karyotype, maternal thrombophilia and/or uterine abnormalities, will be included. Exclusion criteria are current or recent (<3 months ago) pregnancy, breastfeeding or current hormonal contraceptive use, current symptomatic genital infection, BMI>35 kg/m^2 and severe endometriosis (3th–4th stage).

The control group will be recruited at the Centre for Reproductive Medicine of the MUMC+ if they are referred for IVF or ICSI treatment because of a severe male factor (VCM<1), bilateral tubal factor or PGT without concurrent subfertility and are aged between 18 and 38 years old. Women with an uncomplicated previous pregnancy, defined as no preterm delivery, preeclampsia or fetal growth restriction and live birth, are also suitable as controls. Exclusion criteria are a history of RM or RIF, current or recent (<3 months ago) pregnancy, breastfeeding or current hormonal contraceptive use, BMI>35 kg/m^2 and severe endometriosis (3th–4th stage).

Study procedure

The study procedures will be performed 5–8 days after the luteinizing hormone (LH) surge in a natural cycle (luteal phase). The LH surge will be monitored by urine LH testing done daily from approximately cycle day 10 onwards (dependent on cycle length). The tests will be done by the subjects at home.

Peripheral and menstrual blood sampling

A single blood draw will be performed on the day of the endometrial biopsy. For immunology, lymphocytes will be directly isolated from peripheral blood and examined using flow cytometry. For steroid metabolism (intracrinology), serum and plasma are obtained and stored at −80°C. Buffy coat will also be harvested and used for genomic DNA isolation and analyses. Standardised protocols and operating procedures (as recommended by World Endometriosis Research Foundation[16]) will be used to ensure high-quality materials and reproducibility of our results.

Additionally, participants will be asked to collect her menstrual blood during the consecutive menstrual cycle after endometrial biopsy using the Organicup menstrual cup during one 8-hour shift. Patients will be asked to bring the cup to the hospital within 1 day after collection. From the menstrual blood, lymphocytes will be directly isolated and examined using flow cytometry.

Endometrial biopsy

An endometrial biopsy (Pipelle) will be collected by an experienced fertility doctor or gynaecologist. Women will receive clear instructions that they need to ensure they are not pregnant in the cycle during which the biopsy will be obtained. After exposing the uterine cervix and extensive cleaning with sterile water, the endometrial biopsy catheter will be gently introduced through the cervix up to the uterine fundus. The piston will then be drawn back to the end of the biopsy cannula, creating a negative pressure. To cover the entire endometrium, the examiner will apply a slow retraction of the device, while rotating the endometrial biopsy catheter over several ranges of 360°. The procedure will be performed during a maximum of 1–2 min. The obtained tissue will be divided into four parts to allow diverse handling of tissue for further storage. Two tissue parts will be snap-frozen in liquid nitrogen for further storage at −80°C for later determination of the intracrine profile and microbiome. One part is cultured as organoid and stored (biobanking) for future research. One part will be processed for formalin fixation and paraffin embedding (FFPE) or if possible it will directly be used for lymphocyte isolation and flow cytometry analysis.

Vaginal swab

Vaginal swabs for microbiome analyses will be collected by insertion of a Copan flocked swab 3–5 cm beyond the vaginal orifice. The swab will then be moved around the vaginal wall for 10–15 s. After this procedure, the swabs will immediately be placed in reduced transport fluid buffer and stored until further processing in a freezer at −80°C. For patients with RIF, an extra vaginal swab will be collected and stored in Eppendorf tubes filled with eNAT buffer. This eNAT buffer is a buffer conserving nucleic acid for up to 30 days at room temperature.

Data collection

Local steroid metabolism: intracrinology

Steroid profiles in plasma and endometrial tissue will be determined using validated multiplex protocols to assess simultaneously 18 steroids.[13 17–19] These methods are based on LC-MS/MS, which is currently recommended as the gold standard by the Endocrine Society to determine steroid levels, hence, the methods are sensitive, specific, robust and reproducible.[20]

Protein expression of intracrine enzymes, inflammatory and window of implantation markers will be assessed by immunohistochemistry in house using published protocols.[21 22] Total RNA isolated from frozen biopsies will be analysed by global RNA-sequencing to determine mRNA levels of intracrine enzymes. Activity of intracrine enzymes will be determined by HPLC.

Immunology

Lymphocytes will be isolated from peripheral and menstrual blood by density gradient centrifugation. Expression of 20 activating and inhibitory NK cell receptors will be examined by fluorescent surface staining. For surface staining, cells will be washed with fluorescence-activated cell sorting (FACS) buffer (Phosphate-Buffered Saline, 1% Flow Cytometry Staining Buffer) and stained with receptor corresponding fluorescent markers for 30 min on ice in the dark. Flow cytometric analyses will be performed with BD FACS Canto II and data will be analysed with BD FACSDiva Software in order to determine % of NK cells with an activating and inhibitory phenotype based on receptor expression. If flow cytometry is not possible on endometrial tissue due to logistical constraints, the tissue will be processed for FFPE for immunohistochemical analysis of the NK cells.

Microbiome

Microbiome analysis will be performed on frozen endometrial specimens and vaginal swabs.

First, DNA extraction of the vaginal swabs and endometrial specimens will be performed with the Chemagen machine (Chemagen, Baesweiler, Germany) according to the manufacturer's instructions. Amplification of the intergenic spaces (IS) regions will be performed with the IS-pro assay, according to the protocol provided by the manufacturer (inBiome, Amsterdam, the Netherlands). IS-pro is an eubacterial technique based on the detection and categorisation of the length of the 16S-23S rRNA gene IS region. The length and the number of this IS region is specific for each bacterial species. Phylum-specific fluorescently labelled PCR primers will be used for taxonomic classification.[23] Data will be analysed with the IS-pro

proprietary software suite (inBiome, Amsterdam, The Netherlands), and the results will be presented as bacterial profiles. The composition of the microbiome will be specified for among others the following bacteria: *Lactobacillus crispatus, L. Jensenii, L.* spp*, L. iners, Gardnerella vaginalis IST1, proteobacteria* and other. Detailed information on data collection has previously been reported.[12]

Statistical analysis

SPSS statistics V.24 (IBM corp, Armonk, New York, USA) and Excel will be used to perform the statistical analysis. Descriptive analyses will be used to describe outcome variables in the different groups. Data distribution will be evaluated on normality with histograms and the Kolmogorov-Smirnov test. Differences between the three study groups will be analysed by χ^2 tests. Continuous data will be tested with the ANOVA test or Kruskal-Wallis test depending on whether or not data are normally distributed. Continuous, normally distributed variables will be presented as mean with SD, and variables with a skewed distribution as median with the range. Categorical variables will be presented as count and proportions. A probability (p) value of less than 0.05 will be considered statistically significant. The relative contribution of confounding factors of implantation failure, such as embryo quality, age, smoking, body mass index, single versus double embryo transfer and cause of subfertility will be assessed for the individual outcome parameters by univariate and multivariate logistic regression analysis. RNA-sequencing datasets will be analysed using standard bioinformatics and biostatistics methods in 'R' (differentially regulated genes, gene ontology, pathway analyses).

Data management

Data will be collected in an Access database by the coordinating investigator, research nurse and trained research assistant. Data handling will be done anonymously. Missing data will be mentioned along with the specific reason. If the participant did not have her treatment or checks in the MUMC+, a questionnaire will be sent to the participant 12 months after the date of written informed consent to obtain pregnancy results. Data will be preserved for 15 years in compliance with the Dutch Personal Data Protection Act (in Dutch: De Wet Bescherming Persoonsgegevens). There are no restrictions in data access for trial investigators.

Adverse events

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the trial procedure. The end of the study is defined as 1 month after the last study visit. The follow-up period of 1 year and the questionnaire that will be sent after 1 year are not considered as being part of the study period. (Serious) adverse events ((S)AEs) should be reported for all events during the study and 1 months after the last study visit, but not for the total follow-up period. All adverse events reported spontaneously by the subject or observed by the investigator will be recorded by the investigator.

An SAE is any untoward medical occurrence or effect that:
► Results in death.
► Is life-threatening (at the time of the event).
► Requires hospitalisation or prolongation of existing inpatients' hospitalisation; except hospitalisation for pregnancy, labour or hyperemesis gravidarum.
► Results in persistent or significant disability or incapacity.
► Is a congenital anomaly or birth defect.
► Any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based on appropriate judgement by the investigator.

The investigator will report the SAEs through the web portal ToetsingOnline to the accredited Medical Research Ethics Committee (METC) that approved the protocol, within 15 days after the investigator has first knowledge of the SAE. SAEs that result in death or are life-threatening should be reported expedited. The expedited reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse reaction. This is for a preliminary report with another 8 days for completion of the report.

The investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, SAEs/serious adverse reactions, other problems and amendments.

Monitoring

Data monitoring will be performed in compliance with good clinical practice and the rules and regulations to achieve high-quality research and ensure safety of participants. A certified independent party of the Maastricht University Medical Centre+ will monitor the study according to the monitor plan. The monitor is approved by the local medical ethical committee. After the first inclusions, the centre was visited by the monitor, which will followed by a yearly visit. Given the low risk of adverse events, a safety surveillance by a safety monitoring board is not indicated. Interim analysis is not planned so far.

Patient and public involvement

Couples with a desire for a child can feel stressed and anxious if they fail to conceive. Although often a 'keep calm and carry on' approach can lead to success, the repetitive cycle of hope and disappointment can create a believe that something else is needed to become parents. The reality is, however, that treatment options are limited. By doing explorative studies such as the MUltidisciplinary observational cohort study in women with Repeated Implantation failure and recurrent Miscarriage Study, we hope to create a better understanding on reproductive failure and eventually decrease the emotional,

physical and financial burden of this diagnosis. In feedback sessions, patients expressed their wish to contribute to such studies to gain novel insights in reproductive failure, even when new treatment options arising from these studies might no longer be of use for their own trajectories. This has encouraged the authors to design this study. The overall results of the study will be shared with the Dutch patient organisation (Freya; www.freya.nl) and patients will be informed about the ability to participate in this study via patient organisation meetings (Freya Talks).

ETHICS AND DISSEMINATION
This study has been approved by the Medical Ethics Committee of the Maastricht University Medical Centre+, registration number METC 18-040, date of approval 31 December 2018. This study is registered at the Dutch trial registry (https://www.trialregister.nl/trial/7571), registration date 28 February 2019. The investigator obtains written informed consent before study participation from all participants. A trained research nurse will confirm that the participants understands the research and agrees to participate voluntary. All participants will be insured by the sponsor in case of harm due to trial participation. To ensure confidentiality, all participants will be assigned an ID code after study inclusion, which will be used on all documents and data. The study will be conducted according to the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WHO), the Guideline for GCP and other regulatory requirements. All modifications to the protocol, besides minor corrections or insignificant administrative changes, will require formal amendment that has to be approved by the Medical Ethics Committee prior to implementation. Results will be disseminated through peer-reviewed publications and shared with the academic and medical community, patient and funding organisations at international scientific meetings in order to contribute to optimise fertility care.

Author affiliations
[1]Department of Obstetrics and Gynaecology, Maastricht University Medical Centre+, Maastricht, The Netherlands
[2]GROW School for Oncology and Developmental Biology, Maastricht University Faculty of Health Medicine and Life Sciences, Maastricht, Limburg, The Netherlands
[3]Department of Transplantation Immunology, Maastricht University, Maastricht, The Netherlands
[4]Institute of Public Health Genomics, Department of Genetics and Cell Biology, Research Institute GROW, Faculty of Health, Medicine & Life Sciences, University of Maastricht, Maastricht, The Netherlands

ORCID iD
Linda Stevens Brentjens http://orcid.org/0000-0001-7650-8837

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