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General Introduction
BACKGROUND

Human reproduction is a remarkably inefficient process: the average chance of pregnancy is only 20% per cycle 1. Other species have much better fecundity; fertility rates in the baboon can reach 80% per mating and in rabbits bred for meat fertility rates even reach 90% per mating 2-3. With the growing trend to postpone pregnancy, the efficiency of human reproduction is further reduced and referrals for subfertility treatment have increased 4. Nowadays, one in six couples need specialist care because of difficulties to conceive 5 and a large part of those couples will eventually turn to assisted reproductive techniques, such as in-vitro fertilisation (IVF).

Since the beginning of the IVF-era every aspect of the IVF treatment has gone through several developments in controlled ovarian hyperstimulation, fertilization and embryo culture techniques 6. Whereas this has resulted in optimization of the number and quality of obtained embryos, it did not improve endometrial receptivity 6. Although 85% of patients enrolled in an IVF programme reach the phase of embryo transfer with apparently healthy embryos, in only 30% of these patients this results in embryo implantation 7. This suboptimal embryo implantation rate reflects the shortcomings of morphological criteria to predict the embryo’s ability to implant, as well as the importance of endometrial receptivity to achieve successful implantation.

Cavagna et al. 8 define endometrial receptivity as ‘the capacity of the uterine mucosa to facilitate successful embryo implantation’. Endometrial receptivity is influenced by a large number of molecular mediators, involved in the different phases of the embryo implantation process 9. It is difficult to assess embryo implantation in-vivo because human implantation sites are not directly accessible due to technical and ethical obstacles. Furthermore, in natural pregnancies it is difficult to time the exact moment of embryo implantation. Development of functional in-vitro systems have led to a better identification of the molecules involved in the synchronized dialogue between maternal and embryonic tissues 9. However, the degree to which data derived from such in-vitro systems can be extrapolated to clinical practice is yet unclear.

Since in IVF treatment the timing of the implantation process in-vivo can be predicted and the number and quality of transferred embryos is known it offers a practical model to investigate factors associated with increased endometrial receptivity. For this reason, our studies focus on implantation after IVF.

Embryo implantation

Embryo implantation is a highly coordinated event and the most critical step to establish a pregnancy 10-11. It requires the development of a functionally normal blastocyst
competent to implant and a receptive endometrium, followed by a synchronized cross-talk between embryo and endometrium. The series of events resulting in implantation takes place within a small time frame between day 6-10 post ovulation, referred to as ‘the window of implantation’. During this period, the endometrium is optimally receptive to the implanting blastocyst. The accomplishment of a receptive endometrium is mainly driven by progesterone, which influences expression of different apposition and attachment molecules, cytokines and (vascular) growth factors. Some of the many molecular markers of endometrial receptivity are mucin-1, L-selectin, integrins, fibronectin, laminin, pino-podes, leukaemia inhibiting factor, interleukins (IL1, IL6, IL11), transforming growth factor-beta (TGF-b), (heparin binding) epidermal growth factor, insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), colony stimulating factor, prostaglandins and glycodelin A.

The process of embryo implantation can be divided in three subsequent developmental phases: apposition, attachment (adhesion) and penetration (invasion). Apposition denotes the unstable adhesion of a blastocyst to the endometrium. Following apposition, attachment occurs where there is an intimate association between embryo and endometrium preventing the blastocyst from dislocating. Subsequently, the embryo invades into the luminal epithelium to establish complex connections to the maternal circulation to form the placenta. In response to the invasion, the endometrial stromal cells undergo decidualisation. This is the maternal adaptation to implantation and during this phase the endometrium transforms into a well-vascularised receptive tissue, during which vascular expansion through angiogenesis plays a central role.

**Multiple embryo implantation**

Multiple embryo implantation represents the situation in which the embryo implantation process takes places in plural. Although optimal morphological embryo quality seems to be the strongest factor determining multiple embryo implantation, the continuation of multiple implantation becomes more dependent on optimal endometrial environment. Therefore, women who conceive a multiple pregnancy after multiple embryo transfer—in particular those with a multiple pregnancy from all embryos transferred—are probably women with improved endometrial receptivity. Since in IVF treatment information on the number (and quality) of transferred and implanted embryos is known, it allows investigation of factors that associate with multiple embryo implantation.

**Factors that may relate to endometrial receptivity**

**ANGIOGENESIS**

Angiogenesis can be defined as the formation of new blood-vessels from existing ones, through proliferation of endothelial cells and their subsequent migration and differentiation to form new capillary tubes. To create a receptive endometrium and a sufficient functioning of the placenta, angiogenesis plays a pivotal role. Through angiogenesis the uterine vasculature adapts to the rising metabolic demands of the growing foetus. Capillaries in the decidua grow and form a capillary plexus connected to the syncytiotrophoblast.
this way the first vascular system supplying the embryo is developed. Simultaneous with vascular adaptation at the fetomaternal interface, angiogenesis also causes significant changes in the vascular system of the deeper part of the uterus supplied by a branched structure of spiral arteries. During this process, also referred to as spiral artery remodelling, the invasive cytotrophoblast transforms high resistance vessels into a low resistance vascular network by the migration of endovascular trophoblast into the myometrial spiral arteries.

With regard to the processes described above, inadequate angiogenesis in the implantation phase may result into a less receptive endometrium with subsequent implantation failure resulting in first trimester miscarriages or abnormal placenta formation with related pregnancy disorders such as pregnancy induced hypertension (PIH), pre-eclampsia (PE), intrauterine growth restriction and preterm delivery.

VASCULAR ENDOTHELIAL GROWTH FACTOR-A

Endometrial angiogenesis is induced by locally produced growth factors and cytokines that stimulate endothelial cell proliferation and migration in a paracrine way. In the endometrium a broad spectrum of angiogenic factors are synthesized and it is generally assumed that their expression is regulated by the ovarian steroids oestrogen and progesterone. These angiogenic factors include VEGF, fibroblast growth factor, epidermal growth factor, IGF, TGF-alfa and TGF-b, tumor necrosis factor a, thymidine phosphorylase, adrenomedullin and erythropoietin.

Of these factors VEGF-A is one of the most important regulators of angiogenesis. It is the predominant isoform in the endometrium and a member of the VEGF-family that comprises six additional isoforms: VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor. VEGF-A is not only synthesized and secreted by epithelial and stromal cells in the uppermost layer of the endometrium, but it is also expressed by theca cells of antral follicles and by granulosa cells nearest the oocyte in the pre-ovulatory follicle. During early pregnancy, VEGF mRNA is expressed by decidua and trophoblast cells and it has been shown that human embryos synthesize VEGF as well. Expression of VEGF-A is regulated in a temporal and spatial manner during the early stages of implantation and it plays a critical role in evolving pregnancy. VEGF induces endothelial proliferation, modulates the maintenance and integrity of immature blood vessels and affects endothelial permeability through its two specific tyrosine kinase receptors; an up-regulator VEGF-R1 (Flt-1) and a down-regulator VEGF-R2 (KDR). Oxygen tension, various cytokines and growth factors including VEGF itself, as well as placental hormones regulate the expression of VEGF-R1 and VEGF-R2. A soluble variant of VEGF-R1 has been found, antagonizing VEGF action, by reducing the level of free active VEGF-A.

We hypothesize that increased levels of VEGF-A improve endometrial receptivity by stimulating angiogenesis at the site of embryo implantation thereby creating an optimal environment for multiple embryos to implant.
BREAST CANCER
Given that women with a multiple pregnancy from all embryos transferred have elevated levels of VEGF (see CHAPTER 2), which is also involved in breast cancer progression 39-41, it could be speculated that the potential to implant all embryos transferred after multiple embryo transfer may be associated with breast cancer risk.

To date, several studies have reported on breast cancer risk after a multiple pregnancy in the general population 42. These studies have shown inconsistent results and a recent meta-analysis of 17 studies did not reveal a significant association between a multiple pregnancy and breast cancer risk 43

For a natural multiple pregnancy it is not known how many embryos were originally available for implantation. In contrast, IVF pregnancies allow investigation of an association between embryo implantation and breast cancer risk, as both the number of transferred and implanted embryos are known.

The inconsistent results on a multiple pregnancy and breast cancer risk so far and the question whether the potential to implant all embryos transferred affects breast cancer risk prompted us to examine these questions in a nationwide Dutch cohort of IVF-treated women.

MATERNAL BODY COMPOSITION
Body composition plays an important role in the development of many conditions, such as cardiovascular disease, type II diabetes and many musculoskeletal disorders 43-45. Body composition has also been associated with menstrual disorders, infertility and (recurrent) miscarriage 46. Therefore, it is not unreasonable to expect an effect of maternal body composition on endometrial receptivity. As fat tissue secretes angiogenic factors, such as VEGF-A 47 and VEGF-A is also involved in long bone formation 48-49, it could be speculated that increased BMI and maternal height, through increased levels of VEGF-A, associate with improved endometrial receptivity.

For natural dizygotic twinning the association with increased height and body mass index (BMI) has actually been demonstrated 50-52. In natural dizygotic twin pregnancies, however, one is not able to specifically assess the process of multiple implantation as is possible in IVF treatment. Therefore, nothing can be concluded about the effect of BMI and height on multiple embryo implantation.

LOW-DOSE ASPIRIN
In addition to reduced angiogenesis, another cause of impaired uterine receptivity may be found in impaired uterine perfusion 53. Therefore, theoretically, drugs that improve blood flow could improve endometrial receptivity and facilitate successful embryo implantation. Aspirin has been believed to be one of these promising drugs. In obstetrics it is known for its potential to prevent PE 54. Furthermore, it is known to improve the chance on a live birth in women with anti-phospholipid syndrome with a history of recurrent miscarriage 55, although recent studies show that it is not effective in women with unexplained recurrent miscarriage 56.
Hypothetically, one common biological link, possibly increased VEGF concentrations, could be on the basis of the associations investigated in this thesis.
Aspirin is a non-selective inhibitor of the cyclo-oxygenase (COX) enzyme that converts arachidonic acid into prostaglandins and thromboxanes. The COX enzyme exists in two isoforms: COX-1, which is mainly involved in physiological processes and expressed in many cells throughout the body and COX-2, which expression is induced by inflammatory mediators. Whereas both COX-1 and COX-2 are expressed in human endometrium, only COX-2 is expressed at the site of blastocyst attachment and appears to be critical to embryo implantation.

Even though aspirin is a non-selective inhibitor, it more specifically inhibits COX-1, thereby inhibiting troboxane A-2, leading to reduced vasoconstriction and platelet aggregation. Hypothetically this could, firstly, result in increased uterine perfusion and a more receptive endometrium and, secondly, improved trophoblast invasion of the uterine spiral arterioles, resulting in more optimal placental perfusion and reduced chances of developing PIH, PE and preterm delivery.

**SITE OF EMBRYO-IMPLANTATION**

The window of implantation does not only seem to be a temporal, but also a spatial window. The latter becomes evident from both the remarkable constant pattern of intrauterine embryo implantation sites, with embryos generally implanting in the fundal endometrium and the influence of the depth of embryo replacement into the uterine cavity on implantation rates after IVF. Because the expression of factors involved in embryo implantation, such as leptin and MUC-1 differs throughout the endometrium, it could be hypothesized that the expression in the fundal endometrium is increased, making it more receptive for embryo implantation.

Since the introduction of ultrasonic guidance at embryo transfer, it is possible to visualize the transferred air bubbles, being the result of the loading technique of the embryo transfer catheter. These air bubbles are believed to indicate the final position of the embryos and could therefore be used as a marker for their position. It appeared that with the current embryo transfer technique, despite standardization of the transfer protocol (gentle release of the catheter load at a standardized position of the catheter), the final position of the air bubbles is not predictable. This final position is dependent on the injection speed at transfer, which in turn depends on the force to press the plunger and a possible uterine resistance. Theoretically, a standardized injection speed allows more exact positioning of the transferred embryos and therefore higher pregnancy rates.
AIM OF THIS THESIS

The main purpose of this thesis is to investigate factors associated with increased endometrial receptivity as indicated by a multiple pregnancy after multiple embryo transfer. Besides intrinsic (angiogenic) factors directly involved in the embryo implantation process (e.g. VEGF-A), applied factors (low-dose aspirin treatment, pump regulated embryo transfer) and predetermined physical traits (e.g. body composition, breast cancer) are also assessed for their association with endometrial receptivity.

The following questions are addressed in this thesis:
• What is the potential relation between angiogenic factors as VEGF-A, inhibin A, glycodelin A, IGF I, IGF II, IGFBP-1 and IGFBP3 and multiple embryo implantation?
• Are features of maternal body composition (height, weight, BMI) associated with multiple embryo implantation?
• Is there a link between multiple embryo implantation and breast cancer risk?
• Does low-dose aspirin improve endometrial receptivity, resulting in higher implantation rates and a lower incidence of hypertensive pregnancy complications and preterm delivery?
• Will the development of an automated new embryo transfer technique contribute to further standardization of the conventional embryo transfer protocol?
• In CHAPTER 2 in a longitudinal observational study we compare maternal serum concentrations of angiogenic implantation factors in singleton and twin pregnancies conceived after DET to determine their role in multiple implantation.
• In CHAPTER 3 we investigate in a large Dutch nationwide cohort of IVF-treated women whether features of maternal body composition (maternal height, weight and BMI) are associated with multiple embryo implantation after DET.
• In CHAPTER 4 in the same cohort we investigate a possible association between multiple embryo implantation and breast cancer risk.
• In CHAPTER 5 we investigate by means of individual patient data (IPD) meta-analysis, whether the pre-conceptional administration of low-dose aspirin throughout the IVF treatment improves embryo implantation.
• In CHAPTER 6 in an additional IPD meta-analysis we assess low-dose aspirin treatment as prevention of pregnancy complications, such as PIH and PE and preterm delivery.
• In CHAPTER 7 we examine a new pump-regulated embryo transfer technique, developed to standardize injection speed to control the final uterine position of transferred embryos.
• In CHAPTER 8 we summarize and discuss the results of the studies presented in this thesis and provide implications for future research.
REFERENCES


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