GENERAL DISCUSSION
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To achieve a healthy pregnancy three important conditions should be met. First an embryo must adequately develop into the blastocyst stage. Second, there must be an appropriate development of the endometrial cells to attain a receptive endometrium. Finally, there needs to be a perfectly synchronized embryo-maternal interaction leading to attachment and invasion of the blastocyst into the endometrium. Whereas research has focused mostly on embryonic factors, factors that associate with improved endometrial receptivity have been scarcely investigated due to technical and ethical obstacles.

The purpose of this thesis was to further explore factors related to endometrial receptivity. In an in-vitro fertilisation (IVF) setting the moment of embryo implantation can be predicted and it allows a separate assessment of embryonic and endometrial factors. Therefore IVF offers a practical in-vivo model to obtain better insight in the requirements of the human embryo implantation environment.

Women who had a multiple pregnancy after multiple embryo transfer enable us to investigate factors that associate with improved endometrial receptivity. Intrinsic- and applied factors, as well as predetermined physical traits can be assessed for their influence on endometrial receptivity. Improved understanding of the factors that influence endometrial receptivity will be essential for further improvement in the treatment of female subfertility and will hopefully help us to increase pregnancy rates, not only after IVF, but possibly also after natural conception.

Angiogenic factors and multiple embryo implantation

We started this thesis by demonstrating that concentrations of vascular endothelial growth factor-A (VEGF-A) in maternal blood prior to IVF treatment associate with multiple embryo implantation. This finding supports our hypothesis of improved angiogenesis in the endometrium optimizing the endometrial environment for embryo implantation and thus allowing multiple embryos to implant. High maternal VEGF-A concentrations could sustain multiple pregnancy in two ways. Firstly, as VEGF-A modulates angiogenesis it could stimulate microvascular changes in the endometrium at the embryo implantation site, leading to better embryo survival. Secondly, high concentrations of VEGF have been observed in follicles with well-developed vasculature and oocytes deriving from these follicles may have high developmental competence.

Within this study on angiogenic factors and their association with endometrial receptivity, we also discovered that women who conceived twins after double embryo transfer
had a slightly increased body mass index (BMI) compared to women who conceived singletons. As fat tissue secretes angiogenic factors, such as VEGF, increased BMI may in theory improve multiple embryo implantation by elevated levels of VEGF-A.

For natural multiple pregnancies there seems to be a relation with some features of maternal body composition such as maternal height and BMI. As the majority of natural multiple pregnancies primarily result from spontaneous multiple ovulation, it is not clear whether BMI affects multiple ovulation, multiple implantation or even both. Our observations indicate a potential effect on multiple implantation in an IVF population.

In order to confirm our findings we conducted an additional study, using data from a large Dutch nationwide cohort of women who underwent IVF treatment; the OMEGA-study cohort.

**Body composition and multiple embryo implantation**

In the OMEGA-study cohort we further explored the relation between features of maternal body composition (e.g. BMI, weight and height) and multiple embryo implantation. The findings of this study confirmed an association between maternal height and multiple implantation, but we could not confirm such an association for BMI and multiple implantation. Even though an association was suggested by the results of our VEGF study, it was not completely surprising that this association was not proven, as BMI does not seem to reflect the proportion and distribution of body fat accurately and therefore could be misleading. In contrast to BMI, maternal height is an objective parameter not affected by other features of maternal body composition.

The biological association between maternal height and multiple implantation remains to be elucidated. It could be speculated that this association is based on VEGF-A, which is an essential mediator of both angiogenesis and long bone formation. VEGF-A mediated capillary invasion is crucial for growth plate morphogenesis and subsequent bone formation in the growth plate. Therefore, theoretically elevated VEGF-A concentrations might augment this bone formation resulting in height increase. Unfortunately, there is no evidence (yet) to support this hypothesis.

**Breast cancer and multiple embryo implantation**

With the knowledge of the relation between improved angiogenesis and multiple implantation on the one hand and improved angiogenesis and tumour development on the other hand, we aimed to investigate whether women with multiple implantations are at increased risk to develop breast cancer. Therefore, within the same OMEGA-study cohort we compared breast cancer risk in mothers of singletons, mothers of multiples and women who remained nulliparous and found an increased premenopausal breast cancer risk in women who gave birth to multiples (HR 1.44, 95% CI 1.06-1.97).

As the multiple pregnancies in our previous study were the result of implantation of all embryos transferred, we wondered whether it were particularly these pregnancies that...
associate with increased breast cancer risk. Indeed, an additional analysis showed that in particular women with a multiple birth from all embryos transferred were at risk and not women with a multiple birth from part of the embryos transferred (HR 1.86 95% CI [1.01-3.43] and HR 1.31 95% CI [0.76-2.25], respectively). These results support our hypothesis of an association between the potential to successfully implant all embryos transferred and increased breast cancer risk.

Although our results were derived from a large nationwide cohort study, our findings should be replicated in other large studies among IVF-treated women. So far, clinical implications are limited. We have to bear in mind that owing to modern single-embryo transfer strategies, multiple pregnancy rates after artificial reproductive techniques are currently low (around 5%) 16. In the future the potential to successfully implant multiple embryos will therefore not be as easily revealed as in our study cohort. Also, the magnitude of the risk increase for women who had a multiple birth is comparable with effects of established risk factors (i.e. nulliparity, late age at first birth, early age at menarche), which are currently not used to define risk groups for breast cancer screening. Hence, we do not recommend IVF-treated women with a multiple birth from all embryos transferred to undergo routine screening mammography at an earlier age than recommended for the general population.

Our study results may contribute to novel insights into the pathogenesis of breast cancer. Future studies are needed to examine whether the significant association between a multiple birth from all embryos transferred and breast cancer risk shown in our study is based on common angiogenic factors, such as VEGF.

**Low-dose aspirin**

Since the first report of doubled pregnancy rate in IVF patients after the use of low-dose aspirin 16, this treatment is believed to improve the chance of successful implantation during IVF treatment. Thereafter, multiple studies followed investigating the use of low-dose aspirin for subfertile patients undergoing IVF 17-27. Whereas some studies could not demonstrate any benefit of aspirin in IVF, others reported an increase in pregnancy rate, sometimes even statistically significant 17;26-27. Of the four conventional meta-analyses that combined the aforementioned studies 28-31, one reported a significant beneficial effect of the use of low-dose aspirin 31. Thus, despite these conventional meta-analyses, there is still no solid answer to the question whether aspirin is truly effective in IVF.

Therefore, we tried to revisit the question of whether daily low-dose aspirin improves the success of IVF by conducting an individual patient data (IPD) meta-analysis (MA), which is believed to provide more reliable estimates of treatment effect than conventional meta-analyses 32. Instead of adjusting for heterogeneity by applying different statistical models, IPD MA allows for the investigation of heterogeneity through subgroup analysis. Other advantages of IPD MA include the ability to confirm the existence and to check the reliability of the original data, to re-analyze outcomes of original trials and to also include non-published (follow-up) data 33.
In a first IPD MA we demonstrated that aspirin does not improve pregnancy rates in IVF patients. This lack of effect is supported by pathophysiological data from a previous study that demonstrated that aspirin does not improve the uterine blood flow \(^{21}\).

Our conclusion supports three of four previous meta-analyses \(^{28-30}\), in which the small apparent effect of aspirin is likely to be based on chance. Our results also indicate that the apparent effect of aspirin is consistent with the chance effect, although the modest effect in our study is in the opposite direction: a harmful effect of aspirin. The difference in effect reported in previous meta-analyses and obtained with our IPD MA did not arise from a more sophisticated analysis possible with IPD MA, but from the unavailability of data from older studies. This IPD MA could therefore serve as a specific case study, illustrating the need to create a system to ensure preservation of RCT data sets. Medical journals and their editors should, for example, consider handing over the original data as soon as a study is published.

As the beneficial effect of aspirin may not be represented in increasing the chance of implantation, it may be hidden in the improvement of trophoblast invasion of the spiral arteries, thereby reducing related pregnancy complications, such as pregnancy induced hypertension, pre-eclampsia and preterm delivery.

Therefore, in an additional IPD MA we investigated whether low-dose aspirin prevents hypertensive pregnancy complications and preterm delivery and could not find any preventive effect of low dose aspirin \(^{34}\). Our finding of a significantly reduced number of twin pregnancies in the patient group treated with low-dose aspirin confirms the observation in a previous randomised controlled trial \(^{35}\) and indicates a possible negative effect of low-dose aspirin on the embryo implantation process. It could be hypothesized that aspirin treatment, even when administered in low doses does not only inhibit the enzyme cyclo-oxygenase 1 (COX-1), but also cyclo-oxygenase 2 (COX-2), a crucial enzyme for embryo implantation \(^{36}\) [see FIGURE 1]. This possible negative effect of low-dose aspirin on embryo implantation should be further elucidated in future studies.

**Site of embryo implantation**

From previous IVF studies it has become clear that the position of embryo replacement affects pregnancy rates \(^{37-41}\). This final position of the transferred embryos cannot be predicted with the conventional embryo transfer procedure, which is performed manually, using a syringe to ‘launch off’ the embryos into the uterine cavity. This calls for the development of a transfer device that is able to transfer the embryos at an exact position in the endometrium with a controlled speed.

In cooperation with the Delft University of Technology, we developed a new method to standardize injection speed during embryo transfer: pump regulated embryo transfer (PRET). Our results indicate that a PRET device generates a more reliable and reproducible injection speed than is realized with the manually performed embryo transfer. Apparently, it is impossible to control injection speed in manually performed embryo transfers, probably because of the relatively
large syringe to release a small volume and the variable resistance provided by plungers of different syringes.

Therefore, PRET brings new possibilities for further standardization of the embryo transfer procedure \[42\]. Additional research on the device is required to reveal the most optimal injection speed avoiding extra-uterine pregnancies and retained embryos, and to investigate in a randomized clinical trial whether standardization of the injection speed in vitro results in more exact positioning of the transferred embryos in vivo and consequently higher pregnancy rates.

**FIGURE 1**

schematic overview of the effect of aspirin on the cyclooxygenase (COX) enzymes 1 and 2 (COX-1 and COX-2).
This thesis suggests that increased (serum) VEGF-A concentrations stimulate implantation of multiple embryos after IVF. It also demonstrates an association between multiple embryo implantation and breast cancer risk and multiple embryo implantation and increased maternal height. In theory, one common biological link (possibly increased VEGF concentrations) could be on the basis of all the aforementioned associations (see FIGURE 2).

Although IVF studies provide some insight into factors associated with uterine receptivity, for a more direct analysis of the interactions between blastocyst and endometrium in-vitro models for embryo implantation are required. Such models have already been developed by the study group of Macklon et al. and could be useful to examine the molecular basis of endometrial receptivity and to discover endometrial characteristics associated with improved endometrial receptivity resulting in implantation of multiple embryos.

These models could, for example, investigate the expression of VEGF-A in the endometrium of women with multiple implantations. If this expression is higher than in women with single implantations, one could expect a similar association between multiple embryo implantation and other types of cancers. Secondly, these models allow further investigation of the negative effect of low-dose aspirin on embryo implantation, possibly through inhibition of COX-2 resulting in reduced angiogenesis. It could be assessed whether the expression of COX-2 is down-regulated after the administration of low-dose aspirin. Finally, as the position of embryo replacement influences implantation rates, it would be of interest to investigate the expression of implantation markers on different locations in the endometrium.

Women with multiple implantations after IVF may be considered super-fertile women, having improved implantation potential. An association between super-fertility and recurrent miscarriage has been described by Salker et al., possibly through an impaired barrier function of the endometrium, allowing embryos of poorer quality to implant. With this knowledge women with multiple implantations after IVF could also be considered women with a higher risk to experience miscarriages. Future research within the OMEGA cohort, should investigate whether this hypothesis is true.

Since healthy endometrium is able to identify a good quality embryo and to leave...
FIGURE 2
Increased VEGF levels are associated with multiple implantation (CHAPTER 2). In this thesis we also found an association for increased height (CHAPTER 3) and breast cancer (CHAPTER 4) with multiple implantation, possibly through increased levels of VEGF. * Silha et al. 47, **Terman et al. 39, Khosravi et al. 40, Wang et al. 41.
alone the poor quality embryo [7], it will be of interest to investigate the signalling process in case multiple embryos of various quality are transferred.

Finally, at the moment we are conducting a large randomised controlled trial analysing whether pump regulated embryo transfer enables more exact positioning of the embryos in-vivo, and, as a consequence, results in higher pregnancy rates after IVF.
REFERENCES


