BLOOD-BORNE ANGIGENIC FACTORS AND SUSTAINED MULTIPLE IMPLANTATION: A COMPARISON OF SINGLETON AND TWIN PREGNANCIES

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This study aimed to compare longitudinal serum concentrations of angiogenic implantation factors between ongoing singleton and twin pregnancies after double-embryo transfer and to investigate whether these are involved in sustained double implantation. Sixteen patients with an ongoing singleton and nine patients with an ongoing twin pregnancy after double-embryo transfer were included in this prospective observational study. Main outcome measures were concentrations of vascular endothelial growth factor-A (VEGF-A), inhibin A, glycodelin A, insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), insulin-like growth factor binding protein-1 (IGFBP-1) and insulin-like growth factor binding protein-3 (IGFBP-3) at baseline, during the IVF treatment and in early pregnancy.

It appeared that VEGF-A concentrations prior to any treatment and at early implantation as well as body mass index (BMI) were higher in women who conceived a twin pregnancy (P = 0.04). Soon after implantation, inhibin A concentrations were higher in twin pregnancies (P = 0.02). Secretion profiles of glycodelin A and members of the IGF family did not differ between singleton and twin pregnancies. VEGF-A appears to play a role in sustained double implantation. Furthermore a high BMI is associated with ongoing double implantation. Future studies should investigate the predictive value of VEGF-A for having an ongoing singleton or twin pregnancy.
INTRODUCTION

Recent IVF studies have shown that pregnancies with double implantations have a lower chance of pregnancy loss than pregnancies with single implantations, reflected by a lower incidence of complete pregnancy loss and a lower pregnancy loss per gestational sac. While the chance of multiple implantations at 6 weeks of gestation is mainly determined by (morphological) embryo quality, the chance of continuation of multiple pregnancies beyond 6 weeks of gestation becomes more dependent on maternal factors affecting the uterine milieu. Among these are factors involved in angiogenesis, a process which is essential for embryo implantation and, thus, probably also for the maintenance of implanted embryos.

Accordingly, it has been postulated that some of the following angiogenic factors may be involved in sustained pregnancy: vascular endothelial growth factor-A (VEGF-A), inhibin A, glycolelin A, insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II), insulin-like growth factor binding protein-1 (IGFBP-1) and insulin-like growth factor binding protein-3 (IGFBP-3).

VEGF-A is one of the most important regulators of angiogenesis. It is not only synthesised 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.

Inhibin A, a member of the transforming growth factor-b family, has also been implicated in regulation of angiogenesis and embryo implantation. Inhibin A expression shows cycle-dependent variation with an increase towards the window of implantation. During the luteal phase of the menstrual cycle the corpus luteum is the major source of inhibin A production, while during early pregnancy the fetoplacental unit seems to be the main origin.

Furthermore, IGF-I, IGF-II and IGFBP-1 are of great importance during the process of implantation. Because of their spatial and temporal expression in the endometrium, it is suggested that they may participate in angiogenesis. Recently, it was demonstrated that IGFBP-3 plays a role in angiogenesis by up-regulating VEGF. Therefore, it could also have an influence on implantation.
Finally, glycodelin A, although not directly involved in angiogenesis, is present in endometrium during the peri-implantation phase and functions as an immunomodulator at the fetal-maternal interface. In women with reproductive disorders, changes in local and circulating glycodelin concentrations have been observed. Thus, it seems that glycodelin is of potential importance to successful nidation.

The aim of this study was to analyse the potential role of angiogenic factors as VEGF-A, inhibin A, glycodelin A, IGF-I, IGF-II, IGFBP-1 and IGFBP-3 in sustained multiple pregnancy by measuring their serum concentrations in IVF treatments with double-embryo transfer and comparing these concentrations between ongoing singleton and twin pregnancies.
MATERIAL & METHODS

Patients
For this study, patients were selected who had an ongoing singleton or twin pregnancy after double-embryo transfer on day 3 after oocyte retrieval in a previous prospective observational study investigating serial uterine Doppler velocity parameters and human uterine receptivity in IVF/intracytoplasmic sperm injection (ICSI) cycles. For the previous observational study, patients were included using the following inclusion criteria: IVF or ICSI treatment with a long gonadotrophin releasing hormone agonist protocol, age under 39 years, regular menstrual cycle with two normal, non-poly cystic ovaries, FSH <10 IU/l on cycle day 3. Indication for IVF or ICSI was one of the following: idiopathic infertility, male factor, endometriosis American Fertility Society grade I or II. Patients were excluded in the case of uterine surgery and/or apparent endometrial pathology (i.e., polyps, submucous myoma or synechia), systemic disease (such as diabetes mellitus, colitis ulcerosa, Crohn disease or connective tissue disease), hypertension (three diastolic pressure measurements >95 mmHg), smoking, body mass index (BMI) >30 kg/m², endometriosis American Fertility Society grade III or IV, pregnancy and tubal infertility, to avoid potential inadvertent effects of fluids from the abnormal Fallopian tubes on implantation. Each patient was included only once and all patients gave their written informed consent.

Treatment
Patients were stimulated according to the study centre’s standard long agonist protocol with oral contraceptives as pretreatment. Ovarian stimulation was started with an individually adjusted dosage of recombinant FSH depending on age, cycle day 3 FSH and antral follicle count. Patients were monitored with regular transvaginal ultrasound examinations and serum oestradiol concentrations. Urinary human chorionic gonadotrophin (HCG) 10,000 IU (Profasi; Serono, Den-Haag, the Netherlands) was administered to induce final follicular maturation when there was at least one follicle >18 mm and at least three follicles >16 mm. Thirty-six hours after HCG administration, transvaginal ultrasound-guided follicle aspiration was performed. Fertilization was achieved by either IVF or ICSI. On the third day after oocyte retrieval, two embryos were placed into the uterine cavity. The cumulative embryo score of the transferred embryos was calculated as described previously. The luteal phase was supported by 100 mg Progestan (Organon, the Netherlands) two capsules three times daily until a pregnancy test was performed 14–16 days after oocyte retrieval.

To define gestational age, the day of oocyte retrieval was regarded as the day of ovulation and therefore gestational age was defined by date of oocyte retrieval plus 2 weeks. A positive pregnancy test
HCG >50 IU/ml) on day 14–16 after oocyte retrieval was followed by transvaginal ultrasonographic monitoring at 6, 9 and 12 weeks of gestation. If cardiac activity could not be diagnosed at 6 weeks gestational age, ultrasonographic monitoring of the pregnancy was repeated after 1 week.

Biochemical pregnancies were recorded when there were at least two incremental HCG values >4.5 IU/ml without confirmation of intrauterine pregnancy by ultrasound at 6 weeks of gestation. Clinical pregnancy was defined as a positive pregnancy test on day 14–16 post oocyte retrieval followed by intrauterine embryonic sac/parts on ultrasound at 6 weeks of gestation. Ongoing pregnancy was defined as an intrauterine pregnancy showing cardiac activity at 12 weeks of gestation. Spontaneous abortion was defined as an intrauterine pregnancy with fetal cardiac activity at 6 weeks of gestation followed by fetal demise.

**Experimental design**

In order to maximize participation, it was decided to compare serum concentrations instead of endometrial secretion aspirations of various factors involved in angiogenesis. So far, endometrial secretion aspiration procedures to obtain endometrial fluid, which would allow a closer focus on local uterine conditions, have insufficiently warranted safety to justify largescale applications in routine IVF/ICSI treatment. During the observational study, serum samples were obtained prior to the start of the treatment (baseline), on the day of oocyte retrieval, at embryo transfer, 1 and 2 weeks after oocyte retrieval and during early pregnancy (at 5, 6 and 9 weeks of gestational age).

**Assays**

VEGF-A measurements were performed by sandwich enzyme immunoassay (Quantikine; R & D systems Inc., Minneapolis, USA). This assay was designed to measure total VEGF. The intra- and inter-assay coefficients of variation (CV) for VEGF, as indicated by the manufacturer, were 5.4% and 7.3%, respectively. The lower limit of quantification (LOQ) was 31.2 pg/ml.

Inhibin A was measured by an immunometric assay (Serotec limited, Oxford, UK). The inter-assay CV was 10%, 8% and 9% for 20 ng/l, 45 ng/l and 90 ng/l, respectively. The LOQ was 5 ng/l with a CV of 10%.

Measurements of glycodelin A were performed using an immunometric assay (Bioserv Diagnostics, Rostock, Germany). The intra-assay CV was 10% over the entire range. The LOQ was 2 µg/l with a CV of 15%.

IGF-I was measured by an immunometric assay (Immulite 2500, DPC, Los Angeles, USA). The intra-assay and interassay CV were both 5% for the entire range. The LOQ was 3.2 nmol/l with a CV of 5%.

IGF-II concentrations were determined in Sep-Pak C18 extracts of plasma by radioimmunoassay (RIA), as described previously. Native human IGF-II isolated from Cohn fraction IV of human plasma was used as a standard and for 125-iodine labelling. At a mean concentration of 500 ng/ml, the intra-assay and inter-assay CV were 7% and 9%, respectively. The LOQ was 0.09 ng/ml. The RIA was calibrated against the WHO reference preparation of recombinant human IGF-II.
IGFBP-1 was measured by an immunoradiometric assay (DSL, Webster, Los Angeles, USA). The inter-assay CV was 10% at the concentration of 10 µg/l and 6% at the concentration of 6 µg/l. The LOQ was 1.5 µg/l with a CV of 10%. IGFBP-3 was measured by an immunometric assay (Immulite 2500, DPC, Los Angeles, USA). The intra-assay and inter-assay CV were both 6% for the entire range. The LOQ was 0.2 mg/l with a CV of 6%.

**Statistical analysis**

Differences in patient characteristics and treatment aspects between the groups with singleton and multiple implantations were calculated using chi-squared and t-tests. Pearson correlation coefficients between BMI and implantation factors were calculated where applicable.

General estimation equation (GEE) analyses were performed to analyse longitudinal changes in serum concentrations in singleton and twin pregnancies. The dependent markers in this study were the different markers of implantation, namely VEGF-A, inhibin A, glycodelin A, IGF-I, IGF-II, IGFBP-1 and IGFBP-3. As independent variables, dummy variables were used indicating number of implantations (single versus double) as well as time and the interaction between time with number of implantations to investigate whether the effect of time was different between singleton and twin pregnancies. At each time moment, the difference in concentrations of the markers between singleton and twin pregnancies was estimated. Prior to analysis, a logistic transformation was performed on the data to account for a non-normal distribution of the dependent variables.

GEE analysis accounts for dependency in the data, since the same patients were repeatedly measured. It uses all available data, irrespective of the number of repeated measurements, indicating that missing observations are allowed. Furthermore, GEE analysis is capable of dealing with irregularly spaced time intervals. Within GEE, correction for the dependency of observations is performed by adding a ‘within subject correlation structure’ to the regression model. These analyses use an exchangeable correlation structure, meaning that correlations between subsequent measurements are assumed to be the same, irrespective of the length of the period in between. The GEE methodology provides consistent estimators of intercepts and regression coefficients.

Statistical differences were determined at P < 0.05. Statistical analyses were performed using Statistical Package for Social Sciences version 15.0 for windows (SPSS, Chicago, IL, USA).

This study was approved by the ethical committee of the VU University medical centre and the institutional review board of the Department of Obstetrics and Gynaecology of the VU University medical centre, Amsterdam, the Netherlands.
RESULTS

Study population
A total of 102 patients were enrolled into the study (FIGURE 1). Of these, 83 patients reached the stage of embryo transfer, one patient withdrew her informed consent before treatment was started and 18 patients did not have an embryo transfer for one of the following reasons: prevention of ovarian hyperstimulation syndrome, total fertilization failure, IVF conversion to intrauterine insemination, failure of embryo transfer due to impossibility to insert catheter into the uterine cavity and violation of the protocol.

Seventy transfers were double-embryo transfer on day 3 after oocyte retrieval. Thirty-six of these patients had a positive serum pregnancy test on day 14–16 after oocyte retrieval; seven patients had a biochemical pregnancy and 29 patients had a clinical pregnancy. Twenty-five patients had ongoing singleton or twin pregnancies, three patients had a spontaneous abortion and one patient had a vanishing twin pregnancy. The 25 patients with ongoing pregnancies were included for further examination; 16 patients had an ongoing singleton pregnancy and nine patients had an ongoing twin pregnancy. All ongoing twin pregnancies were bichorial–biamniotic.

Patient and treatment characteristics were not significantly different except for BMI (TABLE I). Correlations between BMI and the different implantation factors were not statistically significant (data not shown).
FIGURE I  Flowchart of study population

DET = double-embryo transfer; ET = embryo transfer; IC = informed consent; IUI = intruterine insemination; OHSS = ovarian hyperstimulation syndrome; SET = single-embryo transfer; TFF = total fertilization failure.
### TABLE I: Patient characteristics and treatment aspects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Singletons (n=16)</th>
<th>Twins (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>33.6 ± 3.7</td>
<td>33.4 ± 2.6</td>
</tr>
<tr>
<td>IVF</td>
<td>8 (50)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>ICSI</td>
<td>8 (50)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Male factor</td>
<td>9 (56.3)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>1 (6.3)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>5 (31.3)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Primary infertility</td>
<td>12 (75)</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>Secondary infertility</td>
<td>4 (25)</td>
<td>5 (55.6)</td>
</tr>
<tr>
<td>Average daily dose of FSH</td>
<td>196.5 ± 45.0</td>
<td>175.5 ± 37.4</td>
</tr>
<tr>
<td>Number of stimulation days</td>
<td>12.6 ± 1.5</td>
<td>11.9 ± 2.1</td>
</tr>
<tr>
<td>Number of follicles ≥ 14mm on ultrasound A</td>
<td>14.8 ± 5.7</td>
<td>15.4 ± 10.2</td>
</tr>
<tr>
<td>Oestradiol concentration A</td>
<td>11062.1 ± 5974.5</td>
<td>9761.56 ± 5660.9</td>
</tr>
<tr>
<td>Number of oocytes retrieved</td>
<td>16.4 ± 6.9</td>
<td>13.6 ± 5.8</td>
</tr>
<tr>
<td>Number of embryos formed</td>
<td>9.4 ± 4.8</td>
<td>7.7 ± 3.6</td>
</tr>
<tr>
<td>Cumulative embryo score</td>
<td>21.4 ± 2.6</td>
<td>22.0 ± 3.4</td>
</tr>
<tr>
<td>Endometrial thickness (mm) A</td>
<td>9.8 ± 1.7</td>
<td>10.6 ± 1.9</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>22.4 ± 2.7</td>
<td>25.4 ± 3.6</td>
</tr>
<tr>
<td>Smoking</td>
<td>5 (31.3)</td>
<td>1 (11.1)</td>
</tr>
</tbody>
</table>

Values are mean ± SD or n (%). Chi-squared or independent t-tests were used as applicable.
A  Day of/before human chorionic gonadotrophin.
B  P = 0.03; there were no other statistically significant differences.
VEGF-A

The VEGF-A concentration profiles for singleton and twin pregnancies are shown in FIGURE 2. VEGF-A concentrations at baseline were higher in patients where double implantation occurred (cycle day 3, P = 0.04). These concentrations remained significantly higher until 1 week after oocyte retrieval (on day of oocyte retrieval, P = 0.03; 7 or 8 days after oocyte retrieval, P = 0.04). From the start of the treatment cycle, there was a progressive rise in VEGF-A concentrations in both singleton and twin pregnancies with a peak at 14 days after oocyte retrieval (245.8 pg/ml and 475.7 pg/ml for singletons and twins, respectively). Thereafter, VEGF-A concentrations decreased rapidly, reaching low concentrations at 9 weeks of gestation (14.2 pg/ml and 14.4 pg/ml for singleton and twin pregnancies, respectively).

FIGURE 2 Serum VEGF-A concentrations in singleton and twin pregnancies
Serum vascular endothelial growth factor-A concentrations (mean ± SD) in maternal serum of 25 patients with ongoing singleton (grey bars; n = 16) and twin (black bars; n = 9) pregnancies. CD3 = cycle day 3 after menstrual bleeding; OPU = oocyte retrieval; GA = gestational age; VEGF-A = vascular endothelial growth factor-A. *P < 0.05.
Inhibin A

**FIGURE 3** demonstrates the longitudinal changes in serum inhibin A concentrations. Before pregnancy there were no significant differences in inhibin A between singleton and twin pregnancies. Concentrations of inhibin A were relatively high at the moment of embryo transfer (322.1 ng/l for singleton pregnancies and 371.0 ng/l for twin pregnancies) decreasing directly afterwards to concentrations of 158.9 ng/l and 121.4 ng/l 1 week after oocyte retrieval. During early pregnancy, twin pregnancies developed significantly higher inhibin A concentrations than singleton pregnancies (14 or 15 days after oocyte retrieval, P=0.02; 5 weeks' gestational age, P=0.04; 6 weeks gestational age, P = 0.000; 9 weeks' gestational age P = 0.002). Highest concentrations were found at 9 weeks' gestational age (281.0 ng/l and 398.4 ng/l for singleton and twin pregnancies, respectively).

**FIGURE 3** Serum inhibin A concentrations in singleton and twin pregnancies

Serum inhibin A concentrations (mean ± SD) in maternal serum of 25 patients with ongoing singleton (grey bars; n = 16) and twin (black bars; n = 9) pregnancies. ET = embryo transfer; OPU = oocyte retrieval; GA = gestational age. *P < 0.05.
**Glycodelin A**

The longitudinal profiles of serum glycodelin A in singleton and twin pregnancies are demonstrated in **FIGURE 4**. Before pregnancy, during the window of implantation and during early pregnancy, there were no significant differences in glycodelin A between singleton and twin pregnancies. Seven days after oocyte retrieval, glycodelin A concentrations started to rise both in singleton and in twin pregnancies.

**IGF family**

The secretion profiles of IGF-I, IGF-II, IGFBP-1 and IGFBP-3 did not differ significantly between singleton and twin pregnancies (data not shown).

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**FIGURE 4**  **Serum glycodelin A concentrations in singleton and twin pregnancies**

Glycodelin A concentrations (mean ± SD) in maternal serum of 25 patients with ongoing singleton (grey bars; n = 16) and twin (black bars; n = 9) pregnancies. CD3 = cycle day 3 after menstrual bleeding; OPU = oocyte retrieval; GA = gestational age.
This study demonstrates that before any treatment and during early implantation VEGF-A concentrations are significantly higher in women who achieve an ongoing twin pregnancy compared with women who achieve an ongoing singleton pregnancy. This suggests that maternal VEGF-A is involved in sustained multiple pregnancy.

This study is the first assessing VEGF-A concentrations in IVF patients that included measurements at baseline before start of treatment, followed by serial measurements during treatment and at time of early implantation. It confirms previous findings of VEGF secretory patterns during treatment and early stages of pregnancy showing higher concentrations in women who conceived a multiple pregnancy and that the concentrations become nearly undetectable around 9 weeks of pregnancy. Around this time, the low immunoreactivity of VEGF can be explained by the presence of a soluble VEGF antagonist in maternal serum, called soluble fms-like tyrosine kinase (s-Flt1), which binds free VEGF. It has been suggested that this protein is secreted by the placenta and that it may be important in regulating actions of VEGF during pregnancy.

Higher maternal VEGF-A concentrations could sustain pregnancy in two ways. Firstly, VEGF modulates endometrial angiogenesis. It has been shown that, at the site of embryo implantation, changes in capillary diameter occur. Assuming that VEGF-A is responsible for these microvascular changes, elevated VEGF-A concentrations may result in increased angiogenesis at the implantation site, leading to better embryo survival. Secondly, VEGF regulates ovarian angiogenesis. High concentrations of VEGF have been observed in follicles with well-developed vasculature and oocytes deriving from these follicles result in embryos with high developmental competence.

At this moment, there is no solid explanation why VEGF-A concentrations are elevated prior to pregnancy in women who conceive twins. BMI in this study was significantly higher in women who conceived twins than in women who conceived singletons. Fat tissue secretes angiogenic factors such as VEGF. Therefore, theoretically an increased BMI may cause elevated serum VEGF-A. However, in this study there was no significant correlation between VEGF-A and BMI. This is consistent with the findings of Silha et al. (2005), who did not find a significant correlation between BMI and VEGF-A. Thus, in these data it is not evident that higher VEGF-A concentrations resulted from obesity.

On the other hand, it is remarkable that an increased BMI has shown to be associated with an increased risk of natural dizygotic
twinning. Natural dizygotic twinning derives from multiple follicle growth and ovulation, followed by multiple implantation. Classic factors known to promote dizygotic twinning are an advanced maternal age, increased parity and a positive genetic history. Apparently, the association between BMI and dizygotic twinning, which this study confirms, may lie independent from VEGF-A.

While it has been demonstrated that obesity is a fertility decreasing factor, resulting in retained oocytes of diminished quality, decreased fertilization rates and poor pregnancy outcomes in IVF patients, these data indicate that an increased BMI is associated with double implantation. It must be noted from this study that the BMI of women who conceive twins was not excessively increased (25.4 ± 3.6 kg/m²), indicating that only a few were overweight (25–30 kg/m²). Possibly, some overweight may favour implantation with only minimal or even absent negative implications for fertility.

Concentrations of inhibin A in women with twin pregnancies were significantly higher from early implantation onwards (14–15 days after oocyte retrieval), but not before pregnancy. These higher inhibin A concentrations are likely to be a consequence rather than a cause of double implantation; a larger fetoplacental unit will result in higher concentrations of inhibin A. Studies conducted on inhibin A during early pregnancy support this hypothesis. Lockwood et al. (1997, 1998) showed that inhibin A concentrations in early pregnancy are significantly higher in multiple pregnancies compared with singleton pregnancies, suggesting that inhibin A during early pregnancy derives from the fetoplacental unit. Hwang et al. showed that in IVF patients there was a significant association between the number of fetuses and maternal inhibin A concentrations (P < 0.05), suggesting that the fetoplacental unit is mostly responsible for inhibin A production during early pregnancy. Furthermore, Birdsall et al. (1997) demonstrated higher concentrations of inhibin A in multiple pregnancies, supporting a placental source of inhibin A. The initial high concentrations of inhibin A at the moment of embryo transfer could be explained by multiple corpora lutea secreting inhibin A at that moment.

No significant differences in glycodein A profiles were found between women who conceived of twins and singletons at any moment during the IVF treatment. Glycodein has been proposed as the best marker of endometrial receptivity and low concentrations of glycodein during natural, unstimulated cycles seem to be significantly correlated to implantation and pregnancy during subsequent successive IVF cycles. Moreover, during the IVF cycle itself, glycodein concentrations were lower in women who conceived compared with women who did not. Therefore this study suggests that, although glycodein may have predictive value for the chance of conception and thus may play a role in successive implantation, it does not seem to be involved in development of a sustained pregnancy.

Concentrations of IGF-I, IGF-II, IGFBP-1 and IGFBP-3 were not significantly different between women with singleton and double implantations. So far, no studies have been...
conducted on these factors comparing singleton and twin pregnancies.

Not including women who did not conceive seems a shortcoming of this study, but the aim was to specifically approach the question about singleton versus multiple pregnancy in pregnant women and not in non-pregnant women. It was the particular aim to study factors involved in sustained implantation, given the robust observation that double implantations lead to a higher percentage of sustained pregnancies than single implantations.2

Apparently conception by itself (singleton or multiple) seems to be predominantly dependent on embryo quality. In order to evaluate roles of other factors than embryo quality, one has to eliminate the dominant role of embryo quality with regard to the chance of conception. This can only be done in patients in whom a clinical pregnancy has been established, as this is evidence for good embryo quality. For example, optimal or super optimal endometrial conditions will never be revealed, even if present, when poor embryo quality prevents conception in the first place. Indeed, so far no significant differences in basal serum VEGF concentrations have been observed between conception and non conception cycles, which may be due to the overwhelming presence of non-pregnant data, which may have hampered true novel insight.

Besides the angiogenic factors studied here, there are numerous other factors which may be involved in sustained implantation, for instance prostaglandins. Prostaglandins exert their effect via cyclooxygenase-2 receptors, which are up-regulated at the time of blastocyst attachment. During the implantation window, there is an increased production of prostaglandins, inducing the expression of corticotrophin-releasing factor, which is suggested to be involved in the increase of capillary permeability at implantation sites. Because prostaglandins seem not directly involved in angiogenesis, they were slightly out of the scope of this paper.

In conclusion, these data indicate that maternal VEGF-A may be involved in sustained (multiple) implantation and that a high BMI is associated with double implantation. This observation highlights the need for more research on angiogenic factors which may be involved in optimizing the endometrial conditions for implantation. Future studies should also investigate whether the predictive value of VEGF-A for ongoing singleton or twin pregnancies observed here is due to chance and if not, is a useful clinical test.

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