First trimester serum angiogenic/anti-angiogenic status in twin pregnancies: relationship with assisted reproduction technology

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BACKGROUND: The risk of pre-eclampsia (PE) increases in twin pregnancies, especially when assisted reproduction technologies (ART) are used. The aim of this study was to assess angiogenic/anti-angiogenic factors in maternal serum in the first trimester of twin pregnancies and establish if the mode of conception influences angiogenic status.

METHODS: This prospective study enrolled women with twin (n = 61) and singleton (n = 50) pregnancies. Dichorionic twin pregnancies were divided into two groups according to their mode of conception. Singleton pregnancies were used as the control group. Soluble fms-like tyrosine kinase (sFlt-1), free placental growth factor (PlGF) and soluble endoglin (sEng) concentrations were measured in the first trimester maternal serum.

RESULTS: In the first trimester, women with twin pregnancies had higher serum concentrations of the anti-angiogenic factor sFlt-1 than that with singleton pregnancies (3924 ± 250 versus 2426 ± 162 pg/ml, respectively; P < 0.001). Maternal serum PlGF concentrations were lower in singleton pregnancies than those in twin pregnancies (37 ± 3.7 versus 59 ± 5.6, respectively; P < 0.001). Serum concentrations of sFlt-1 were higher in twin pregnancies conceived by ART than those in spontaneous twin pregnancies (4313 ± 389 versus 3522 ± 300 pg/ml, respectively; P < 0.05). No differences between groups were observed for sEng.

CONCLUSIONS: In the first trimester, twin pregnancies conceived using ART showed a heightened anti-angiogenic status that could explain the increased risk of PE in these cases.

Key words: twin pregnancy / angiogenic factors / anti-angiogenic factors / IVF / pre-eclampsia

Introduction

The reported incidence of hypertensive disorders in twin pregnancies ranges from 7.6 to 37%, which is 2–3 times higher than that in singleton pregnancies (Spellacy et al., 1990; Fischer et al., 1995; Santema et al., 1995; Ros et al., 1998; Suzuki and Igarashi, 2005). Twin pregnancies are at greater risk of pre-eclampsia (PE)-related complications, such as abruptio placentae, eclampsia and HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome. Perinatal mortality is also higher, mainly related to prematurity-related complications and fetal growth restriction (Sibai et al., 2000). Exactly why twin pregnancies have a higher incidence of PE is unclear but risk factors include chronic hypertension, primiparity, maternal age and conception by assisted reproduction technologies (ART; Erez et al., 2006). Concern has been raised regarding the rise in the twin birth rate related to the use of ART in the last three decades. A recent

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report from the USA showed that the twin birth rate exceeded 30 per 1000 live births in 2002 with an overall increase of 65% since 1980 (Martin et al., 2003).

There is mounting evidence that ART poses greater obstetric risks (perinatal mortality, preterm delivery, low birthweight and PE) than spontaneously conceived pregnancies (Maman et al., 1998; Jackson et al., 2004; Källen et al., 2005; Chen et al., 2009; Sun et al., 2009). It remains unclear whether this increased risk in IVF is a direct effect of the procedure itself (Olivennes et al., 1997; Sundstrom et al., 1997) or reflects other underlying factors implicated in the infertility of the couple (Williams et al., 1991; Henriksen et al., 1997; McElrath and Wise, 1997). Indeed, more recent studies have shown that infertility per se, unrelated to treatment, is associated with an increased risk of adverse obstetric outcome (Basso and Baird, 2003).

Recently, it has been described that between the first and second trimesters of singleton pregnancies, changes in maternal plasma concentrations of angiogenic and anti-angiogenic factors, or their ratios, confer a higher risk of delivering a small-for-gestational age (SGA) neonate and/or developing PE (Rana et al., 2007; Erez et al., 2008). There are, however, few studies that describe the levels of circulating angiogenic factors during twin or multiple pregnancies (Bdolah et al., 2008; Maynard et al., 2008) and, to our knowledge, no information on serum concentrations of these angiogenic factors during early twin pregnancy.

Therefore, we aimed to ascertain whether differences exist in angiogenic and anti-angiogenic factors in maternal serum from the first trimester of twin pregnancies compared with spontaneously conceived singleton pregnancies and whether differences in these angiogenic and anti-angiogenic factors occur in twin pregnancies depending on the mode of conception.

**Materials and Methods**

**Patients and blood samples**

This was a prospective study on women with twin pregnancies from whom serum samples were obtained between June 2008 and December 2010 at the time of first trimester screening for fetal abnormalities. All pregnant women being followed at the Maternal–Fetal Medicine Unit, Department of Obstetrics, Hospital Universitari Vall d’Hebron, Barcelona, Spain were requested to give a serum sample from their routine blood test for a serum bank to be created. The study protocol was approved by the hospital Ethics Committee and written informed consent was obtained from all participants in accordance with the Helsinki declaration. Routine pregnancy blood tests were performed between 9 and 13.6 weeks of gestation for first trimester screening for Down’s syndrome.

A first trimester scan was performed in all patients. In this scan, crown rump length measurement to date the pregnancy, nuchal transluency and chorionicity were determined in twin pregnancies. All sonographers were obstetricians specialized in fetal ultrasound, certified by the Fetal Medicine Foundation. Maternal history risk factors were obtained prospectively at the time of the first trimester scan. Patients were asked to complete a questionnaire on maternal age, race, height, weight, smoking status during pregnancy, obstetric history (previous PE, SGA, abruptio placenta or stillbirth) and medical history including chronic hypertension or diabetes. The BMI was calculated from the reported pre-pregnancy weight. The use of ART [artificial insemination, IVF, ICSI or oocyte donation (OD)] was also recorded.

Demographic characteristics were entered into the database of the prenatal screening program. Pregnancy outcomes were ascertained from hospital medical records by independent assessors who were blinded to biochemical results.

Criteria for the definition of PE were those of the International Society for the Study of Hypertension in Pregnancy (Brown et al., 2001). PE was diagnosed if a previously normotensive woman had diastolic blood pressure >90 mmHg measured twice (4 h apart) and also proteinuria >300 mg in a 24-h urine specimen or, in its absence, urinary dipstick of 2+ or more in two repeated measurements (4 h apart) after the 20th week of gestation. Intrauterine growth restriction (IUGR), defined as failure of the fetus to achieve its growth potential (Resnik, 2002), was estimated when fetal weight was below the 10th percentile and impedance increased on umbilical artery Doppler (Soothill et al., 1999). In all cases, estimated fetal birthweight was confirmed as below the 10th percentile after birth (Breeze et al., 2007).

Venous blood samples were drawn during routine blood tests on the same day and processed within 1 h. Serum and plasma were separated by centrifugation at 1400g for 10 min at 4°C, and sample aliquots were immediately stored at −80°C until assayed.

**Serum PPAP-A and free-βHCG**

Serum concentrations of pregnancy-associated plasma protein-A (PPAP-A) and free β-HCG were measured using a AutoDelfia platform (Perkin Elmer Life Science, Boston, MA, USA). Concentrations of biochemical parameters were entered into the database of the first trimester prenatal screening program and automatically converted into MoMs (multiple of the median) by the laboratory information management system using underlying reference values based on the Spanish population and were constantly monitored. The MoMs were corrected for maternal weight. All laboratory methods were continually assessed by internal and external quality assurance program.

**Serum levels of angiogenic and anti-angiogenic factors**

Soluble fms-like tyrosine kinase (sFlt-1), free placental growth factor (PIGF) and soluble endoglin (sEng) concentrations were measured under pregnancy type-blinded conditions. Enzyme-linked immunosorbent assays for human sFlt-1, PIGF and sEng were performed in duplicate using commercial kits (R&D Systems Europe Ltd, Abington, UK) following the manufacturer’s instructions. Minimum detectable values in the assays were 3.5 pg/ml for sFlt-1, 7 pg/ml for PIGF and 0.007 ng/ml for sEng. In all the kits, intra-assay precision was always <5% and inter-assay precision <10%. Linear regression coefficients of the standard curves were never <0.99%.

**Statistical analysis**

Statistical analyses were performed using Prism software (GraphPad, version 5.02, San Diego, CA, USA). Demographic and clinical data were compared by independent-sample Student’s t-test or Fisher’s exact test, as appropriate. Results are presented as mean ± SEM. Distribution of variables was tested for normality by the D’Agostino and Pearson omnibus K2 test. When the assumption of normality was satisfied, data were analyzed by Student’s unpaired t-test; otherwise, the non-parametric Mann–Whitney U-test was used. A sample size of eight for each group achieved power of 80% to detect a difference of 600 pg/ml in sFlt-1 levels between the null hypothesis, that both group means are 2000, and the alternative hypothesis, that the mean of group 2 is 2600, with estimated group standard deviations of 400 and 400 and with a significance level (alpha) of 0.05 using a two-sided two-sample t-test. Two-way analyses of variance followed by the Bonferroni post hoc test were performed to
determine whether maternal age and previous reproductive history could affect sFlt-1, PlGF and sEng concentrations. Inter-groups differences were considered statistically significant when the \( P < 0.05 \).

## Results

One hundred and twenty-four pregnant women, 50 singleton pregnancies, 73 twin pregnancies and 1 triplet pregnancy were enrolled. Chorionicity was known in all twin pregnancies: 12 monochorionic (16%) and 61 dichorionic (84%). In order to avoid biased results, the monochorionic twin pregnancies and the triplet pregnancy were excluded from the study.

Demographic and clinical characteristics of singleton and all dichorionic twin pregnancies compared in the present study are shown in Table I. No differences in terms of gestational age, BMI or parity were found between groups. Maternal age was lower in singleton than in twin pregnancies.

### Table I Demographic and clinical characteristics of the population.

<table>
<thead>
<tr>
<th></th>
<th>Singleton ( (n = 50) )</th>
<th>Twins ( (n = 61) )</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>31.1 ± 0.81</td>
<td>34.1 ± 0.70</td>
<td>0.01</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>29 (58)</td>
<td>40 (66)</td>
<td>0.43</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.8 ± 0.66</td>
<td>23.8 ± 0.51</td>
<td>0.28</td>
</tr>
<tr>
<td>GA (weeks)</td>
<td>11.6 ± 0.15</td>
<td>11.9 ± 0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>Smoker</td>
<td>10 (20)</td>
<td>6 (10)</td>
<td>0.17</td>
</tr>
<tr>
<td>Previous PE</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>0.48</td>
</tr>
<tr>
<td>Previous IUGR</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Previous miscarriage</td>
<td>12 (24)</td>
<td>18 (29)</td>
<td>0.67</td>
</tr>
<tr>
<td>(≤22 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous fetal death</td>
<td>0 (0)</td>
<td>4 (6)</td>
<td>0.30</td>
</tr>
<tr>
<td>(&gt;22 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM or as number (percentage). GA, gestational age; PE, pre-eclampsia; IUGR, intrauterine growth restriction. Independent-sample Student’s t-test, Mann–Whitney U-test or Fisher’s exact test, as appropriate.

Soluble F1t-1 concentrations in twin pregnancies were 1.6 times higher than in singleton pregnancies \((3924 ± 250 \text{ versus } 2426 ± 162 \text{ pg/ml, respectively; } P < 0.001; \text{ Fig. 1A})\). In singleton pregnancies, PlGF serum concentrations were significantly lower than those in twin pregnancies \((37 ± 3.7 \text{ versus } 59 ± 5.6, \text{ respectively; } P < 0.001; \text{ Fig. 1B})\). Soluble endoglin concentrations were slightly, but not significantly, higher in maternal serum samples from twin \((6.53 ± 0.41 \text{ ng/ml})\) than from singleton \((5.79 ± 0.39 \text{ ng/ml})\) pregnancies (Fig. 1C).

Weight-corrected MoM PAPP-A and free β-hCG were significantly higher in twin pregnancies than in singleton pregnancies \((P < 0.001)\).

Dichorionic twin pregnancies were divided into two groups according to their mode of conception: spontaneous conception (S-twins) or ART conception (ART-twins). Within the ART-twin group, 26 were conceived after IVF with autologous oocytes (AOs) and 5 were conceived after OD.

Demographic and clinical characteristics of dichorionic twin pregnancies are shown in Table II. Maternal age and nulliparity were lower in S-twins than in ART-twin pregnancies. No differences were found in serum concentrations of angiogenic factors with respect to maternal age or parity (Fig. 2).

Serum concentrations of sFlt-1 were higher in twin pregnancies conceived by ART than those in spontaneous twin pregnancies \((4313 ± 389 \text{ versus } 3522 ± 300 \text{ pg/ml, respectively; } P < 0.05; \text{ Fig. 3A})\). However, no differences were found in PlGF levels between groups of the twin pregnancies analyzed \((59 ± 10.46 \text{ in S-twins versus } 59.2 ± 4.88 \text{ pg/ml in ART-twins; Fig. 3B})\). Soluble endoglin concentrations were similar between groups \((6.78 \text{ ng/ml ± 0.48 in S-twins versus } 6.28 \text{ ng/ml in ART-twins; Fig. 3C})\). When ART-twins conceived after IVF with AO or after OD were compared, no differences in angiogenic factors were detected \((sFlt-1, AO: 4326 ± 437 \text{ versus OD: } 4241 ± 917, P = 9358; \text{ PlGF, AO: } 59.6 ± 5.69 \text{ versus OD: } 57.0 ± 7.26, P = 0.8469; \text{ sEng, AO: } 5.86 ± 0.67 \text{ versus OD: } 8.47 ± 2.25, P = 0.1326)\). We also analyzed samples from monochorionic twins (M-twins) and levels of angiogenic factors were similar to those of dichorionic twins (D-twins): \((sFlt-1, M-\text{twins: } 3374 ± 282 \text{ versus D-\text{twins: } 3522 ± 300, } P = 0.5464; \text{ PlGF, M-\text{twins: } 37 ± 3.9 \text{ versus D-\text{twins: } 59 ± 10.4, } P = 0.2334; \text{ sEng, M-\text{twins: } 5.57 ± 0.58 \text{ versus D-\text{twins: } 6.78 ± 0.48, } P = 0.2220). \)No differences were observed in weight-corrected MoM PAPP-A and free β-HCG values.
between pregnancies conceived by ART and those conceived spontaneously (data not shown).

Maternal outcomes of singleton and twin pregnancies are shown in Table III. Mean gestational age at delivery was higher in singleton pregnancies than that in both types of twin pregnancies (35.7 ± 0.76 in S-twins and 34.8 ± 0.76 in ART-twins compared with 39.4 ± 0.33 weeks for singletons; both P < 0.001). The same trend was observed for birthweight: 2299 ± 88 g in S-twin and 2145 ± 90 g in ART-twin pregnancies compared with 3358 ± 94 g in singleton pregnancies (P < 0.001). Preterm delivery rate tended to be higher in ART-twin (14%) than that in singleton pregnancies (2%); however, this difference was not statistically significant (P = 0.0775). The incidence of PE was higher in ART-twins than that in singleton pregnancies (19% in ART-twins versus 2% in singletons, P = 0.0282). Moreover, the difference in the incidence of IUGR among pregnancies was: 6% in S-twins and 13% in ART-twins versus 0% in singletons (P = 0.1655 and P = 0.0252, respectively).

Finally, serum sFlt-1 concentrations of women who later developed PE and/or IUGR were compared with those who had normal outcome in relation to placental disease (Table IV). In our singleton pregnancies only one patient developed PE; thus, it was not possible to draw meaningful conclusions, although sFlt-1 levels were higher in that particular patient compared with those in the normal outcome group. In all twin gestations, patients who developed placental-related

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**Table II Demographic and clinical characteristics of dichorionic twin pregnancies.**

<table>
<thead>
<tr>
<th></th>
<th>S-twins (n = 30)</th>
<th>ART-twins (n = 31)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>31.9 ± 0.79</td>
<td>36.3 ± 1.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>15 (50)</td>
<td>25 (81)</td>
<td>0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.9 ± 0.82</td>
<td>23.8 ± 0.62</td>
<td>0.83</td>
</tr>
<tr>
<td>GA (weeks)</td>
<td>11.8 ± 0.25</td>
<td>12.2 ± 0.17</td>
<td>0.23</td>
</tr>
<tr>
<td>Smoker</td>
<td>3 (10)</td>
<td>3 (10)</td>
<td>1.00</td>
</tr>
<tr>
<td>Previous miscarriage (≤22 weeks)</td>
<td>6 (20)</td>
<td>12 (39)</td>
<td>0.16</td>
</tr>
<tr>
<td>Previous fetal death (&gt;22 weeks)</td>
<td>1 (3)</td>
<td>3 (9)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM or as number (percentage). S-twins, twins conceived spontaneously; ART-twins, twins conceived following the use of assisted reproduction technology. Independent-sample Student’s t-test, Mann–Whitney U-test or Fisher’s exact test, as appropriate.

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**Figure 2** Maternal serum levels of sFlt-1 (pg/ml) (A), PIGF (pg/ml) (B) and sEng (ng/ml) (C) in the first trimester twin nulliparous (N, n = 40) and non-nulliparous (Non-N, n = 21) pregnancies, in women aged <36 (n = 39, white bars) or ≥36 years (n = 22, black bars). Data are presented as mean with SEM. Two-way analysis of variance, P > 0.05.

**Figure 3** Maternal serum sFlt1 (pg/ml) (A), PIGF (pg/ml) (B) and sEng (ng/ml) (C) in the first trimester twin pregnancies conceived spontaneously (S, n = 30) or by assisted reproduction technology (ART, n = 31). Data are presented as mean with SEM. *P < 0.05, compared with spontaneous twin pregnancies.
disease tended to have higher sFlt-1 levels than healthy controls (5001 ± 672 versus 3608 ± 234 pg/ml, P = 0.1330). When twin pregnancies were distinguished according to mode of conception, only pregnant women with ART twins who developed PE and/or IUGR had higher sFlt-1 levels compared with mothers with normal outcome ART pregnancies; however, no differences were found in women with either S-twin pregnancies that developed placental-related disease or those with normal outcome (Table IV). When patients with normal perinatal outcome were analyzed independently, those with twin pregnancies were found to have higher sFlt-1 levels compared with those with singleton pregnancies (3608 ± 234 versus 2407 ± 164 pg/ml, P < 0.0001). Between twin-pregnancy groups, sFlt-1 levels were similar in patients with normal outcome (S-twins: 3562 ± 349 pg/ml versus ART-twins: 3661 ± 311 pg/ml, P = 0.4949).

### Discussion

In this study, in the first trimester, serum concentrations of angiogenic (PIGF) and anti-angiogenic (sFlt-1) factors were found to be higher in mothers with twin pregnancies than in those with singleton pregnancies. In the third trimester of twin pregnancies, Bdolah et al. (2008) found serum sFlt-1 concentrations to be 2.2-fold higher than those measured in singleton pregnancies. Interestingly, Bdolah et al. (2008) found that sFlt-1 levels correlated with increased placental weight, suggesting that the greater risk of PE in twin pregnancies may be related to increased placental mass. In another work, Maynard et al. (2008) reported that maternal serum sFlt-1 levels and the sFlt-1:PIGF ratio were higher in women with twins or triplets compared with high-risk singleton pregnancies in the late second and third trimesters, although neither study provided information on the mode of conception.

The salient finding of our study was that differences existed between twin pregnancies according to the mode of conception; twins conceived by ART (ART-twins) presented higher plasma sFlt-1 levels than those conceived spontaneously (S-twins). However, no differences were observed in PIGF levels between the twins groups. Therefore, women with ART presented an imbalance between angiogenic and anti-angiogenic markers which tended towards an anti-angiogenic state in the first trimester of pregnancy.

PIGF is highly expressed by trophoblastic cells and is known to be involved in the regulation of placental vascular development. sFlt-1 (also known as soluble vascular endothelial growth factor EGFR receptor 1) is a potent antagonist of PIGF that prevents its interaction with cell receptors (Ahmad and Ahmed, 2004). sEng, a truncated form of endoglin (a receptor for several of the transforming growth factor-β (TGF-β) superfamily members; Guerrero-Esteo et al., 2002), is a protein that inhibits TGF-β1 signalling in vasculature (Venkatesha et al., 2006). These proteins have been widely reported to act as controllers of angiogenesis during pregnancy (Lam et al., 2005; Smith and Wear, 2009; Wang et al., 2009). In PE, ischemia and placental underperfusion were found to be caused by failure of perivascular and endovascular trophoblastic invasion into the spiral arteries (De Wolf et al., 1980; Meekins et al., 1994), which points to angiogenic modulator involvement in the development of the disease. Increased circulating sFlt-1 levels and decreased PIGF levels were reported in PE (Maynard et al., 2003) and these changes have been detected 5–14 weeks before PE was clinically diagnosed (Levine et al., 2004; Crispi et al., 2008). Elevated circulating sEng levels have also been found in patients with PE (Venkatesha et al., 2006). It is thought that sEng acts together with sFlt-1 to induce this pathological process and, as with sFlt-1, circulating sEng levels are elevated weeks before PE onset (Levine et al., 2006; Romero et al., 2008).

Our data showed that, whereas in S-twins increases are found in both angiogenic and anti-angiogenic factors, in the ART-twins studied here, there was only an increase in anti-angiogenic factors. Therefore, in addition to the risks of twinning itself, the fact that the risk of pregnancy complications is higher in ART-twin pregnancies may be related to the imbalance in angiogenic/anti-angiogenic factors. These alterations in the angiogenic factors may reflect early failure in trophoblast invasion that might be related to the initial fertility problems. A possible underlying mechanism associated with sterility is endothelial dysfunction and maternal predisposition to endothelial disease. This possible pathologic process present in the mother before conception could determine abnormal implantation and trophoblast invasion in the early stages of placentation, which in turn would provoke placental underperfusion, ischemia and increases in sFlt-1 and sEng released from the placenta. However, is not possible to distinguish whether sFlt-1 or sEng are synthesized in the endothelium or in the placenta, therefore, we speculate that both sources could explain the rise in sFlt-1 and sEng in these cases.
As ART became more widely used, knowledge of perinatal outcomes in these pregnancies has progressively increased. A meta-analysis of 12,283 IVF singleton gestations found significantly higher odds of perinatal mortality, preterm delivery, low birthweight and PE (Jackson et al., 2004). Other studies also pointed out the relationship between ART and PE and other complications derived from placental insufficiency (Maman et al., 1998; Källén et al., 2005; Sun et al., 2009). Moreover, a more recent study found a higher incidence of PE among pregnancies conceived by IVF; however, no significant association was found between intrauterine insemination and ovulation induction (Chen et al., 2009). In view of these results, the National Institute of Child Health and Human Development established that couples undergoing ART should be counselled regarding the increased frequency of obstetric complications, including SGA fetuses, maternal PE or both (Reddy et al., 2007).

Among women with ART-twin pregnancies, those that developed placental-related disease had significantly higher sFlt-1 levels than those without placental-related disease; however, similar sFlt-1 levels were found in women with S-twin pregnancies who developed placenta-related disease and those with normal outcome. This finding suggests that the higher risk of placental-related disease observed in ART-twin pregnancies could be related to impairment in the process of first-wave trophoblastic invasion that involves the decidual portion of spiral arteries and begins at 8 weeks of gestation (Pijnenburg, 1983). In S-twin pregnancies, other features, such as high placental volume, and consequently high anti-angiogenic factors at the end of pregnancy (Badoloh et al., 2008; Maynard et al., 2008), could induce endothelial dysfunction and PE syndrome in otherwise normally developed placenta. Therefore, the pathogenic features that lead to PE syndrome appear to differ in ART-twins and S-twins.

Few data regarding twin pregnancies have been reported in the literature. One of the main drawbacks when ART-twin and S-twin pregnancies are compared is that monochorionic twins are usually included, mainly within the spontaneous conception group. In the present study, although no differences in angiogenic and anti-angiogenic factors were found between monochorionic and dichorionic twins, monochorionic twins were excluded from our cohort to avoid such a bias. Monochorionic twins are a different entity from dichorionic twins in whom perinatal results are affected by complications derived from sharing the placenta, which significantly worsens the results (twin–twin transfusion syndrome, selective IUGR, TRAP-Twin Reversed Arterial Perfusion sequence). Our study aimed to analyze twin gestations according to their form of conception and spontaneously conceived dichorionic twins are more comparable to twins obtained by ART.

Although significant differences in maternal age and frequency of nulliparity were found between mothers with S-twins and ART-twins in this study, these differences did not appear to affect serum concentrations of sFlt-1, PI GF and sEng. A previous study has also reported no correlation between older maternal age and sFlt-1 levels in normotensive pregnant women (Staff et al., 2009). In fact, some authors have reported that maternal age and parity do not exert an adverse influence on outcomes in twin/multiple pregnancies (Prapas et al., 2006; Fox et al., 2009; Kathiresan et al., 2011).

Our data add valuable information on angiogenic and anti-angiogenic factors in the first trimester of twin pregnancies and their relationship with placental impairment reflected by biochemical markers, such as PPAP-A. Pregnancies achieved by ART have been shown to be associated with changes in biochemical serum screening markers (Tul and Novack-Antoloc, 2006), although whether the underlying fertility or fertility treatments cause these changes remains unknown. In twin pregnancies, low PAPP-A is associated with discordant inter-fetal growth and hypertensive disorders (Chasen et al., 2009). In the present study lower PAPP-A levels were found in the first trimester of ART-twin pregnancies, although the differences did not reach statistical significance. This finding was consistent with an angiogenic imbalance in these cases, and thus adds further support to the hypothesis that placental impairment ‘per se’ is the source from which stem the obstetric complications observed in ART pregnancies.

One limitation of this study was that we only looked for changes in maternal circulation; therefore we could not know the mechanisms by which ART affects angiogenic phenotype. Nevertheless, a particular strength of this study is that we addressed the relationship between ART and angiogenic factors in women with twin pregnancies.

To our knowledge, this is the first study that evaluates angiogenic factors during early twin pregnancy and their relationship with ART. Overall, our data suggest that an anti-angiogenic environment prevails as early as the first trimester of pregnancy in ART-twins. Our results offer a plausible explanation for how ART affects the risk of PE and IUGR by linking this effect to the angiogenic imbalance, which is one of the underlying mechanisms implicated in placental insufficiency. By being aware of the negative effects of an anti-angiogenic environment in these cases, we would be able to take preventive measures to avoid complications derived from this pathology.

**Details of ethics approval**

The study was approved by the Ethics Committee of Vall d’Hebron University Hospital on 21 March 2006, reference number IP061312.

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**Authors’ roles**

O.S. carried out the laboratory work, the statistical analysis of the results, contributed to data analysis and interpretation and wrote part of the manuscript. E.L. came up with the idea, designed the study objectives and work plan, contributed to data analysis and interpretation and wrote part of the manuscript. G.M. organised the inclusion of patients and was responsible for the verification and digitalisation of clinical data and of maternal and perinatal results. C.D. was in charge of laboratory analyses, contributed to data analysis and interpretation of the results and redaction of the manuscript. C.A. was in charge of laboratory analyses of first trimester biochemical screening markers from Down syndrome. M.A.S.-D. contributes to the inclusion of patients. M.G. contributes to the inclusion of patients. J.A.-R. contributed in the redaction of the manuscript. E.C. provided a critical review of the manuscript. L.C. provided a critical review of the manuscript.
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Conflict of interest
None declared.

References