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ORIGINAL ARTICLE *Early pregnancy*

The effect of a 'vanishing twin' on biochemical and ultrasound first trimester screening markers for Down's syndrome in pregnancies conceived by assisted reproductive technology

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BACKGROUND: Previous studies have found that 1 in 10 *in vitro* fertilization (IVF) singletons originates from a twin gestation. First trimester Down's syndrome screening markers are altered in assisted reproductive techniques (ART) pregnancies compared with spontaneously conceived pregnancies. The presence of a perished embryo may further complicate prenatal screening among women pregnant after ART. The aim of this study was to assess the impact of a 'vanishing twin' on first trimester combined biochemical and ultrasound screening in pregnancies conceived after IVF and intracytoplasmatic sperm injection.

METHODS: From a national prospective cohort study concerning first trimester combined screening among women pregnant after ART, 56 cases of pregnancies with a vanishing twin were identified. As control group 897 cases of ART singleton pregnancies were used. All women completed a first trimester combined ultrasound and biochemical screening programme comprising serum PAPP-A and free β-hCG together with nuchal translucency (NT) measurement.

RESULTS: There were no significant differences in geometric mean MoM free β-hCG and PAPP-A between pregnancies with an early (gestational week <9, EVT) or late vanishing twin (gestational week 9–13, LVT) or singleton pregnancies (0.98, 1.13 and 0.95 for free β-hCG and 0.84, 0.80 and 0.74 for PAPP-A, respectively). Likewise, no difference was seen for NT measurements. The gestational age at the time of blood sampling and NT scan was similar for the three groups. The proportion of EVT pregnancies with a PAPP-A and free β-hCG $\log_{10}$MoM value below the 5th%iles and above the 95th%iles of the value in the singleton pregnancies were 4.3%, 4.3%, 6.4% and 8.5%, respectively, which did not constitute a significant difference from singletons. The corresponding values for LVT pregnancies were 0%, 22.2%, 0% and 11.1%, respectively; however, these numbers were too small to allow for statistical calculations.

CONCLUSIONS: First trimester biochemical screening markers in women pregnant after ART, and with a vanished twin diagnosed at early ultrasound, do not differ from those of other ART singleton pregnancies. In cases where the fetal demise was first diagnosed at the time of the NT scan, it is doubtful whether the serum risk assessment is as precise as it is in singleton ART pregnancies. No difference was seen for NT measurements.

Key words: vanishing twin / first trimester screening / ART / PAPP-A / β-hCG

Introduction

The increasing use of high-quality ultrasound in early pregnancy has demonstrated that spontaneous reduction of a twin pregnancy to a singleton pregnancy, the so-called vanishing twin phenomenon, is a rather frequent incident (10–40%) (Landy and Keith, 1998; Dickey *et al*., 2002). In Europe, twin pregnancies conceived by assisted reproductive techniques (ART) occur in 22% of all pregnancies due to transfer of more than one embryo (Pinborg, 2005). Despite increasing use of elective single-embryo transfer, double (or more) embryo transfer was in 2004 performed in more than 80% of all ART procedures (Andersen *et al*., 2008). To confirm that fertility treatment has been successful, an early ultrasound scan (around pregnancy Week 8) is an obligatory procedure at all fertility clinics in order to demonstrate the presence of a live fetus; consequently pregnancies with a (diagnosed) 'vanishing twin' are not likely to be overlooked in ART pregnancies. It has been estimated that the vanishing twin phenomenon occurs in 10% of ART pregnancies with a live born *in vitro* fertilization (IVF) singleton (Pinborg *et al*., 2005).

Screening for Down's syndrome in the first trimester using biochemical and ultrasonographic markers has become a part of the antenatal care in many countries. Using the biochemical markers free β-hCG and PAPP-A ('the double test') in combination with the sonographic marker nuchal translucency (NT), it has been demonstrated that 90% of singleton pregnancies with a Down's syndrome fetus can be detected, with a false positive rate of 5% (Spencer, 2007) or even lower (Wøjdemann *et al*., 2005). Screening for chromosomal abnormalities in twin pregnancies is complex; on average, the biochemical markers are twice that in normal singleton pregnancies, but it is difficult to interpret data when the fetuses are either concordant or discordant for a chromosomal aberration. NT measurements are not affected by the number of fetuses and ultrasound is, in twin pregnancies, the predominant method for risk assessment in clinical practice (Spencer, 2007). Prenatal screening among women pregnant after ART is complicated by several factors: the women are generally older, conception by the use of intracytoplasmatic sperm injection (ICSI) seems to involve an increased risk of chromosomal abnormalities (Gjerris *et al*., 2008a,b), the biochemical markers are altered (Liao *et al*., 2001; Tul and Novak-Antolic, 2006; Gjerris *et al*., 2008a,b) and women pregnant after ART seem to be reluctant to have invasive diagnostic testing performed (Gjerris *et al*., 2008a,b). The presence of a vanishing twin is another parameter that might complicate prenatal screening among women pregnant after ART.

The aim of this study was to assess the impact of a 'vanishing twin' on first trimester combined biochemical and ultrasound screening in pregnancies conceived after IVF and ICSI.

Materials and Methods

A nationwide prospective cohort study, concerning first trimester Down's syndrome screening among women pregnant after ART, carried out in Denmark from 1 April 2004 to 31 January 2006 included 1666 pregnant women meeting the following criteria: only singleton or twin pregnancy, the pregnancies should be conceived after IVF, ICSI or frozen embryo replacement (FER) and the participants should reside in Denmark to allow follow-up. The overall study population is described in detail in a recent publication (Gjerris, 2008a). Eighteen Danish public and private fertility clinics and 10 departments of fetal medicine participated in the study. Women were invited to enter the study at the time of early ultrasound. They were offered (and completed) a first trimester combined screening programme with double test and NT scan. At the fertility clinic, data on fertility treatment and demographics were recorded and entered into a database. Data on pregnancy outcome were obtained by a self-administered questionnaire given to the women immediately after inclusion and returned after the end of pregnancy. In those cases where the questionnaire was not returned, information was retrieved from the fertility clinic or from hospital records.

From this cohort, we identified 78 cases of singleton pregnancies with an originally described co-twin. These pregnancies were either described with one viable and one demised fetus or with two viable fetuses, but then later found to have only one viable fetus. Cases where two embryo sacs but only one fetus was described were excluded.

We excluded all pregnancies conceived after FER (13 cases) because their first trimester screening markers have been shown to be significantly different from the screening markers in IVF/ICSI pregnancies, and are instead comparable with spontaneous pregnancies (Gjerris *et al*., 2008a,b), resulting in the exclusion of 13 cases of vanishing twin. Of the remaining 65 cases, three did not have their blood sample analysed at Statens Serum Institut (SSI) and in another three cases, the spontaneous reduction occurred after the risk assessment. Additionally, in three cases, 'the vanished twin' was a result of an elective reduction due to either prenatal diagnosis of Down's syndrome, a severe malformation found by ultrasound, or twin-to-twin-transfusion syndrome. Thus, the study group included 56 cases.

As controls, we identified all IVF/ICSI singleton pregnancies without chromosomal abnormalities with blood samples analysed at SSI ($n = 897$).

All women gave written informed consent and the Local Scientific Ethics Committee (Jr. nr. KF 01-218/03) as well as the Data Protection Agency approved this study.

Vanishing twin cases

The vanishing twin cases were further subdivided according to the time of spontaneous reduction. In 47 cases, the vanished twin was diagnosed already at the early ultrasound at a median crown-rump length (CRL) of 17 mm corresponding to a mean gestational age (GA) of the surviving twin of 8 week + 1 day (Robinson and Flemming, 1975): GA in days = $8.052 \times \mathrm{CRL}1/2 + 23.75$), these are referred to as 'early vanishing twin' (EVT) pregnancies. In the remaining nine cases, the spontaneous reduction occurred between the early ultrasound scan and the time of NT scan. These constitute 'late vanishing twin' (LVT) pregnancies. In 11 EVT cases, a CRL of the non-viable fetus was measured and could be used to estimate the time of embryonic demise. In the remaining EVT cases, no exact time of embryonic demise was obtained. No data concerning chorionicity in the vanishing twin pregnancies were available from the fertility clinics.

Screening markers and gestational age

All physicians, nurses or midwives performing the NT scans were certified according to the Fetal Medicine Foundation (Snijders *et al*., 1998). Data on NT scan were registered at the departments of Fetal Medicine on registration forms which were collected and entered into a database. Biochemical measurements of PAPP-A and free β-hCG were determined in serum samples as part of the routine first trimester prenatal screening programme at SSI, Copenhagen. Briefly, the concentrations of the analytes were measured using either the Kryptor platform (Brahms, Henningsdorf, Berlin) or the AutoDelfia platform (Perkin Elmer Life Science, USA).

Concentrations of biochemical parameters were entered into the database of the first trimester prenatal screening program at SSI and automatically converted into MoMs by the laboratory information management system using underlying reference values based on a log-regression of parameters on GA in the Danish population and continuously monitored. The MoMs were calculated separately for the Kryptor and AutoDelfia platforms and were weight corrected. Internal and external (UKNEQUAS) quality assurance programmes continually assessed all laboratory methods and the laboratory is accredited by Danak (http://webtool.danak.dk/Plone/english/) in agreement with the specifications from the Danish National Board of Health.

GA at the time of blood sampling was calculated based on CRL and GA at the time of delivery was calculated based on the date of oocyte aspiration.

Statistics

For serum markers, the MoM values were calculated as described above.

The distributions of markers were tested by Kolmogorow–Smirnov test of fit. When the assumption of normality was not satisfied, non-parametric analysis was used, i.e. Kruskal–Wallis test for comparison between >2 groups and Mann–Whitney *U*-test for comparison between two groups. When normality was satisfied, the one-way ANOVA test and *t*-test were used.

Categorical data were compared with the χ^2 test. All analyses were made using SAS Enterprise Guide v 9.2. Statistical significance was defined as $P < 0.05$.

Results

Demographic characteristics of the study populations and controls are presented in Table I, showing that the groups are comparable with respect to baseline parameters. There were no significant differences between geometric mean MoM values of free β-hCG and PAPP-A in the three groups (Table II). The correlation coefficients between PAPP-A and free β-hCG log MoM values were 0.344, −0.082 and 0.288 for EVT, LVT and singletons, respectively. This further supports that there is no difference between the serum screening markers in EVT and singletons, whereas the small number of LVT cases precludes any such conclusion for that group. The GA at the time of blood sampling and NT scan together with the NT measurements were similar for the three groups (Table II). The NT measurements were also similar (Table II).

In Table III, the proportions of EVT and singleton pregnancies with a PAPP-A and free β-hCG $\log_{10}$MoM value above or below the 95th%iles and 5th%iles, respectively, of the value in the singleton pregnancies are shown. There was no significant difference between the distribution of extreme values of PAPP-A and β-hCG between the two groups. The corresponding values for LVT pregnancies were 0% below the 5th%iles and 22.2% above the 95th%iles of PAPP-A $\log_{10}$MoM and 0% below the 5th%iles and 11.1% above the 95th%iles of free β-hCG $\log_{10}$MoM; however, these numbers were too small to allow for statistical calculations.

Information on the occurrence of bleeding during first trimester was available in almost 80% of the cases in all groups. The incidence of bleeding in first trimester of the pregnancy did not differ between the groups: 6/36 (16.7%), 1/6 (16.7%) and 123/699 (18.7%) for EVT, LVT and singleton pregnancies, respectively, and there were no significant differences in the mean $\log_{10}$MoM values of PAPP-A and free β-hCG between pregnancies with and without bleeding.

Table I Demographic data on the different ART pregnancies

	Early vanishing twin (*n* = 47)	Late vanishing twin (*n* = 9)	ART singleton (*n* = 897)
Maternal age (years)			
Mean (SD)	33.6 (4.3)	32.6 (4.9)	32.8 (4.0)
Median (range)	34 (24–41)	33 (24–39)	33 (20–43)
Maternal weight (kg)			
Mean (SD)	68.7 (12.4)	65.6 (11.2)	68.2 (12.1)
Median (range)	66 (50–118)	64 (55–97)	66 (39–125)
Gravidity			
Mean (SD)	0.9 (1.2)	1.3 (1.6)	0.8 (1.2)
Median (range)	1 (0–5)	1 (0–4)	0 (0–9)
Parity			
Mean (SD)	0.3 (0.6)	0.2 (0.4)	0.3 (0.6)
Median (range)	0 (0–3)	0 (0–1)	0 (0–5)
Birthweight (g)			
Mean (SD)	3290 (609)	3028 (537)	3383 (615)
Median (range)	3200 (1880–3200)	2929 (2400–4150)	3430 (267–5410)
GA at delivery (days)			
Mean (SD)	276.1 (15.0)	270.2 (19.5)	275.4 (16.3)
Median (range)	279 (197–294)	270 (255–282)	279 (156–301)
Sex of the child (% boys)	55.3	55.6	49.1

MoM, multiple of the mean; SD, standard deviation; GA, gestational age.

Table II First trimester screening parameters for ART pregnancies with an early vanished twin, late vanished twin and singleton

	Early vanishing twin (n = 47)	Late vanishing twin (n = 9)	ART singleton (n = 897)
B-hCG MoM			
Mean (SD)	1.19 (0.78)	1.28 (0.72)	1.12 (0.70)
Median (range)	0.92 (0.24–3.65)	1.13 (0.46–2.91)	0.95 (0.09–6.42)
Log mean (SD)	−0.01 (028)	0.05 (0.23)	−0.02 (0.25)
Geometric mean	0.98	1.13	0.95
PAPP-A MoM			
Mean (SD)	1.02 (0.60)	1.17 (1.08)	0.90 (0.61)
Median (range)	0.92 (0.19–2.44)	0.85 (0.26–3.20)	0.77 (0.01–0.60)
Log mean (SD)	−0.08 (0.29)	−0.10 (0.40)	−0.13 (0.29)
Geometric mean	0.84	0.80	0.74
GA BC (days)			
Mean (SD)	65.5 (6.9)	69.9 (5.6)	67.3 (7.9)
CRL NT (mm)			
Mean (SD)	64.5 (7.7)	63.3 (8.7)	65.2 (7.7)
GA CRL (days)			
Mean (SD)	90.0 (3.9)	89.4 (4.6)	90.3 (3.9)
NT (mm)			
Mean (SD)	1.6 (0.6)	1.5 (0.26)	1.6 (0.5)

MoM, multiple of the mean; GA BC, the gestational age at the time of blood sampling; CRL NT, the measured crown-rump length at the nuchal translucency scan; GA CRL, the calculated gestational age based on crown-rump length at the time of nuchal translucency scan.

There was no association between the CRL of the surviving twin or the vanished twin and the serum screening markers, as shown in Fig. 1. Likewise, there was no association between the GA at the time of blood sampling and serum screening markers (Fig. 2).

A scatter plot illustrating the association between PAPP-A and free β-hCG for singleton and vanishing twin pregnancies is shown in Fig. 3. The points representing the vanishing twin pregnancies are evenly distributed among the controls and do not constitute a separate cluster.

Table III The proportions of pregnancies with an early vanished twin and singleton pregnancies with a PAPP-A and free β-hCG $\log_{10}$MoM value above or below the 95th%iles and 5th%iles

	Early vanishing twin (n = 47)	ART singletons (n = 897)	P-value
PAPP-A <5th%iles	4.3%	5%	1.00†
PAPP-A >95th%iles	4.3%	5%	1.00†
β-hCG <5th%iles	6.4%	5%	0.50†
β-hCG >95th%iles	8.5%	5%	0.29†

†Fisher's exact test.

This again supports that the serum screening markers do not differ significantly between the groups.

Discussion

The main result of this study is that first trimester serum markers in ART pregnancies are not affected by a vanishing twin, if this is diagnosed at an early ultrasound scan (<pregnancy Week 9). The early ultrasound scan is routinely performed at the fertility clinics to establish the presence of a live fetus *in utero*. In spontaneously conceived pregnancies, an early ultrasound scan is not usually mandatory, thus the vanishing twin phenomenon is less frequent diagnosed in early pregnancy. However, it is likely that the same will be the case in these pregnancies.

Our results are in contrast with the only other—to our knowledge—study on vanishing twins and serum markers by Chasen *et al.* (2006), who concluded that recent spontaneous reduction is associated with higher values of free β-hCG and PAPP-A in primarily IVF pregnancies compared with spontaneously conceived pregnancies. However, this study did not take into account the significant changes associated with the mere use of ART (Liao *et al.*, 2001; Tul and Novak-Antolic, 2006; Gjerris *et al.*, 2008a,b) which makes interpretation difficult.

One study concerning hCG as a tool for monitoring early pregnancy in fertility patients found that maternal serum hCG in pregnancies with a vanishing twin exhibited a slower rise of the serum concentration of hCG already from Day 12 of pregnancy when compared with normally

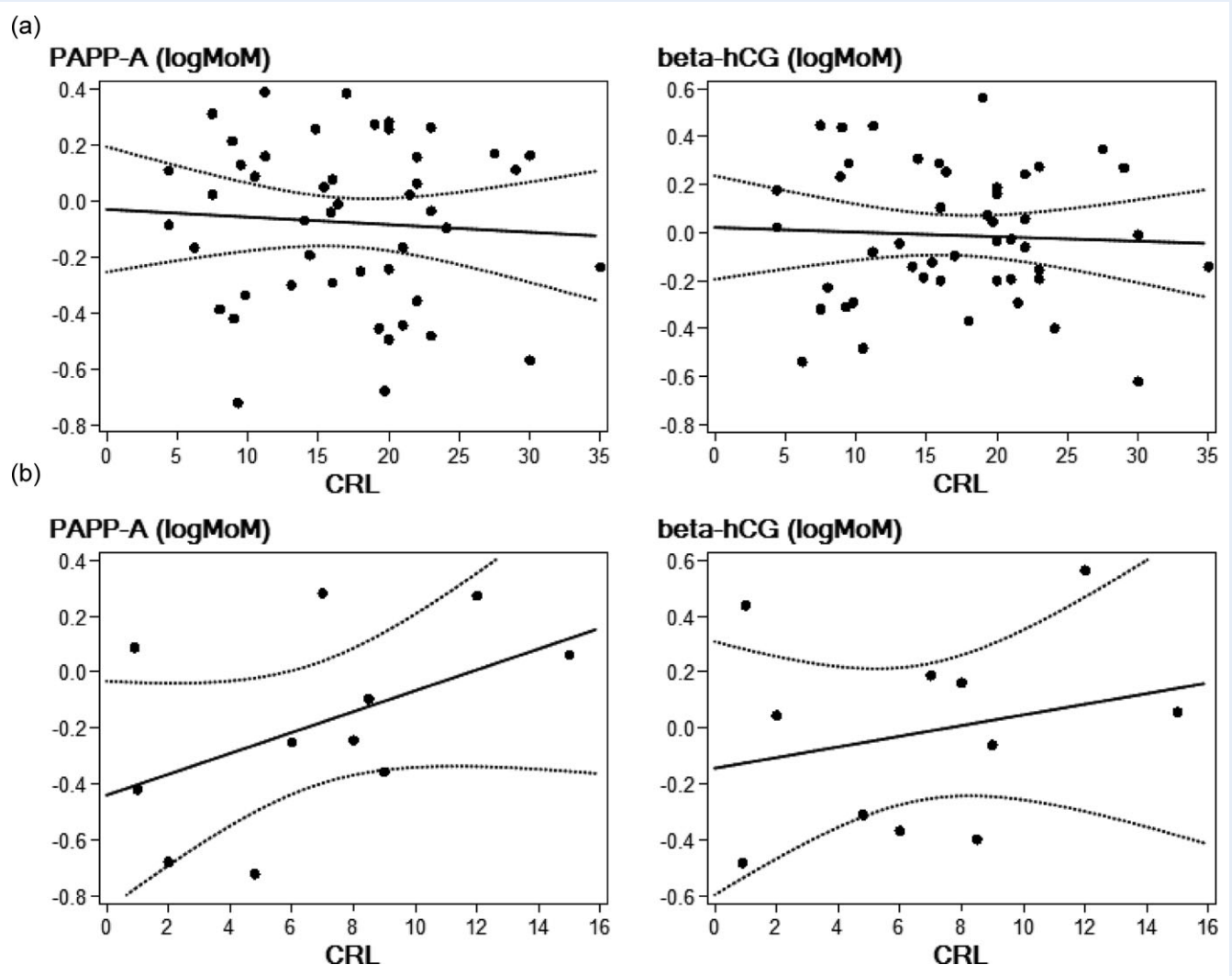

Figure 1 (**a**) LogMoM values of PAPP-A and β-hCG as a function of CRL for the 'surviving twin' at the time of early ultrasound and detection of a vanished co-twin.
The regression line and the 95% confidence interval are shown. Log_{10}PAPP-A $= -0.03 - 0.0023 \times$ CRL, $P = 0.65$; log_{10}β-hCG $= 0.02 - 0.0019 \times$ CRL, $P = 0.74$. (**b**) LogMoM values of PAPP-A and β-hCG as a function of CRL for the 11 cases of 'vanished twin' with an estimated CRL. The regression line and the 95% confidence interval are shown. Log_{10}PAPP-A $= -0.44 - 0.0373 \times$ CRL, $P = 0.13$ Log_{10}β-hCG $= -0.14 + 0.019 \times$ CRL, $P = 0.49$.

progressing twin pregnancies. From Day 44, the rate of serum hCG change became similar, but with absolute hCG concentrations of less than half in vanishing twin pregnancies compared with normally progressing twin pregnancies (Kelly *et al.*, 1991). These findings are in agreement with our results.

Another study regarding the second trimester serum screening marker α-fetoprotein (AFP) in pregnancies following elective multifetal pregnancy reduction demonstrated elevated maternal serum levels of AFP in these pregnancies, suggesting that AFP was released from the dead autolysed fetus(es) into the maternal circulation after reduction (Lynch and Berkowitz, 1993). This marker is produced by the fetus and yolk sac in contrast to free β-hCG and PAPP-A, which are produced by the placenta, making it unlikely that the same process should take place with these markers.

Residual placental tissue from the perished embryo might produce hormones causing higher than expected values of β-hCG and PAPP-A. However, our data showed that the markers were not significantly increased compared with singleton ART pregnancies. In the rare case of a surviving fetus with Down's syndrome, this might 'hide' a pathologically low serum level of PAPP-A related to the ongoing, chromosomally abnormal feto-placental unit. However, if this should happen it would most likely be associated with an elevated level of hCG which would entail an increased risk estimate for Down's syndrome using the standard algorithms.

In the case of a surviving fetus with trisomy 13/18, in which both markers will be decreased, there might be a small risk of overlooking this. However, these conditions are much rarer than trisomy 21, and the diagnosis is usually made or confirmed by ultrasound due to a very

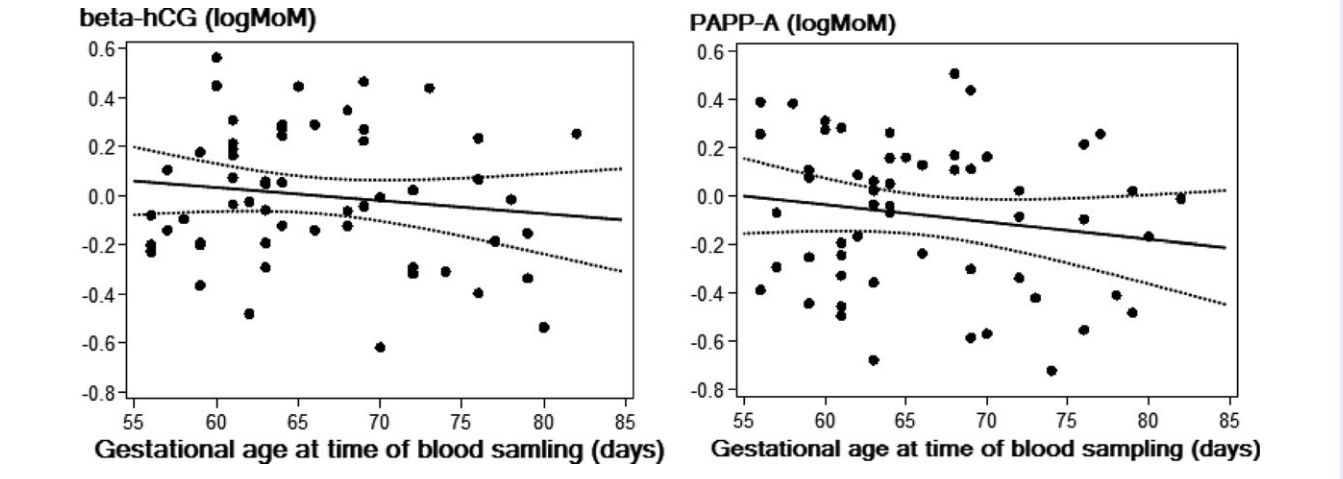

Figure 2 LogMoM values of PAPP-A and β-hCG as a function of the GA at the time of blood sampling.
The regression line and the 95% confidence interval are shown. Log_{10}PAPP-A = 0.39 − 0.0071 × days, $P = 0.23$; log_{10}β-hCG = 0.35 − 0.0053 × days, $P = 0.31$.

large NT and/or the presence of other fetal structural malformations characteristic of these conditions.

The vanished twin may have perished because of a chromosomal abnormality as it is well known that a large fraction of early spontaneous miscarriages occur due to chromosomal aberrations (Macklon *et al*., 2002). This might affect the serum markers *per se* and differentiate them from chromosomally normal ART twins and singletons, i.e. there may be a lower level of PAPP-A and higher beta-hCG (in the case of a Down's fetus) or low levels of both markers (in the case of a trisomy 13/18 fetus). If this occurrence has major impact on the screening parameters, it would be expected that the points representing the vanishing twin pregnancies in Fig. 3 should appear as separated clusters, which was not the case.

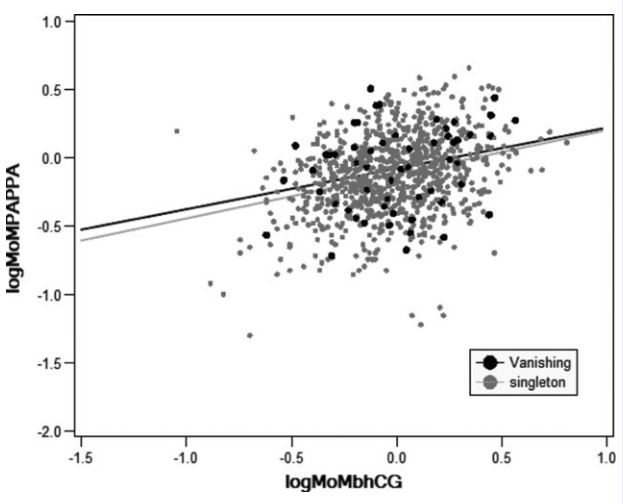

Figure 3 A scatter plot illustrating the association between PAPP-A and free β-hCG for singleton and vanishing twin pregnancies.

It is conceivable that the influence of the presence of a vanishing twin on the serum marker levels would depend on the size of the fetus at the time of embryonic demise. In this study, however, we demonstrate that if the embryonic demise occurred at least before Week 9, there is no relation between either increasing GA of the surviving twin (Fig. 1a) or size of the vanished fetus (Fig. 1b) and the first trimester serum screening marker levels expressed in MoMs. However, the number of cases in Fig. 1b is very small.

Bleeding during first trimester of pregnancy has been related to the demise of a co-twin (Saidi, 1988; De *et al*., 2006). Additionally bleeding has been demonstrated to influence first trimester biochemical markers causing increased levels of free β-hCG (De *et al*., 2003). We found no difference in the prevalence of bleeding during first trimester between the groups with normal singleton pregnancies and a vanishing twin, which is in line with a recent study by Pinborg *et al*. (2007). Likewise, we found no difference in the levels of first trimester serum markers between pregnancies with and without bleeding during first trimester. Thus, it seems that bleeding in first trimester is neither an indicator of the presence of a vanished twin nor influences first trimester serum markers significantly.

Several studies have demonstrated that a vanished twin is associated with poorer obstetric outcome such as preterm birth, low birthweight and small for GA (Pinborg *et al*., 2005,2007). Some of these conditions are also associated with a decreased level of PAPP-A (Barrett *et al*., 2008; Pihl *et al*., 2008) and it is likely that first trimester serum markers might be used as components in a risk assessment, e.g. pre-eclampsia and intrauterine growth restriction in the near future. Thus, it is possible that detection of high-risk pregnancy conditions by first trimester markers might be influenced by a vanished twin, and hence, not be a valuable tool in these particular pregnancies.

Chorionicity in twin pregnancies seems to impact the levels of maternal serum PAPP-A, with PAPP-A MoM values in monochorioinic

twin pregnancies being ~20% lower than in dichorionic twin pregnancies (Spencer *et al*., 2008). Several studies have found a higher rate of monochorionic twins in pregnancies conceived by ART compared with spontaneously conceived twins; however, this rate is still relatively small (Aston *et al*., 2008; Spencer *et al*., 2008). In a study concerning 15 644 ART single-embryo transfer cycles, a 2.3% rate of zygotic splitting was found (Blickstein *et al*., 2003). Unfortunately, we did not have information about chorionicity in the vanishing twin pregnancies. However, as estimated by the above described figures, one case with a 20% lower PAPP-A MoM levels is not likely to have any impact on the overall results.

The occurrence of the vanishing twin phenomenon is dependent on the rate of twin gestations, and as a consequence closely related to the use of ART. Several studies have demonstrated that first trimester serum screening markers for Down's syndrome are affected by mode of conception (Liao *et al*., 2001). It is still unknown why these markers are altered, and therefore it is also uncertain whether or not it is appropriate to correct them. However, the overall altered levels of serum screening markers and the associated risk of a higher false positive rate should be taken into consideration when interpreting the results of risk assessment for use in genetic counselling in ART pregnancies.

Thus, it seems, although based on a limited number of cases, that women pregnant after ART, who are diagnosed with a vanished twin at early ultrasound in Weeks 8–9, can have first trimester combined screening for Down's syndrome performed using the same risk calculation algorithm as in singleton ART pregnancies. In cases where the vanished twin is first diagnosed later in pregnancy, i.e. at the time of the NT scan, it is doubtful whether the serum risk assessment is as precise as it is in singleton ART pregnancies. For these later diagnoses, we consider it more appropriate to base the risk assessment on ultrasonographic markers solely, which are unaffected by the number of embryos. However, if the serum markers can be shown to accurately predict adverse pregnancy outcome, they may supplement the ultrasound evaluation also in these particular pregnancies.

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