PLACENTAL DEVELOPMENT IN ONGOING PREGNANCY AND MISCARRIAGE

Averil D. Reus

Placental development in ongoing pregnancy and miscarriage

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a.reus@erasmusmc.nl

PLACENTAL DEVELOPMENT IN ONGOING PREGNANCY AND MISCARRIAGE

De ontwikkeling van de placenta
in doorgaande zwangerschappen
en miskramen

Proefschrift

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Copromotor:	Dr. N. Exalto

Paranimfen:	Drs. N.C.J. Peters Drs. M.C. de Wit

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GENERAL INTRODUCTION

INTRODUCTION AND AIMS OF THE THESIS

Providing adequate nutrition to the fetus is essential for a successful pregnancy. Uterine glands are an important source of nutrients during organogenesis, when placental blood flow is not yet established and metabolism is essentially anaerobic. They therefore play a major role in early human pregnancy and their malfunction could be factor in early pregnancy loss.[1] The glands also release cytokines that may exert a powerful influence on placental development. Up to 9 weeks of gestation, fetal nutrition appears mainly to depend on uterine glandular secretions that are delivered into the intervillous space. Molecules diffuse through, or are transported by, the decidua and the trophoblast of the chorionic villi into the exo-coelomic cavity. From here they are absorbed by the secondary yolk sac. At the end of the first trimester the secondary yolk sac degenerate, and the exo-coelomic cavity is progressively obliterated by the increase of the amniotic cavity.[2]

The development of the placenta starts at the end of the first week after conception, during implantation. After implantation is complete, the original trophoblast surrounding the embryo has undergone differentiation into two layers: the inner cytotrophoblast and the outer syncytiotrophoblast. Lacunae in the rapidly expanding trophoblast have filled with uterine gland secretions. Late in the second week, defined cytotrophoblastic projections called primary villi begin to take shape. When a mesenchymal core appears within an expanding villus it is called a secondary villus. Surrounding the mesenchymal core of the secondary villus is a complete layer of cytotrophoblastic cells, and outside of that is the syncytiotrophoblast. The secondary villus becomes a tertiary villus toward the end of the third week of pregnancy when blood vessels arise in the mesenchymal core. Extravillous cytotrophoblastic cells, migrating out from the anchoring villi, invade the spiral arteries and cause major modifications of their walls. As a result, the arteries become wider and when the blood escapes from their open ends at the end of organogenesis it leaves at a low pressure. During these modifications endovascular trophoblast cells migrate into the spiral arteries in such way that their lumens are effectively plugged.[2-4] This prevents arterial blood to enter the intervillous space and makes that the embryo

can develop in a hypoxic environment. At the end of the first trimester the trophoblastic plugs occluding the utero-placental arteries are gradually dislocated, allowing maternal blood to flow into the intervillous space, and the uterine glands involute. Ultrasound and anatomical studies have demonstrated that the intervillous circulation starts in the periphery of the placenta at around 9 weeks of gestation and that it becomes continuous and diffuse after 12 weeks.[5, 6] These major anatomical transformations modify relationships between the maternal tissues and the developing embryo, and the materno-embryonic exchange pathways.[2] Overall these findings reveal that the architecture of the human first trimester gestational sac is designed to limit fetal exposure to oxygen to that which is strictly necessary for its development, and that during early pregnancy alternative nutritional pathways are in use.[2] Incomplete plugging of the arteries is associated with early onset of the maternal circulation into the placenta and failure of pregnancy.[4, 7-10] This excessive entry of maternal blood into the intervillous space has a direct mechanical effect on the villous tissue and an indirect oxidative stress effect, which contributes to cellular dysfunction and cell damage. Underdeveloped vascularization of the chorionic villi as a result of early deficient villous vasculogenesis and/or angiogenesis may result in miscarriage, early fetal growth restriction and preeclampsia.[11-14] In about two-thirds of early pregnancy failures there is anatomical evidence of defective placentation, which is mainly characterised by a thinner and fragmented trophoblast shell, and reduced cytotrophoblast invasion of the lumen at the tips of the spiral arteries.[8, 10, 15-17] Correlation of in vivo and in vitro data suggests that overwhelming oxidative stress of placental tissues represents a common pathophysiological mechanism for different etiologies of early pregnancy loss.[10] On the other hand the intraplacental oxygen concentration increases rapidly from 10 to 12 weeks of gestation with the onset of the maternal intervillous circulation.[9] This physiological burst of oxidative stress may play an important physiological role in triggering normal placental differentiation.[18]

In this thesis three-dimensional ultrasound, three-dimensional power Doppler ultrasound, virtual reality and histologic examination of the chorionic villous vascularization were used to investigate early placental development in normal ongoing pregnancy as well as miscarriage. The research objectives were:

1. To establish the reproducibility of trophoblast volume measurements using 3D ultrasound and to investigate a possible difference in trophoblast volume and growth between ongoing pregnancies and pregnancies ending in a miscarriage (**chapter 2**).

2. To establish the reproducibility of a new placental bed volumetric measurement using 3D power Doppler and virtual reality and to investigate a possible difference in placental bed vascular volume between spontaneously conceived pregnancies and pregnancies brought about by artificial reproductive technology (**chapter 3**).

3. To study the correlation between chorionic villous vascularization, ultrasound findings and corresponding chromosomal analyses in early miscarriage specimens from a cohort of recurrent pregnancy loss patients (**chapter 4**).

4. To investigate the relationship between the severity of Chronic Histiocytic InterVillositis (CHIV) and the outcome of pregnancy, and to compare the immune response between CHIV patients and controls to explore an immunological origin of CHIV (**chapter 5**).

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2

EARLY FIRST-TRIMESTER **TROPHOBLAST VOLUME** IN PREGNANCIES THAT RESULT IN LIVE BIRTH OR **MISCARRIAGE**

A.D. Reus

H. el-Harbachi

M. Rousian

S.P. Willemsen

R.P.M. Steegers-Theunissen

E.A.P. Steegers

N. Exalto

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ABSTRACT

Objectives To assess the validity of trophoblast volume measurements on three-dimensional ultrasound (3D-US) with Virtual Organ Computer-aided AnaLysis (VOCAL™), to create reference values between 6 and 12 weeks of gestation and to compare trophoblast volume between pregnancies ending in miscarriage and those resulting in live birth.

Methods In a prospective periconceptional cohort, we performed weekly 3D-US in 112 singleton pregnancies resulting in a non-malformed live birth and in 56 ending in miscarriage. Scans were performed between 6 and 12 weeks. Trophoblast volumes were calculated by subtracting the gestational sac volume from the volume of the total pregnancy. The interobserver and intraobserver agreement of measurements were determined to assess validity. Reference values were created for trophoblast volume in relation to crown-rump length and gestational age.

Results A total of 722 3D-US examinations were available for offline VOCAL measurements, but measurements could be performed in only 53% of these due to non-targeted scanning and incomplete framing. Interobserver and intraobserver agreement for trophoblast volume measurements were excellent, with intraclass correlation coefficients >0.97. Trophoblast volumes of pregnancies ending in miscarriage were significantly smaller ($P<0.01$) than were those of pregnancies that resulted in live birth. Trophoblast growth in pregnancies ending in miscarriage was also reduced compared with that in pregnancies that resulted in live birth.

Conclusions VOCAL is a valid technique for measuring trophoblast volume during the early first trimester of pregnancy. Pregnancies ending in miscarriage have smaller trophoblast volumes as well as reduced trophoblast growth compared with those that result in live birth.

Keywords Trophoblast volume; early first trimester; three dimensional ultrasound; VOCAL; miscarriage; reproducibility; placental volume.

INTRODUCTION

It is well known that successful first-trimester trophoblast invasion and growth are essential for second- and third-trimester placental function and subsequent outcome of pregnancy [1,2]. In about two thirds of cases of early pregnancy failure there is anatomical evidence of defective placentation, which is characterized mainly by a thin and fragmented trophoblast shell and reduced cytotrophoblast invasion of the lumen of the spiral artery [3]. This is associated with premature onset of maternal–placental circulation, resulting in an increased risk of miscarriage [4,5]. Precise trophoblast volume measurements during the early first trimester may therefore improve our knowledge regarding the success of early placentation and subsequent placental function, and their association with embryonic and fetal growth. Traditionally, embryonic and trophoblast volumes are measured using two-dimensional ultrasonography. Although this is a valid technique [6,7], it is time-consuming and not useful for routine clinical practice. Volume calculation from three-dimensional ultrasound (3D-US) examinations may be more applicable clinically, and measurement of in-vitro structures such as the placenta performed in 3D-US datasets has been shown to be valid using both conventional methods and the more recent Virtual Organ Computer-aided AnaLysis (VOCAL™; GE Medical Systems, Zipf, Austria) [8]. VOCAL has the advantage that it allows rotation of the 3D dataset around a central axis in a number of steps, the angle of which is defined by the user. Several studies have shown that the rotational technique is significantly more reliable than is the conventional measurement method [8,9]. In fact, the VOCAL measurement technique is now considered to be the 'gold standard' for sonographic volume measurements [10]. Before the 10th week of pregnancy the trophoblastic villi cover the entire surface of the gestational sac. In previous studies only the thickest part, rather than the complete trophoblast, was measured [6,11].

The aims of this study, therefore, were to assess the validity of 3D-US trophoblast volume measurements using VOCAL, to create reference values between 6 and 12weeks of gestation and to investigate possible differences in trophoblast volume between pregnancies resulting in live birth and those ending in miscarriage.

METHODS

For this study we used first-trimester 3D-US scans from patients recruited for the Rotterdam Predict Study, a prospective periconceptional birth cohort study [12–14]. In 2009, 141 patients between 6+0 and 8+0weeks entered the study, having weekly transvaginal ultrasound scans up to 12 gestational weeks. Of these, 17 were excluded from the study and 124 included, of which 112 were liveborn without any congenital anomaly and 12 ended in miscarriage (Figure 1). In 2009, an additional seven miscarriages from our early pregnancy unit were included and we continued including miscarriages from the Predict Study until July 2011 (n = 37). In the 56 pregnancies ending in miscarriage, a total of 127 scans (mean, 2.02; range, 1–6 scans per pregnancy) was performed, and in the 112 pregnancies ending in live birth, there was a total of 595 scans (mean, 5.3; range, 3–7 scans per pregnancy).

Written informed consent was obtained from all patients and the medical ethics review board approved the study. Each patient underwent a weekly 3D-US examination, performed in a clinical setting by a single researcher (M.R.), using a Voluson E8 (GE Medical Systems) ultrasound machine with standard settings and a 6–12-MHz transvaginal probe. Trophoblast volume measurements were performed offline with VOCAL by a single researcher (H.E.H.) using specialized 3D software (4D View, GE Medical Systems). A sequence of 12 sections of the trophoblast was obtained using a rotational step of 15° (Figure 2). Trophoblast and myometrium can be distinguished by their difference in echogenicity, and measurements were performed in a dark room, making this difference clearer for the examiner to see. In each section the contours of both the total pregnancy (Figure 2b) and the gestational sac (Figure 2a) were traced manually, and from these the computer provided a reconstruction of the trophoblast and the calculated volume [15,16], by subtracting the gestational sac volume from the total pregnancy volume. All measurements were performed three times and the mean values were used for further analysis.

The crown–rump length (CRL) was measured very precisely using a validated virtual reality application, 'I-Space' [12,17]. Gestational age (GA) was determined by the first day of the last menstrual period (LMP) in cases

that conceived spontaneously if they had a regular cycle (28 ± 3 days), by the day of oocyte retrieval plus 14 days in cases of in-vitro fertilization/ intracytoplasmic sperm injection and by the day of embryo transfer plus 17 or 18 days in pregnancies originating from transfer of cryopreserved embryos, depending on the number of days between oocyte pick-up and cryopreservation of the embryo. In spontaneously conceived pregnancies, if the first day of the LMP was unknown, or if the observed CRL differed by more than 6 days from the expected CRL, the pregnancy was excluded from the study (n = 6). In cases of embryonic death, only the measurements obtained when there was a positive heart beat were used for further analysis; in cases of empty sac miscarriage, all measurements were used. Of the 595 scans in the pregnancies that resulted in live birth, 322 were excluded from analysis, as were 20 of the 127 scans in pregnancies ending in miscarriage (47% in total) because parts of the trophoblast could not be visualized, the trophoblast was located too far away from the high-resolution transvaginal probe due to an intermediate position of the uterus or fibroids, acoustic shadowing or artifacts caused by fetal movement. Therefore, available for analysis were 273 (46%) scans from 112 pregnancies that resulted in live birth and 107 (84%) scans from 56 pregnancies ending in miscarriage. For the evaluation of intraobserver agreement, 3D volumes were measured twice by one observer (H.E.H.) in 24 pregnancies selected arbitrarily (four from each week of gestation). The time interval between the two measurements was at least 1 week. For the evaluation of interobserver agreement, another observer (A.R.) performed the same measurements once. The observers were blinded to each other's results. As for those obtained for the primary analysis, all measurements were performed three times on each occasion, with the mean value used for further analysis.

Statistical analysis

Initial data analysis was performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). The intraclass correlation coefficient (ICC) was used to quantify the intra- and interobserver agreement. Good agreement was defined by an ICC of 0.90 or higher. We created Bland–Altman plots to

assess the agreement between and among the measurements of the two observers. The difference between the measurements of the two observers and the difference between the measurements of the same observer performed at least 1 week later were plotted against the mean of all measurements.

In pregnancies that resulted in live birth we analyzed the longitudinal measurements using a linear mixed model in SAS PROC MIXED (release 9.2, SAS Institute Inc, Cary, NC, USA) to create first-trimester reference curves for trophoblast volume. We used log-transformed models for both axes describing the relationships between trophoblast volume and GA or CRL in order to obtain approximate normal distributions. To analyze trophoblast volume (TV) in relation to CRL, we used the equation log2 (TV) = a + b $\times$ $\log_2$(CRL) with random effects for subject-specific slope and intercept. The same model equation was used for the analysis of GA by replacing CRL with GA. $P \leq 0.05$ (two sided) was considered the limit of statistical significance. In order to compare the average trophoblast growth profile of pregnancies ending in miscarriage and pregnancies that resulted in live birth, we estimated a joint model of trophoblast volume and survival using WinBUGS (MRC Biostatistics Unit, Cambridge, UK)[18]. Growth was modeled using a linear model with subject-specific intercept and slope. A piecewise constant hazard function was used to model the probability of miscarriage. For each time point (t) we calculated the expected trophoblast volume for the pregnancies that were ongoing at time (t) but ultimately ended in miscarriage. We did this by taking a weighted average over the predicted volumes, where the weights were the expected probabilities of miscarriage occurring beyond that time point. In a similar way we calculated the expected volume for those pregnancies that were ongoing at time (t) and did not result in miscarriage. To check for the significance of differences we calculated the Z-scores of the measured trophoblast volume (TV) as: Z-score = (measured TV–expected TV)/SD, and compared the Z-scores of the pregnancies that resulted in live birth to those of the pregnancies ending in miscarriage using an independent samples t-test.

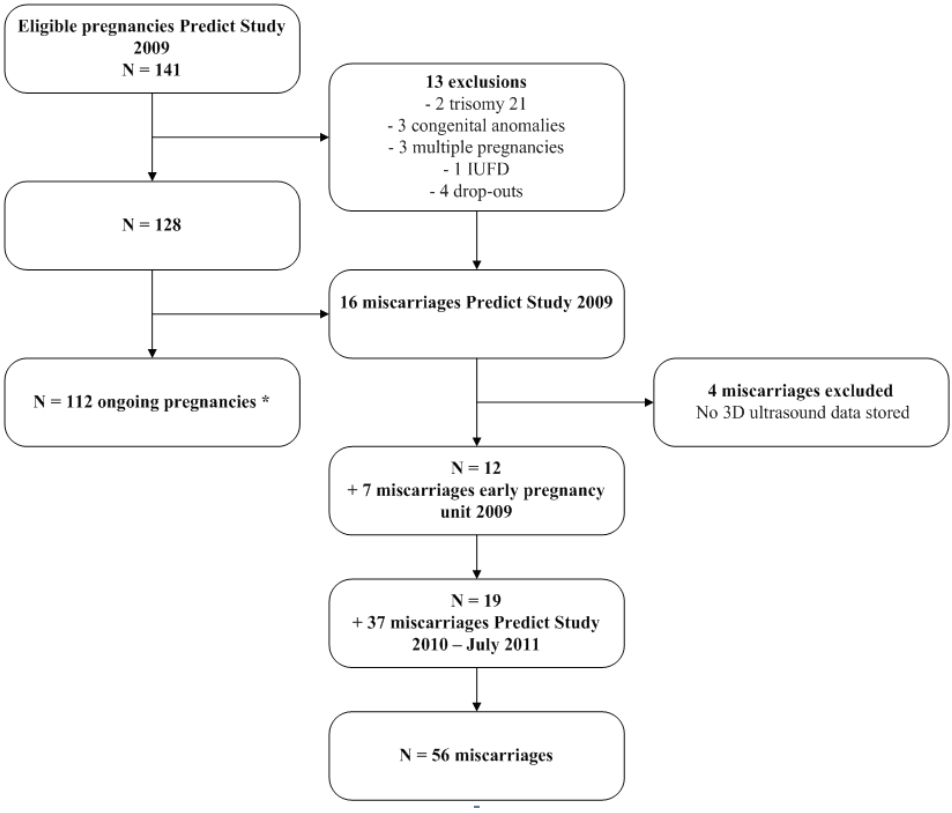


Figure 1: *Flowchart showing inclusion of patients in the study. *To calculate relation between gestational age (GA) and trophoblast volume we excluded six patients in whom GA was adjusted for crown–rump length. †One patient dropped out because of religious background, two were unable to participate on a weekly basis and one was lost to follow-up. 3D, three-dimensional; IUFD, intrauterine fetal death.*

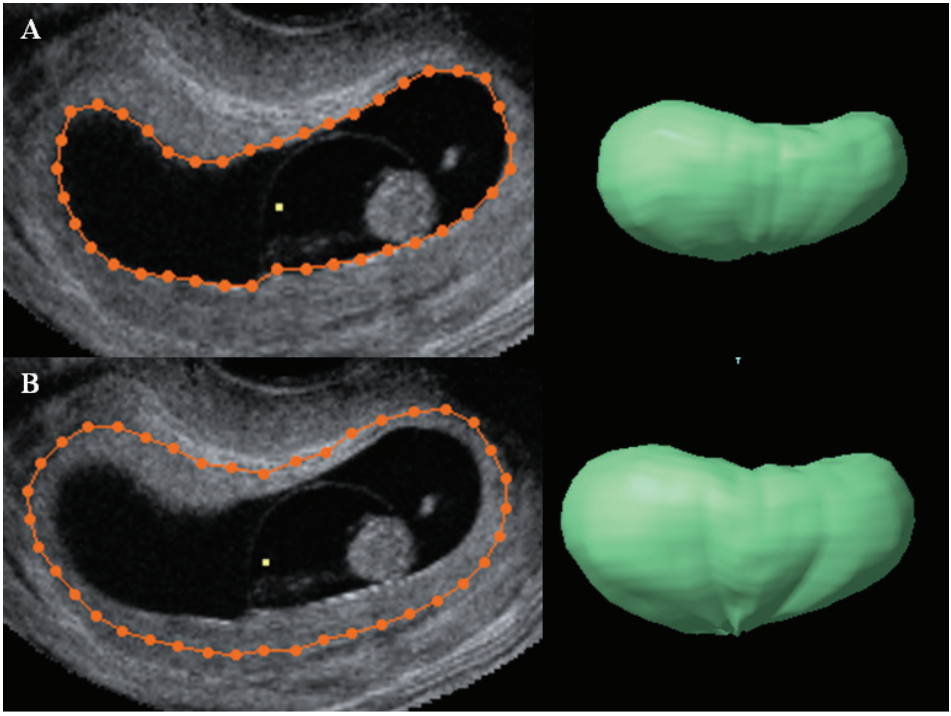


Figure 2: *Trophoblast volume was calculated by subtracting gestational sac volume* ***(a)*** *from total volume of pregnancy* ***(b)****, using Virtual Organ Computer-aided AnaLysis (VOCAL™).*

RESULTS

Demographic and pregnancy characteristics of the study group are shown in Table 1. The women in the miscarriage group had a significantly greater number of previous pregnancies compared with those women in the live-birth group ($P<0.01$). There were two women who smoked during pregnancy and one who consumed alcohol. The trophoblast volume ranged from 0.6 to 142.5 (median, 19.2) cm^3 in the 273 scans of pregnancies that resulted in live birth, and from 0.1 to 90.5 (median, 5.0) cm^3 in the 107 scans of pregnancies ending in miscarriage. Details of the trophoblast volume measurements obtained per gestational week are shown in Table 2. The CRL measurements for these scans ranged from 2.5 to 67.8 (median, 24.9) mm. Both the inter- and intraobserver agreement for trophoblast volume measurements were good, with ICCs of 0.976 and 0.997, respectively. The inter- and intraobserver ICCs and 95% CIs of the trophoblast volume measurements per gestational week are shown in Table 3. The ICC values for the gestational sac volume measurements were 0.999 for both inter- and intraobserver agreement. Bland–Altman plots of the differences between pairs of measurements are given in Figure 3. Scatterplots of trophoblast volume vs GA and CRL for pregnancies that resulted in a live birth in combination with plotted curves for the median and 2.5^{th} and 97.5^{th} percentiles derived from the linear mixed model analysis showed good fit of the data to the model, in which log-transformation of all variables resulted in approximate linear relationships ($P<0.001$) (Figure 4). Following the model structure described, for CRL: $a \pm SE = -1.5396 \pm 0.1924$ and $b = 1.4372 \pm 0.03827$, $P<0.01$; and for GA: $a = -26.8107 \pm 0.9218$ and $b = 5.3055 \pm 0.1525$, $P<0.01$.

The study included 112 pregnancies that resulted in live birth without any congenital abnormality. In the 56 pregnancies ending in miscarriage, there were 42 fetal deaths and 14 empty sac miscarriages. The trophoblast volume measurements in pregnancies ending in miscarriage were significantly smaller compared with those in pregnancies that resulted in live birth (mean ± SD Z-scores, –1.28 ± 1.23 vs −0.09 ± 0.99, $P<0.01$) (Figure 5). Trophoblast growth in pregnancies ending in miscarriage was also reduced compared with that in pregnancies that resulted in live birth (Figure 5).

Table 1: *Demographic and pregnancy characteristics of women included in the study, according to pregnancy outcome of live birth or miscarriage.*

Parameter	Ongoing pregnancies (n= 112)	Miscarriages (n = 56)	P-value
Gravidity	2.0 (1 – 9)	3.5 (1 – 15)	0.001
Parity	0 (0 – 3)	0 (0 – 3)	0.60
Nulliparous %	68 (60.7)	31 (55.4)	
Maternal age (years)	33.0 (19 – 42)	32.0 (24 – 45)	0.48
Body mass index (kg/m²)	23.8 (19.1 – 38.3)	23.3 (18.2 – 38.3)	0.68
IVF/ICSI (%)	32 (28.6)	15 (26.8)	0.86
GA at delivery or miscarriage (weeks)	39+4 (26+4 – 42+0)	8+0 (5+0 – 16+2)	
Birth weight (g)	3390 (450 – 4700)		
Female neonate (%)	59 (52.7)		

Data are given as median (range) or n (%). There were no missing values. GA, gestational age; ICSI, intracytoplasmic sperm injection; IVF, in-vitro fertilization; NA, not applicable; NK, not known.

Table 2: *First-trimester total trophoblast volume (TV) measurements in singleton pregnancies, according to outcome of live birth (n = 112) or miscarriage (n = 56) and gestational age (GA) at scan.*

			TV measurement (cm^3)		
Outcome/GA at scan	Available 3D volumes (n)	Success of TV measurement (n(%))	Mean	SD	Range
Live birth					
GA at scan					
6 weeks	66	56 (84.8)	6.0	3.4	0.6-13.6
7 weeks	102	68 (66.7)	13.6	8.0	1.5-39.6
8 weeks	103	63 (61.2)	27.1	17.3	2.8-80.6
9 weeks	107	44 (41.1)	45.2	20.7	9.3-101.7
10 weeks	108	30 (27.8)	50.0	24.1	19.4-142.5
11 weeks	109	12 (11.0)	69.1	22.3	35.0-110.8
6 – 11 weeks (total)	595	273 (45.9)	26.7	23.7	0.6-142.5
Miscarriage					
GA at scan					
6 weeks	42	38 (90.5)	2.6	2.3	0.1-11.3
7 weeks	38	35 (92.1)	6.9	7.1	1.2-41.9
8 weeks	29	21 (72.4)	10.7	6.2	0.7-26.0
9 weeks	8	5 (62.5)	22.1	10.2	9.0-32.7
10 weeks	5	4 (80.0)	44.0	4.3	40.6-50.4
11 weeks	5	4 (80.0)	56.5	23.0	39.7-90.5
6 – 11 weeks (total)	127	107 (84.3)	10.1	14.1	0.1-90.5

Table 3: *Inter- and intraobserver agreement for first-trimester total trophoblast volume measurements in 24 singleton pregnancies that resulted in live birth, according to gestational age (GA) at scan.*

	GA	N	Mean difference (95% CI)	Limits of agreement	ICC (95%CI)
Interobserver	6	4	-0.40 (-2.75 to 1.94)	-2.55 – 2.55	0.884 (0.025-0.992)
Intraobserver	6	4	-0.06 (-0.72 to 0.59)	-0.89 – 0.47	0.993 (0.897-1.000)
Interobserver	7	4	-1.19 (-3.46 to 1.07)	-4.04 – 1.66	0.969 (0.611-0.998)
Intraobserver	7	4	0.83 (-2.05 to 3.70)	-2.78 – 4.44	0.949 (0.426-0.997)
Interobserver	8	4	2.65 (-7.78 to 13.08)	-10.45– 15.76	0.928 (0.272-0.995)
Intraobserver	8	4	0.49 (-0.91 to 1.90)	-1.28 – 2.26	0.998 (0.969-1.000)
Interobserver	9	4	6.30 (-9.58 to 22.18)	-3.68 – 16.28	0.911 (0.163-0.994)
Intraobserver	9	4	0.16 (-0.91 to 1.23)	-1.19 – 1.50	0.999 (0.990-1.000)
Interobserver	10	4	-0.35 (-7.46 to 6.77)	-9.29 – 8.60	0.977 (0.695-0.998)
Intraobserver	10	4	0.98 (-2.59 to 4.55)	-3.51 – 5.47	0.995 (0.932-1.000)
Interobserver	11	4	2.32 (-5.47 to 10.10)	-7.46 – 12.10	0.980 (0.728-0.999)
Intraobserver	11	4	3.69 (-1.04 to 8.42)	-5.24 – 12.61	0.993 (0.894-1.000)
Interobserver	Total	24	1.56 (-0.82 to 3.93)	-12.8 – 9.71	0.976 (0.945 to 0.989)
Intraobserver	Total	24	1.01 (0.17 to 1.86)	-3.01 – 5.04	0.997 (0.992 to 0.999)

N= number of pregnancies, GA= gestational age, SD= standard deviation. Mean difference = first measurement minus second measurement, Limits of agreement = mean difference ± 2SD.

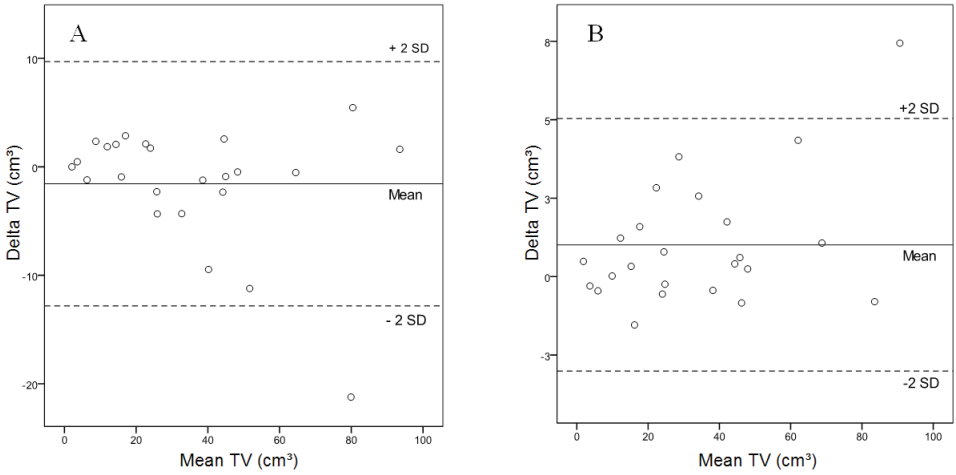


Figure 3: *Interobserver (a) and intraobserver (b) Bland–Altman plots for trophoblast volume (TV) measurements in 24 pregnancies that resulted in live birth. Lines indicate mean difference and 95% limits of agreement, calculated as mean difference ±2 SD.*

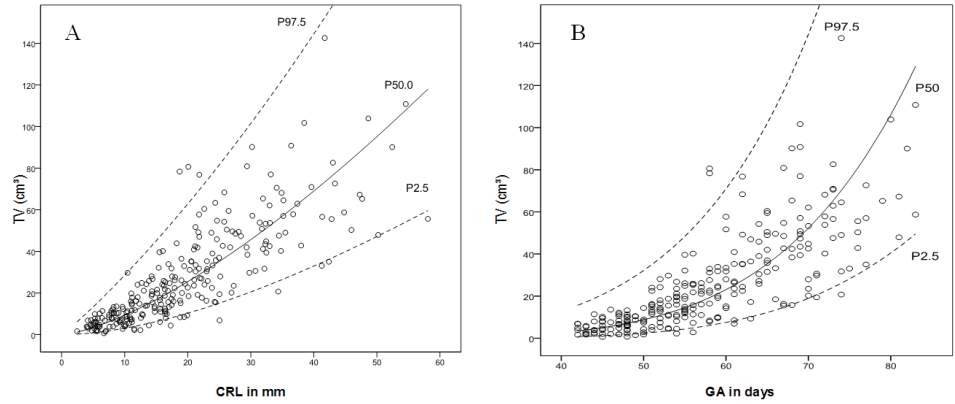


Figure 4: *Scatterplots of trophoblast volume (TV) measurements in relation to: (a) crown–rump length (n=112) and (b) gestational age (n=106), in pregnancies that resulted in live birth. Lines indicate mean and 95% prediction interval derived from linear mixed model analysis.*

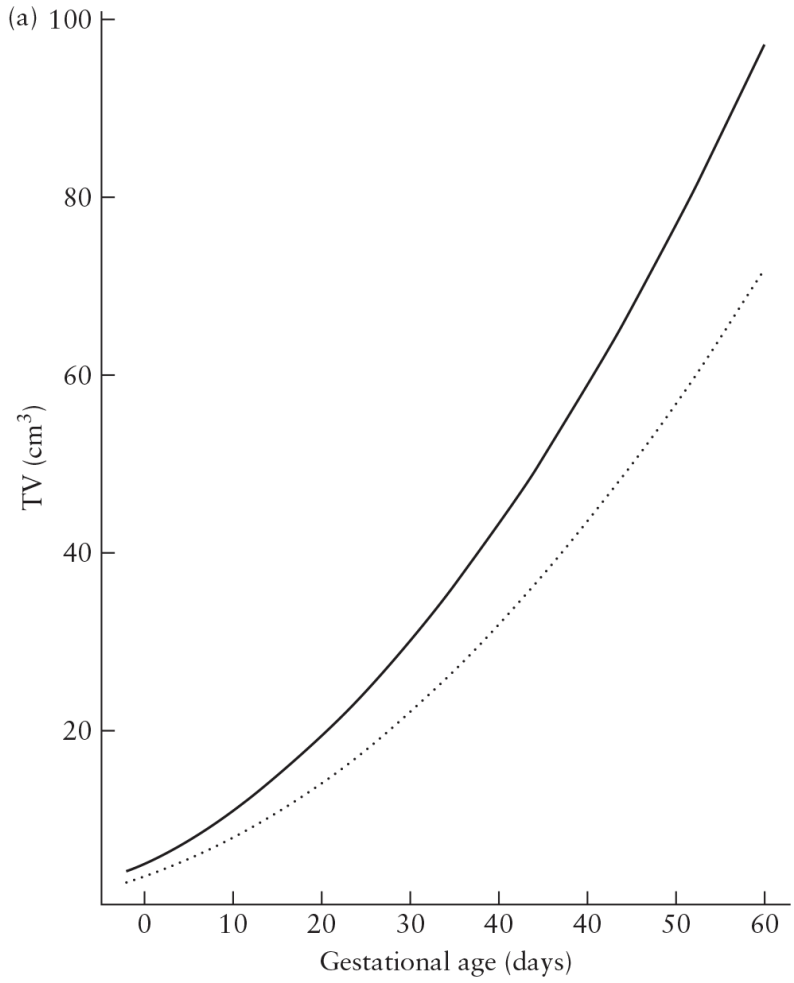


Figure 5 (A): *Trophoblast growth curves by the WinBUGS joint model of pregnancies resulting in a live birth (black line) and ongoing pregnancies (i.e. at time t) that ultimately result in a miscarriage (dotted line). The lines represent the mean values.*

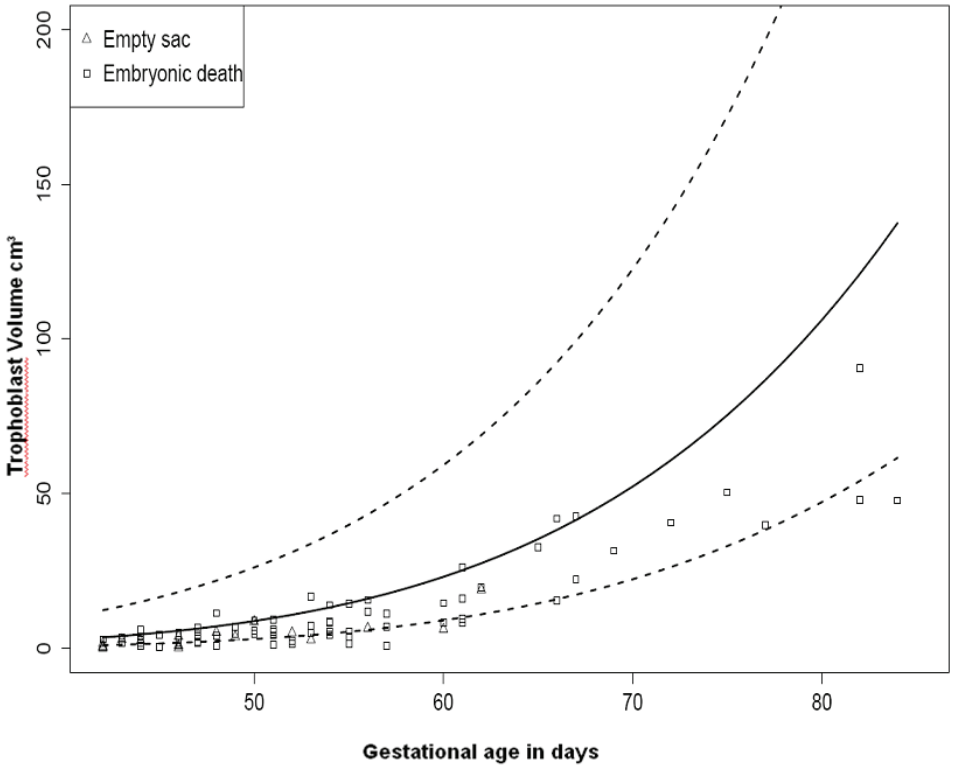


Figure 5 (B): *The mean value of the ongoing pregnancies is represented by the solid line and the 90% prediction intervals are presented by the dotted lines. The embryonic death measurements are represented by the quadrangle markers and the empty sac miscarriages by the triangles.*

DISCUSSION

We found smaller first-trimester trophoblast volume as well as reduced trophoblast growth in pregnancies ending in miscarriage compared with those resulting in live birth. To the best of our knowledge, trophoblast volume measurements have only been performed previously at the end of the first trimester and in the second trimester of pregnancy. Like our measurements at 6–12 weeks, these measurements were also shown to be reproducible [15,19,20]. Fetal volumes and placental volumes in late first and early second trimesters have also been used in screening for fetuses with chromosomal abnormalities [15,21] and intrauterine growth restriction (IUGR), as well as in pregnancies complicated by pre- eclampsia [9,19,22–24]. The placentae of women with high-resistance uterine perfusion in the second trimester and in fetuses that were subsequently found to be small-for-gestational age (SGA) were shown to be already remarkably small at 12 weeks of gestation, suggesting that placental growth is reduced early in IUGR, becoming apparent later in pregnancy [24]. In our study only five patients delivered a SGA child, so we are unable to reflect on any associations with trophoblast volume. Mercé et al. found that intervillous circulation is increased abnormally when a miscarriage is diagnosed [25]. Premature onset of intervillous blood flow may increase oxidative stress on the early placental tissue and subsequently impair placental development [26–28]. This impaired development may be associated with smaller trophoblast volume, as seen in our study. These findings indicate the potential for the development of new diagnostic techniques for the early detection of placenta-associated complications [29]. As shown in our study, one such technique may be first-trimester trophoblast volumetry, which may also reflect the success of trophoblast invasion [30]. Because of the design of this exploratory study, the oversampling of miscarriages and the relatively small sample size of the study population, the value of a receiver–operating characteristics (ROC) curve is limited. However, to give an initial indication regarding the accuracy of trophoblast volume measurements to predict miscarriage, we created a ROC curve for trophoblast volume measurements obtained at 8 gestational weeks, and it was found to have an area under the curve of

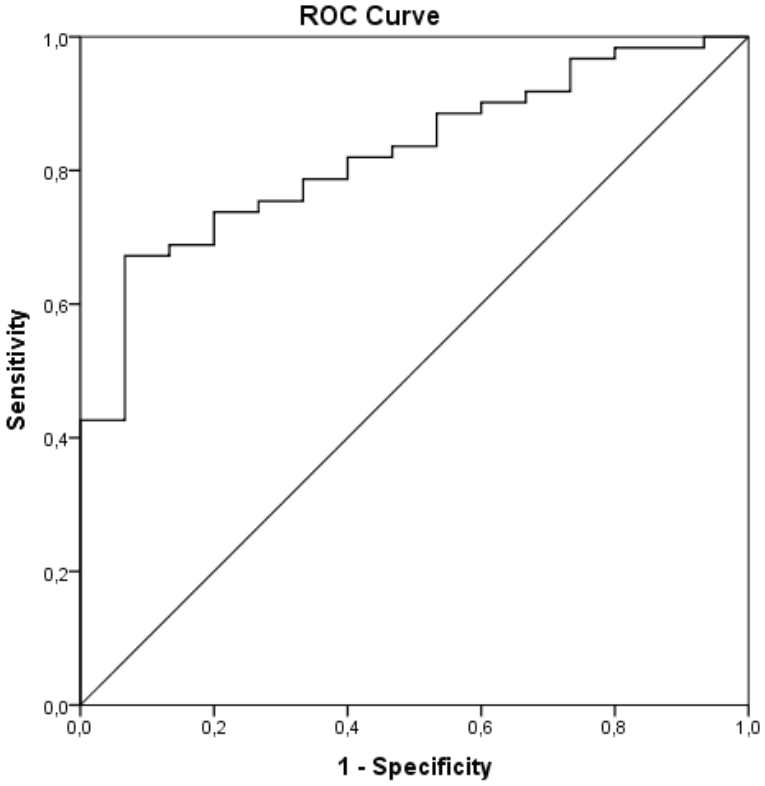
0.82 (Figure S1). In order to be able to evaluate correctly the sensitivity, specificity and predictive value of serial trophoblast volume measurements, we are continuing with this sub-analysis of eligible patients in the ongoing Rotterdam Predict study. Our study shows high validity of trophoblast volume measurements performed with VOCAL between 6 and 12 weeks' gestation, as reflected by the high interobserver and intraobserver reliability. Nowak et al.[6] and Nardozza et al.[11] have previously shown that VOCAL is a valid technique for placental measurements in the first trimester of pregnancy. We chose to start the trophoblast volume measurements at 6 weeks' gestation, because this is the earliest time in pregnancy at which the trophoblast is clearly visible. After 11weeks we were unable to measure the whole trophoblast volume using a transvaginal probe. Nowak et al.[6] and Nardozza et al.[11], who measured trophoblast volumes from 7 until 10+6weeks' gestation, did not measure the entire trophoblast volume, thereby underestimating the true volume. Between the 6th and 10th weeks of pregnancy, villi cover the entire surface of the gestational sac. In week 10 and week 11, we observed lateralization, with a thinning of the trophoblast layer on one side and thickening at the other. We measured the thinner site of the total pregnancy volume as close as possible to the gestational sac. In our opinion, this method will measure the true trophoblast volume in the most appropriate way, but this remains a matter of discussion. Although our measurements were shown to be reproducible, the difference in echogenicity between the trophoblast and the myometrium is hard to see, and measurements can be performed properly only in a dark room. Another limitation of the study is that it is not known exactly how the decidua fits into this measurement; one can only tell that there is a difference in echogenicity between trophoblast and myometrium that can be measured, and not whether this difference is at the trophoblast–decidua or the decidua–myometrium interface. Previous studies have also used VOCAL for placental volumetry in the first trimester [15,16]. Nowak et al.[6] proved that placental volumetry between 7 and 10 weeks' gestation using VOCAL with a 30° rotation step was just as accurate as was that performed using VOCAL with 12° steps and the multiplanar method (1-mm interval). They found a strong

correlation between GA and placental volume estimated using all (VOCAL 12°, VOCAL 30° and multiplanar) methods. These findings are relevant since evaluation of placental volume using the VOCAL method with a 30° rotation step is more practical and faster to perform than it is with the other methods, but apparently just as accurate. However, Cheong et al.[7] demonstrated that, in the in-vitro setting, measurements performed using VOCAL 30° were significantly less valid were than those performed using VOCAL 12°. There is some evidence from in-vitro studies that the volume of an irregular object can be measured better with the rotational technique of VOCAL than by the conventional technique [8,9]. Since the trophoblast is an irregular structure and because VOCAL is considered to be the 'gold standard' for sonographic volume measurements, we used VOCAL with a 15° rotation step to measure the trophoblast volume. Only two of 168 women included in the study smoked during pregnancy. A possible explanation for this low number may be the large number of patients that entered the study from our fertility clinic, where they are counseled about lifestyle factors and their consequences. Another explanation is the high socioeconomic status of a large number of the patients, which may have biased these results. A limitation of trophoblast volume measurements performed with VOCAL is that, due to incomplete or poor scan quality as a result of the position of the uterus or fetal movements and maternal bowel movements, only 53% of the stored 3D-US examinations could be used. As a result, the number of trophoblast volumes that could be measured decreased with increasing GA. In many cases the uterus was too large to fit in one 3D-US dataset. This problem may be solved using transvaginal ultrasound probes with a lower frequency. Additionally, in the miscarriage group the numbers were small after 9 weeks of gestation because a large proportion of the cases miscarried at an early stage. This should be taken into account when interpreting these results.

We conclude that trophoblast volume measurements using the VOCAL measurement application in 3D-US datasets obtained during the first trimester of pregnancy are reproducible. This may be a first step towards

implementation of a technique in clinical practice for identifying women at risk for the development of placenta related pregnancy complications. Whether trophoblast volume can be measured depends on the quality of the 3D-US scan as determined by GA and the size and position of the uterus. Pregnancies ending in a miscarriage show smaller trophoblast volumes as well as reduced trophoblast growth compared with pregnancies resulting in live birth. Future studies should establish the predictive value of first-trimester trophoblast volume with regards to other pregnancy complications.

Supplement figure: *receiver operating characteristic (ROC) curve.*



The area under the curve of 0.82 gives an idea of the accuracy of the trophoblast volume measurements as a predictor for miscarriage at a 8 weeks gestational age.

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3

EXAMINING PLACENTAL VASCULATURE USING THREE DIMENSIONAL VIRTUAL REALITY

CHAPTER 3.1

EARLY PREGNANCY **PLACENTAL BED AND FETAL VASCULAR** VOLUME MEASUREMENTS USING **3-D VIRTUAL** REALITY

A.D. Reus

J. Klop – van der Aa

M.S. Rifouna

A.H.J. Koning

N. Exalto

P.J. van der Spek

E.A.P. Steegers

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ABSTRACT

Objectives Quantification of placental vascularization by performing measurements on three-dimensional Power Doppler (3DPD) ultrasound datasets may enhance our insight in normal and abnormal uteroplacental anatomy and placentation. V-Scope is a new Virtual Reality application which enables visualization of all three dimensions in 3DPD ultrasound. The aims of this study were to introduce a new technique for examining placental and uterine vasculature and anatomy, to assess validity of placental bed vascular volume (PBVV) and fetal vascular volume (FVV) measurements with V-Scope at 12 weeks of gestation and to investigate possible associations of PBVV and FVV with embryonic volume, crown-rump length, fetal birth weight and maternal parity.

Methods We analyzed 100 3DPD datasets of 100 pregnancies at 12 weeks of gestation using V-Scope in the I-Space virtual reality system. Volume measurements with V-Scope on placental vasculature were performed with semi-automatic pre-defined parameters. The interobserver and intraobserver agreement by two researchers was determined to assess validity. We correlated PBVV and FVV to embryonic volume, crown-rump length, birth weight and maternal parity.

Results Both the interobserver and intraobserver agreement was found to be excellent with all ICCs >0.93. PBVV of multiparous women were significantly larger than the PBVV of primiparous women ($p = 0.008$). No other correlations were found.

Conclusions V-Scope offers a reproducible method for measuring placental bed vascular volume at 12 weeks of gestation. Maternal parity influences PBVV. The clinical value of such measurements needs to be further studied.

Keywords Placental vascularization; placental bed vascular volume; 3D Power Doppler Ultrasonography; Barco I-Space; Virtual Reality.

INTRODUCTION

Development of the human placental bed is a unique process starting after fertilization[1,2]. Abnormalities in this development in first trimester can lead to pregnancy-associated complications like preeclampsia and fetal growth restriction and also to poor obstetric outcome[3-5]. If, however, we could develop a reproducible method to quantify the vascularization of the placenta in vivo, this may enable a more adequate description of normal and abnormal placental development. In the future this may also contribute to prediction models to identify 'at-risk' women in the first trimester of pregnancy[6].

Already in 1954 Kloosterman concluded that fetal growth and birth weight depend on placental size. Placentas of multiparous women appeared to be heavier compared to the placentas of primigravid women[7, 8]. Recent advances in three-dimensional Power Doppler (3DPD) ultrasound offer evaluation of the architecture of the placental vasculature and quantification of placental vascularization and perfusion. Vascular indices, like placental bed vascular index (PBVI), calculated using Virtual Organ Computer-aided AnaLysis (VOCAL) software, provide assessment of the placental vascularization[9-11]. Calculation of these indices in first trimester proved to be reproducible[12]. However, standardization of 3DPD indices is needed before their introduction into clinical practice[13, 14].

To provide depth perception and thus allow a better visualization of the placental bed vascularization we visualized three-dimensional (3D) ultrasound datasets in an innovative Virtual Reality (VR) system. 3D 'holograms' were created with the V-Scope volume rendering application in the Barco I-Space (Barco N.V., Belgium) at the Erasmus MC Rotterdam[15]. V-Scope is a newly developed 3D volume visualization application using a Barco I-Space virtual reality system[16]. This technique allows efficient measurement of the total placental vascularization, where the VOCAL approach is either used to measure vascular indices in a spherical region of interest or requires labor-intensive manual delineation of the placenta.

The aims of this study were to introduce a new technique and different method for examining placental and uterine vasculature and anatomy, to

assess validity of placental bed vascular volume (PBVV) and fetal vascular volume (FVV) measurements with V-Scope at 12 weeks of gestation and to investigate possible associations of PBVV and FVV with embryonic volume (EV), crown-rump length (CRL), fetal birth weight, BMI and maternal parity.

MATERIALS AND METHODS

Patient selection

From November 2009 until December 2010, 133 healthy, pregnant women enrolled in this study. After exclusion of pregnancies with congenital anomalies, miscarriages, twin pregnancies and chromosomal abnormalities 132 patients remained in our study cohort, consisting of 84 spontaneously conceived pregnancies and 48 patients who conceived by artificial fertilization treatment. Gestational age (GA) was calculated using the first day of the last menstrual period, and in cases of an unknown last menstrual period or a discrepancy of more than a week, the GA was determined by the CRL measurements performed in the first trimester. For the IVF/ICSI pregnancies, the GA was based on the date of oocyte retrieval. Length and weight of the patients were recorded for BMI calculation, probably influencing the intensity of the Doppler signal. After delivery all patients were contacted to inquire about the pregnancy outcome. Written informed consent was obtained from all participants. The study was approved by the Medical Ethics review board of Erasmus Medical Center and is part of an ongoing prospective longitudinal cohort study, called the Rotterdam Predict Study[17-20]. In the Predict Study patients are included between 6+0 and 8+0 weeks, they receive a weekly 3D transvaginal ultrasound scans up to 12 gestational weeks. A transabdominal 3DPD ultrasound scan is made only at 12 weeks of gestation.

3-D ultrasonography and the Barco I-Space

Experienced operators, with a transabdominal probe (4-8 MHz), used a GE Voluson E8 (General Electrics Medical Systems, Zipf, Austria) to make

ultrasound scans. The 3D scans were performed transabdominally at 12 weeks of gestation using Power Doppler. Prenatal exposure to Power Doppler ultrasound waves is considered safe at 12 weeks of gestation.[21, 22] During the ultrasound the thermal index and the mechanical index stayed completely within limits for safety (MI: 1.2, TI: 0.2). Every scan was made with standard settings of the ultrasound machine: PRFs (pulse repetition frequencies) of 0.9 kHz, Gain -2.0, Quality "high", WMF (wall motion filter) "low". Patients were asked to hold their breath while the scan was made to limit disturbance by movement of the abdomen. Obtaining the scan takes approximately 30 seconds.

A total of 243 3DPD ultrasound scans from 132 patients were saved as Cartesian volumes using 4D view (Kretz, Zipf, Austria). Patients were only scanned on one occasion but in most cases more than one scan during the same consult was made to obtain good quality, with a mean of 1.84 ultrasound scans per patient. These datasets were transferred to the Barco I-Space at the department of Bioinformatics. In this four-walled CAVE™-like Virtual Reality (VR) system 'holograms' can be viewed with depth perception by wearing a pair of stereo glasses with polarizing lenses. The measurements of the placental vasculature were performed by using V-Scope. This application allows datasets to be manipulated in all three dimensions [15]; the volumes can be resized, turned and clipped to provide the optimal view of the vascularization. Semi-automatic volume measurements of the placental vasculature were obtained by thresholding the Doppler data [23], followed by manual correction of the segmentation to remove artefacts and select only the structures of interest. For this study, we set the lower-doppler level threshold at 100 for the most appropriate visualization of the vasculature in all scans. This means that all voxels with a grey value between 100 and 250 are colored and counted in the volume calculation. We visualized the vasculature in the I-Space by making the typical ultrasound signal of the data set temporary fully transparent. The following parameters of early pregnancy vasculature were determined: 'Complete Volume' (CV), 'Total Volume' (TV), 'Placental bed vascular volume' (PBVV) and 'Fetal Vascular Volume' (FVV). We measured the CV, meaning the vessels of the placental bed, the fetal

vasculature, the uterine vessels and artifacts. After this we performed a visual inspection to determine which structures were artifacts and consequently removed these with the help of a virtual eraser, to ensure the volume would not be overestimated, leaving us the TV. The artifacts can be recognized by their stripe-like appearance. Then we removed the vasculature of the fetus and measured the volume again. We calculated the FVV by subtracting the volume after erasing the vasculature of the fetus from the volume with the fetal vasculature. By removing all uterine vessels up to the point where they split in the placental bed with the virtual brush, we were able to measure the volume of the PBVV. The definitions and measurements of the different structures are shown in Figure 1. When images could not be optimally visualized because of incomplete framing, low image quality or too many artifacts, they were excluded. In 168 3DPD datasets of 100 patients we performed the measurements. For statistical analyses, we used only one dataset from each patient and chose the first scan that was made. Therefore, a total of 100 datasets, each of a different patient, remained for further analysis.

Statistical analysis

The results were analyzed using SPSS (Release 18.0., SPSS Inc, USA). The intra-class correlation coefficient (ICC) was used to quantify the intra-observer and inter-observer agreement. For intra-observer variability, one operator (J.A.) measured VR volumes twice with a time interval of at least one week in 20 at random selected datasets. For inter-observer variability, another operator (A.R) performed the same measurements. A single measurement consists of three separate attempts, and then the mean was used for further calculations. The observers were blinded for one another's results. For a good agreement, the ICC has to be 0.90 or higher. The CRL and the EV were already measured using VR in the Rotterdam Predict Study according to the method described earlier[24]. Bland-Altman plots [25] were created to assess the agreement between and among the two examiners because a flawed impression of reliability can be acquired by using intra-class correlation alone. Limits of agreement were calculated (mean difference

± 2 SD) and the difference between the measurements of the same operator at different times and the difference between the measurements of both operators were plotted against the mean of all measurements.

Spearman's rank correlation coefficients were calculated of PBVV and FVV and CRL at 12 weeks of gestation, EV at 11 weeks of gestation and fetal birth weight. We used the EV at 11 weeks instead of 12 weeks of gestation because EV measurement is generally only possible up to 11 weeks. After 11 weeks the success rate rapidly decreases because of fetal movements and large size of the fetus. To use the fetal birth weight for comparison, we adjusted for gender and gestational age at time of delivery.[26]

We used the Mann-Whitney U test to discover a correlation between PBVV and maternal parity. We also calculated the influence of BMI on the measured PBVV by calculating the Spearman's rank correlation coefficient. A p-value of 0.05 (two-sided) was considered the limit of significance.

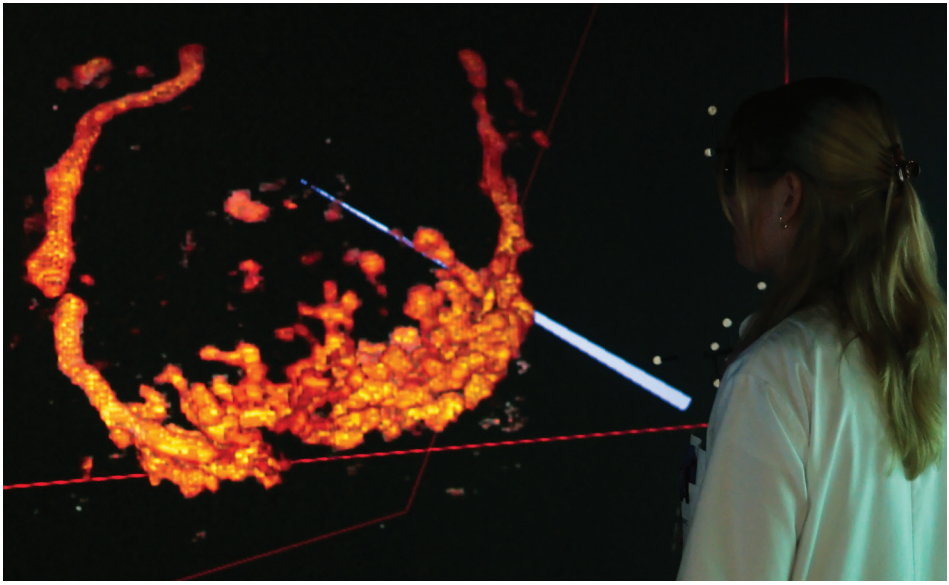

Figure 1a: *Photograph of an observer in the I-Space. An image of a placenta of 12+3 weeks' gestational age is projected on the wall.*

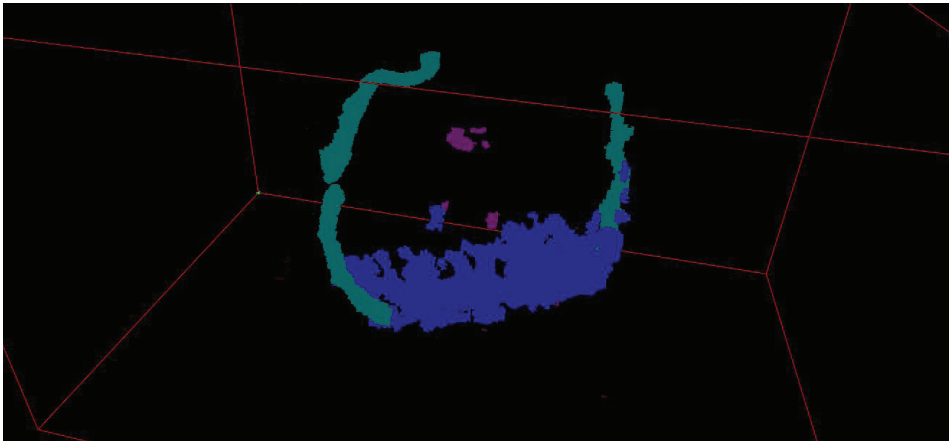

Figure 1b: *Shows how the different structures were defined and measured. Braches of the uterine arteries are shown in mint, the fetal vascular volume in purple and the placental bed vascular volume in blue, these structures together represent the Total Volume.*

RESULTS

A total of 132 patients were included in this study. The scans of 32 patients were excluded from analysis because of incomplete framing, low image quality or too many artifacts, leaving 100 patients. Clinical characteristics of the pregnancies are shown in Table 1. Of the 100 included pregnancies, all pregnancies resulted in the birth of a healthy child, except for one intra uterine fetal death occurring at 38 weeks of gestation. Autopsy in the case of intrauterine fetal death showed normal growth of the child according to gestational age and no congenital anomalies. The placenta showed signs of fetal thrombotic vasculopathy and recent ischemia. PBVV at 12 weeks of gestation was 13.77 cm^3 and did not differ more than 1 SD from the mean (15.26 cm^3) of all measurements. No chromosome results were available. One child had micrognathia and retrognathia. Three children were born premature, and three children had a birth weight below the 5^{th} percentile. In addition, two patients were lost to follow up. Ten women developed pregnancy-induced hypertension (n=4), preeclampsia (n=4) or hemolysis elevated liver enzymes and low platelets syndrome (n=2).

Results of the intra observer and inter observer variability are shown in Table 2 and 3. All ICC values were >0.93, indicating good agreement for all parameters. The mean differences between the measurements by the same operator and between two different observers and the limits of agreement are also shown in Table 2 and 3.

Bland-Altman plots of the difference between the measurements are given in Figure 2. The measurements of the TV between the two operators (Figure 2a) show a large difference in two of the datasets. In these datasets large artifacts were visible and were not removed to the same extent, resulting in large differences between the values of this parameter. This did not affect the overall agreement (ICC = 0.93). Differences between the placental vasculature measurements did not depend on the size of the volumes measured.

Table 4 shows the measured volumes of all parameters. There are not significant associations between PBVV and CRL (r_s= 0.183, p = 0.07),

EV (r_s= 0.142, p = 0.33) or between PBVV and birth weight (r_s= 0.183, p = 0.07). Neither were correlations found between FVV and CRL (r_s= 0.090, p = 0.38), EV (r_s= 0.049, p = 0.74) or between FVV and birth weight (r_s= 0.057, p = 0.58).

PBVVs of multiparous women (n = 31, mean 19.15 cm^3) were significantly larger than those of primiparous women (n = 69, mean 12.83cm^3) (p = 0.01) (Figure 3.). No differences were found in FVV (p = 0.36). The mean PBVV of pregnancies complicated by hypertensive disease (PIH, PE and HELLP, n = 10) did not differ from the mean PBVV of normal pregnancies (n = 90) (13.75 vs 15.93; p = 0.85). BMI shows to have an inverse relation with PBVV (r_s = -0.21, p = 0.04).

Table 1: *Study group characteristics.*

Parameter	Characteristics (n=100)
Gravidity (median, range)	2.0 (1 – 9)
Parity (median, range)	0.0 (0 – 3)
Gestational age (days) at US (median, range)	87.0 (84 – 90)
Embryonic volume (cm^3)(mean, SD)	8.4 (2.3)
Gestational age (weeks) at birth (median, range)	39.2 (31.3 – 42.1)
Maternal age (years) at US (mean, SD)	31.2 (4.9)
BMI (kg/height^2) (mean, SD)	24.6 (3.7)
ART %	39
Folate %	95
Smoking % (periconception/current)	13/6
Alcohol use% (periconception/current)	23/1
Birth weight (g) (mean, SD)	3249 (513)
Female sex %	54

US: ultrasound, SD: standard deviation, BMI: body mass index, ART: assisted reproductive technology.

Table 2: *Intra observer differences between the first and second measurements made by examiner 1.*

Parameter	Mean difference†	95% CI for mean difference	Limits of agreement‡	ICC	95% CI
TV	-0.30	-0.56 to -0.05	-1.40 to 0.80	1.000	0.999-1.000
PBVV	-0.03	-0.46 to 0.52	-2.08 to 2.13	0.994	0.985-0.998
FVV	0.07	-0.08 to 0.22	-0.57 to 0.72	0.964	0.913-0.986

TV = Total Volume; PBVV = Placental bed vascular volume; FVV = Fetal Vascular Volume; ICC = intra-class correlation coefficient; CI = confidence interval; SD = standard deviation.
† Mean difference = first measurement minus second measurement
‡ Limits of agreement = mean difference ± 2 SD

Table 3: *Inter observer differences between the measurements made by the two examiners.*

Parameter	Mean difference†	95% CI for mean difference	Limits of agreement‡	ICC	95% CI
TV	3.88	-1.41 to 9.17	-18.74 to 26.49	0.928	0.829-0.971
PBVV	-0.64	-1.25 to -0.03	-3.23 to 1.95	0.988	0.965-0.995
FVV	0.18	-0.04 to 0.40	-0.74 to 1.10	0.930	0.828-0.972

TV = Total Volume; PBVV = Placental bed vascular volume; FVV = Fetal Vascular Volume; ICC = intra-class correlation coefficient; CI = confidence interval; SD = standard deviation.
† Mean difference = first measurement minus second measurement
‡ Limits of agreement = mean difference ± 2 SD

Table 4: *Vascular volume measurements (cm3) at 12 weeks of gestation in normal pregnancy.*

Parameter	Mean (SD)	Median	Range
CV	63.05 (32.21)	57.63	7.56-180.53
TV	58.46 (30.17)	52.33	7.44-164.06
PBVV	14.79 (10.58)	12.70	1.46-76.79
FVV	2.84 (1.37)	2.66	0.08-6.90

CV = Complete Volume; TV = Total Volume; PBVV = Placental Bed Vascular Volume; FVV = Fetal Vascular Volume. SD = standard deviation, Data presented in mean, SD and range.

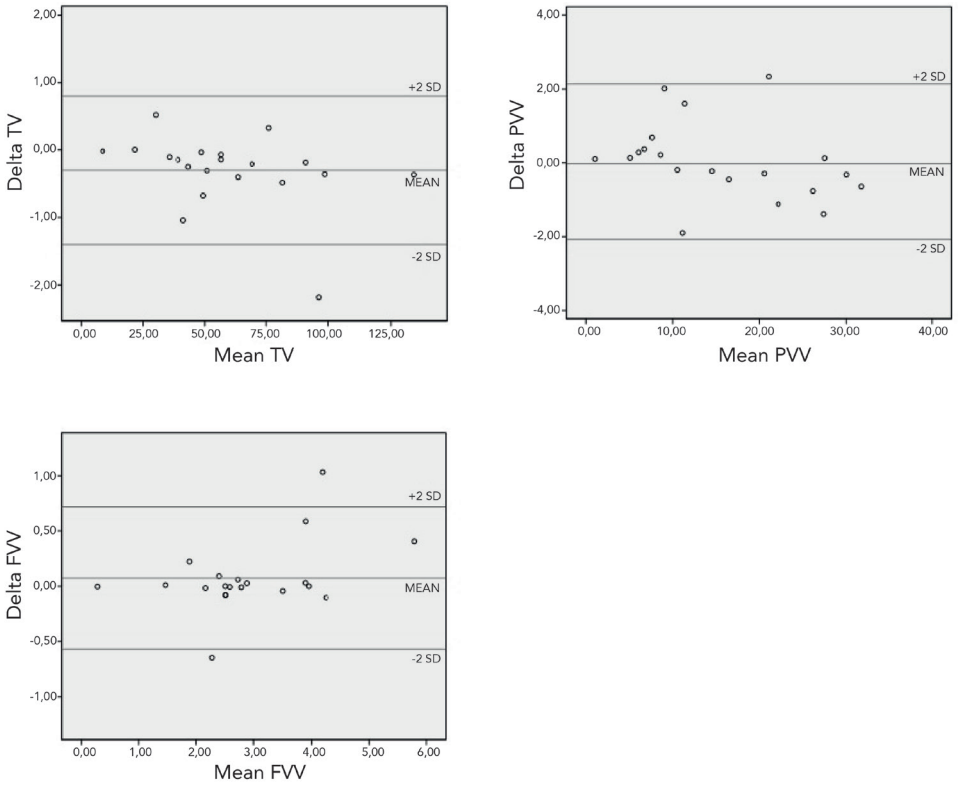

Figure 2a: *Bland-Altman plots comparing placental vasculature volume measurements within one observer (intra-observer variability). The lines represent the mean difference and the limits of agreement, calculated as mean difference ±2 SD. TV refers to Total Volume, PBVV refers to Placental bed vascular volume, FVV refers to Fetal Vascular Volume.*

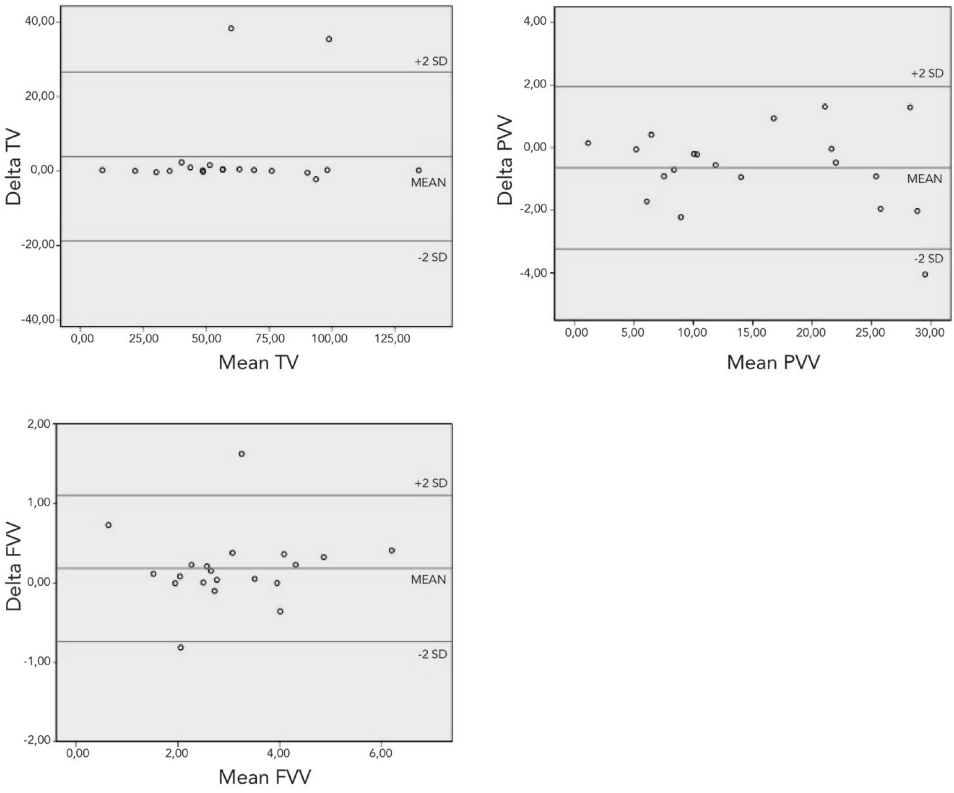

Figure 2b: *Bland-Altman plots comparing placental vasculature volume measurements between the two observers (inter-observer variability). The lines represent the mean difference and the limits of agreement, calculated as mean difference ±2 SD. TV refers to Total Volume, PBVV refers to Placental bed vascular volume, FVV refers to Fetal Vascular Volume.*

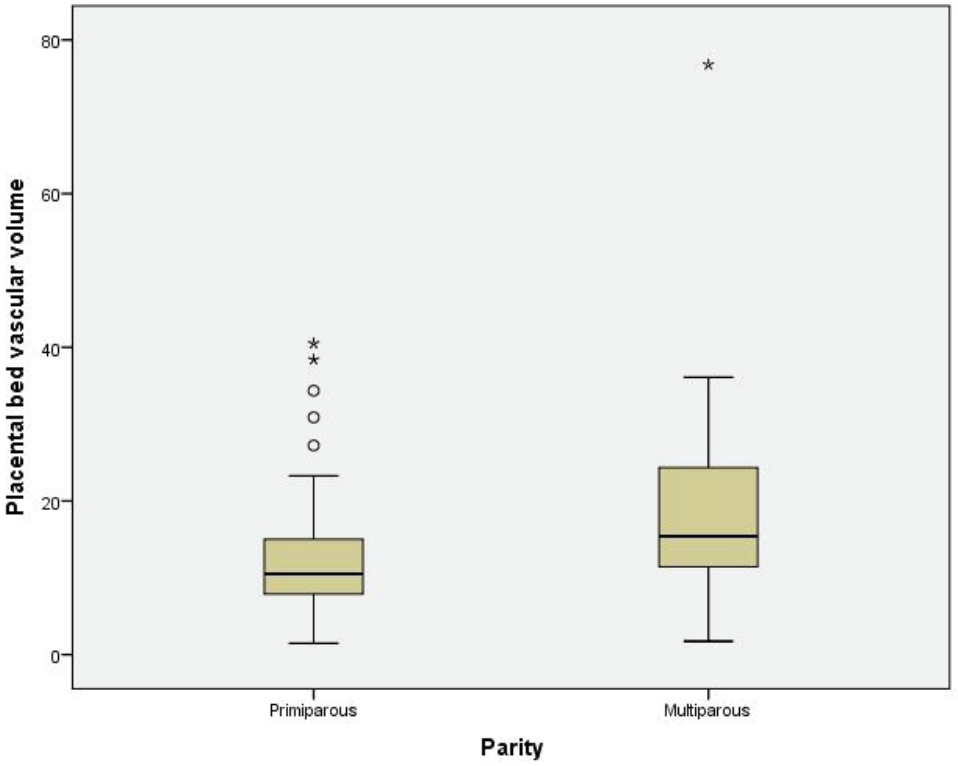

Figure 3: *Boxplot of the measured Placental bed vascular volume (cm³) at 12 wk of gestation in primiparous (n = 69) and multiparous (n = 31) women. Represented in Median, 25 and 75 percentile and range.*

DISCUSSION

In this study we introduced a new approach to examining placental and uterine vasculature and anatomy. With our virtual reality system, we visualized and measured anatomical structures in a different way compared with known techniques, such as VOCAL. In 76% of the patients the measurements were performed. We have demonstrated that early pregnancy vasculature volume measurements performed with V-Scope in the I-Space are highly reproducible. Our study confirms earlier findings of larger PBVV in multiparous women. This is of interest because placental vascular complications are more common in nulliparous women.[27] This may be explained by the hypothesis of Kloosterman et al. who stated that remodeling of the maternal vasculature in former pregnancies provides a more favorable environment for both placental development and placental function in multiparous women.[8, 28, 29]

The I-Space provides the opportunity to improve our knowledge about development of placental vasculature and about the differences in anatomy of the uterine and placental vessels among women. Interaction with the whole volume-rendered ultrasound datasets and perception of depth allows more precise visualization and accurate interpretation of this complex structure, as well as efficient and precise removal of artifacts to allow accurate measurements. The 3D visualization allows a more detailed view than 2D visualization. Another advantage of the I-Space is the way the measurements are performed. The reliability of this system in standard human embryonic biometry, and the good accuracy and reliability of V-Scope measurements have been demonstrated in the past[15]. With the V-Scope application, the sum of all voxels with a Doppler value above a certain threshold represents an absolute amount of vessels. It is not necessary to use a 'region-of-interest' or quantify the vascular density because all vessels in the scan can be evaluated. The time it takes to perform a measurement in the Barco I-Space in comparable to those of other techniques without special user expertise[16]. In this study, we measured the total amount of fetal and maternal vessels, including branches of the uterine artery and part of the uterine artery itself. In this way, we measured the size of the placental

vasculature. To allow integration into routine medical care, a prototype desk top system for 3-D Virtual Reality, is being developed using the same V-Scope volume rendering application.
The vascular indices obtained using VOCAL are the vascular index, flow index and vascularization flow index[10]. They are assumed to reflect the blood flow and vascularization, by using the ratio of voxels that have a Doppler value above a certain threshold to all voxels within a region-of-interest. These indices from sonobiopsy have a good correlation with those from the entire placenta[30]. Although these vascular indices are generally recognized as a usable method to quantify the placental vascularization, their real clinical value is not yet known. In-vitro studies [31,32] show that these indices are highly dependent on machine settings, flow rate, concentration of particles in the fluid and attenuation[14].

Our results show that quantifying placental vasculature needs strict definitions for different parameters, including removal of artifacts. Because it takes approximately 30 seconds to perform the scan, multiple heartbeats of the patient cover one scan, and it is unlikely that this influences the measured placental bed vascular volume. BMI shows to have a correlation with the measured size of the PBVV, likely because the Doppler signal is getting weaker with increasing BMI. Another explanation can be unfavorable influence of high BMI on the cardio-vascular status and on the placental vessels in these patients. The abdominal route should be taken into account when analyzing these parameters, while the movements of the women or fetus influence the quality of ultrasound. Fetal movements and movements of the patient cause artifacts and in that way interfere with the 3DPD ultrasound scan. These technical problems are responsible for exclusion of 24% of the patients. We chose 12 weeks of gestation because of safety reasons[33]. However, at this gestational age the whole placenta does not fit in the scan when using a vaginal ultrasound probe, we used an abdominal probe. Given the reliability of our measurements, this innovative VR system is a step forward in examining placental and uterine vasculature and anatomy. Our measurements are reproducible, but we do not know if the volume we measure represents the true volume of the vasculature because there is no

gold standard with which to compare our measurements. So further studies are needed if establishment of absolute vascular volume is required.

CONCLUSION

V-Scope offers a new reproducible method for measuring PBVV and FVV at 12 weeks of gestation. Maternal parity influences PBVV. The clinical value of such measurements needs to be further studied, especially because we are unsure whether the volume we measure represents the true volume of the vasculature.

ACKNOWLEDGMENT

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CHAPTER 3.2

FIRST TRIMESTER **TROPHOBLAST AND PLACENTAL BED VASCULAR** VOLUME MEASUREMENTS IN **IVF OR IVF/ICSI** PREGNANCIES

M.S. Rifouna

A.D. Reus

A.H.J. Koning

P.J. van der Spek

N. Exalto

E.A.P. Steegers

J.S.E. Laven

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ABSTRACT

Objectives Assisted reproductive technology (ART) pregnancies have been associated with an increased risk for placenta related adverse pregnancy outcomes. It is unclear whether these effects originate from infertility or from the technique itself. Therefore the aim of this study was to compare trophoblast volume (TV) and placental bed vascular volume (PBVV) in early pregnancy between IVF or IVF/ICSI pregnancies and spontaneously conceived pregnancies.

Methods We performed a retrospective cohort study in which 154 pregnant patients qualified for participation. Out of 154 pregnant patients, 84 conceived spontaneously and 70 after IVF or IVF/ICSI. We determined the TV at 10 weeks GA by Virtual Organ Computer-aided AnaLysis (VOCAL) measuring application and the PBVV at 12 weeks GA by the virtual reality operating system of BARCO I-Space in both subgroups. The investigators were blinded to the mode of conception during the measurements. Analysis was limited to singleton pregnancies with only one sac even detectable.

Results There were no differences in TV (mean 42.7, SD 15.9 vs mean 41.2, SD 13.9, p=0.70) and PBVV (mean 27.6, SD: 16.9 vs mean 24.8, SD: 19.9, p=0.20) between IVF or IVF/ICSI pregnancies and spontaneously conceived pregnancies. There is a significant correlation between TV and PBVV (r_s=0.283, p= 0.004).

Conclusions The possible abnormal placentation in IVF or IVF/ICSI pregnancies, in comparison with spontaneously conceived pregnancies, is not detected by a difference in PBVV or TV at an early gestational age. TV and PBVV are significantly correlated, indicating an association of trophoblast growth and uterine maternal vascularisation.

Keywords assisted reproductive technology; IVF; ICSI; trophoblast volume; placental bed vasculature volume; virtual reality; VOCAL.

INTRODUCTION

Up to 4 % of children are born after assisted reproductive technology (ART)[1]; however, there are issues raised concerning the impact of ART on the outcome of pregnancy. ART pregnancies are at higher risk of preterm birth, fetal growth restriction, low birth weight and preeclampsia (PE)[2-7]. A favourable outcome of pregnancy largely depends on an adequate function of the placenta. Several studies describe differences between placentas of ART and spontaneously conceived pregnancies.

Increased placental weight as well as an increased placental weight/fetal weight ratio have been described in ART pregnancies[8, 9]. Microscopic examination of placentas showed significant increase of villous oedema and an increased incidence of microcalcification in placentas of ART pregnancies[10]. Moreover, an increase in thickness of the placental blood barrier is found in ART pregnancies, suggesting a reduced materno-fetal exchange following ART treatment[11]. A study on the association of abnormal placentation and ART has displayed significantly increased placental thickness, which has previously been linked to increased perinatal risk[12]. Also more placental haematomas have been found in ART pregnancies[12]. In other studies, no difference in first trimester placentation between ART and spontaneously conceived pregnancies has been observed.[13]

Given the fact that low birth weight and abnormal placental development are associated, we hypothesized that placental growth and/or placental bed vascular growth is influenced by IVF or IVF/ICSI. We compared the Trophoblast Volume (TV) and Placental Bed Vasculature Volume (PBVV) at an early gestational age (GA) between IVF or IVF/ICSI and spontaneously conceived pregnancies using the latest techniques of three dimensional virtual reality (3D VR) and 3D ultrasound (3D US)[14-19]. Thus, the aim of this study was to compare TV and PBVV in early pregnancy between IVF or IVF/ICSI pregnancies and spontaneously conceived pregnancies.

MATERIALS AND METHODS

Patient selection

From November 2009 until December 2012, a total of 244 healthy pregnant women were enrolled in the study. Patients were recruited from the OB/GYN outpatient clinic and fertility unit at the Erasmus Medical Center. Patients who conceived spontaneously had no known history of infertility. The analysis was limited to singleton pregnancies with only one sac ever detectable. After exclusion of pregnancies with suboptimal quality of ultrasound images due to incomplete framing or too many artefacts (n= 81) and pregnancies conceived with intrauterine insemination (n = 9), 154 patients remained in our study cohort, consisting of 84 spontaneously conceived pregnancies and 70 IVF (n = 41) or IVF/ICSI (n = 29) pregnancies. Forty of these patients (37 IVF or IVF/ICSI and 3 spontaneously conceived pregnancies) also participated in our previous study in which PBVV measurements were validated[18]. Fresh embryo transfers (n = 59) as well as cryopreserved embryo transvers (n = 11) were included. Two patients become pregnant by oocyte donation. In 60 patients, 1 embryo was transferred and in 10 patients 2 embryos were transferred; all embryos were transferred on Day 3. GA was determined by the first day of the last menstrual period or by the day of oocyte retrieval, in case of IVF or IVF/ICSI. There were no patients with large and/or multiple fibroids or known uterine anomalies.

Ethical approval

Written informed consent was obtained from all participants. The study was approved by the Medical Ethics review board of Erasmus Medical Center and is part of an ongoing, prospective, periconception cohort study, called the Rotterdam Predict Study[20].

Placental Bed Vascular Volume (PBVV)

To measure the PBVV, we performed a 3D Power Doppler ultrasound scan at 12 weeks of gestation using a 4-8 MHz transabdominal probe of the GE Voluson E8 (General Electrics Medical Systems, Zipf, Austria). We chose 12 weeks of gestation because of safety reasons[21]. Every scan was

performed with standard settings of the ultrasound machine: pulse repetition frequencies of 0.9 kHz, Gain -2.0, Quality "high", wall motion filter "low". Patients were asked to hold their breath while the scan was done to limit disturbance by movement of the abdomen.

3D VR is an imaging technique that actually uses all three dimensions, in contrast to traditional 3D reconstructions displayed on a 2D screen. A fully immersive virtual reality system, the Barco I-Space, is operating at the department of Bioinformatics of the Erasmus MC Rotterdam. The V-Scope volume rendering application creates a hologram of the 3D US volume for optimal depth perception and an intuitive interaction for enlargement and rotation for length and volume measurements[15].

For the offline measurement of the vascular volumes in the I-Space, we first set the lower-Doppler threshold at 100 for better perception of the vasculature. This means that all voxels with a grey value between 100 and 250 are colored and counted in the volume calculation. We removed the normal US signal by making this channel fully transparent, retaining the coloured power Doppler signal of the vasculature. After removing artefacts, fetal vasculature and the uterine vessels, we measured the PBVV (Figure 1), as described elsewhere[18]. The measurements were performed three times by one experienced investigator (M.S.R) and the mean values were used for further calculations. In case of more than one US dataset was available the first dataset was used unless this was technically inappropriate. The investigators were blinded to the mode of conception during the measurements.

Trophoblast Volume

To measure the TV, a 3D-US scan was performed at 10 weeks of gestation using a transvaginal probe (6-12 MHz) of the GE Voluson E8 (General Electrics Medical Systems, Zipf, Austria). All scans were performed with standard settings of the ultrasound machine and evaluated offline using specialized 3D software (4Dview, GE medical Systems), using the Virtual Organ Computer-aided AnaLysis (VOCAL) measuring application[17]. We decided to use the datasets of 10 weeks being the optimal period for

placental volume measurements. The 15° rotational angle was used to perform trophoblast volume (TV) measurements. A sequence of 12 sections of the trophoblast was obtained, each after a 15° rotation from the previous one. In each plane, the contour was traced manually and at the end the computer provided the reconstruction of the trophoblast and the volume. We first measured the Total Pregnancy Volume (TPV) by tracing the total pregnancy along the outside of the placental contour (Figure 2). Subsequently Gestational Sac Volume (GSV) was measured in the same way. The TV was calculated by subtracting the GSV from the TPV. The VOCAL measurements were performed once.

Statistical analysis

Data analysis was performed using the SPSS software (version 20, Chicago, IL, USA). The Mann-Whitney U test was used for evaluating differences between patient characteristics and periconceptional data in our two groups. To check for differences in TV and PBVV between the two groups, the Mann-Whitney U test was used. The Kruskal-Wallis test was used to check for differences in PBVV and TV between the different groups of gravidity and parity. Spearman's rank correlation coefficients were calculated to check for associations between the measured data. The impact of BMI and age to TV and the PBVV was tested by using the Spearman's rank test. Differences were supposed to be significant in case of a p-value of < 0.05.

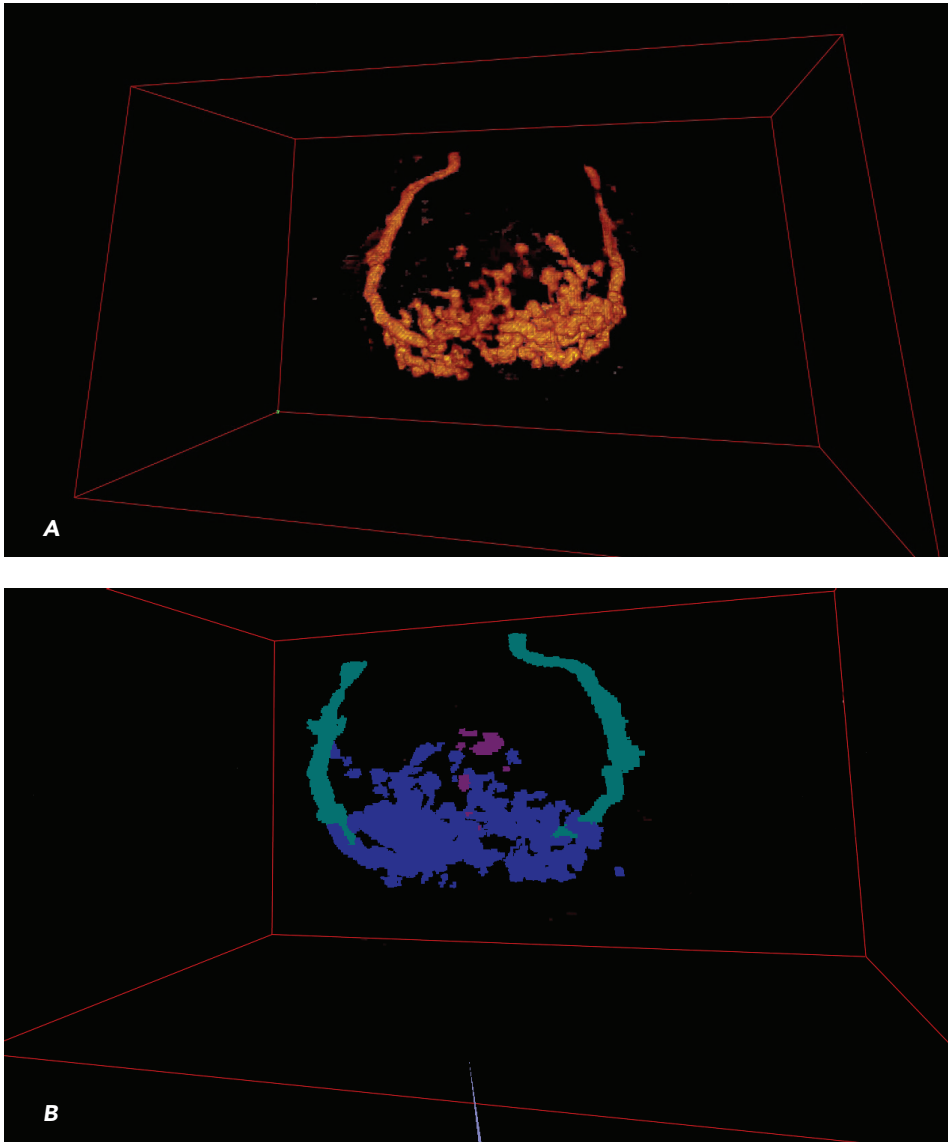

Figure 1: (a) *An image of a placenta of 12+3 weeks GA is projected on the wall of the I-Space.* ***(b)*** *Different structures were defined and measured: braches of the uterine arteries are shown in mint, the fetal vascular volume in purple and the placental bed vascular volume in blue; these structures together represent the 'Total Volume'.*

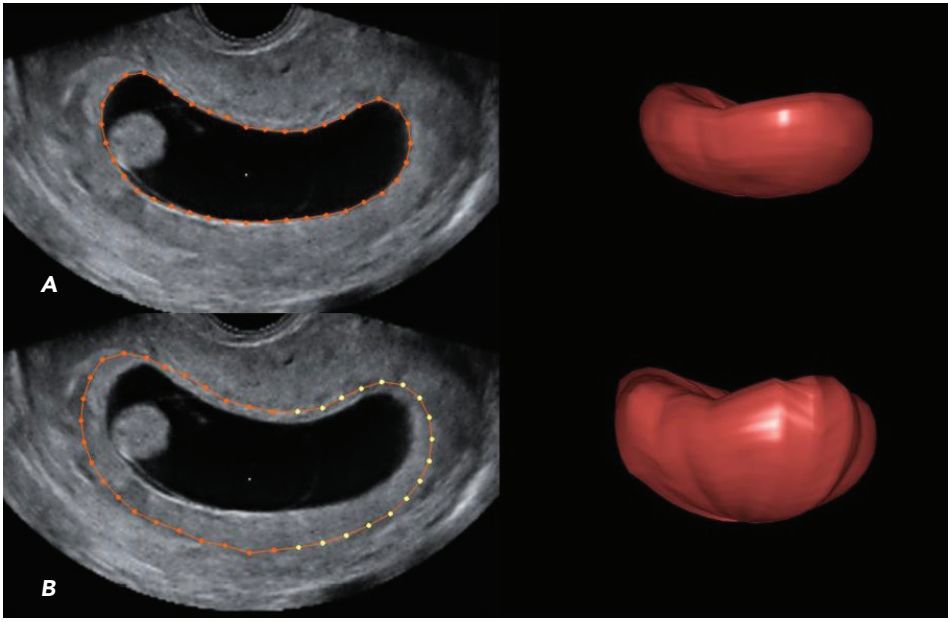

Figure 2: *Image illustrating total TV measured by the VOCAL method. The TVs were calculated by subtracting the volume of the gestational sac* ***(a)*** *from the volume of the total pregnancy* ***(b)*** *by using VOCAL.*

RESULTS

There were 282 ultrasound datasets obtained from 154 pregnant patients with a mean of 1.83 ultrasound scan per patient (ranging from 0 to 4 datasets) at their 10th week of GA. Additionally, 231 ultrasound datasets were obtained at 12th week of GA with a mean of 1.50 scan per patient (ranging from 0 to 4 datasets). Patient characteristics, periconceptional data and treatment characteristics of the IVF and IVF/ICSI are summarized in Table 1. There were no significant differences in patient characteristics between in included and excluded patients (data not shown). Women who conceived spontaneously had been pregnant more often and had more children compared to women who conceived by IVF or IVF/ICSI. No other significant differences were found. Five women developed either pregnancy-induced hypertension (PIH, n=2), or preeclampsia (PE, n=2) or hemolysis elevated liver enzymes and low platelets syndrome (HELLP, n=1). One of the patients who developed PIH conceived spontaneously, the other four became pregnant after IVF or IVF/ICSI. Four children were born premature and two of these pregnancies were conceived spontaneously, while three children, all conceived spontaneously, had a birth weight below the 5th percentile. The numbers of these abnormal pregnancies are small, but the results of the PBVV and TV measurements did not differ from the other pregnancies.

There was no difference in PBVV between the different groups of gravidity ($p = 0.18$) and parity ($p = 0.37$), neither was there a difference in TV between these groups (gravidity $p = 0.40$ and parity $p = 0.61$). There was no difference in PBVV between nulliparous women and multiparous women. PBVV and TV measurements are presented in Table 2 for the IVF or IVF/ICSI group and the spontaneously conceived group. There were no significant differences in TV (mean 42.7, SD 15.9 vs mean 41.2, SD 13.9, $p = 0.70$) and PBVV (mean 27.6, SD: 16.9 vs mean 24.8, SD 19.9, $p = 0.20$) between IVF or IVF/ICSI pregnancies and spontaneously conceived pregnancies. Nor was there a significant difference in PBVV between the IVF and IVF/ICSI group (mean 25.1, SD 16.4 vs mean 30.6, SD 17.2, $p = 0.163$). A borderline difference was found in TV between the IVF and IVF/ICSI group (mean 39.6,

SD 15.6 vs mean 47.1, SD 15.5, p = 0.051). There was no difference in PBVV (p = 0.49) or in TV (p = 0.16) between the fresh or cryopreserved embryo transfers.

There was a significant correlation between TV and PBVV (r_s=0.283, p= 0.004) when taking all the patients together. This significant correlation was also present in the IVF or IVF/ICSI group separately (r_s= 0.351, p= 0.012), whereas this was absent in the group of patients who conceived spontaneously ($r_{s=}$ 0.244, p= 0.081). Similarly, a significant correlation between TV and PBVV among the nulliparous women in the group of patients who conceived spontaneously ($r_{s=}$ 0513, p=0.012) was found, whereas such a relationship was absent in the group of multiparous women ($r_{s=}$ 0.030, p= 0.789). In the IVF or IVF/ICSI group we found the same significant correlation among the nulliparous women ($r_{s=}$ 0.397, p= 0.011) and absence among the multiparous women ($r_{s=}$ 0.227, p= 0.502).

The mean PBVV of pregnancies complicated by hypertensive disease (PIH, PE and HELLP, n = 5) did not differ from the mean PBVV of normal pregnancies (n = 90) (27.53 vs 26.16; p = 0.58). Neither significant correlations were found between BMI and PBVV (r_s = -0.132, p= 0.163) or TV (r_s = 0.115, p = 0.176) nor between age and PBVV (r_s = 0.076, p = 0.419) or TV (r_s = -0.038, p = 0.649).

Table 1: *Patient characteristics, periconceptional data and treatment characteristics of IVF or IVF/ICSI and spontaneous pregnancies.*

Parameters	IVF or IVF/ICSI	Spontaneous	p-value
	N=70	N=84	
Maternal age (years) at US	33.0 (24 – 44)	31.0 (22 – 42)	0.149
BMI (kg/height^2)	23.8 (18.6 – 35.4)	23.2 (17.8 – 38.5)	0.665
Gravidity	1 (1 – 7))	2 (1 – 12)	0.001
Parity	0 (0 – 2)	1 (0 – 2)	0.001
Birth Weight (g)	3380 (1475 – 4270)	3395 (1200 – 4650)	0.378
Gestational age at birth (weeks)	39 3/7 (31 3/7 – 41 4/7)	39 2/7(27 4/7 – 41 3/7)	0.844
Cigarettes (n)	2	0	
Alcohol consumption (n)	0	2	
Number of oocytes retrieved	8 (1 – 23)		
Number of embryos created	3 (1 – 13)		

The characteristics are given by median and range. Abbreviations: g = gram; N = number

Table 2: *PBVV (cm^3) at 12th and TV (cm^3) at 10th weeks of gestation in IVF or IVF/ICSI and spontaneously conceived pregnancies, given in median, interquartile range and range.*

	Parameter	Median	P25	P75	Range
IVF or IVF/ICSI	PBVV	22.8	14.8	37.6	6.4 – 82.4
	TV	40.2	30.0	51.9	16.8 – 79.8
Spontaneous	PBVV	19.5	13.8	34.1	2.7 – 133.2
	TV	39.8	29.7	50.4	14.3 – 78.8

Abbreviations: PBVV = placental bed vascular volume; TV = trophoblast volume; P = percentile

DISCUSSION

ART pregnancies have been associated with an increased risk of placenta-related adverse pregnancy outcomes such as fetal growth restriction and PE[4, 22, 23]. In this study, we tried to provide further insight, with novel techniques, into early parameters of placentation, including TV as well as PBVV at 10th and 12th weeks of gestation. Our previous study on PBVV in the first trimester of pregnancy did show larger volumes in multiparous women[18]. In the present study, no difference was found in PBVV between IVF or IVF/ICSI and spontaneously conceived pregnancies, but among the spontaneously conceived pregnancies were significantly more multiparous women. It is possible that the difference in PBVV is nullified by the difference in parity between the two groups. Another explanation for not finding a difference in PBVV may be that the study size is too small to detect the difference. We did find a significant correlation between TV and the PBVV, suggesting that larger placentas are supplied by a more pronounced adaptation of maternal vessels. Nevertheless, no significant differences were found between the IVF or IVF/ICSI-women and those naturally conceived. This is in accordance with previous studies in which uterine artery pulsatility index (PI), as a reflection of placentation in first trimester, was compared between ART and spontaneously conceived pregnancies[22,24]. In these studies, no difference was found in PI indicating that the increased risk of placenta related complication as early preeclampsia in ART pregnancies is not clinically measurable in this way. Moreover, there seems to be no discernible difference in trophoblastic invasion of the spiral arteries between ART and spontaneously conceived pregnancies[22, 24]. However, the number of the number of children born premature or with a low birth weight in this study are too small to reflect on any associations with PBVV or TV.

In an effort to elucidate the pathogenic basis of the relationship between ART and increased pregnancy complications, several histopathologic [8-12, 25] and molecular[26, 27] placental studies have been performed. Although altered proteomic and gene expression profiles in ART-derived placentas were identified [26, 27], results were rather inconsistent with regard to histopathologic and macroscopic features. Haavaldsen et al. found larger placentas and a

higher placental weight/birthweight ratio among pregnancies conceived by ART [9] while others did not find this difference[8, 25]. Different studies have shown an increased placental thickness in ART pregnancies[8, 12]. Lalosevic et al. found an increase of villous oedema and an increased incidence of microcalcifications in placentas of ART pregnancies[10]. Zhang et al. found a difference with respect to the placental blood barrier [11] while others did not find a difference in histopathological features of the placenta of ART and spontaneously conceived pregnancies[8, 25].

Although the limitations of the present study concern the small size of the study groups, our findings suggest that IVF or IVF/ICSI conception itself seems not to be associated with abnormal early placentation. Given the reported difference in placental weight independent of GA at delivery between ART and spontaneous pregnancies [9], future research should delineate the mechanisms responsible for such differences in placental growth.

CONCLUSIONS

The possible abnormal placentation in IVF or IVF/ICSI pregnancies, in comparison with spontaneously conceived pregnancies, is not detected by a difference in PBVV or TV at an early GA. TV and PBVV are significantly correlated, indicating an association of trophoblast growth and uterine maternal vascularisation.

AUTHOR'S ROLES

M.S.R contributed to data-collection, analyzed and interpreted the data, drafted and revised the paper. A.D.R. was involved in the data-collection, data analysis and revision of the manuscript. A.H.J.K and P.J.S respectively developed the V-Scope software and supported 3D virtual reality and both contributed to the revision of the manuscript. J.S.E.L was responsible for IVF

or IVF/ICSI patients and N.E supervised the TV and PBVV measurements. Both contributed to the design of the study and revision of the manuscript. E.A.R.S. initiated the 3D US and I-Space facilities for embryonic measurements and revised the manuscript. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

None declared.

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4

CHORIONIC VILLOUS VASCULARIZATION

RELATED TO PHENOTYPE AND GENOTYPE IN FIRST TRIMESTER MISCARRIAGES IN A RECURRENT PREGNANCY LOSS COHORT

Averil D. Reus

Mary D. Stephenson

Frederique M. van Dunne

Ronald R. de Krijger

Marieke Joosten

Eric A.P. Steegers

Niek Exalto

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ABSTRACT

Objectives At least half of first trimester miscarriages are due to embryopathogenesis associated with chromosome errors and/or major congenital anomalies, resulting in an empty sac, a yolk sac or an embryonic miscarriage. Absent and decreased chorionic villous vascularization is usually present in these pregnancies. The aim of this study is to compare the results of this validated histological classification for early pregnancy chorionic villous vascularization with ultrasound findings and chromosome results of early miscarriages from a cohort of well-characterized recurrent pregnancy loss patients.

Methods For this retrospective study, 60 hematoxylin and eosin slides of miscarriage tissue of less than 10 weeks gestational age were collected from an academic institution. All patients were seen in consultation between July 2004 and October 2009. Chorionic villous vascularization was determined using a previously published classification. The results were validated and compared with the ultrasound findings and corresponding chromosome results.

Results There were 53 embryonic miscarriages, 5 yolk sac miscarriages and 2 empty sac miscarriages. Chromosome results were obtained in 59 of the 60 miscarriages; 37.3% were euploid and 62.7% were noneuploid. Validation of the vascularization score between observers was reasonable to good (Kappa 0.47–0.76), and 59% of the cases were classified as avascular. The vascularization score did not differ between euploid or noneuploid miscarriages, or between embryonic, yolk sac or empty sac miscarriages. Avascular villi were seen more frequently in miscarriages trisomic for chromosome 16, when compared with miscarriages with other trisomies (6 out of 7 versus 8 out of 22, $P = 0.04$).

Conclusions We did not find a significant difference in vascularization scores of chorionic villi between embryonic, yolk sac or empty sac miscarriages, or between euploid and noneuploid miscarriages. Avascular villi may indicate abnormal early placentation as a part of embryopathogenesis.

Keywords Recurrent miscarriage, vascularization, chromosome results, ultrasound, pregnancy loss.

INTRODUCTION

At least half of first trimester miscarriages are associated with chromosome abnormalities[1]. In addition, there are miscarriages with embryonic developmental defects that have normal chromosome results[2-4]. Miscarriage implies an intrauterine pregnancy loss, whereas early miscarriage is defined as an intrauterine pregnancy loss of less than 10 weeks gestational age. These first trimester miscarriages can be classified as anembryonic (empty sac), yolk sac (gestational sac with yolk sac but no embryo) or embryonic miscarriage. We do not understand why miscarriages occur if embryopathogenesis is not associated with chromosome errors and or major congenital anomalies. We hypothesize that a defective vascularization of the placenta may be involved in the underlying cause.

Morphological examination and documentation of abnormal development is difficult at this early stage of pregnancy. However, it is important in determining why pregnancy loss occurred, particularly in cases of recurrent miscarriage[5-8]. Although ultrasound may document the gestational age at the time of demise, it does not provide the underlying cause of the pregnancy loss.

Vasculogenesis starts from the hemangioblastic cell cords that are the precursors of both capillary endothelium and hematopoietic stem cells. These structures are easily identified in trophoblastic tissue of empty sac miscarriages and complete hydatidiform mole pregnancies[9, 10]. Vasculogenesis of normal chorionic villi is then characterized by the maturation of these hemangioblastic cell cords into luminized vessels and their margination to peripherally located vessels at the end of organogenesis, to finally form the vasculosyncytial membrane[11]. Defective vascularization has been documented in embryonic miscarriages, but is more pronounced in empty sac miscarriages[10-12]. Hypovascularization of chorionic villi appears to be associated with early miscarriage and is unaffected by a prolonged intrauterine retention[12]. The changes in vascular parameters in the first trimester chorionic villi of miscarriage, such as the mean functional vascular area, the number of vessels with a lumen and whether

hemangiogenetic cords are peripherally or centrally located, are the result of defective development due to abnormal vasculogenesis, rather than to postmortem changes[9].

In 1969, Philippe and Boué were the first to study the phenotype–karyotype relationship using microscopic investigation of chorionic villi from miscarriage specimens[13, 14]. They described a case of polyploidy in which the trophoblast showed hydropic degeneration of the chorionic villi with numerous vesicles. Microscopic examination revealed microcysts that are the result of invagination of the trophoblast into edematous, poorly vascularized villous stroma. Since then, several studies have been performed, and an association between placental histology and chromosome results has been found in triploid miscarriages[15-17]. For example, trisomy 16 and 22 are most often found in anembryonic (empty sac) miscarriages. Additionally, monosomy X (45, XO) is usually associated with an embryonic or fetal miscarriage,[18, 19] and a smaller than expected embryo was found to be associated with aneuploidy[19-21]. Others found that some types of chromosomal abnormalities, such as trisomy 22, seem to be miscarriage at a later stage than those with trisomy 16, multiple trisomies and unusual other variants[20, 22]. Additionally, villous morphology in miscarriages has previously been studied, partly based on hypovascularization of peripheral villi. This was found to be an inaccurate indicator of chromosomal errors[15, 23].

In 2006, a histological classification for early pregnancy chorionic villous vascularization (Grade: I, normal; IIA, mild hypoplasia; IIB, severe hypoplasia and III, avascular) was developed and validated for clinical use[24]. This scoring system may be helpful to determine whether miscarriages occur due to abnormal embryonic and/or chorionic development.

The aim of this study is to compare the results of this validated histological classification for early pregnancy chorionic villous vascularization with ultrasound findings and chromosome results of early miscarriages from a cohort of well-characterized recurrent pregnancy loss patients.

MATERIALS AND METHODS

The study is an analysis of prospectively collected data and samples. These subjects were identified through the University of Chicago Recurrent Pregnancy Loss Database, created and managed by one of the authors (M.D.S.). All subjects signed a written consent for inclusion in the recurrent pregnancy loss database and use of excess miscarriage tissue for future research purposes. Institutional research board approval was obtained from the University of Chicago.

Patient selection

A patient was included, if she met the following criteria:

1. Seen in consultation by the author (M.D.S.) between July 2004 and October 2009.

2. A history of recurrent early pregnancy loss, defined as 2 or more miscarriages of less than 10 weeks gestation, based on ultrasound findings.

3. Miscarriage tissue was sent to University of Chicago Cytogenetics for chromosome testing and to Pathology for histological assessment.

Ultrasound diagnosis of miscarriage

An empty sac miscarriage was defined by a mean sac diameter of greater than 8 mm without visualization of a yolk sac; in these cases, an embryonic arrest at a gestational age of 28 days was assigned. A yolk sac miscarriage was defined by a mean sac diameter of greater than 16 mm with the presence of a yolk sac, but no embryo; for this study, an embryonic arrest at a gestational age of 35 days was assigned. An embryonic miscarriage was defined as an embryo of at least 5 mm without cardiac activity; the moment of embryonic demise was based on the crown rump length measurement after the cardiac activity had stopped.

Standard miscarriage chromosome testing

Chromosome testing was performed using conventional cytogenetic analysis[25]. If conventional cytogenetic analysis failed, comparative genomic hybridization (CGH) was performed using cryopreserved miscarriage tissue. In case of a 46, XX result, EDTA blood was obtained from the patient and microsatellite analysis was performed and the result would have been excluded, if the sample was of maternal origin.

Standard histological testing

Miscarriage tissue was sent to the University of Chicago Pathology Laboratory; chorionic villi were isolated and embedded in paraffin, and the slides were stained with hematoxylin and eosin (H&E) for interpretation.

Chorionic villous vascularization studies

For each miscarriage specimen in this cohort, unstained and H&E slides were prepared from the paraffin blocks stored in Pathology. The slides and the demographic data for all subjects were coded for anonymity. One coded slide from each miscarriage was sent to four independent observers who were blinded to the subject's clinical history and miscarriage results. Each observer classified the slides in one run. Two of the observers were experienced, and two were inexperienced, in assessing chorionic villous vascularization. Each observer assessed the chorionic villous vascularization using the following scoring system from Hakvoort et al., 2006:

'*Grade 0*: unknown. There are insufficient number of villi available for evaluation.

Grade I: normal. Vessels with nucleated blood cells are present in almost every (at least nine of 10) villus, have a very clear appearance and are located centrally as well as peripherally (in contact with the trophoblastic layer). In some villi, the number of vessels is even numerous (>5).

Grade IIA: mild hypoplasia. Vessels with nucleated blood cells are not present in all villi, less numerous and predominantly located centrally.

Grade IIB: severe hypoplasia. Villi are predominantly avascular; however, in a single villus, a vessel is present with one or more nucleated blood cells.

Grade III: avascular. All villi are avascular, although sporadically a very small

vessel, with or without a nucleated blood cell, may be present.' Features including fibrosis, hydropic degeneration, trophoblast inclusions and abnormal trophoblast proliferation were recorded, if present to the opinion of the observer, without using criteria other than usual in cases of routine microscopic examination of chorionic villi.

Analysis of data

The scores of the experienced and inexperienced observers were compared by calculating the Kappa value. The overall vascularization scores were determined by counting the scores of the two experienced observers twice and the scores of the two inexperienced observers once and dividing by six. The vascularization scores were compared with the type of early miscarriage, the retention time (time between embryonic demise and collection of the tissue) and the chromosome results.

Data analysis was performed using the SPSS software (version 17.0, Chicago, Illinois). Chi-square test was used for categorical variables. Spearman's rank correlation coefficient was used to determine the relationship between the chromosome results and the number of previous miscarriages. Mann–Whitney U-test was used to determine whether there was a relation between the vascularization score and gestational age at the time of miscarriage.

RESULTS

A total of 60 miscarriages from 57 patients met the inclusion criteria and were used for microscopic evaluation of the vascularization score. In 1 of the 60 miscarriages, the chromosome testing was not successful; therefore, a total of 59 miscarriage specimens from 56 patients with recurrent early pregnancy loss could be analyzed. The miscarriage tissue was obtained by dilatation and curettage using office manual vacuum aspiration (51 cases) or under general anesthesia in (7 cases), whereas 1 patient passed the tissue spontaneously.

The demographics of the 56 women are shown in Table 1. This cohort had a total of 197 prior pregnancies. Of the 59 miscarriages studied, 53 were conceived spontaneously and 6 of the pregnancies were conceived through IVF. Characteristics of the studied miscarriages are shown in Table 2.

Phenotype–genotype relation

Embryonic miscarriage was more frequent than empty sac or yolk sac miscarriage (53 versus 2 versus 5). Chromosome testing was successful in 59 out of 60 miscarriages; 22 were euploid and 37 were noneuploid, as shown in Table 3. Among the euploid miscarriage, there were 15 male karyotypes and 7 female karyotypes (ratio 2.1; $P = 0.19$). This difference is not significant probably as a result of our small sample size. Euploid and noneuploid chromosome results were observed in all types of miscarriages, and there were no significant differences. Trisomy 16 was the most frequent noneuploid result.

Evaluation of the scoring system

Validation of the vascularization score between the experienced and inexperienced observers turned out to be reasonable to good (Kappa 0.47–0.76), as shown in Table 4. In 10 of the 60 cases, there was an insufficient number of villi (<10) to evaluate, and they were all given a vascularization score 0 by all observers. Villi were mainly avascular (score III: 29 out of 50) when compared with the other grades (score IIB: 7 out of 50, score IIA: 11 out of 50 and score I: 3 out of 50). In five patients, hydropic degeneration was found. In three of these cases, the villi were avascular, one case showed mild and one case showed severe hypoplasia. Four of them had an euploid chromosome result, and one of the avascular miscarriages was trisomic for chromosome 16. In only one case, fibrosis was seen; this case showed severe hypoplasia and had monosomy X. Trophoblast inclusion and abnormal trophoblast proliferation were not found in any of the cases. No difference was found in the vascularization score between empty sac or yolk sac miscarriages and embryonic miscarriages: grade I (0 out of 6 versus 3 out of 43), grade IIA (1 out of 6 versus 9 out of 43), grade IIB (0 out of 6

versus 7 out of 43) or grade III (5 out of 6 versus 24 out of 43)(P = 0.67). The vascularization score did not significantly differ between miscarriages with an euploid or noneuploid chromosome result: grade I (1 out of 19 versus 2/30), grade IIA (2 out of 19 versus 8 out of 30), grade IIB (4 out of 19 versus 3 out of 30) or grade III (12 out of 19 versus 17 out of 30) (P = 0.51). All the chromosome results of the miscarriages and their corresponding vascularization scores are shown in Table 5. In the trisomy 16 miscarriages, all of which were embryonic, grade III vasculization was more frequent when compared with the other trisomies (6 out of 7 versus 8 out of 22, P = 0.04). There was no correlation between the number of previous miscarriages and the chromosome results (r_s = 20.04, P = 0.76).

Gestational age and vascularization

The gestational age at the time of miscarriage was known in 57 cases and ranged from 4^{+0} to 9^{+5} weeks of gestation (median: 6^{+2}, SD 1^{+2} weeks of gestation). There was no significant difference between the gestational age in days at time of miscarriage between the miscarriages with a euploid result and the miscarriages with a noneuploid chromosome result (euploid: median 46.0, SD 10.5, range 28.0–68.0, noneuploid: median 43.5, SD 8.1, range 28.0–68.0, P = 0.50).

Retention time and vascularization score

The miscarriages avascular villi did not show a longer retention time than miscarriages with more normal vascularization (difference between a normal vascularization score and an avascular score, P = 0.96), as shown in Table 6.

Table 1: *Demographics of the recurrent early pregnancy loss subjects (n=56 patients with a total of 197 prior pregnancies).*

	Mean (SD)	Range
Gravidity	4.5 (1.7)	1 – 8
Number of prior live births (n = 19)	0.3 (0.5)	0 – 2
Number of prior miscarriages <10 wks (n = 144)	2.6 (1.3)	0 – 6
Number of fetal demise ≥10 wks (n = 13)	0.2 (0.6)	0 – 2
Preterm delivery (n = 7)	0.1 (0.3)	0 – 1
Number of neonatal deaths (n = 3)	0.05 (0.2)	0 – 1
Elective abortion (n = 9)	0.2 (0.4)	0 – 2
Genetic termination (n = 2)	0.04 (0.2)	0 – 1
Ethnicity		
Caucasian	49	
African American	5	
Asian	2	
Smokers	0	
Alcohol use in pregnancy	0	
Recreational drug use	0	
First born male	11	
First born female	8	

Table 2: *Patient characteristics of subsequent miscarriages (n=59)* *******.

	Mean (SD)	Range
Maternal age at miscarriage (years)	36.0 (4.2)	25.0 – 46.0
Body mass index (kg/height^2) at miscarriage	27.5 (6.4)	20.0 – 47.0
Gestational age (days) at demise*	46.6 (9.0)	28.0 – 68.0
Retention time (days) between demise and tissue collection*	14.4 (8.3)	1 – 34

** In one miscarriages no dating ultrasound was performed and in another case no ultrasound was performed at demise so the gestational age at time of demise and retention time were not available.*

Table 3: *Type of miscarriage according to chromosome results (n=59).*

Chromosome results	Total	Empty sac miscarriage	Yolk sac miscarriage	Embryonic miscarriage
Euploid				
46,XY (one with inversion)	15	1	1	13
46,XX *	7	0	1	6
Monosomy				
45,X	2	0	0	2
-21	1	0	0	1
Trisomy				
+3	2	0	0	2
+8	1	0	0	1
+9	1	0	0	1
+13 (one with inversion)	3	0	0	3
+14 (one with der(13;14) (q10;q10))	3	0	0	3
+15	4	0	0	4
+16	7	0	0	7
+20	2	0	2	0
+22	6	0	0	6
Polyploidy				
69,XXX	1	0	0	1
92,XXXX	1	0	0	1
Other				
68,XXX,-11	1	1	0	0
68,XXY,-13	1	0	1	0
68,XXY,del(1)(q11),-4[4]/ 68,XXY,-4[3]/69,XXY[2]	1	0	0	1
Total	59	2	5	52

** Confirmed to be of chorionic origin by microsatellite analysis.*

Table 4: *Reproducibility between experienced (exp) and inexperienced (inexp) observers 1 and 2, expressed as the observed and expected proportion of agreement and kappa values, for the histological vascularization score in 59 specimens.*

Observed pair	Observed proportion of agreement	Expected proportion of agreement	Kappa (Outcome)
Exp 1/ Exp 2	0,68	0,25	0,58 (Reasonable)
Exp 1/ Inexp 1	0,80	0,25	0,73 (Good)
Exp 1/ Inexp 2	0,82	0,25	0,76 (Good)
Inexp 1/ Inexp 2	0,72	0,25	0,62 (Good)
Exp 2/ Inexp 1	0,70	0,25	0,60 (Reasonable)
Exp 2/ Inexp 2	0,60	0,25	0,47 (Reasonable)

Table 5: *Final vascularization score for 59 miscarriages grouped by chromosome results.*

		Vascularization score				
Chromosome result	Normal	Mild hypoplasia	Severe hypoplasia	Avascular	Insufficient number of villi	Total
46,XY	1	1	2	9	2	15
46,XX	0	1	2	3	1	7
Trisomy 15	2	1	0	1	0	4
Trisomy 16	0	0	1	6	0	7
Trisomy 22	0	3	0	2	1	6
Other trisomies	0	3	1	5	3*	12
Monosomies	0	1	1	0	1	3
Polyploidies	0	0	0	1	1	2
Other	0	0	0	2	1	3
Total	3	10	7	29	10	59

** In all miscarriages trisomic for chromosome 14 (n = 3) an insufficient number of villi was available for evaluation, resulting in score 0.*

Table 6: *Analysis of mean retention time by vascularisation score (49 miscarriages*).*

	Retention time		
Vascularization score groups	Mean (SD)	Median	Range
Normal (I; n=3)	18.7 days (5.1)	20.0 days	13.0 – 23.0
Mild hypoplasia (IIA; n=10)	7.1 days (6.6)	5.5 days	2.0 – 24.0
Severe hypoplasia (IIB; n=7)	10.0 days (7.9)	8.0 days	1.0 – 21.0
Avascular (III; n=29)	18.1 days (8.0)	17.0 days	1.0 – 34.0

** In 10 of the 59 cases there was an insufficient number of villi (<10) to evaluate and they were all given a vascularization score 0 by all observers and these cases were not used for this analysis.*

DISCUSSION

Our study shows that the histological scoring system we used for the classification of chorionic villous vascularization is a reproducible tool, as reflected by the Kappa value ≥0.47. The villi were found to be mainly avascular in this study. In addition, we show that the chorionic villous vascularization of empty sac or yolk sac miscarriages does not differ from that of embryonic miscarriages. The high number of embryonic miscarriages in our study (52 out of 59; 88%) when compared with other studies (181 out of 272; 67%) [26] may be due to ultrasound examination performed earlier in pregnancy. According to the studies of Byrne et al. [18] and Canki et al. [19], who demonstrated that 45,X miscarriages are usually embryonic, the two 45,X cases in our study were also embryonic. Trisomy 16 was the most frequent noneuploid result that is what was expected, based on other published studies[1, 7, 27]. There was no difference in vascularization scores between euploid and noneuploid miscarriages; however, we saw high rates of avascular villi in trisomy 16 miscarriages. We also showed that the retention time has no significant influence on the vascularization score.
In our study, 63% of the miscarriages were aneuploid. Stephenson et

al. found chromosomal errors in about half of 420 recurrent miscarriage specimens[27]. This difference may be explained by the fact that the mean age of the women in our study was above 35 years. It is known that the number of chromosomal errors rapidly increases after that age[28]. It should also be stated that our numbers are too small to give a good representation of the frequency of chromosomal errors in recurrent miscarriage.

One of the possible causes of recurrent early pregnancy loss is lethal submicroscopic chromosomal changes[2]. These submicroscopic chromosomal changes, also termed DNA copy number variants (CNVs), can be detected by array CGH. With array-CGH, it is possible to evaluate the whole genome at a much higher resolution than with conventional cytogenetic analysis[29-32]. Previous studies of CNVs in miscarriages with the use of array-CGH indicate that small chromosomal changes are present in 1–13% of miscarriages[29, 31- 37]. These studies have the potential to identify CNVs that could lead to developmental failure and in this way find possible 'miscarriage CNVs'. Rajcan-Separovic et al. [37] identified inherited CNVs that contain genes that impact early pregnancy, specifically TIMP2 (metalloproteinases-2 inhibitor) and CTNNA3 (a-T-catenin), both of which act as inhibitors of trophoblast invasion and are only maternally expressed in the placenta [38-42]; thus, the authors proposed them as candidate miscarriage genes. The finding of avascular chorionic villi in miscarriages with euploid chromosome results in our study may yet indicate the presence of CNVs that interfere with trophoblast invasion and early trimester development and in this way cause miscarriage. We plan to investigate the presence of these and other possible miscarriage CNVs in euploid miscarriages with avascular chorionic villi.

We found a high male/female ratio in this study (2.1; 15 males and 7 females), probably due to our small numbers, but a possible explanation could be paternally expressed imprinted X chromosome CNVs[43-45]. However, others have not found an association between skewed X-chromosome inactivation and recurrent miscarriage [46, 47] or a skewed sex ratio in recurrent miscarriages[48]. Another explanation found in the literature for a high male/female ratio in euploid miscarriages is that of an immunologic cause, described as an abnormal immune reaction against male-specific minor

histocompatibility H-Y antigens[49-51]. Nielsen et al. state that sex ratios prior and subsequent to secondary recurrent miscarriage show that birth of a boy predisposes to secondary recurrent miscarriage and male fetuses are more likely to be miscarried. However, agenesis of chorionic villous vascularization, resulting in avascular villi, is more likely to be an embryopathogenic abnormality during organogenesis, rather than the result of an abnormal immunologic reaction.

CONCLUSIONS

No significant difference in vascularization scores of chorionic villi was found between euploid and noneuploid miscarriages, or between empty sac, yolk sac and embryonic miscarriages. Although the number of samples was limited, we did find the interesting result that among the euploid miscarriages, there were twice as many males as females and that avascular villi were predominantly present in trisomy 16 miscarriages. Avascular villi may indicate abnormal early placentation as a part of embryopathogenesis. Further study is warranted to determine whether a genetic cause can be found to explain these results.

AUTHORS' ROLES

M.D.S, N.E, and A.D.R. contributed to the study concept and design. A.D.R., M.D.S, F.M.D, R.R.K and N.E. participated in the study execution and acquisition of data. A.D.R., M.D.S, M.J. and N.E participated in analysis of the data. All authors participated in the interpretation of the data, manuscript drafting and critical discussion and gave final approval of the submitted manuscript. The study was supervised by M.D.S, E.A.P.S and N.E.

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5

AN IMMUNOLOGICAL BASIS FOR **CHRONIC HISTIOCYTIC INTERVILLOSITIS** IN RECURRENT FETAL LOSS

Averil D. Reus

Nicole M. van Besouw

Nikki M. Molenaar

Eric A.P. Steegers

Willy Visser

Ronella P. de Kuiper

Ronald R. de Krijger

Dave L. Roelen

Niek Exalto

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ABSTRACT

Objectives Chronic histiocytic intervillositis (CHIV) is a rare type of placental pathology that is associated with reproductive loss at all gestational ages. The aim of the study was to investigate the relationship between the severity of CHIV and the outcome of pregnancy and to compare the immune response between CHIV patients and controls to explore an immunological origin of CHIV.

Methods Microscopic slides were reviewed and scored according to a previously published grading system in 30 pregnancies of 22 CHIV patients. Partner- specific mixed lymphocyte reactions, cytotoxic T-lymphocyte precursor frequencies (CTLpf), and anti-HLA antibodies were determined in four patients and seven controls.

Results Higher CHIV scores are associated with worse pregnancy outcome. CHIV patients demonstrated a higher CTLpf against their partner compared to non-complicated pregnancies ($P = 0.03$). The CTLpf was extremely high in 75% of the patients. Anti-paternal HLA antibodies were only present in 75% of the CHIV patients compared to none of the controls ($P = 0.02$).

Conclusions CHIV scores seem to be associated with the severity of adverse pregnancy outcome. High antipaternal cellular (T-cell) and humoral (B-cell) response to partner-specific CTLpf and the presence of anti-HLA antibodies directed to the partner suggest an immunologic origin of CHIV.

Keywords Adverse pregnancy outcome, CHIV, Chronic histiocytic intervillositis, Immunology, Miscarriage.

INTRODUCTION

Chronic histiocytic intervillositis (CHIV) is a rare type of placental pathology[1]. It is characterized by an intervillous infiltrate of maternal mononuclear cells. The exact incidence is unknown. One study reported an overall prevalence of 9.6 per 1000 miscarriages and in 0.6 per 1000 placentas in the second and third trimester [2,3]. Another study showed lesions in 4.4% of first trimester miscarriages [4]. CHIV is frequently associated with intervillous and perivillous fibrinoid deposition at the materno-fetal interface, anchoring villi, and adjacent decidua[5]. This pathologic condition was initially described by Labarrere et al.[6] as massive chronic intervillositis. CHIV is a placental lesion of unknown pathogenesis associated with reproductive loss at all gestational ages[1,2]. When CHIV is found in the placenta, children were born with lower birth weight and lower crown-heel length as compared to children with only focal villitis in their placentas[6]. CHIV has a high rate of recurrence in the next pregnancy, varying between 67 and 100%[2,3,6]. A high rate of fetal loss is seen in all trimesters of pregnancy[7]. Although a non-determined placental infection cannot be ruled out completely, an immunological cause seems to be present[2,8]. Parant et al.[1] described the correlation between infiltrating cells of mononuclear origin ($CD45^+$ and $CD68^+$) and the severity of CHIV. Semi-quantitative grading was used to classify the intensity of chronic inflammatory infiltrates and the amount of fibrinoid deposition in the intervillous space.

During pregnancy, women are tolerant to their semi-allogenic fetus. There is a change in the human immune response from a predominant Th1-type immunity to a Th2 cell type response, being protective for the fetus. Both recurrent miscarriages and CHIV are associated with a predominant Th1-like immune response[4,9]. Th1 cells mainly produce the proinflammatory cytokine IFN-γ[10]. The number of IFN- γ -producing cells is often used as marker for the number of cytotoxic T-lymphocytes (CTL). However, the actual endpoint of cytolytic activity remains the gold standard[11]. In a recent paper, we described an increased cytotoxic T-lymphocyte precursor frequency (CTLpf) directed to paternal antigens during pregnancy in patients with pre-

eclampsia compared to patients without preeclampsia, while no difference was found in proliferative capacity to paternal antigens[12]. In materno–fetal alloimmunization, anti- HLA antibodies are also described. Umapathy et al.[13] reported a higher frequency of anti-HLA antibodies in women with recurrent miscarriage compared to those without. Others describe that local immunotolerance at the fetal–maternal interface during pregnancy is more likely[14–16]. Tilburgs et al.[17] suggest that local $CD8^+$ T-cell differentiation may play a crucial part in maintenance of maternal immune tolerance to the fetus during human pregnancy. Because the exact pathogenesis of the CHIV is still unknown and an immunological origin of this rare entity is possible, we hypothesize that women with recurrent miscarriages and CHIV could have an abnormal immune response against paternal antigens. The aim of this study was twofold. First, the relationship between the severity of CHIV and outcome of pregnancy was studied. A secondary aim was to explore an immunologic origin of CHIV by comparing both the cellular (T-cell) and humoral (B-cell) immune response between patients with CHIV and other patients with recurrent fetal loss without CHIV (controls).

MATERIAL AND METHODS

Study Population

We retrospectively collected all patients (n = 22), diagnosed with placental CHIV between 2000 and 2010, from the database at the Department of Pathology at the Erasmus Medical Centre in Rotterdam. Written informed consent was obtained from all patients. Microscopic slides were available from 30 of 105 pregnancies of these 22 patients. Patient characteristics and details about the outcome of all pregnancies of these patients were collected from clinical records. Birth weight percentiles were calculated using the Netherlands Perinatal Registry data[18]. Most patients with recurrent miscarriages had already been screened for abnormal parental karyotype, uterine anomaly, antiphospholipid syndrome, and thrombophilia.

CHIV Scoring

In 16 of the 22 patients one placenta was available, in four patients two, and in two patient three placentas. Microscopic slides of the 30 specimen were stained with hematoxylin and eosin (HE, Klinipath, Duiven, the Netherlands) and with anti-CD68 antibodies ($CD68^+$; Dako, Glostrup, Denmark) for the detection of the mononuclear origin of cells in the intervillous space. For a comparison of the scores based on anti-CD68 with the scores based on HE stained slides, we compared the total sum of both scores. The anti-CD68 stained slides were used for further analysis. The CHIV classification described by Parant et al.[1] was used. Additionally, we added grade 0 for the absence of CHIV because we expected that CHIV was probably not present in all specimen.

The lesions were classified as 0: absent, 1: focal (<10% of the slide), 2: moderate (10–50%), or 3: severe or massive (more than 50%). The slides were scored independently by two investigators (NMM and RK), who were blinded to all relevant clinical information and prior histological results.

Proliferative Response and Cytotoxic T-cell Response

In patients who still wish to conceive, partner-specific mixed lymphocyte reactions (MLR) and cytotoxic T-lymphocyte precursor frequency (CTLpf) were determined in peripheral blood mononuclear cells (PBMC) from women with CHIV (n = 4) and women who had experienced recurrent miscarriages without CHIV serving as controls (n = 7). The results were also compared with women with non-complicated pregnancies from our earlier report[12]. In brief, MLR were set up in triplicate wells using 5×10^4 responder PBMC and 5×10^4 irradiated (40 Gy) partner PBMC. After 7 days, proliferation was measured by incorporation of ^{3}H-thymidine added during the last 8 hr of culture. The stimulation index (SI) was calculated by the ratio of the counts per minute obtained in the presence of antigen to the counts per minute obtained in the absence of antigen. For the CTLpf, limiting dilution cultures were performed. Briefly, 12 replicates of graded number of PBMC were titrated in 7-step double dilution starting from 5×10^4 to 781 PBMC/well in the presence of recombinant IL-2. After 7 days of culture, each well was

tested for cytolytic activity of Europium-labeled target cells. After 4 hr of incubation, the supernatant was harvested, and the fluorescence of the released Europium was measured. The CTLpf was expressed as number of CTLp per million PBMC[12,19].

Anti-HLA Antibodies

Anti-HLA antibodies in patient sera were initially detected by Lambda Antigen Tray (One Lambda, Canoga Park, CA, USA) for ELISA HLA class I and II. The sera were further tested for HLA antibody specificities by complement- dependent cytotoxicity against a panel of peripheral blood cells from 54 different donors in the absence and presence of dithiothreitol (DTT), a reducing agent that breaks down disulfide bonds of pentameric IgM, but has minimal effect when used at low concentration on IgG[19]. Specificities were verified, and, in some cases, additional specificities were found using flow cytometry or ELISA[20]. Based on the HLA antibody specificities, a value for virtual panel reactive antibodies (PRAs) was calculated (http://www.etrl.org/etrlpra/webform1.aspx)[21]. This virtual PRA reflects the chance that a cross-match with a potential donor will be positive. Only patients with positive anti-HLA antibodies were HLA typed to determine whether the antibodies were directed to the partner.

Analysis of the Data

The intra-individual difference in classification of the CHIV score between the two observers was assessed by calculating the weighted kappa value between the scores of the observers[22]. The final conclusion for the CHIV score was based upon agreement between the two investigators. Further data analysis was performed using SPSS software (version 17.0; Chicago, IL, USA). The Wilcoxon signed rank test was used to determine differences in CHIV scores between HE and CD68 staining. The Fischer's exact test was used to determine the difference in CHIV scores between groups with different outcome of pregnancy. The correlation between CHIV score and birth weight and birth weight percentiles were calculated with a Spearman's rank correlation coefficient (r_s). We also used the Fischer's exact test to

determine differences in CTLpf and MLR response between CHIV patients and patients with and without pre-eclampsia and between CHIV patients and patients with recurrent miscarriage without CHIV.

RESULTS

HE and CD68 staining (Figure 1) was performed on placental slides from 22 patients who had 30 pregnancies, including one twin pregnancy. Patient characteristics are summarized in Table 1. Only 10 of 30 pregnancies (33.3%) ended in the birth of a healthy child. Details about the outcome of the pregnancies are summarized in Table 2. Among the miscarriages, there were four early miscarriages (<12 weeks of gestation) and two late miscarriages (12–16 weeks of gestation). In the clinical history of the 22 patients, we found one patient with diabetes, one with hypertension, one developed pre-eclampsia, four patients had an allergy, and four patients smoked. Eleven of the 22 patients were screened for antiphospholipid syndrome and thrombophilia. Only one patient turned out to be heterozygous for a factor V Leiden mutation.

The CHIV score of observer NMM and RK differed between 9 of 30 HE stained slides and 9 of 30 CD68 stained slides. The calculated weighted Kappa of these scores between both observers was 0.544. This is a reasonable measure of agreement according to the reproducibility score of Landis and Koch[22]. After combined revision of the slides, both observers reached agreement resulting in a final conclusion of CHIV score. The sum of the scores in CD68 (total: 62) is significantly higher compared to HE staining (total: 54) ($P = 0.02$). The CD68 staining was used for the classification of CHIV[1]. CHIV was absent in 10% (3/30) of the slides, focal in 13.3% (4/30), moderate in 36.7% (11/30), and severe/massive in 40.0% (12/30) of the slides. A higher CHIV score was associated with a shorter duration of pregnancy ($r_s = -0.37$, $P = 0.04$). CHIV score 3 was significantly more often seen in miscarriages (MISC; 4/6; 66.7%; $P = 0.008$), intrauterine fetal death (IUFD; 4/6; 66.7%; $P = 0.008$), and neonatal death (NND; 3/7; 42.9%; P =

0.05), while none of the pregnancies resulting in a living child had a CHIV score 3 (Figure 2). Birth weight was available in 21 of 30 cases, and percentiles could be calculated in 15 cases with CHIV being present. Lower birth weight (r_s = -0.53, P = 0.01; Table 3) and a tendency to lower birth weight percentiles (r_s = -0.49, P = 0.07) were seen in patients with higher CHIV scores.

Neither statistical significant correlation was observed between CHIV score and number of previous pregnancies (r_s = -0.01, P = 0.94) nor between CHIV score and the number of previous miscarriages (r_s = 0.35, P = 0.06).

Abnormal Immune Response in CHIV Patients

At the moment of the study, only four patients were still visiting our outpatient clinic because of a strong wish to conceive.

Patient A

Patient A is a 37-year-old woman with a history of three pregnancies without having living children. Her first pregnancy was terminated at 23 weeks because of extreme growth retardation, and CHIV was found in the placenta. Her second pregnancy, a dichorionic twin pregnancy, resulted in fetal death at 10 and 13 weeks, respectively. Both fetuses had already growth retardation at the time of fetal death, and again, CHIV was found in both placentas. The third pregnancy of this patient ended in spontaneous miscarriage after fetal death at 10 weeks, and placental tissue was not available for pathologic examination. Recently, this patient became mother of a healthy twin. Pregnancy was achieved by in vitro fertilization with the gametes of the patient and her husband and was successfully carried until term by a surrogate mother.

Patient B

Patient B is a 32-year-old woman with 15 pregnancies in the obstetric history and no living children. Her first pregnancy was a premature delivery at 30 weeks of gestation. The baby died because of hydrocephaly and associated congenital anomalies. After this pregnancy, she experienced 11 early miscarriages (<12 weeks) and three late miscarriages (12–16 weeks). CHIV was found in the placenta of an intrauterine fetal death at 15 weeks and also

in the placenta of her last miscarriage at 16 weeks of gestation. Placentas of the other pregnancies were not available for pathological examination.

Patient C

Patient C is a 35-year-old woman with 10 pregnancies in the obstetric history and three living children. She had two living children, and she experienced one early miscarriage with her first husband. With her new husband, she experienced two early miscarriages: one ectopic pregnancy and one late miscarriage. Their fifth pregnancy ended in the birth of a healthy daughter. After this healthy child, she experienced two late miscarriages again. CHIV was found in the placenta of one of the two late miscarriages. Placentas of the other pregnancies were not available for pathological examination.

Patient D

Patient D is a 33-year-old woman with a history of four pregnancies. Her first pregnancy ended in a term delivery of a healthy daughter. All three pregnancies thereafter were characterized by premature rupture of the membranes at 21, 18, and 14 weeks, respectively. The second child was born at 31 weeks with lung hypoplasia but survived. The other two pregnancies were terminated at 19 and 15 weeks. CHIV with CD68 and CD3 positive cells was present in all three placentas.

Peripheral blood from these four women was obtained. In PBMC, we determined the proliferative and cytolytic response to paternal antigens. Anti- HLA antibodies were determined in plasma. Seven women with recurrent miscarriages without signs of CHIV served as controls. CHIV patients had comparable proliferative responses to their partners compared to controls [median and range: 78 (6–173) versus 9 (2–123) P = 0.16]. The partner-specific MLR response from CHIV patients was comparable with women without preeclampsia from our earlier report[12]. The CTLpf directed to paternal antigens was found to be extremely high (>400/10^6 PBMC) in three of four patients with CHIV (Figure 3). Only one of the seven control women with multiple miscarriages without signs of CHIV had high partner-specific CTLpf (P = 0.09, Fischer's Exact Test). We found significantly higher partner-directed CTLpf in CHIV women compared with women with non-complicated pregnancies [695 (66–895) versus. 67 (9–235); P = 0.03] (Figure 3)[12]. In three

of four CHIV patients, anti-HLA antibodies were found compared to none of seven controls (P = 0.02). All three CHIV patients had partner-directed anti-HLA class I IgG antibodies, and one of these three patients also had circulating anti-HLA class II IgG antibodies directed to her partner.

Table 1: *Patient characteristics of 22 patients with CHIV at the moment of the last pregnancy. The characteristics are presented in numbers (n), mean, standard deviation (SD) and range.*

Parameter	Mean (SD)	Range
Gravidity n = 22	4.5 (3.1)	1 – 13
Parity n = 22	2.3 (1.9)	0 – 8
Maternal age (years) n = 22	31.8 (4.9)	22 – 45
Gestational age (weeks) n = 22	25+6 (10+2)	8+0 – 40+3
Birth weight (gram) n =16	1295.3 (1171.1)	55 – 3940
Placental weight (gram) n = 19	164.5 (128.6)	12 – 383

Table 2: *Morphology of the outcomes of the 30 studied pregnancies.*

	Outcome					
	Term	Immature	Premature	miscarriage	TOP	Total
IUFD	0	4	2	0	0	6
NND	0	5	2	0	0	7
Miscarriage	0	0	0	6	0	6
TOP	0	0	0	0	1	1
Alive	3	1	6	0	0	10
Total	3	10	10	6	1	30

IUFD = intra uterine fetal death; NND = neonatal death; TOP = termination of pregnancy; alive = children who survived.

Table 3: *Relations between CHIV score (0-3) and birth weight (gram) in 30 pregnancies. 0: absent CHIV, 1: focal (less than 10% of the slide), 2: moderate (10-50%) or 3: severe or massive (more than 50%).*

		Birth weight (gram)		
Score	N	Mean	SD	Range
0	2	1595	2093	115 - 3075
1	4	1590	720	875 - 2310
2	9	1371	1171	80 - 3940
3	6	291	209	55 - 580
Total	21	1125	1076	55 - 3940

CHIV = Chronic histiocytic intervillositis; SD = Standard deviation.

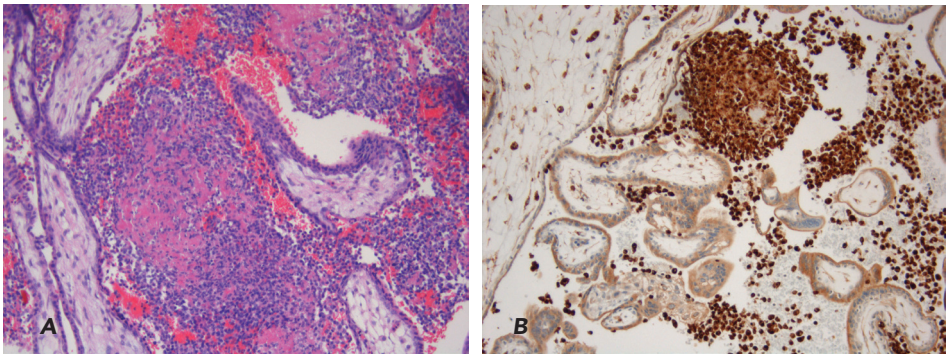

Figure 1: *Chronic histiocytic intervillositis (CHIV) with macrophages and fibrinoid depositions present in the intervillous space, observed in hematoxylin and eosin (HE) stained* ***(A)*** *and CD68 stained* ***(B)*** *microscopic slides of the placental villi of a representative case.*

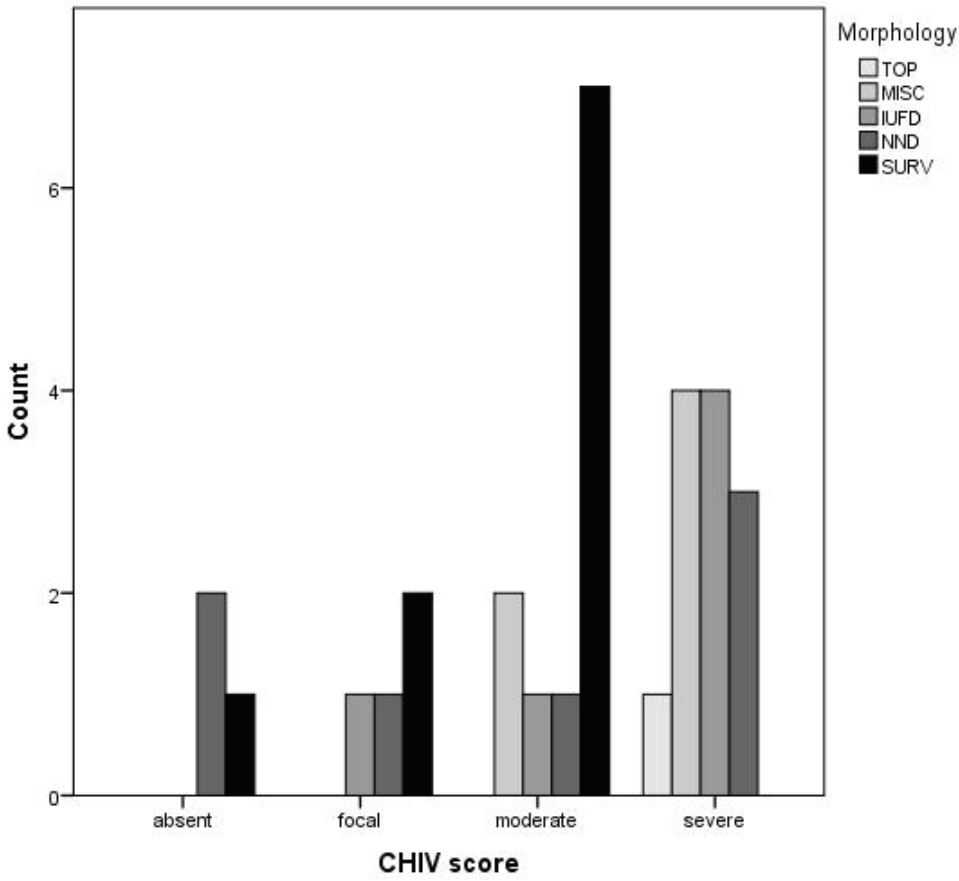

Figure 2: *CHIV score in relation to pregnancy outcome (n=30). TOP = termination of pregnancy; MISC = miscarriages; IUFD = intra uterine fetal death, NND = neonatal death; SURV = children who survived.*

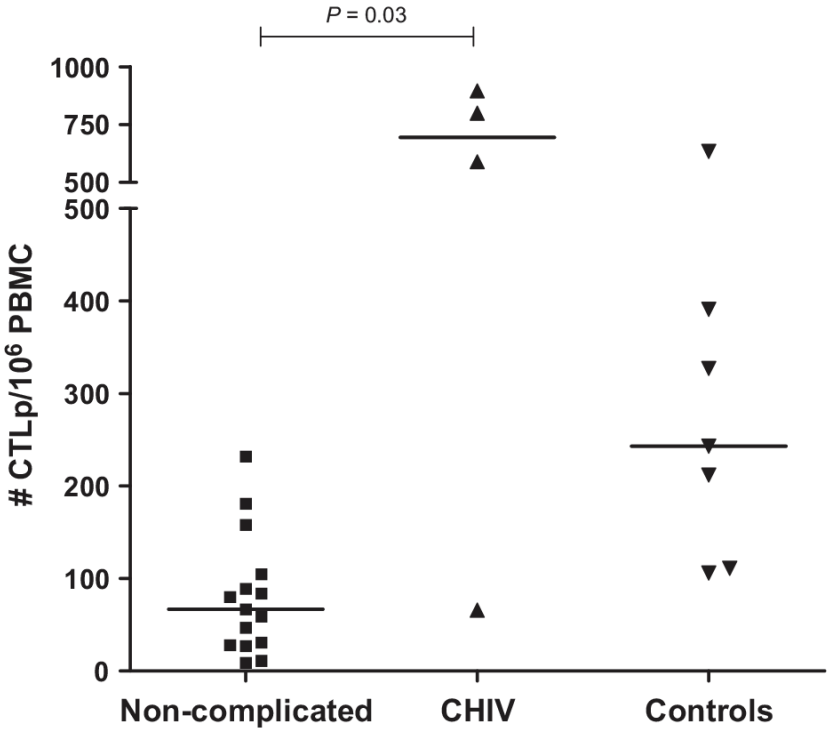

Figure 3: *Cytotoxic T-lymphocyte precursor frequency (CTLpf) against the partner was measured in peripheral blood mononuclear cells (PBMC) from women with non-complicated pregnancies12, from women with signs of CHIV during pregnancy (CHIV) and from women who had experienced recurrent miscarriages without CHIV (controls).*

DISCUSSION

In our study, CHIV was found to be associated with a high fetal loss rate. These findings are comparable with other studies[1,3,7]. Although the data in our retrospective study are not complete, our results are indicative for an immunologic origin of CHIV. CHIV was not associated with other diseases like diabetes, hypertension, and pre-eclampsia. Several studies have shown that HLA antibodies develop during progressing pregnancy[23] and can therefore be found more often in both normal women and women with recurrent miscarriage who have previously given birth[24]. Nielsen et al.[25] showed before that the presence of HLA antibodies in recurrent miscarriage patients is associated with a reduced chance of a live birth.

We demonstrated that the CHIV classification for microscopic scoring is a reliable technique. We observed a reasonable measure of agreement between investigators. The specimens were scored by an experienced and an inexperienced investigator. Therefore, the kappa value might have been even higher when the scoring would have been done by experienced investigators only. Higher score in CD68 stained microscopic slides as compared to HE staining was found. Therefore, we conclude that in case of unexplained (recurrent) fetal loss, CHIV should be part of the differential diagnosis, and microscopic examination of the materno–fetal interface after immunohistochemical staining for CD68 positive cells is of additional value for the diagnosis and scoring of CHIV. We used staining with anti-CD68 antibodies, because CD68 is specific for macrophages and has proven to be useful in the diagnosis of CHIV, as a significant difference was found between the counts of CD68 positive cells in cases and controls. Although this does not rule out an unrecognized infectious process completely, an infectious origin is most unlikely. Infections are only seen incidentally in a single miscarriage, and there is no evidence that infections are involved in the origin of recurrent miscarriages[26,27]. To our opinion, the scoring system of Parant et al.[1] was best defined and the easiest system to use. In our study, a high CHIV score was also related to more severe adverse pregnancy outcome. High CHIV scores were associated with earlier fetal loss, and live births were only observed in patients with low CHIV scores. Boyd and

Redline[2] define CHIV as monomorphic infiltration of the placental intervillous space by cells identifiable as belonging to the mononuclear phagocyte lineage (histiocytes) by morphologic criteria. They graded CHIV on a 1–3 qualitative scale for the number of histiocytes and the amount of fibrinoid material in the intervillous space, but they did not specify when a score 1, 2, or 3 was given. In another article, Redline defines two grades, focal or diffuse chronic villitis[28]. CHIV also occurs in primigravidas, which tells us that the abnormal immune response may develop in early pregnancy, probably as a reaction to paternal antigens on cytotrophoblast cells in the maternal circulation or even before pregnancy as a result of maternal contact with paternal sperm cells.

Our limited data on CTLpf and MLR in four patients who still visit our outpatient clinic suggest an immunologic underlying cause of CHIV, based on a mismatch between donor (fetal–paternal antigens) and graft recipient (mother). Nevertheless, the CTLpf is a very robust and reproducible assay[29]. Some studies reported that patients benefit from prophylactic treatment with prednisone 20–40 mg/day in combination with aspirin (100–160 mg/day) starting from the beginning of pregnancy, while others conclude that there is no statistical significant difference in live birth rate between treated and non-treated patients[1,3,8,30]. Further studies are needed to assess the efficiency of such treatment and to confirm the immunologic origin of this entity.

One of the limits of this study is the retrospective design of our study with possible biases caused by, for example, referral policy to our tertiary center of only cases with recurrent pregnancy loss. The disease is very rare, and therefore, the number of patients is small, especially of patients who still visit our outpatient clinic.

In conclusion, CHIV is associated with adverse pregnancy outcome, and CHIV scores seem to be associated with the severity of adverse pregnancy outcome. High anti-paternal cellular (T-cell) and humoral (B-cell) response to partner-specific CTLpf and the presence of anti-HLA antibodies directed to the partner suggest an immunologic origin of CHIV.

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6 GENERAL DISCUSSION

Human reproduction seems inefficient due to a large number of early pregnancy loss[1]. Miscarriage is a very common occurrence for otherwise healthy women[2]. An estimated 30% of human conceptions are lost prior to implantation and a further 30% following implantation but before the missed menstrual period[3]. About 15% of clinically recognized pregnancies fail to result in a live birth[4] with a significant variation according to maternal age (Figure 1). The incidence of failures ranges from 10% in women aged 20-24 years to 51% in women aged 40 to 44 years[1, 5]. Recurrent miscarriage is a reproductive disorder distressing many couples. Depending on the definition used, recurrent pregnancy loss affects up to 5% of couples trying to establish a family [1, 4, 6, 7]. Despite its frequency, pathological and genetic evaluation for the cause is not being performed very often[2]. In contrast to a sporadic miscarriage, which is often caused by structural malformations or chromosomal abnormalities of the fetus, the causes of recurrent miscarriage are far from completely understood[1, 6]. Defective vascularization of the placenta may be involved in the underlying cause of miscarriage.

The aim of this thesis is to improve our knowledge regarding early placental development and subsequent placental function in normal early pregnancy and in miscarriage using 3D ultrasound, an innovative virtual reality application and microscopic histology of miscarriage specimen.

We focus on four topics:

1 The reproducibility of trophoblast volume measurements using Virtual Organ Computer-aided AnaLysis (VOCAL) and the construction of trophoblast volume growth charts in ongoing pregnancy and to compare those with trophoblast volumes of pregnancies ending in a miscarriage.

2 The reproducibility of a new technique for measuring placental bed and fetal vascular volume using virtual reality to quantify these vascular systems and the differences in these measurements between IVF and IVF/ICSI and spontaneously conceived pregnancies.

3 Defective vascularization of the placenta as the underlying cause of miscarriage in patients with recurrent miscarriage.

4 The relation between the severity of Chronic Histiocytic InterVillositis (CHIV) and the outcome of pregnancy and the study of an immunological basis for CHIV.

VOCAL is considered the 'gold standard' for sonographic volume measurements. It is a disadvantage that in previous studies not the whole trophoblast volume was measured, but only the thickest part, while between the 6th and 10th week of pregnancy the villi cover up the entire surface of the gestational sac[8, 9]. In order to overcome this problem a new method is described in this thesis to measure the whole trophoblast by using VOCAL. With this method it is possible measure the true trophoblast volume in the most appropriate way. Another difficulty of placental volume measurements is that the border between trofoblast and decidua is not well defined in terms of echogenicity. There only is a difference in echogenicity between trophoblast and myometrium that can be used for measurements. It is unknown whether this difference in echogenicity is most prominent at the trophoblast–decidua or the decidua–myometrium interface. Despite this uncertainty our measurements are highly reproducible. About half of the 3D-US scans in our study could be used for trophoblast volume measurements. This due to incomplete or poor scan quality as a result of the size and position of the uterus or due to non-targeted scanning. The number of trophoblast volumes that could be measured decrease with increasing gestational age. First trimester trophoblast volumes of pregnancies ending is a miscarriage are smaller and these pregnancies show reduced trophoblast growth compared with those resulting in live birth. These smaller trophoblast volumes may indicate an impaired placental development. If trophoblast volume measurements reflect on the success of trophoblast invasion, these measurements have the potential for the development of new diagnostic

techniques for the early detection of placenta-associated complications in pregnancy[10, 11].

After measuring trophoblast volume in 3D-US datasets we developed a new method for measuring the maternal and fetal vasculature of the placenta in virtual reality. Quantifying the vascularization of the placenta in vivo may enable a more adequate description of normal and abnormal placental development. We did this by using V-Scope in the Barco I-Space. In previous studies the Barco I-Space was used to investigate embryonic growth and development[12-20]. The biometric measurements involved (length, volume and morphology) have shown to be highly accurate and reproducible. With the perception of depth and the interaction with the whole volume-rendered ultrasound dataset the I-Space allows for a more precise visualization and accurate interpretation of complex structures as the placental bed and fetal placental vascular tree as compared to conventional techniques. After studying the reproducibility of this new method we investigated associations with embryonic growth parameters, birth weight and maternal parity. We found significantly larger placental bed vascular volumes in multiparous women. This may be due to persistent effects of remodeling of the maternal vasculature in former pregnancies providing a more favorable environment for both placental development and placental function in multiparous women [21-23]. This is also in correspondence with the fact that placental vascular complications are more common in nulliparous women [24]. Also a difference between placentas of assisted reproductive technology (ART) and spontaneously conceived pregnancies is described in the literature[25-28]. With our newly developed technique we investigated the influence of ART on the placental bed vascular volume (PBVV) and fetal vascular volume (FVV). However the suggested abnormal placental development in IVF or IVF/ICSI pregnancies could not be confirmed by a difference in PBVV of FVV in the first trimester of pregnancy. We found that FVV and PBVV are significantly correlated, indicating an association of uterine maternal vascularization and trophoblastic growth.

In this thesis we have shown that the trophoblast volumes of pregnancies ending in a miscarriage are smaller and show reduced growth as compared

to ongoing pregnancies and we discuss the hypothesis that a defective vascularization of the placenta may be involved in the underlying cause of these miscarriage. Besides the use of ultrasound we investigated the vascularization by histopathological examination of miscarriage tissue. We found that the villi in the miscarriage specimen in our cohort were mainly avascular, confirming defective vascularization of the placenta. There was no difference in vascularization between miscarriages with either a euploid or aneuploid chromosomal analysis. The finding of avascular chorionic villi in miscarriages with euploid chromosome results in our study may be related to the presence of DNA copy number variants (CNVs) that interfere with trophoblast invasion and early trimester development resulting in miscarriage[29]. Previous studies of CNVs in miscarriages with the use of array-CGH indicate that small chromosomal changes are present in 1–13% of miscarriages[30-37]. These studies have the potential to identify CNVs that could lead to developmental failure of vascularization and in this way find possible 'miscarriage CNVs'. We found a high male/female ratio in our recurrent miscarriage cohort. A possible explanation could be paternally expressed imprinted X chromosome CNVs[38-40]. Another explanation found in the literature for a high male/female ratio in euploid miscarriages is that of an immunologic cause, described as an abnormal immune reaction against male-specific minor histocompatibility H-Y antigens [41-43].

In this thesis we also investigated a possible immunological cause of Chronic Histiocytic InterVillositis (CHIV), a rare type of placental pathology that is associated with reproductive loss and of which the exact pathogenesis is unknown. CHIV patients have a stronger immune response against their partners compared to controls. When the cause of recurrent miscarriage is unknown, CHIV should be part of the differential diagnosis.

The placenta must continually adapt, maximizing maternal supply and fetal extraction of nutrients without compromising the integrity of the maternal and fetal circulatory systems[44]. Placental development is a carefully orchestrated process regulated by hormones, growth factors, oxygen tension, and other local factors such as cytokines. Maternal factors preventing normal vascular remodeling are not fully understood[44].

Antiphospholipid antibody (aPL) syndrome (APS), uterine anomalies and abnormal chromosomes in either partner are established causes of recurrent miscarriage[45-47]. Also immunological aberrations might be a cause of miscarriage[48]. Only about 30% of cases of recurrent miscarriages have an identifiable cause[46], and the cause remains indeterminate in over a half of the cases[49]. According to Sugiura-Ogasawara et al., abnormal embryonic karyotype is found in about 40% of subjects in whom no conventional causes of miscarriage could be identified (Figure 2)[50]. Therefore, the percentage of cases with recurrent miscarriage of truly unexplained cause may not exceed 25%. Embryonic aneuploidy is often regarded to be the most important cause of miscarriage before 10 weeks of gestation. However, this cannot be conclusive, because miscarriages are seldom karyotyped clinically[51]. The larger the number of previous miscarriages, the lower the live-birth rate is and the higher the number of normal embryonic karyotypes[52]. The live-birth rate of patients with a previous abnormal embryonic karyotype is significantly higher, which suggests that the embryonic karyotype can be a good predictor of a subsequent successful pregnancy[51]. It is likely that some unexplained early losses are due to as yet undefined subchromosomal genetic abnormalities impairing early development of the pregnancy[48, 53]. Paternal immunization, or treatment with low-dose aspirin and heparin has no effect of improving the live-birth rate in women with unexplained recurrent miscarriage[54, 55]. These treatments are expensive and may have serious side effects. Although women may have a desire to receive medication, they should be spared the pain and grief associated with false expectations that an ineffective treatment might work[48]. Psychological support with tender loving care may be the most important strategy to encourage couples to continue to conceive until a live birth occurs[51].

In this thesis we tried to improve our knowledge regarding early placentation and subsequent placental function in normal early pregnancy and in miscarriage using 3D ultrasound datasets, an innovative virtual reality application and by histological testing of miscarriage specimen. We developed methods to measure the success of early placentation and subsequent placental function and their association with embryonic and fetal

growth and miscarriage. This may be a first step towards implementation of a technique in clinical practice for identifying women at risk for the development of placenta related pregnancy complications in the future [56]. The I-Space provides the opportunity to improve our knowledge about development of placental vasculature and the differences in anatomy of the uterine and placental vessels among women. With the development of an affordable and small sized 3D-VR desktop system that can be placed in the ultrasound room, having the same biometric properties, the 3D-VR technique is becoming available for large scale clinical research[57]. Further studies are needed if establishment of absolute vascular volume is required since we do not know if the volume we measure represents the true volume of the vasculature because there is no gold standard with which to compare our measurements.

The cause of pregnancy complications and miscarriage is multifarious and complex. A lot is still unknown on early placental development, function and cause of miscarriage. Avascular villi may indicate abnormal early placentation as a part of embryopathogenesis. Further study is warranted to determine whether a genetic cause can be found to explain these results. To establish definitively or to rule out the efficacy of any proposed treatment for recurrent pregnancy loss, randomized controlled trials with adequate numbers of participants are needed as well as more complete knowledge of the pathophysiology involved[48].

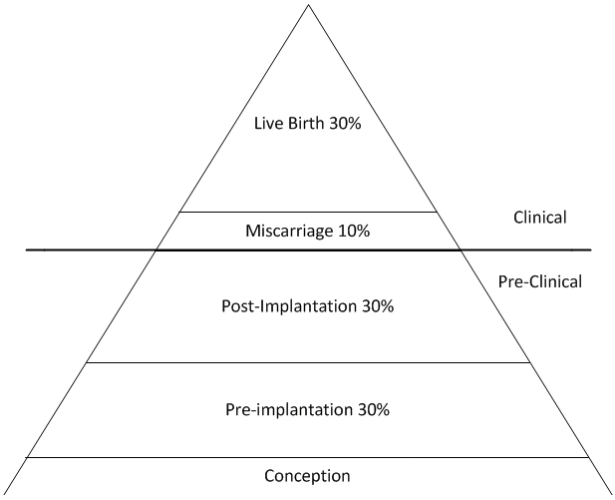

Figure 1: *A total of 70% of spontaneous human pregnancies are lost prior to live birth. The majority of those losses occur prior to the time of the missed menstrual period, and are not clinically recognized. Adapted from Macklon et al. 2002[3].*

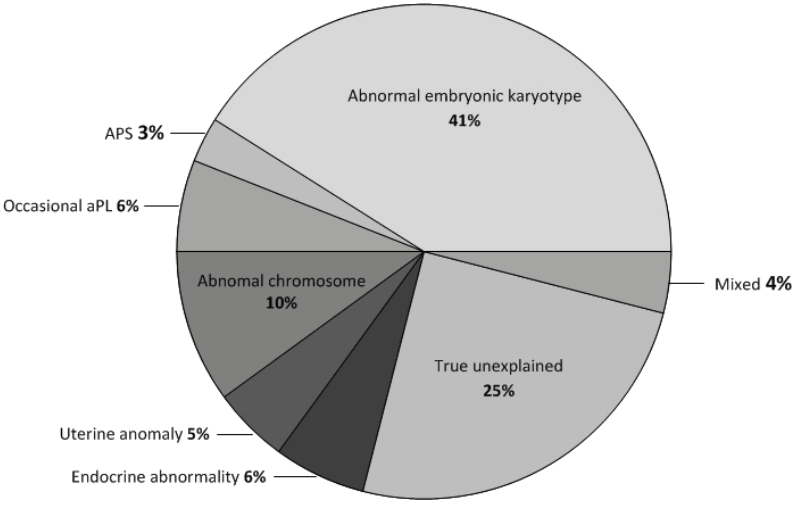

Figure 2: *The distribution of causes in patients with recurrent miscarriage. aPL: antiphospholipid antibody; APS: antiphospholipid syndrome. Adapted from Sugiura – Ogasawara et al. 2012[50].*

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7

SUMMARY
SAMENVATTING

SUMMARY

The overall aim of this thesis is to examine the development of the placental vasculature in ongoing early pregnancy and miscarriage by using three-dimensional ultrasound, three-dimensional power Doppler ultrasound, virtual reality and histologic examination of the chorionic villous vascularization. **Chapter 1** provides a general introduction describing the physiological development of the human placenta.

In **chapter 2** the validity of three-dimensional ultrasound (3D-US) trophoblast volume (TV) measurements by the use of Virtual Organ Computer-aided AnaLysis (VOCAL) are assessed. VOCAL has the advantage above conventional techniques that it allows rotation of a 3D dataset around a central axis in a number of steps, of which the user defines the angle. Interobserver and intraobserver agreement for these trophoblast volume measurements proved to be excellent. First-trimester reference curves for trophoblast volume between 6 and 12 weeks of gestation are created. The average trophoblast growth profile of pregnancies ending in a miscarriage is compared to pregnancies that resulted in live birth. Pregnancies ending in a miscarriage show smaller trophoblast volumes as well as reduced trophoblast growth compared with the reference pregnancies.

In **chapter 3** three-dimensional Power Doppler (3DPD) and 3D Virtual reality (3D-VR) are used to examine placental and uterine vasculature. With V-Scope volume rendering application in the Barco I-Space 3D 'holograms' are created which enables visualization of all three dimensions in 3DPD ultrasound. The volumes can be resized, turned and clipped to provide the optimal view of the vascularization. Quantification of placental vascularization by performing measurements on three-dimensional Power Doppler (3DPD) ultrasound datasets may enhance our insight in normal and abnormal uteroplacental anatomy and placentation. In **chapter 3.1** the validity of placental bed vascular volume (PBVV) and fetal vascular volume (FVV) measurements is tested with the use of V-Scope at 12 weeks of gestation. Measurements are performed with semi-automatic pre-defined parameters. Possible associations of PBVV and FVV with embryonic volume,

crown-rump length, fetal birth weight and maternal parity are investigated. Both the interobserver and intraobserver agreement was found to be excellent. PBVV of multiparous women are significantly larger than the PBVV of primiparous women. No other correlations were found. V-Scope offers a reproducible method for measuring placental bed vascular volume at 12 weeks of gestation. PBVV is influenced by maternal parity. In **chapter 3.2** the difference in PBVV and TV between IVF or IVF/ICSI pregnancies and spontaneously conceived pregnancies is investigated. No evidence was found for a possible abnormal placentation in IVF or IVF/ICSI pregnancies. Compared with spontaneously conceived pregnancies in early gestation no difference in PBVV or TV was found. There was found a significant correlation between TV and PBVV indicating a possible association between the degree of maternal uterine vascularisation and growth of the trophoblast.

In **chapter 4** the degree of chorionic villous vascularization is studied in correlation with ultrasound findings and corresponding chromosome results in early miscarriage specimens from a cohort of patients with recurrent pregnancy loss. Miscarriage tissue of less than 10 weeks of gestation is collected in an academic institution and the degree of chorionic villous vascularization is reviewed in hematoxylin and eosin stained microscopic slides. The results were validated and compared with the ultrasound findings and corresponding chromosome results. Validation of the vascularization score between observers was reasonable to good. The vascularization score did not differ between euploid or noneuploid miscarriages, or between embryonic, yolk sac or empty sac miscarriages. Avascular villi were seen more frequently in miscarriages trisomic for chromosome 16, when compared with miscarriages with other trisomies. Avascular villi may indicate an abnormal placental development as a part of embryopathogenesis.

In **chapter 5** findings in Chronic histiocytic intervillositis (CHIV) are described. CHIV is a rare type of placental pathology that is associated with reproductive loss as well. In contrast to the previous chapter in CHIV there is a normal development of the placenta. The relationship between the severity of CHIV and the outcome of pregnancy is investigated and the immune response between CHIV patients and controls was compared to explore

an immunological origin of CHIV. There is an association between CHIV scores and the severity of adverse pregnancy outcome. High antipaternal cellular (T-cell) and humoral (B-cell) response to partner-specific cytotoxic T-lymphocyte precursor frequency (CTLpf) and the presence of anti-HLA antibodies directed to the partner suggest an immunologic origin of CHIV.

Chapter 6 provides a general discussion on the combined results of the studies in a broader perspective.

SAMENVATTING

Het doel van dit proefschrift is het bestuderen van de ontwikkeling van de placentavasculatuur in doorgaande zwangerschappen en miskramen door middel van driedimensionale echografie, driedimensionale power Doppler echografie, virtuele realiteit en histologisch onderzoek van de chorion villus vascularisatie. **Hoofdstuk 1** geeft een algemene beschrijving van de fysiologische ontwikkeling van de menselijke placenta. In **hoofdstuk 2** wordt de validiteit van driedimensionale echografie trofoblast volume (TV) metingen met behulp van Virtual Organ Computer-aided AnaLysis (VOCAL) onderzocht. VOCAL heeft in vergelijking met conventionele echoscopische technieken het voordeel dat het rotatie van een 3D dataset rond zijn as in stappen mogelijk maakt waarbij het aantal graden kan worden bepaald door de gebruiker. De inter-onderzoeker en intra-onderzoeker reproduceerbaarheid van deze trofoblast volume metingen was uitstekend. Er werd een eerste trimester referentiecurve geconstrueerd van het trofoblastvolume bij een zwangerschapsduur van 6 tot 12 weken. Van zwangerschappen eindigend in een miskraam werd het gemiddelde trofoblast groeiprofiel vergeleken met zwangerschappen die eindigden in de geboorte van een levend kind. De trofoblast volumes van zwangerschappen eindigend in een miskraam waren kleiner en de groeisnelheid van het trofoblast volume was verminderd in vergelijking tot die van de referentie zwangerschappen.

In **hoofdstuk 3** wordt gebruik gemaakt van driedimensionale Power Doppler (3D-PD) en driedimensionale virtuele realiteit (3D-VR) voor het bestuderen van de placentaire en uterine vasculatuur. Met V-Scope rendering applicatie in de Barco I-Space worden 3D 'hologrammen' gecreëerd hetgeen visualisatie van alle drie de dimensies van de 3D-PD echoscopie mogelijk maakt. De grootte van de volumes kunnen worden aangepast, ze kunnen worden gedraaid en geknipt om de vasculatuur optimaal in beeld te kunnen brengen. Kwantificatie van de placentavasculatuur door het meten van 3D-PD echoscopische datasets kunnen ons inzicht in normale en abnormale uteroplacentaire anatomie en placentatie verbeteren. In **hoofdstuk 3.1** wordt de validiteit van het placentabed vasculaire volume (PBVV) en foetaal vasculaire volume (FVV) metingen getest met behulp van V-Scope bij een zwangerschapsduur van 12 weken. De metingen worden verricht met semi-automatische vooraf gedefinieerde parameters. Mogelijke associaties tussen PBVV en FVV met het embryonaal volume, kop-stuit lengte, geboortegewicht en maternale pariteit worden onderzocht. Zowel de inter-onderzoeker als de antra-onderzoeker reproduceerbaarheid van de metingen was uitstekend. PBVV van multipara vrouwen zijn significant groter dan die van primipara vrouwen. Er werden geen andere correlaties gevonden. V-Scope biedt een reproduceerbare methode voor het meten van PBVV bij een zwangerschapsduur van 12 weken. Het PBVV wordt beïnvloed door de pariteit van de zwangere. In **hoofdstuk 3.2** wordt het verschil in PBVV tussen IVF en IVF/ICSI zwangerschappen en spontane zwangerschappen onderzocht. Voor een mogelijke abnormale placentatie bij IVF of IVF/ICSI zwangerschappen werden geen aanwijzingen gevonden. In vergelijking tot spontane zwangerschappen werden vroeg in de zwangerschap geen verschil in PBVV of TV gevonden. Er is wel een significante correlatie tussen TV en PBVV hetgeen wijst op een mogelijke associatie tussen de mate van maternale uterine vascularisatie en groei van de trofoblast.

In **hoofdstuk 4** wordt de mate van vascularisatie van de chorionvlokken onderzocht in relatie tot de echoscopische bevindingen en de resultaten van chromosoom onderzoek bij vroege miskramen uit een cohort van patiënten

met herhaalde miskramen. Miskraamweefsel met een zwangerschapsduur van minder van 10 weken werden verzameld in een academisch centrum en de mate van vascularisatie van de villi werd beoordeeld in microscopische coupes gekleurd met haematoxyline en eosine (HE). De resultaten werden gevalideerd en vergeleken met de echoscopische bevindingen en overeenkomstige resultaten van het chromosoomonderzoek. De validatie van de vascularisatie score tussen de verschillende onderzoekers was redelijk tot goed. Er is geen verschil in vascularisatiescore tussen euploïde en aneuploïde miskramen, of tussen embryonale miskramen, miskramen met dooierzak of miskramen met een lege vruchtzak. Avasculaire villi werden meer gezien in miskramen met een trisomie 16 in vergelijking met miskramen met een andere trisomie. Avasculaire villi wijzen mogelijk op een afwijkende aanleg van de placenta als onderdeel van embryopathogenese.

In **hoofdstuk 5** worden de bevindingen bij chronische histiocytaire intervillositis (CHIV) beschreven. CHIV is een zeldzame vorm van placenta pathologie welke eveneens geassocieerd is met verlies van zwangerschappen. In tegenstelling tot het vorige hoofdstuk is er bij CHIV wel sprake van een normale aanleg van de placenta. De relatie tussen de ernst van CHIV en de uitkomst van de zwangerschap wordt onderzocht als ook de immuun response tussen CHIV patiënten en controle patiënten om een immunologische oorsprong van CHIV te onderzoeken. Er is een verband gevonden tussen CHIV scores en zwangerschapsuitkomsten. Hoge anti-paternale cellulaire (T-cel) en humorale (B-cel) reactie tegen partner specifieke cytotoxic T-lymphocyte precursor frequency (CTLpf) en de aanwezigheid van anti-HLA antilichamen gericht tegen de partner wijzen op een immunologische basis van CHIV.

Hoofdstuk 6 bevat de algemene discussie over de resultaten beschreven in dit proefschrift, waarbij deze resultaten in een breder perspectief worden geplaatst.

8 ADDENDUM

AUTHORS AND AFFILIATIONS

From the department of Obstetrics and Gynaecology, Division of obstetrics and Prenatal Medicine, Erasmus MC, University Medical Center Rotterdam, The Netherlands:

Eric A.P. Steegers
Niek Exalto
Regine P.M. Steegers-Theunissen
Willy Visser
Melek Rousian
Josine Klop – van der Aa
Maria S. Rifouna
Nikki M. Molenaar
Hakima el-Harbachi

From the department of Obstetrics and Gynaecology, Division of Reproductive Medicine, Erasmus MC, University Medical Center Rotterdam, The Netherlands:

Joop S.E. Laven

From the department of Bioinformatics, Erasmus MC, University Medical Center Rotterdam, The Netherlands:

Anton H.J. Koning
Peter J. van der Spek

From the department of Biostatistics, Erasmus MC, University Medical Center Rotterdam, The Netherlands:

Sten P. Willemsen

University of Chicago Recurrent Pregnancy Loss Program, Department of Obstetrics and Gynaecology, University of Chicago, Chicago, Illinois:

Mary D. Stephenson

From the department of Department of Obstetrics and Gynecology, Medical Centre Haaglanden, The Hague, The Netherlands:
Frederique M. van Dunné

Department of Clinical Genetics, Erasmus MC, University Medical Centre Rotterdam, The Netherlands:
Marieke Joosten

Department of Internal Medicine-Transplantation, Erasmus MC, University Medical Center, Rotterdam, The Netherlands:
Nicole M. van Besouw
Ronella de Kuiper

Department of Pathology, Reinier de Graaf Hospital, Delft, The Netherlands:
Ronald R. de Krijger

Department of Immunohaematology and Blood Transfusion, Leids University Medical Center, Leiden, The Netherlands:
Dave L. Roelen

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: Averil D. Reus Erasmus MC Department: Obstetrics and Gynaecology,, subdivision of prenatal Medicine Research School: Netherlands Institute for Health Sciences (NIHES)	PhD period: 2011 – 2015 Promotor: Prof. dr. Eric A.P. Steegers Supervisor: Dr N. Exalto

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- Course Scientific Writing in English for Publication	2011	2.0
- Course SPSS	2013	0.3
- Course systematic literature searching in PubMed and other databases	2013	0.3
- Research Integrity course	2014	0.3
Specific courses (e.g. Research school, Medical Training)		
- Digitale Individuele Nascholing Prenatale Screening	2010	0.3
- Certificaat Structureel Echoscopisch Onderzoek – FMF	2010	0.3
- Course Sequential Segmental Analysis of Malformed Hearts - Boerhaave, Leiden	2010	0.6
- Borderline Hypoplastic Left Ventricle – Boerhaave, Leiden	2010	0.3
- Intensive Fetal Cardiology Course – UCL Institute of Child Health, London, UK	2010	0.3
- Cursus foetale echocardiografie – Vumc, Amsterdam	2011	0.3
- Certificate 13 weeks Scan – Nuchal Translucency measurement – FMF	2011	0.9
- Symposium Werkgroep foetale Echoscopie – AMC, Amsterdam	2012	1.0
- Course Introduction to Clinical Research – NIHES	2012	1.9

- Course Biostatistics for Clinicians – NIHES	2012	1.0
- Course Regression Analysis for Clinicians – NIHES	2012	1.9
- Course Survival Analysis for Clinicians – NIHES	2012	1.9
- Cursus Foetale Neurosonografie – FMF	2012	0.3
Seminars and workshops		
- PhD-Day, Education and research	2010	0.2
- ESHRE workshop: Evidence based early pregnancy care, Amsterdam	2012	0.6
- Prof Wladimiroff Symposium, Rotterdam	2013	0.2
- Weekly research meeting of the department of Obstetrics and gynecologyn including two oral presentations	2010-2014	5.0
- Weekly meeting of the department of prenatal medicine	2010-2014	2.0
- Weekly multidisciplinary obstetrics-paediatrics meeting	2010-2014	2.0
- Monthly fetal pathology meeting	2010-2014	1.5
(Inter)national conferences		
- SGI – Society for Gynecologic Investigation, Miami, USA (poster presentation)	2011	1.0
- Werkgroep jonge zwangerschap NVOG, Utrecht (oral presentation)	2011	1.0
- European Society of Human Reproduction & Embryology (ESHRE), Stockholm, Sweden (poster presentation)	2012	1.0
- International Society of Ultrasound in Obstetrics and Gynecology, Copenhagen, Denmark (two oral posters)	2012	1.0
- Symposium jonge zwangerschap, Rotterdam (oral presentation)	2013	1.0
- Research meeting Moeder & kind centrum Erasmus MC(oral presentation)	2013	1.0
- Weekly research meeting of the department of Obstetrics and gynecology, oral presentation	2011-2013	1.0

Other -		
2. Teaching		
	Year	**Workload (Hours/ECTS)**
Lecturing		
- Vaardigheidsonderwijs - Prenatale screening op foetale structurele afwijkingen aan geneeskunde studenten	2010-2014	2.0
- Vaardigheidsonderwijs – Anatomie, fysiologie en pathologie van de placenta aan geneeskunde studenten	2011	1.0
- Assistenten onderwijs: Fysische grondslagen en basisbegrippen van Doppler	2011	1.0
Supervising practicals and excursions, Tutoring		
- Supervising elective research programs (21 weeks) of medical student: Hakima el-Harbachi, Josine Klop-van der Aa	2010-2012	2.0
- Substitute supervisor of foreign research assistant Maria Rifouna	2011-2013	1.0
- Visitatie voor NT en SEO beoordeling voor de Stichting Prenatale Screening Zuidwest Nederland in verschillende echocentra.	2012-2015	1.0
- Hands-on training masterclass SEO	2013-2014	0.5

ABOUT THE AUTHOR

Averil Reus was born in Hoorn on September 9th 1983. She passed her secondary school at 'Martinus college' secondary school in Grootebroek, where she graduated in 2000. That year she was not selected to enroll in medicine and started the study Biomedical Sciences at the Vrije Universiteit and moved to Amsterdam, where she met Jorrit. After one year she made a switch to study medicine and started her research at the department of Obstetrics and Gynecology at the VU, first for a period of 5 months. During her study she did practical work at the Upper West Regional Hospital Wa (Wa, Ghana). In 2010 she graduated as a doctor and started her first job at the Department of Obstetrics and Gynaecology, Division of Obstetrics and Prenatal Medicine at the Erasmus MC, University Medical Center in Rotterdam. She started her PhD research under the supervision of prof.dr. E.A.P. Steegers and dr. N. Exalto. In this period she presented results of her research at several international symposia, amongst others at the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) World congress and European Society of Human Reproduction & Embryology (ESHRE). April 2014 she gave birth to her son, Ben.

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Nina Leonie Melek **Ben** Hein
Margreet Irene Titia Ernst Isabella Fieke Karin
Ronald PREDICT study Leonie Anneke Ria Petra
Charlotte Tilly Attie Alex Maarten
Krista Ingrid Eline Bero Annemarie Jerome Niek
Eric Hans Marjolein Hilmar **Professor Steegers**
Regine Steegers-Theunissen Anton Koning
Peter van der Spek Afdeling Bioinformatica
Hajo Nicolle van Besouw Willy Visser Joop Laven
Josine Hakima Joke
Robert **Niek** **Exalto** Nienke
Marieke Joosten Karin
Diderich Luthgarde Govaerts Sten Willemsen Mary
Stephenson **Paulien** Frederique van Dunne
Dave Roelen Ronella de Kuiper Cor **Mam** Greet
Roderik Esther Leo Marty Anton Mieke Douwe
Koen **Jorrit** Andrew Lysanne
Bart Denise Michel Hanneke Chris Gerie Laura
Pap Sjaak Kleis Anke Mimi Rebecca
Michel Jeroen Lisa Marcel **Michelle** Daphne
Marc Dominique Denise Martijn Mandy Marco
lees- en grote commissie