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ORIGINAL ARTICLE *Early pregnancy*

Embryonic morphological development is delayed in pregnancies ending in a spontaneous miscarriage

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STUDY QUESTION: Is there a difference in embryonic morphological development between ongoing pregnancies and live pregnancies ending in a miscarriage?

SUMMARY ANSWER: Embryonic morphological development, assessed by the Carnegie stages, is delayed in live pregnancies ending in a miscarriage compared to ongoing pregnancies.

WHAT IS KNOWN ALREADY: Pregnancies ending in a miscarriage tend to have smaller embryos and slower heart rates.

STUDY DESIGN, SIZE, DURATION: Between 2010 and 2018, 644 women with singleton pregnancies, in the periconception period, were enrolled in a prospective cohort study with follow up until 1 year after delivery. A miscarriage was registered as a non-viable pregnancy before 22 weeks gestational age, defined by an absent heartbeat by ultrasound for a previously reported live pregnancy.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Pregnant women with live singleton pregnancies were included and serial three-dimensional transvaginal ultrasound scans were performed. Embryonic morphological development was assessed by the Carnegie developmental stages and evaluated using virtual reality techniques. The embryonic morphology was compared to clinically used growth parameters (i.e. crown-rump length (CRL) and embryonic volume (EV)). Linear mixed models were used to evaluate the association between miscarriage and the Carnegie stages. Logistic regression with generalized estimating equations was used to calculate the odds of a miscarriage after a delay in Carnegie stages. Adjustments were made for potential confounders or covariates and include age, parity, and smoking status.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 611 ongoing pregnancies and 33 pregnancies ending in a miscarriage were included between 7 + 0 and 10 + 3 weeks gestational age, resulting in 1127 assigned Carnegie stages for evaluation. Compared to an ongoing pregnancy, a pregnancy ending in a miscarriage is associated with a lower Carnegie stage ($\beta_{Carnegie} = -0.824$, 95% CI −1.190; −0.458, $P < 0.001$). A live embryo of a pregnancy ending in a miscarriage will reach the final Carnegie stage with a delay of 4.0 days compared to an ongoing pregnancy. A pregnancy ending in a miscarriage is associated with a smaller CRL ($\beta_{CRL} = -0.120$, 95% CI −0.240; −0.001, $P = 0.049$) and EV ($\beta_{EV} = -0.060$, 95% CI −0.112; −0.007, $P = 0.027$). The delay in Carnegie stage increases the odds of a miscarriage by 1.5% per delayed Carnegie stage ($OR_{Carnegie} = 1.015$, 95% CI 1.002; 1.028, $P = 0.028$).

LIMITATIONS, REASONS FOR CAUTION: We included a relatively small number of pregnancies ending in a miscarriage from a study population that is recruited from a tertiary referral centre. Furthermore, results of genetic testing on the products of the miscarriages or information on the karyotype of the parents were not available.

WIDER IMPLICATIONS OF THE FINDINGS: Embryonic morphological development, assessed by the Carnegie stages, is delayed in live pregnancies ending in a miscarriage. In the future, embryonic morphology may be used to estimate the likelihood of a pregnancy continuing to the delivery of a healthy baby. This is of crucial importance for all women but in particular for those at risk of a recurrent pregnancy loss. As part of supportive care, both women and their partners may benefit from information on the prospective outcome of the pregnancy and the timely identification of a miscarriage.

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Introduction

Approximately 12–15% of all live pregnancies will end in miscarriage, resulting in an emotional burden for both women and their partners (Magnus *et al.*, 2019; Quenby *et al.*, 2021). Various genetic and environmental factors have been associated with miscarriage but the underlying condition remains unknown in nearly 40% of cases (Stephenson, 1996). The presence of an embryonic heartbeat seen during an ultrasound examination does not guarantee an ongoing pregnancy (Naert *et al.*, 2022). Based on 2D first-trimester ultrasound parameters, such as the size of the gestational sac and embryo and the presence of a heartbeat, a likelihood of ongoing pregnancy can be estimated (Preisler *et al.*, 2015). However, overall small size of early pregnancy parameters and the use of 2D ultrasound equipment and low-frequency transabdominal transducers with concomitant suboptimal resolution may limit the value of early first-trimester 2D ultrasound imaging. State-of-the-art imaging techniques may provide novel insight into the understanding of pregnancies developing into a miscarriage.

These state-of-the-art imaging methods may include three-dimensional (3D) ultrasound, obtained with high-resolution transvaginal ultrasound probes, and virtual reality (VR) techniques. By generating a hologram using 3D VR, biometric and volumetric measurements can be performed with high accuracy and precision (Rousian *et al.*, 2018). For example, 3D VR provides easy to perform semi-automated volumetric measurements, such as the embryonic volume (EV), enabling earlier detection of first-trimester growth restriction in aneuploid foetuses (Rousian *et al.*, 2018). Next, 3D VR allows an in-depth evaluation of external and internal structures, such as the position of limbs and development of the brain cavities (Rousian *et al.*, 2021). The external and internal features of the embryo can be related to the embryonic position, providing the unique opportunity to examine embryonic morphology *in utero*. The Carnegie stages are used to assess embryonic morphology, partially based on the development and position of upper and lower limbs, shape and length of the brain, and the curvature of the embryo (O'Rahilly and Müller, 2010). Finally, by examining embryonic morphology and studying development rather than growth, reflected by crown-rump length (CRL) and EV, the Carnegie staging system allows us to combine multiple parameters in a single gestational age (GA)-independent value (Rousian *et al.*, 2018). Consequently, 3D VR allows us to study embryonic morphology from live pregnancies continuing to the delivery of a baby, but also from live pregnancies ultimately ending in a miscarriage.

At present, data on embryonic morphology of live pregnancies ultimately ending in a miscarriage are absent. However, we hypothesize that embryonic morphology using the Carnegie staging system and VR techniques, will provide new understanding in the aetiology of pregnancies ending in miscarriage. The aim of this study was to find associations between the embryonic developmental stages in live pregnancies ending in a miscarriage, and to compare these developmental stages to the stages in live pregnancies ultimately resulting in the delivery of a child. Moreover, the embryonic developmental stages in both groups were compared to previously studied and clinically used embryonic growth parameters (i.e. CRL and EV). Finally, we aimed to assess the chance of a live pregnancy to end in a miscarriage based on the embryonic developmental stages.

Materials and methods

Study population

The data used for this study was collected as part of the Rotterdam Periconception Cohort (Predict Study). This is an ongoing, prospective, and tertiary hospital-based study embedded in the outpatient clinic of the Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Centre, Rotterdam, the Netherlands (Steegers-Theunissen *et al.*, 2016; Rousian *et al.*, 2021). All women attending the outpatient clinic for preconception or antenatal care before 8 weeks of gestation with an age of $\geq$18 years were eligible to participate in the Predict Study.

Data collection

For this study, the ultrasound data of participants between 7 + 0 and 10 + 3 weeks GA were included. Participants in this study were recruited between 2010 and 2018.

A non-viable pregnancy before 22 weeks GA, defined by an absent heartbeat by ultrasound for a previously reported live pregnancy, was registered a miscarriage (Kolte *et al.*, 2015; ESHRE Working Group on Ectopic Pregnancy *et al.*, 2020). Pregnancies were considered ongoing when the pregnancy continued beyond 22 weeks GA and did not end as a foetal or neonatal death before 28 days of life. Viability was assessed by a dating scan at 10–12 weeks GA and a structural anatomy scan between 18 and 20 weeks GA. Pregnancy outcomes were collected from obstetric clinics and midwifes. Recurrent pregnancy loss

is defined as the loss of two or more pregnancies (Bender Atik et al., 2018).

Exclusion criteria were pregnancies without available 3D ultrasound datasets, pregnancies ending in a miscarriage without preceding confirmation of viability by the ultrasonographic detection of a heartbeat, pregnancies with structural anomaly or genetic abnormalities who underwent a termination of pregnancy, and pregnancies with the following adverse outcomes: foetal death beyond 22 weeks GA, neonatal death, congenital anomaly, or small for GA (SGA) foetuses. Finally, ongoing pregnancies were excluded in case of a self-reported irregular cycle (<21 or >35 days), unknown last menstrual period (LMP) or if the GA based on LMP differed more than 6 days from GA based on CRL.

Pregnancy dating

GA in natural pregnancies was calculated from the first day of the LMP. Pregnancies achieved after IUI, artificial insemination with donor sperm or by means of controlled ovarian stimulation and hormone treatment were classified into the subgroup of natural pregnancies. In case of IUI or donor insemination, GA was calculated using the insemination date. In IVF and ICSI pregnancies achieved after fresh embryo transfer (ET), GA was calculated from the oocyte retrieval day plus 14 days. In IVF or ICSI pregnancies achieved after frozen–thawed ET, GA was calculated from the day of ET plus 19 days. The calculation of the GA of cryopreserved embryo transfers depended on the number of days between oocyte retrieval and embryonic cryopreservation.

Ultrasound measurements

All women received serial transvaginal 3D ultrasound examinations performed by trained examiners using a 6–12 MHz high-resolution probe of a GE Voluson E8 ultrasound machine (GE, Zipf, Austria). From 2010 to 2013, weekly scans were performed between 6 and 12 weeks GA. After 2013, 3D ultrasound examinations were performed at 7, 9, and 11 weeks GA (Steegers-Theunissen et al., 2016; Rousian et al., 2021). All 3D ultrasound data were transformed into Cartesian (rectangular) volumes to enable offline analysis. The V-Scope software (Erasmus MC, Rotterdam, the Netherlands) is used for the rendering of the embryonic holograms in the BARCO I-Space, a VR room, offering real depth perception using stereoscopic glasses (Rousian et al., 2018). First, the morphology was assessed with 3D VR by determining the Carnegie stages based on the development and position of upper and lower limbs, curvature of the embryo and morphological features of the pros-, mes-, and rhombencephalon, as described previously (O'Rahilly and Müller, 2010). Morphological development was visually compared to the Carnegie stages 13–23 described by O'Rahilly and Müller (2010). Supplementary Table SI provides an overview and description of the Carnegie stages 13–23. The Carnegie stages are represented by images created for the Human Atlas project (with permission of de Bakker et al. (2016)). Also, 3D VR images of the Carnegie stages are depicted. Second, the growth parameters CRL and EV were assessed in the same first-trimester 3D ultrasound datasets, using previously described methods (Rousian et al., 2009, 2021).

Questionnaires

At enrolment, all participants filled in a self-administered general questionnaire on maternal characteristics, providing details of age, ethnic background, education level (according to Dutch classification (Statistics Netherlands (CBS), 2016)), medical and obstetric history, BMI, and lifestyle behaviours (i.e. smoking, alcohol consumption, folic acid supplement use). During the intake visit at the outpatient clinic, experienced researchers and research nurses check the questionnaires in a standardized manner for completeness and consistency (Steegers-Theunissen et al., 2016).

General data

At enrolment, blood pressure, weight, and height were measured. Trained researchers registered the anthropometric measurements. Data on the infant's birth outcomes, including GA at delivery and birthweight, were obtained from medical records.

Statistical analysis

Baseline maternal characteristics between ongoing pregnancies and pregnancies ending in a miscarriage were compared using Student's *t*-test or Mann–Whitney *U*-test for continuous variables and Chi-square or, when appropriate, Fisher's exact test for categorical variables. Potential confounders were identified from recent studies. Variables considered as confounders or covariates are based on previously identified factors with possible influence on the pregnancy outcome and include parity, ethnicity, socio-economic status, alcohol use, smoking, folic acid/multivitamin supplement use, maternal age, BMI, and mode of conception (Rousian et al., 2018).

The Carnegie stages were treated as a continuous variable with stage 23 reflecting the final stage of embryonic morphologic development. Student's *t*-test was used to compare Carnegie stages between ongoing pregnancies and pregnancies ending in a miscarriage. With linear mixed models, Carnegie stages, CRL and EV were compared between ongoing pregnancies and in pregnancies ultimately ending in a miscarriage. With a linear mixed model, repeated measurements of the same participant can be included in one analysis, and correction for multiple confounders or covariates is allowed. At first, a model analysis (Model 1) with GA and miscarriage (yes/no) was performed. Finally, the complete model (Model 2) additionally included all known confounders or covariates, as previously described. All confounders were entered simultaneously into the model together with a random intercept. Since embryonic growth differs according to the conception method, a subgroup analysis with stratification for conception method was performed (Berntsen et al., 2019). An interaction term for miscarriage (yes/no) and GA was introduced in the linear mixed model to calculate the difference in GA between ongoing pregnancies and pregnancies ending in a miscarriage. The calculated difference can be expressed as a delay in reaching the final Carnegie stage, Stage 23.

The odds of a miscarriage per Carnegie stage were calculated using a two-step approach. First, we fitted a mixed model for the longitudinal Carnegie scores. Using this model, we were able to generate predictions of the Carnegie stage for given GAs given all observations before this time point. This predicted Carnegie stage is now used in a logistic regression model estimated with generalized estimating

equations (GEE) with an independent working correlation to predict the event of a miscarriage. The GEE is used to assess the odds of a miscarriage. The GEE analysis was performed for Model 1 and the fully adjusted Model 2.

A joint model was used to assess the chance of a miscarriage based on the delay of Carnegie stage per pregnancy. A joint model incorporates multiple longitudinal measurements and time-to-event outcomes (Papageorgiou *et al.*, 2019). The joint longitudinal model utilizes all the repeated measurements on pregnancy, irrespective of timing and number of observations, without using imputed data. The model combines linear mixed modelling and survival analysis, and is expressed as a hazard ratio (HR) for the event miscarriage. First, a linear mixed model is constructed for Carnegie stages as a predictor for miscarriage. Then the time-to-event is incorporated into the joint model. The random effects in the linear mixed model account for both the association between the longitudinal repeated measurements and the time-to-event outcome (Papageorgiou *et al.*, 2019). This analysis was performed for Model 1 and the fully adjusted Model 2 using 150 000 iterations.

All statistical analyses within this study were performed using IBM SPSS Statistics for Windows, version 24.0 (SPSS Inc., Chicago, IL, USA) and R-Studio, version 3.6.1 (The R Foundation for Statistical Computing). A *P*-value <0.05 was considered to indicate statistical significance.

Ethical approval

The study protocol was approved by the Erasmus MC Institutional Review Board (MEC-2004-227) and all participating women and their partners signed written informed consent at enrolment.

Results

In Fig. 1, the flowchart of the study population is depicted. In total, 716 out of 1360 pregnancies with available 3D ultrasound datasets were excluded from analysis. A total of 644 live pregnancies, of which 611 ongoing pregnancies and 33 pregnancies ending in a miscarriage, were included in the final analysis. Of the 611 ongoing pregnancies, 1068 3D ultrasound datasets were available to assign Carnegie stages. Of the 33 pregnancies ending in a miscarriage, a total of 59 3D ultrasound datasets were available to assign Carnegie stages. Baseline characteristics for both groups are shown in Table I. At baseline, significant differences were noted between the ongoing pregnancies and the pregnancies ending in a miscarriage for maternal age (32.5 years versus 34.7 years, respectively, $P=0.010$), history of recurrent pregnancy loss (16.8% versus 39.4, respectively, $P=0.001$), and mode of conception ($P=0.017$).

The Carnegie stages were different between the ongoing pregnancies and the pregnancies ending in a miscarriage ($P<0.001$). In Table II, the association between miscarriage and Carnegie stages is demonstrated. Compared to an ongoing pregnancy, a pregnancy ending in a miscarriage is associated with a lower than expected score of the Carnegie stages in Model 1 ($\beta_{Carnegie} = -1.061$, 95% CI -1.354; -0.768, $P<0.001$) and in Model 2 ($\beta_{Carnegie} = -0.824$, 95% CI -1.190; -0.458, $P<0.001$).

Table III shows the association between miscarriage and embryonic growth parameters reflected by CRL and EV. Compared to an ongoing pregnancy, a pregnancy ending in a miscarriage is associated with a smaller CRL in Model 1 ($\beta_{CRL} = -0.273$, 95% CI -0.367; -0.179, $P<0.001$) and in the fully adjusted Model 2 ($\beta_{CRL} = -0.120$, 95% CI -0.240; -0.001, $P=0.049$). In addition, EV is smaller in pregnancies ending in a miscarriage, expressed as a lower than expected measurement of EV in Model 1 ($\beta_{EV} = -0.122$, 95% CI -0.163; -0.081, $P<0.001$) and in the fully adjusted Model 2 ($\beta_{EV} = -0.060$, 95% CI -0.112; -0.007, $P=0.027$).

In Fig. 2A, the delay in Carnegie stages is displayed, comparing ongoing pregnancies and pregnancies ending in a miscarriage. A live embryo of a pregnancy ending in a miscarriage will reach the final Carnegie stage with a delay of 4.0 days compared to an ongoing pregnancy. Supplementary Fig. S1 shows that the Carnegie stages of individual cases ranged from +3.1 days (i.e. advanced morphology) to −7.8 days (i.e. delayed). For 16 (48%) of the pregnancies ending in miscarriage, the first measurement of morphological development, reflected by the Carnegie stage, is below the 95% CI of the ongoing pregnancies. Using repeated measurements, approximately half of the pregnancies ending in miscarriage show a normal morphological development, whereas the remaining embryos demonstrate deviated morphological development, reflected by a delayed or advanced development or they started (far) below the 95% CI of the ongoing pregnancies. Figure 2B and C, respectively, the CRL and EV growth trajectories of the ongoing pregnancies compared to the pregnancies ending in a miscarriage. In Supplementary Fig. S1A–C, the curves of the Carnegie stages and the growth trajectories of the individual pregnancies ending in a miscarriage are shown.

The odds ratio (OR) for miscarriage according to Carnegie stage is shown in Table IV. In Model 1, the delay in Carnegie stage increased the odds of a miscarriage by 2.7% per delayed Carnegie stage ($OR_{Carnegie} = 1.027$, 95% CI 1.001; 1.044, $P=0.004$). For the fully adjusted Model 2, the odds of a miscarriage increased by 1.5% per delayed Carnegie stage ($OR_{Carnegie} = 1.015$, 95% CI 1.002; 1.028, $P=0.028$).

Supplementary Table SII depicts an HR according to delay in Carnegie stages. The HR did not show an association between a delay in Carnegie stages and the chance of a miscarriage in Model 1 ($HR_{Carnegie} = 1.180$, 95% CI 0.891; 1.549, $P=0.130$) but could not be estimated for the fully adjusted model because model convergence could not be achieved.

Discussion

This is the first study to examine morphological development *in utero* using Carnegie stages of live pregnancies ending in a miscarriage. We have shown that embryonic development differs between ongoing pregnancies and live pregnancies ending in a miscarriage. Using the Carnegie staging system, we show a 4.0-day delay (range +3.1; −7.8 days) in reaching the final stage of embryonic morphological development in pregnancies ending in a miscarriage. Furthermore, embryonic growth, expressed by serial CRL and EV, in pregnancies ending in a miscarriage is decreased in the unadjusted model. In this

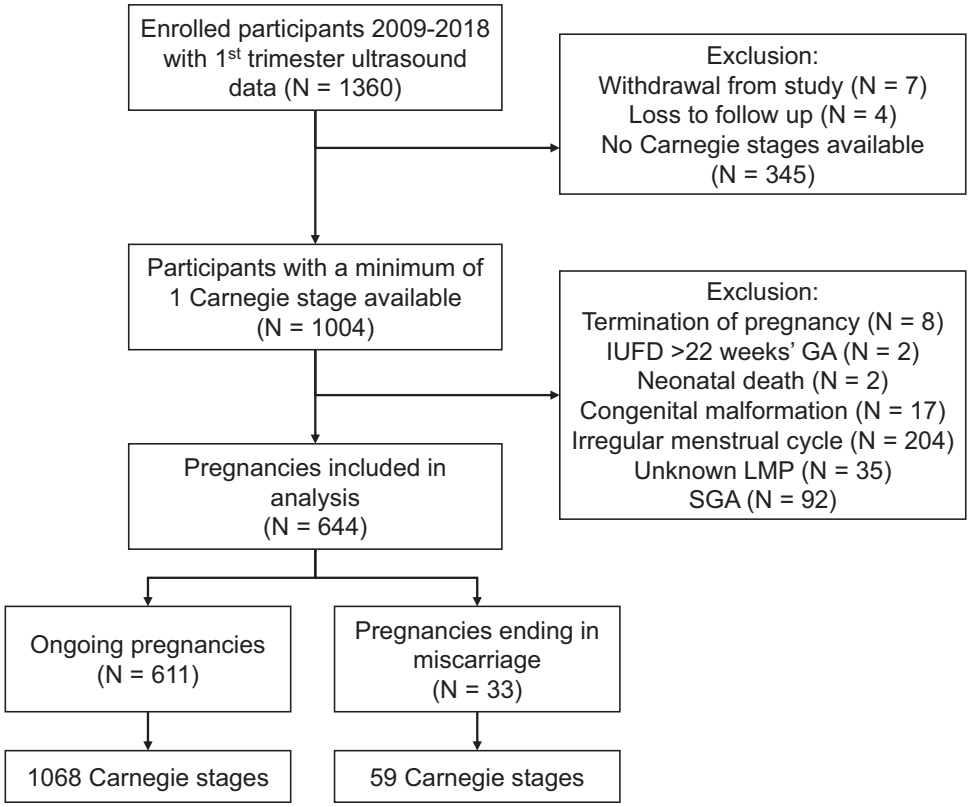

Figure 1. Flowchart of total study population. IUFD, intrauterine foetal demise; GA, gestational age; LMP, last menstrual period; SGA, small-for-gestational age.

small cohort of miscarriages, we showed that the delay of one Carnegie stage increased the odds of a miscarriage by 1.5%.

Studies in ART have shown that accelerated and delayed cell division is associated with a lower pregnancy rate (Pennetta *et al.*, 2018). In our study, we solely found delayed embryonic morphology in pregnancies ending in a miscarriage. However, we cannot exclude a miscarriage is preceded by accelerated morphological development as we only included live pregnancies from 7 + 0 weeks of gestation onwards. Direct inspection of miscarriages by transcervical embryoscopy, performed following confirmed non-viability, has shown that up to 91% of embryos have morphological abnormalities (Rajcan-Separovic *et al.*, 2010; Feichtinger *et al.*, 2018). Morphological abnormalities included growth disorganization or external defects but did not describe embryonic morphology using the Carnegie staging system. In this study, we used the Carnegie stages to assess morphological development and did not take the evaluation of external defects into account.

In a previous study, investigating the embryonic curvature using 3D VR ultrasound, our group showed the curvature of live embryos ending in a miscarriage was not significantly different from ongoing pregnancies (Bogers *et al.*, 2018). As far as we know, Bogers *et al.* (2018) were the first to investigate morphology of live embryos ending in a miscarriage using *in vivo* measurements (Bogers *et al.*, 2018). In their cohort of 33 miscarriages and 202 ongoing pregnancies, embryonic curvature, a component of the Carnegie staging system, was compared to CRL. In contrast to the study by Bogers *et al.* (2018), we did not quantify solely the curvature of the embryos but considered all components of the Carnegie staging system when performing our morphologic assessment. By assessing all components of the Carnegie classification, we were able to find a significant difference between pregnancies ending in miscarriage and ongoing pregnancies.

Our findings for decreased embryonic growth in miscarriages are in line with the previous study by Bogers *et al.* (2018) and other studies using 2D ultrasound data. Shaamash *et al.* (2020) have studied foetal heart rate, CRL and gestational sac diameter to predict miscarriage. They show that a smaller CRL, a smaller gestational sac and slower heart rate are associated with miscarriage. In a study by Preisler *et al.*, a smaller CRL is also associated with miscarriage (Preisler *et al.*, 2015). Both studies used transvaginal ultrasound examination, with only 2D ultrasonography. In our study, using 3D VR, on average we show smaller CRL values for pregnancies ending in miscarriage. We also observed embryos with increased volume and size, which may be indicative of hydropic changes, suggesting a miscarriage is likely to occur in the near future. Two studies have shown that an increased nuchal

Table I Baseline characteristics of ongoing pregnancies and pregnancies ending in a miscarriage.

Maternal characteristics	Ongoing pregnancies N = 611	Missing	Pregnancies ending in miscarriage N = 33	Missing	*P*
Maternal age, years, median (IQR)	32.5 (29.3–35.6)	0	34.7 (29.5–37.7)	0	**0.010***
Nulliparous, n (%)	326 (53.4)	1 (0.1%)	13 (40.5)	0	0.131
Recurrent pregnancy loss,¶ n (%)	102 (16.8)	7 (1.1%)	13 (39.4)	0	**0.001***
Geographical origin		0		0	0.052
Western, n (%)	525 (85.9)		24 (72.7)		
Non-Western, n (%)	86 (14.1)		9 (27.3)		
Education level		13 (2.1%)		8 (24%)	0.142
High, n (%)	360 (60.1)		11 (44.0)		
Intermediate, n (%)	196 (32.7)		10 (40.0)		
Low, n (%)	43 (7.2)		4 (16.0)		
BMI, kg/m^2, median (IQR)	24.1 (21.8–27.8)	0	24. (22.0–27.3)	0	0.737
Periconceptional alcohol use, n (%)	193 (31.6)	0	6 (18.2)	0	0.141
Periconceptional smoking, n (%)	76 (12.4)	0	3 (9.1)	0	0.606
Periconceptional folic acid, n (%)	529 (86.6)	0	27 (81.8)	0	0.328
Vitamin use, n (%)	408 (67.1)	3 (0.5%)	20 (62.5)	1 (4%)	0.590
Mode of conception		0		0	**0.017***
Natural, n (%)	304 (49.8)		24 (72.7)		
IVF/ICSI, n (%)	297 (48.6)		8 (24.2)		
Oocyte donation, n (%)	10 (1.6)		1 (3.0)		
Birth outcomes					
Birthweight (g), median (IQR)	3440 (3160–3736)	55 (9.0%)	–		
GA at birth (wk), median (IQR)	39 + 1 (38 + 1 to 40 + 2)	54 (8.9%)	–		
Males, n (%)	296 (49.8)	17 (2.8%)	–		
Preterm birth, n (%)	40 (7.1)	54 (8.9%)	–		

IQR, interquartile range; GA, gestational age.
*$P < 0.05$ (bold text).
¶The loss of two or more pregnancies. Baseline characteristics were compared using Student's *t*-test or Mann–Whitney *U*-test for continuous variables and Chi-square or Fisher's exact test for categorical variables.

Table II Association between miscarriage and Carnegie stages.

Miscarriage	Model 1[a] Effect estimate (β), 95% CI	*P*	Model 2[b] Effect estimate (β) (95% CI)	*P*
No	0 (Reference)		0 (Reference)	
Yes	−1.061 (−1.354; −0.768)	**<0.001***	−0.824 (−1.190; −0.458)	**<0.001***

Carnegie stages were compared to ongoing pregnancies.
[a]Crude model with gestational age as time predictor.
[b]Fully adjusted model with gestational age as time predictor; adjusted for alcohol use, educational level, ethnicity, folic acid supplement use, maternal age, maternal BMI, mode of conception, parity, smoking, and vitamin use.
*$P < 0.05$ (bold text).

translucency and/or a reversed blood flow in the ductus venosus, as proxies for genetic abnormalities, were associated with an increased risk of miscarriage (Akolekar *et al.*, 2011; Yan *et al.*, 2022). Hence, to identify pregnancies at risk of a miscarriage embryonic growth as a sole marker is insufficient. The combination of embryonic growth and morphology may offer a solution. With the availability of additional

Table III Association between miscarriage and embryonic growth parameters.

	Miscarriage	Model 1[a] Effect estimate (β) 95% CI	P	Model 2[b] Effect estimate (β) 95% CI	P
Crown-rump length	No	0 (Reference)		0 (Reference)	
	Yes	−0.273 (−0.367; −0.179)	**<0.001***	−0.120 (−0.240; −0.001)	**0.049***
Embryonic volume	No	0 (Reference)		0 (Reference)	
	Yes	−0.122 (−0.163; −0.081)	**<0.001***	−0.060 (−0.112; −0.007)	**0.027***

Crown-rump length and embryonic volume were compared to ongoing pregnancies.
[a]Crude model with gestational age as time predictor.
[b]Fully adjusted model with gestational age as time predictor; adjusted for alcohol use, educational level, ethnicity, folic acid supplement use, maternal age, maternal BMI, mode of conception, parity, smoking, and vitamin use.
*$P < 0.05$ (bold text).

information on morphological development, we can distinguish two entities within the group of pregnancies ending in a miscarriage. We identify a group with a smaller CRL and concomitant delayed morphological development, and another group showing a CRL within normal ranges developing delayed morphology during later stages. Unfortunately, we were unable to predict the time-to-miscarriage by HRs for ongoing pregnancies. We did find an increased OR for miscarriages in pregnancies with a delayed morphological development. For example, the odds for a miscarriage would increase by 4.6% (1.015^3) if the development is three Carnegie stages delayed. Further, it would have been interesting to predict the chance of a miscarriage in ongoing pregnancies, especially in the group with smaller CRL and concomitant delayed morphological development, and to compare this to the group with normal CRL and delayed morphological development to identify pregnancies at risk of miscarriage. The identification of these two entities in which growth and development seem to be regulated differently, may represent different causes of miscarriage for which future research may elucidate additional details. However, the group sizes were limited, and additional analyses could not be performed. The limited group sizes may serve as an explanatory factor for the insufficient statistical power to predict the risk of a miscarriage by HRs.

This is the first prospective cohort study to describe repeated measurements of embryonic morphological development in ongoing pregnancies ultimately ending in miscarriage. Pregnancies without a live embryo were excluded because information on the exact timing of the demise was lacking. Furthermore, non-viable embryos may decrease in size and volume, or become hydropic: either way this may have significant influence on our measurements of embryonic size and evaluation of morphology. Hence, we strictly included parameters of live pregnancies in the final analysis. Another strength of this study is the certainty of the pregnancy dating owing to exclusion of pregnancies with irregular menstrual cycles or uncertain LMP. Lastly, the serial, longitudinal assessment of embryonic morphology and growth using 3D VR, a validated technique with high accuracy and reproducibility of measurements of embryonic growth, can also be seen as an important strength (Rousian *et al.*, 2018).

In many miscarriages, aneuploidy of the embryo is the reason for the event (Bender Atik *et al.*, 2018). Unfortunately, we do not have information on the results of genetic testing on the products of the miscarriages or on the karyotype of parents, as such investigations are not routinely performed according to existing guidelines. The *in vivo* 3D VR assignment of the Carnegie stages is performed using the same criteria used by O'Rahilly and Müller (2010). We are limited to examine the external morphological development and internal development of the brain, compared to *ex vivo* studies that may include histological confirmation. Another limitation was the small sample size of this study. The sample size was not sufficient to predict the event of a miscarriage, based on embryonic morphology, or to allow correction for different confounders or covariates. Women included in this study were mainly recruited from a tertiary referral hospital also including many women with a high level of education and who used folic acid supplementation. External validity is therefore limited. Finally, the study is embedded in the outpatient clinic, therefore it is not feasible to record participation rate, and this may be a potential risk for confounding caused by selection bias.

Our advanced non-invasive imaging technique, 3D VR ultrasound, may be of value for parents in terms of acquiring early information on delayed morphological development of the embryo and providing support to prepare for an early adverse pregnancy outcome. Managing expectations and thus including reliable counselling regarding prognosis as part of supportive care, especially for couples with recurrent miscarriages, is considered of utmost importance for women and their partners during early pregnancy (van den Berg *et al.*, 2018). Future research should focus on a larger cohort to study the value of embryonic morphology in the understanding of miscarriages. In a previous study, our group showed that delayed morphological development was associated with delayed growth in the second trimester (Parisi *et al.*, 2019). Perhaps the *in utero* assessment of growth and the Carnegie stages may contribute to the etiologic understanding of a miscarriage.

In conclusion, embryonic morphological development, expressed by the Carnegie stages, is delayed in live pregnancies ending in a miscarriage. Interestingly, the delay in embryonic morphology was independent of embryonic growth. In the future, embryonic morphology may be used as a parameter for estimating the likelihood of a pregnancy continuing to the delivery of a healthy baby, which is of crucial importance for all women but in particular for those at risk of a recurrent pregnancy loss. As part of supportive care, both women and their partners may benefit from information on the prospective outcome of the pregnancy and the timely identification of pregnancies at risk of miscarriage.

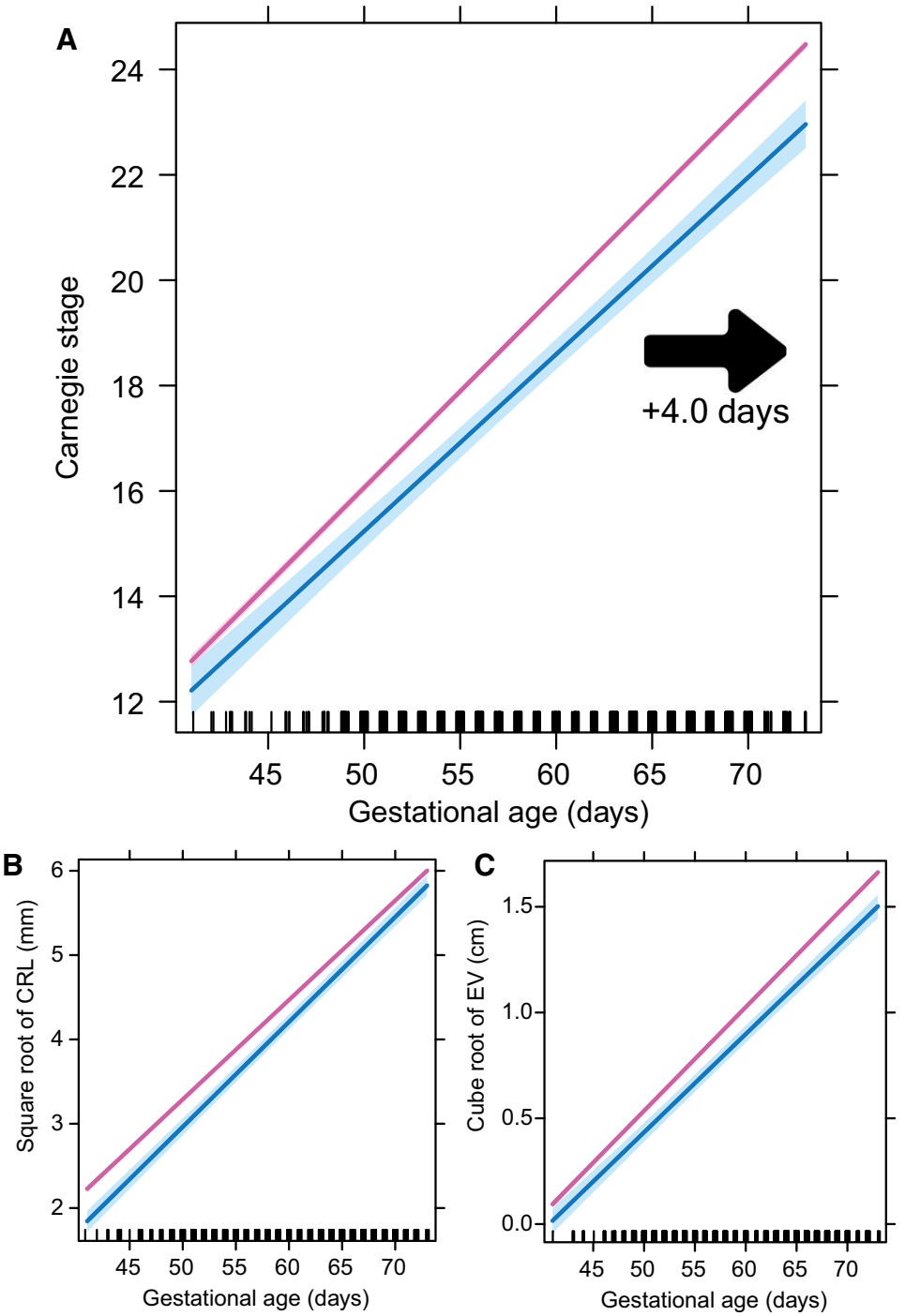

Figure 2. Characteristics of ongoing pregnancies and pregnancies ending in miscarriage. Embryonic morphological development (**A**: Carnegie stages), growth trajectories (**B**: crown-rump length: (CRL)), and embryonic volume (**C**: EV) of the included pregnancies depicted by gestational age. The pink coloured line indicates the ongoing pregnancies (N = 611) and the blue lines indicate the pregnancies ending in a miscarriage (N = 33), with their 95% CI.

Table IV Odds ratio of delay in Carnegie stages and miscarriage.

Miscarriage	Model 1[a] Odds ratio, 95% CI	*P*	Model 2[b] Odds ratio, 95% CI	*P*
No	0 (Reference)		0 (Reference)	
Yes	1.027 (1.001–1.044)	**0.004***	1.015 (1.002–1.028)	**0.028***

Odds ratio of miscarriage per delay in Carnegie stage compared to ongoing pregnancies.
[a]Crude model with gestational age as time predictor.
[b]Fully adjusted model with gestational age as time predictor; adjusted for alcohol use, educational level, ethnicity, folic acid supplement use, maternal age, maternal BMI, mode of conception, parity, smoking, and vitamin use. A linear mixed model and logistic regression with generalized estimating equations were used to calculate the odds.
*$P < 0.05$ (bold text).

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

Authors' roles

C.S.P., S.P.W, A.G.M.G.J.M., and M.R. contributed to the study concept and design. C.S.P. and A.C.A. performed data collection. C.S.P., N.G., and M.R. performed embryonic morphological measurements. C.S.P. and S.P.W. conducted statistical analyses. C.S.P., N.G., and B.S.B. contributed to the images. All authors contributed to data interpretation and manuscript writing, approved the final version of the manuscript and vouch for data accuracy.

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Conflict of interest

No conflict of interest has to be declared by any of the authors regarding the material discussed in the article.

References

Akolekar R, Bower S, Flack N, Bilardo CM, Nicolaides KH. Prediction of miscarriage and stillbirth at 11-13 weeks and the contribution of chorionic villus sampling. *Prenat Diagn* 2011;**31**:38–45.

Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, Nelen W, Peramo B, Quenby S, Vermeulen N *et al.* ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open* 2018;**2018**:hoy004.

Berntsen S, Soderstrom-Anttila V, Wennerholm UB, Laivuori H, Loft A, Oldereid NB, Romundstad LB, Bergh C, Pinborg A. The health of children conceived by ART: 'the chicken or the egg?' *Hum Reprod Update* 2019;**25**:137–158.

Bogers H, van Uitert EM, van Ginkel S, van der Mooren EDH, Groenenberg IAL, Eilers PHC, Exalto N, Steegers EAP, Steegers-Theunissen RPM. Human embryonic curvature studied with 3D ultrasound in ongoing pregnancies and miscarriages. *Reprod Biomed Online* 2018;**36**:576–583.

de Bakker BS, de Jong KH, Hagoort J, de Bree K, Besselink CT, de Kanter FE, Veldhuis T, Bais B, Schildmeijer R, Ruijter JM *et al.* An interactive three-dimensional digital atlas and quantitative database of human development. *Science* 2016;**354**:1019.

ESHRE Working Group on Ectopic Pregnancy, Kirk E, Ankum P, Jakab A, Le Clef N, Ludwin A, Small R, Tellum T, Toyli M, Van den Bosch T *et al.* Terminology for describing normally sited and ectopic pregnancies on ultrasound: ESHRE recommendations for good practice. *Hum Reprod Open* 2020;**2020**:hoaa055.

Feichtinger M, Reiner A, Hartmann B, Philipp T. Embryoscopy and karyotype findings of repeated miscarriages in recurrent pregnancy loss and spontaneous pregnancy loss. *J Assist Reprod Genet* 2018;**35**:1401–1406.

Kolte AM, Bernardi LA, Christiansen OB, Quenby S, Farquharson RG, Goddijn M, Stephenson MD; ESHRE Special Interest Group EP. Terminology for pregnancy loss prior to viability: a consensus statement from the ESHRE early pregnancy special interest group. *Hum Reprod* 2015;**30**:495–498.

Magnus MC, Wilcox AJ, Morken NH, Weinberg CR, Haberg SE. Role of maternal age and pregnancy history in risk of miscarriage: prospective register based study. *BMJ* 2019;**364**:l869.

Naert MN, Khadraoui H, Muniz Rodriguez A, Fox NS. Stratified risk of pregnancy loss for women with a viable singleton pregnancy in the first trimester. *J Matern Fetal Neonatal Med* 2022;**35**:4491–4495.

O'Rahilly R, Müller F. Developmental stages in human embryos: revised and new measurements. *Cells Tissues Organs* 2010;**192**:73–84.

Papageorgiou G, Mauff K, Tomer A, Rizopoulos D. An overview of joint modeling of time-to-event and longitudinal outcomes. *Annu Rev Stat Appl* 2019;**6**:223–240.

Parisi F, Rousian M, Koning AHJ, Willemsen SP, Steegers EAP, Steegers-Theunissen RPM. Effect of human embryonic morphological development on fetal growth parameters: the Rotterdam Periconceptional Cohort (Predict Study). *Reprod Biomed Online* 2019;**38**:613–620.

Pennetta F, Lagalla C, Borini A. Embryo morphokinetic characteristics and euploidy. *Curr Opin Obstet Gynecol* 2018;**30**:185–196.

Preisler J, Kopeika J, Ismail L, Vathanan V, Farren J, Abdallah Y, Battacharjee P, Van Holsbeke C, Bottomley C, Gould D *et al.* Defining safe criteria to diagnose miscarriage: prospective observational multicentre study. *BMJ* 2015;**351**:h4579.

Quenby S, Gallos ID, Dhillon-Smith RK, Podesek M, Stephenson MD, Fisher J, Brosens JJ, Brewin J, Ramhorst R, Lucas ES *et al*. Miscarriage matters: the epidemiological, physical, psychological, and economic costs of early pregnancy loss. *Lancet* 2021;**397**:1658–1667.

Rajcan-Separovic E, Qiao Y, Tyson C, Harvard C, Fawcett C, Kalousek D, Stephenson M, Philipp T. Genomic changes detected by array CGH in human embryos with developmental defects. *Mol Hum Reprod* 2010;**16**:125–134.

Rousian M, Hop WC, Koning AHJ, van der Spek P J, Steegers EAP. First trimester brain ventricle fluid and embryonic volumes measured by three-dimensional ultrasound with the use of I-Space virtual reality. *Hum Reprod* 2013;**28**:1181–1189.

Rousian M, Koster MPH, Mulders A, Koning AHJ, Steegers-Theunissen RPM, Steegers EAP. Virtual reality imaging techniques in the study of embryonic and early placental health. *Placenta* 2018;**64**(Suppl 1):S29–S35.

Rousian M, Schoenmakers S, Eggink AJ, Gootjes DV, Koning AHJ, Koster MPH, Mulders A, Baart EB, Reiss IKM, Laven JSE *et al*. Cohort Profile Update: the Rotterdam Periconceptional Cohort and embryonic and fetal measurements using 3D ultrasound and virtual reality techniques. *Int J Epidemiol* 2021;**50**:1426–1427l.

Rousian M, Verwoerd-Dikkeboom CM, Koning AH, Hop WC, van der Spek PJ, Exalto N, Steegers EA. Early pregnancy volume measurements: validation of ultrasound techniques and new perspectives. *BJOG* 2009;**116**:278–285.

Shaamash AH, Aly HA, Abdel-Aleem M, Akhnowkh SN. Clinical and ultrasound evaluation of early threatened miscarriage to predict pregnancy continuation up to 28 weeks: a prospective cohort study. *J Ultrasound Med* 2020;**39**:1777–1785.

Statistics Netherlands (CBS). *The Dutch Standard Classification of Education 2016*, 2016. https://www.cbs.nl/nl-nl/onze-diensten/methoden/classificaties/onderwijs-en-beroepen/standaard-onderwijsindeling–soi–/standaard-onderwijsindeling-2016 (30 May 2021, date last accessed).

Steegers-Theunissen RP, Verheijden-Paulissen JJ, van Uitert EM, Wildhagen MF, Exalto N, Koning AH, Eggink AJ, Duvekot JJ, Laven JS, Tibboel D *et al*. Cohort Profile: the Rotterdam Periconceptional Cohort (Predict Study). *Int J Epidemiol* 2016;**45**:374–381.

Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. *Fertil Steril* 1996;**66**:24–29.

van den Berg MMJ, Dancet EAF, Erlikh T, van der Veen F, Goddijn M, Hajenius PJ. Patient-centered early pregnancy care: a systematic review of quantitative and qualitative studies on the perspectives of women and their partners. *Hum Reprod Update* 2018;**24**:106–118.

Yan X, Xu H, Li J, Xu Z, Niu Y, Wang Y. Subchorionic hematoma and risk of preterm delivery: a systematic review and meta-analysis. *Am J Obstet Gynecol MFM* 2023;**5**:100791.