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The impact of IVF culture medium on post-implantation embryonic growth and development with emphasis on sex specificity: the Rotterdam Periconceptional Cohort

BIOGRAPHY

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KEY MESSAGE

Human embryos cultured in SAGE 1-Step, especially males, grow and develop faster prenatally than those cultured in Vitrolife G-1 PLUS or naturally conceived, although pregnancy outcomes are comparable. Further research should focus on the impact of culture media on postnatal development and the susceptibility to non-communicable diseases.

ABSTRACT

Research question: Are there (sex-specific) differences in first-trimester embryonic growth and morphological development between two culture media used for IVF and intracytoplasmic sperm injection (ICSI) treatment?

Design: A total of 835 singleton pregnancies from a prospective hospital-based cohort study were included, of which 153 conceived after IVF/ICSI treatment with Vitrolife G-1™ PLUS culture medium, 252 after culture in SAGE 1-Step™ and 430 were naturally conceived. Longitudinal three-dimensional ultrasound examinations were performed at 7, 9 and 11 weeks of gestation for offline biometric (crown rump length, CRL), volumetric (embryonic volume) and morphological (Carnegie stage) measurements.

Results: Embryos cultured in SAGE 1-Step grew faster than those cultured in Vitrolife G-1 PLUS ($beta_{EV}$ 0.030 $\sqrt[3]{ml}$ [95% CI 0.008–0.052], $P = 0.007$). After stratification for fetal sex, male embryos cultured in SAGE 1-Step demonstrated faster growth than those cultured in Vitrolife G-1 PLUS ($beta_{EV}$ 0.048 $\sqrt[3]{ml}$ [95% CI 0.015–0.081], $P = 0.005$). When compared with naturally conceived embryos, those cultured in SAGE 1-Step grew faster ($beta_{EV}$ 0.040 $\sqrt[3]{ml}$ [95% CI 0.012–0.069], $P = 0.005$). This association was most pronounced in male embryos ($beta_{EV}$ 0.078 $\sqrt[3]{ml}$ [95% CI 0.035–0.120], $P < 0.001$).

Conclusions: This study shows that SAGE 1-Step culture medium accelerates embryonic growth trajectories compared with Vitrolife G-1 PLUS and naturally conceived pregnancies, especially in male embryos. Further research should focus on the impact of culture media on postnatal development and the susceptibility to non-communicable diseases.

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KEYWORDS

Crown rump length
Culture media
Embryonic development
Fetal weight
IVF

INTRODUCTION

Globally, over 10 million children have been conceived through IVF treatment since the birth of the first IVF baby in 1978 (*De Geyter et al., 2018*; *Steptoe and Edwards, 1978*). Success rates have increased to a live birth rate of 28% per oocyte aspiration, which can be attributed to several advances, such as intracytoplasmic sperm injection (ICSI) and improved culture conditions (*Behr and Wang, 2004*; *Wong et al., 2014*). Moreover, pregnancies after IVF/ICSI still have an increased risk of several complications, such as preterm birth (PTB) and congenital anomalies, despite all efforts to optimize culture conditions (*Pandey et al., 2012*). Interestingly, it has also been suggested that these complications are associated with fetal sex (*Broere-Brown et al., 2020*). Although the pathophysiology of these complications is complex and not yet fully understood, investigating the role of culture conditions may provide useful insights to reduce the prevalence of pregnancy complications after IVF/ICSI pregnancies.

Improvements in culture conditions aim to mimic the physiological in-vivo environment, as the in-vitro milieu exposes the preimplantation embryo to stressors not present in the in-vivo milieu (*Behr and Wang, 2004*; *Chronopoulou and Harper, 2015*; *Kovacic and Vlaisavljevic, 2008*). Regarding culture media formulations, two main protocols can be distinguished. In the sequential protocol, different media are used for fertilization, cleavage and blastocyst stages. In the single-step protocol, a single medium is used for all stages. Although the exact composition of culture media is usually not disclosed by the manufacturers, considerable differences in glucose, lactate, pyruvate, methionine and other amino acid concentrations between culture media have been observed after mass spectrometry or colorimetry analysis (*Morbeck et al., 2014*, *2017*; *Sunde et al., 2016*). Interestingly, clinical outcomes such as pregnancy rate per oocyte aspiration are similar for the two protocols (*Mantikou et al., 2013*).

A second important issue is that variations in IVF/ICSI procedures can impact not only success rates, but also post-implantation development. For example, the culture medium used during the first 3 days post-fertilization can affect fetal size, birthweight and even postnatal weight at 2 years of age (*Kleijkers et al., 2014*, *2016*; *Nelissen et al., 2013*). However, less is known about the impact of culture media on embryonic growth and morphological development in the first trimester of pregnancy. This is an important period in which rapid cell division, proliferation and differentiation for organogenesis takes place (*Steegers-Theunissen et al., 2013*). Appropriate growth and development in the first trimester are crucial for a healthy pregnancy and life course. This is substantiated by studies demonstrating associations between morphological development and fetal growth rate, and between first-trimester crown rump length (CRL), birthweight and cardiovascular outcomes in childhood (*Jaddoe et al., 2014*; *Parisi et al., 2019*; *Salomon et al., 2011*; *Vafaei et al., 2012*). First-trimester embryonic growth and morphological development can be meticulously studied by combining three-dimensional ultrasound techniques with virtual reality. This allows in-depth perception and precise assessment of novel volumetric and morphological measurements (*Verwoerd-Dikkeboom et al., 2008*).

Moreover, there is also some evidence that IVF/ICSI procedures affect the sex ratio in favour of males (*Bonduelle et al., 2002*; *Menezo et al., 1999*). A possible explanation is that male and female preimplantation embryos respond differently to the additional stress of the in-vitro procedures. In rats, exposure to prenatal stress affects males and females differently in terms of gene expression and cognitive and hormonal responses (*Bowman et al., 2004*; *Mychasiuk et al., 2011*). In humans, less is known about whether exposure to culture medium also impacts post-implantation embryonic growth and development in a sex-specific manner.

In this study, the primary aim was to compare embryonic growth and morphological development in IVF/ICSI pregnancies conceived after culture in two different, widely used culture media, for the total study population as well as for male and female embryos separately. The secondary aim was to compare second-trimester size and perinatal outcomes. The final aim was to compare post-implantation growth and development after culture in these two media with natural conception.

MATERIALS AND METHODS

Study population

This study is embedded in the Rotterdam Periconception Cohort, an ongoing prospective tertiary care hospital-based study, conducted since November 2009 at the outpatient clinic of the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center, Rotterdam, the Netherlands (*Steegers-Theunissen et al., 2016*). The data used for this study were obtained between November 2009 and August 2018. Women aged ≥18 years with an ongoing intrauterine singleton pregnancy less than 10+0 weeks pregnant were eligible to participate. Women pregnant after IVF/ICSI were recruited from the reproductive medicine outpatient clinic; women pregnant after natural conception were recruited from the obstetrics outpatient clinic. Reasons for referral to this outpatient clinic varied, from complications in a previous pregnancy (e.g. severe post-partum haemorrhage) to non-pregnancy-related conditions (e.g. endometriosis, rheumatic disease). During the inclusion period, patients were allowed to participate more than once.

For this study, only women who conceived via IVF/ICSI treatment and women who conceived naturally with a regular cycle were selected. Pregnancies conceived after oocyte donation, intrauterine insemination or hormonal therapy and pregnancies resulting in a miscarriage, fetal death, fetuses or neonates with congenital malformations, and terminated pregnancies were excluded (FIGURE 1). The prevalence of fetal death, congenital malformations and terminated pregnancies was comparable between the two media (Supplementary Table 1).

Ethical approval

This study was approved on 15 October 2004 by the local Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center, Rotterdam, the Netherlands (MEC-2004-227). All participants provided written informed consent.

IVF and culture procedures

Ovarian stimulation, oocyte retrieval, subsequent IVF/ICSI procedures and

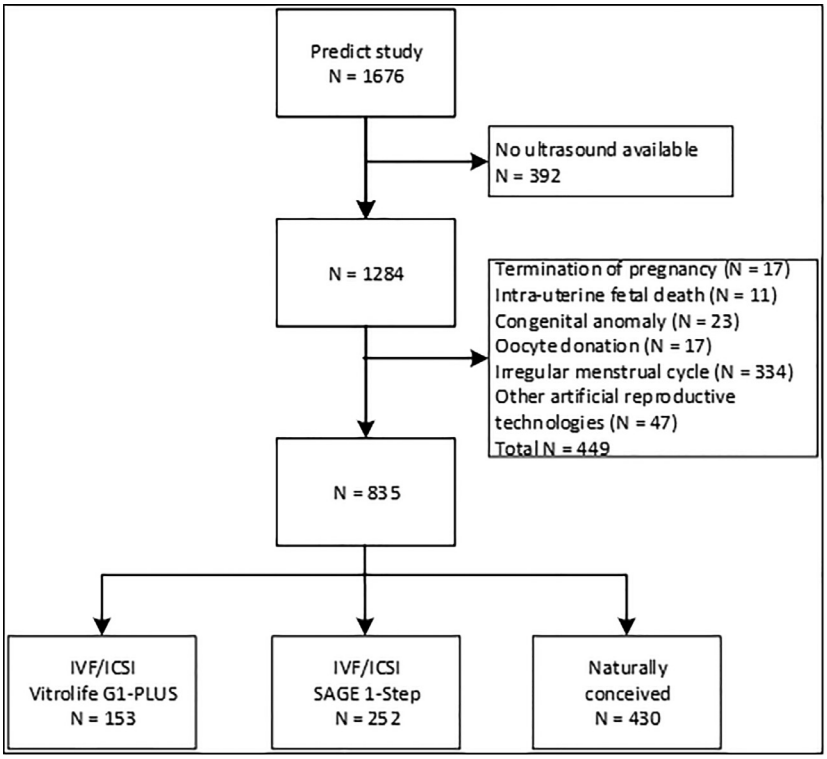

FIGURE 1 Flow chart of the study population.

embryo morphology assessment were performed as described previously (*Heijnen et al., 2007*; *Hohmann et al., 2003*). During the period from 1 January 2010 to 17 November 2014, retrieved oocytes were submitted to routine IVF or ICSI procedures and cultured in G-IVF™ PLUS (G5series, Vitrolife, Goteborg, Sweden) overnight for fertilization. After fertilization, embryos were transferred to G-1™ PLUS cleavage stage medium (Vitrolife). On day 3, surplus embryos were transferred to a fresh dish containing G-2™ PLUS medium for further culture to day 4. From 17 November 2014 until the end of the study period, SAGE 1-Step™ medium (Origio/Cooper Surgical, Trumbull, CT, USA) was used for culture from day 0 until day 4. During the study period, embryo transfer was performed on day 3 after fertilization and supernumerary embryos of adequate quality, based on number of blastomeres and amount of compaction, were cryopreserved on day 4 by slow freezing (*Saragusty and Arav, 2011*). During the study period, it was routine care in most IVF clinics, including the study clinic, to perform fresh transfer of cleavage embryos. No other modifications to the procedures than the change in culture medium were made during the study period.

Study parameters

At enrolment, standardized anthropometric measurements were performed. Women filled out questionnaires regarding general characteristics and periconceptional nutrition and lifestyle. Geographic origin was categorized into Western or non-Western and educational level was categorized according to the definition of Statistics Netherlands (CBS) (https://www.cbs.nl/en-gb/news/2018/20/well-being-not-distributed-equally/education-level). For pregnancies conceived after IVF/ICSI, gestational age was based on the conception date, which is the oocyte retrieval date minus 14 days for pregnancies after fresh embryo transfer and the embryo transfer date minus 19 days for pregnancies after frozen–thawed embryo transfer. For naturally conceived pregnancies, gestational age was based on the last reported menstrual period.

First-trimester growth was expressed by serial CRL and embryonic volume measurements, and morphological development was assessed by Carnegie stages, based on internal and external characteristics (*O'Rahilly and Muller, 2010*). These measurements provide more detailed information on growth and development than the CRL only, which is a length measurement performed in one single plane. For the volume and morphological measurements all three dimensions, including depth of the embryo, are taken into account, which may be more sensitive parameters for embryonic growth (*Rousian et al., 2010*). Second-trimester estimated fetal weight, head circumference, biparietal diameter, abdominal circumference and femur length were retrieved from the second-trimester anomaly scan. Birth outcomes such as sex, gestational age, weight at birth and pregnancy complications were retrieved from medical records. Birthweight <10th percentile was classified as small for gestational age (SGA) and birthweight >90th percentile was classified as large for gestational age (LGA). PTB was defined as delivery <37+0 weeks of gestational age.

Ultrasound data

Ultrasound examinations were performed in gestational weeks 7, 9 and 11 by trained sonographers with a 6–12 MHz transvaginal probe using GE Voluson™ E8 equipment and 4D View software (General Electric Medical Systems, Chicago, IL, USA). Afterwards, three-dimensional ultrasound data were transferred to the Barco I-Space (a CAVE [Cave Automatic Virtual Environment]-like virtual reality system) to optimally

TABLE 1 BASELINE CHARACTERISTICS OF THE STUDY POPULATION, STRATIFIED FOR CULTURE MEDIUM USED DURING IVF/ICSI TREATMENT AND NATURALLY CONCEIVED PREGNANCIES

Characteristic	IVF/ICSI Vitrolife G-1 PLUS *n* = 153	IVF/ICSI SAGE 1-Step *n* = 252	Naturally conceived pregnancies *n* = 430	*P*-value	Missing
Age, years	32.8 (30.1–35.9)	33.0 (29.8–36.2)	31.9 (29.0–35.1)	0.002[a,b]	
BMI, kg/m^2	24.2 (21.7–27.7)	24.4 (21.7–27.8)	24.4 (22.2–28.5)	0.183	36
Nulliparous	92 (60.1)	185 (73.4)	156 (36.3)	<0.001[a,b,c]	
Western geographic origin	125 (81.7)	207 (82.1)	360 (83.7)	0.794	
Level of education				0.055	20
Low	8 (5.4)	19 (7.9)	34 (8.0)		
Middle	62 (41.6)	91 (37.6)	126 (29.7)		
High	79 (53.0)	132 (54.5)	264 (62.3)		
Alcohol, yes[d]	31 (20.5)	57 (22.9)	163 (38.3)	<0.001[a,b]	9
Smoking, yes[d]	15 (9.9)	34 (13.7)	73 (17.1)	0.084	9
Folic acid supplements, yes[e]	145 (96.7)	240 (96.4)	302 (70.7)	<0.001[a,b]	9
Embryo transfer, frozen–thawed	46 (30.1)	83 (32.9)	N/A	0.548	

All data are given as *n* (%) or mean (IQR). *P*-values <0.05 indicate statistical significance.

[a] Significantly different between pregnancies after culture in Vitrolife G-1 PLUS and naturally conceived pregnancies after pairwise comparisons.

[b] Significantly different between pregnancies after culture in SAGE 1-Step and naturally conceived pregnancies after pairwise comparisons.

[c] Significantly different between pregnancies after culture in Vitrolife G-1 PLUS and in SAGE 1-Step after pairwise comparisons.

[d] Any use during the 14 weeks prior to 10 weeks after conception.

[e] Daily use during the 14 weeks prior to 10 weeks after conception.

BMI = body mass index; ICSI = intracytoplasmic sperm injection; IQR = interquartile range; N/A = not applicable; IVF = in vitro fertilisation.

use the third dimension (*Rousian et al., 2011*). By using the V-scope volume rendering software, an interactive virtual reality hologram was created, allowing true depth perception. The technique, accuracy and reliability of CRL and embryonic volume measurements and Carnegie classification have been previously described (*O'Rahilly and Muller, 2010*; *Rousian et al., 2010*; *Verwoerd-Dikkeboom et al., 2008*).

Statistical analyses

The study population was stratified in three groups of pregnancies: (i) after IVF/ICSI treatment with G-1 PLUS cleavage stage medium; (ii) after IVF/ICSI treatment with SAGE 1-Step medium and (iii) naturally conceived. Baseline characteristics were compared by Kruskal–Wallis tests for continuous variables (because not all variables were parametrically distributed) and by chi-squared tests for categorical variables. If these tests yielded significant results, post-hoc tests for pairwise comparisons, i.e. chi-squared tests and Dunn–Bonferroni tests, were performed so that the underlying significant difference could be identified.

The associations between culture media and first-trimester growth and morphological development were assessed by linear mixed models. Prior to analysis, CRL was root-transformed and embryonic volume was cube root-transformed to approximate linearity. Analyses were performed using Vitrolife G-1 PLUS as reference category. Model 1 adjusted for gestational age only. Model 2 was additionally adjusted for maternal age, body mass index (BMI), parity, geographical background, level of education and periconceptional use of alcohol, cigarettes and folic acid supplements. Additionally, these analyses were performed using naturally conceived pregnancies as reference category. To create graphs of embryonic growth trajectories, the embryonic volume was estimated per week of gestational age, based on model 1, and retransformed to the original values by cubing.

Percentiles of estimated fetal weight, head circumference, biparietal diameter, abdominal circumference and femur length were calculated from population-based fetal growth charts (*Gaillard et al., 2011*; *Papageorghiou et al., 2014*). Differences were studied using Kruskal–Wallis tests. Birthweight was expressed in percentiles based on Dutch reference curves, which take gestational age at birth and fetal sex into account (*Hoftiezer et al., 2016*). Differences in birthweight percentiles, birthweight and gestational age at delivery were analysed by the Kruskal–Wallis test as well and differences in prevalence of PTB, SGA and LGA by chi-squared test. Sub-analyses were performed for each fetal sex separately for first- and second-trimester growth and development and for type of embryo transfer, i.e. fresh or frozen–thawed. *P*-values ≤0.05 were considered significant. All analyses were performed using SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Baseline characteristics

The study population comprised 153 pregnancies after IVF/ICSI treatment with Vitrolife G-1 PLUS culture medium and 252 after culture in SAGE 1-Step and 430 naturally conceived pregnancies (FIGURE 1). A total of 42 women participated twice, of which six patients participated with both a naturally conceived and an IVF/ICSI pregnancy, and three with pregnancies after IVF/ICSI treatment with Vitrolife G-1 PLUS culture medium as well as after treatment with SAGE 1-Step medium. Women pregnant after IVF/ICSI treatment with Vitrolife G-1 PLUS culture medium were less often nulliparous than those after IVF/ICSI treatment with SAGE 1-Step culture medium (60.1% and 73.4%, $P < 0.001$) (TABLE 1). The other characteristics were comparable between these two groups.

TABLE 2 FIRST-TRIMESTER EMBRYONIC GROWTH AND MORPHOLOGICAL DEVELOPMENT AFTER CULTURE IN SAGE 1-STEP, RELATIVE TO CULTURE IN VITROLIFE G-1 PLUS, IN THE TOTAL POPULATION AND STRATIFIED FOR FETAL SEX

Population	Parameter	Model 1			Model 2		
		Vitrolife G-1 PLUS	SAGE 1-Step		Vitrolife G-1 PLUS	SAGE 1-Step	
			Beta (95% CI)	*P*-value		Beta (95% CI)	*P*-value
Total population *n* = 405	CRL, √mm	Reference	0.017 (–0.022, 0.056)	0.339	Reference	0.026 (–0.015, 0.067)	0.213
	EV, $\sqrt[3]{}$ml	Reference	0.025 (0.004, 0.045)	0.017	Reference	0.030 (0.008, 0.052)	0.007
	Carnegie stage	Reference	0.035 (–0.132, 0.202)	0.682	Reference	0.099 (–0.082, 0.280)	0.283
Females *n* = 194	CRL, √mm	Reference	0.022 (–0.044, 0.088)	0.508	Reference	0.038 (–0.037, 0.114)	0.313
	EV, $\sqrt[3]{}$ml	Reference	0.010 (–0.023, 0.042)	0.554	Reference	0.009 (–0.026, 0.046)	0.596
	Carnegie stage	Reference	0.184 (–0.099, 0.467)	0.119	Reference	0.340 (–0.012, 0.692)	0.058
Males *n* = 199	CRL, √mm	Reference	0.038 (–0.016, 0.092)	0.167	Reference	0.049 (–0.012, 0.109)	0.113
	EV, $\sqrt[3]{}$ml	Reference	0.044 (0.014, 0.074)	0.004	Reference	0.048 (0.015, 0.081)	0.005
	Carnegie stage	Reference	0.281 (–0.156, 0.307)	0.520	Reference	0.083 (–0.172, 0.338)	0.907

P-values <0.05 indicate statistical significance.

Model 1: Adjusted for gestational age.

Model 2: Adjusted for gestational age, maternal age, BMI, parity, geographical background, level of education, periconceptional use of alcohol, cigarettes and folic acid supplements. Fetal sex was unknown in 12 pregnancies.

BMI = body mass index; CRL = crown rump length; EV = embryonic volume.

Comparison of post-implantation growth and development between the two culture media

Embryos cultured in SAGE 1-Step grew significantly faster regarding embryonic volume than embryos cultured in Vitrolife G-1 PLUS in both models (model 1: beta 0.025 [95% CI 0.004–0.045], *P* = 0.017; model 2: beta 0.030 [95% CI 0.008–0.052], *P* = 0.007; TABLE 2). Interestingly, after stratification, this was only observed in embryos after fresh embryo transfer (model 1: beta 0.036 [95% CI 0.010–0.062], *P* = 0.007; model 2: beta 0.031 [95% CI 0.004–0.059], *P* = 0.024; Supplementary Table 2). Furthermore, growth patterns of embryos after IVF and embryos after ICSI were comparable (Supplementary Figure 1). After stratification for fetal sex, this finding was only observed in male embryos (model 1: beta 0.044 [95% CI 0.014–0.074], *P* = 0.004; model 2: beta 0.048 [95% CI 0.015–0.081], *P* = 0.005). No significant differences were observed for CRL and the Carnegie stages in the total population as well as after stratification for fetal sex.

In the total study population, percentiles of estimated fetal weight, head circumference, biparietal diameter, abdominal circumference and femur length were comparable between the two culture media (TABLE 3). After stratification for fetal sex, similar results were observed for both female and male fetuses.

Absolute and relative birthweight, gestational age at birth and fetal sex ratio were comparable in pregnancies after culture in Vitrolife G-1 PLUS and culture in SAGE 1-Step (TABLE 4). Furthermore, the prevalence of PTB, SGA and LGA in pregnancies after culture in either Vitrolife G-1 PLUS or SAGE 1-Step was comparable. Finally, rate of pregnancy-induced hypertension, pre-eclampsia and gestational diabetes mellitus were comparable between both media.

Comparison of post-implantation growth and development between the two culture media and naturally conceived pregnancies

Embryos cultured in SAGE 1-Step showed faster embryonic growth than naturally conceived pregnancies, depicted by CRL, embryonic volume and morphological development in model 1 ($beta_{CRL}$ 0.053 [95% CI 0.005–0.101], *P* = 0.031; $beta_{EV}$ 0.043 [95% CI 0.018–0.068], *P* = 0.001; $beta_{Carnegie}$ 0.214 [95% CI 0.049–0.379], *P* = 0.011, respectively) (TABLE 5). In model 2, this was only significant for embryonic volume (beta 0.040 [95% CI 0.012–0.069], *P* = 0.005).

After stratification, it was observed that female embryos cultured in SAGE 1-Step medium showed faster morphological development than naturally conceived female embryos in model 2 (beta 0.292 [95% CI 0.001–0.584], *P* = 0.049), but this was not reflected in embryonic growth. Male embryos cultured in SAGE 1-Step medium grew and developed morphologically faster compared with naturally conceived embryos ($beta_{CRL}$ 0.083 [95% CI 0.011–0.155], *P* = 0.024; $beta_{EV}$ 0.078 [95% CI 0.041–0.115], *P* < 0.001; $beta_{Carnegie}$ 0.272 [95% CI 0.017–0.526], *P* = 0.036) according to model 1, with similar effects for embryonic volume in model 2 ($beta_{EV}$ 0.078 [95% CI 0.035–0.120], *P* < 0.001). Male embryos cultured in Vitrolife G-1 PLUS also showed faster embryonic growth, depicted by embryonic volume and morphological development, than those naturally conceived ($beta_{EV}$ 0.047 [95% CI 0.006–0.088], *P* = 0.025; $beta_{Carnegie}$ 0.284 [95% CI 0.018–0.551], *P* = 0.036) according to model 1, with similar effects for embryonic volume in model 2 ($beta_{EV}$ 0.049 [95% CI 0.002–0.095], *P* = 0.040).

After retransformation, the embryonic volume of embryos cultured in SAGE 1-Step was on average 0.52 ml higher (6.7%) at 11 weeks of gestational age than naturally conceived embryos, whereas for embryos cultured in Vitrolife G-1 PLUS this was 0.25 ml (3.2%) (FIGURE 2). In female embryos these estimates were smaller for SAGE 1-Step: 0.14 ml (1.8%) and there was no difference for Vitrolife G-1 PLUS: 0.00 ml (0.0%), whereas for males these were larger (SAGE 1-Step: 0.92 ml [12.6%]; Vitrolife G-1 PLUS: 0.53 ml [7.3%]).

TABLE 3 PERCENTILES OF SECOND-TRIMESTER FETAL GROWTH PARAMETERS OF PREGNANCIES AFTER IVF/ICSI WITH VITROLIFE G-1 PLUS OR SAGE 1-STEP AND NATURALLY CONCEIVED PREGNANCIES, IN THE TOTAL POPULATION AND STRATIFIED FOR FETAL SEX

Population	Parameter	IVF/ICSI Vitrolife G-1 PLUS *n* = 153	IVF/ICSI SAGE 1-Step *n* = 252	Naturally conceived pregnancies *n* = 430	*P*-value	Missing
Total population *n* = 835	EFW	69.0 (44.6–85.9)	71.6 (49.2–89.0)	68.2 (42.9–87.4)	0.270	65
	HC	58.7 (36.4–77.5)	58.5 (37.6–76.8)	58.1 (32.7–76.1)	0.734	63
	BPD	52.1 (28.1–75.6)	48.6 (23.0–74.9)	44.8 (18.7–70.4)	0.072	75
	AC	78.4 (56.5–90.1)	78.5 (58.8–92.7)	77.1 (57.1–91.0)	0.632	64
	FL	61.1 (39.3–81.3)	69.5 (45.7–82.5)	63.1 (39.1–82.8)	0.357	64
Females *n* = 405	EFW	60.3 (37.1–80.5)	62.5 (44.9–84.1)	64.1 (40.5–85.2)	0.693	30
	HC	48.1 (32.0–67.3)	45.8 (26.5–63.3)	50.5 (26.6–71.0)	0.418	30
	BPD	37.1 (24.9–64.5)	34.3 (13.4–57.8)	35.6 (12.4–61.8)	0.210	35
	AC	68.7 (47.7–83.9)	70.7 (50.0–88.2)	72.2 (52.3–88.5)	0.615	30
	FL	56.9 (38.6–78.6)	65.5 (43.1–83.1)	63.1 (39.8–83.1)	0.526	30
Males *n* = 410	EFW	76.8 (51.9–90.8)	83.4 (61.7–92.2)	71.1 (44.6–89.9)	0.033[a]	29
	HC	66.7 (48.5–81.0)	72.0 (57.1–84.7)	64.6 (40.7–81.6)	0.047[a]	27
	BPD	63.5 (37.7–85.0)	65.4 (37.9–87.2)	56.6 (28.6–79.0)	0.014[a]	32
	AC	83.4 (63.4–93.3)	86.7 (68.7–95.0)	79.7 (60.4–92.3)	0.114	28
	FL	64.0 (45.2–87.5)	69.6 (46.1–81.7)	61.3 (38.2–82.6)	0.322	28

Data are presented as median (IQR). *P*-values <0.05 indicate statistical significance.

EFW was calculated using the Hadlock formula: log(EFW) = 1.326 − 0.00326 × AC × FL + 0.0107 × HC + 0.0438 × AC + 0.158 × FL. Fetal sex was unknown in 20 pregnancies.

[a] Significantly different between pregnancies after culture in SAGE 1-Step and naturally conceived pregnancies after pairwise comparisons.

AC = abdominal circumference; BPD = biparietal diameter; EFW = estimated fetal weight; FL = femur length; HC = head circumference; ICSI = intracytoplasmic sperm injection; IQR = interquartile range; IVF = in vitro fertilisation.

The second-trimester growth parameters were not significantly different between pregnancies after culture in Vitrolife G-1 PLUS or SAGE 1-Step and naturally conceived pregnancies. After stratification for fetal sex, it was observed that male fetuses cultured in SAGE 1-Step had a higher estimated fetal weight (83.4 and 71.1, respectively, $P = 0.033$), head circumference (72.0 and 64.6, respectively, $P = 0.047$) and biparietal diameter (65.4 and 56.6, respectively, $P = 0.014$) than those naturally conceived (TABLE 3). Other growth parameters were comparable.

Gestational age was shorter in naturally conceived pregnancies than pregnancies after IVF/ICSI treatment with culture in either Vitrolife G-1 PLUS or SAGE 1-Step (272, 276 and 276 days, respectively, $P = 0.001$). No differences were observed in other birth outcomes or

TABLE 4 PERINATAL OUTCOMES STRATIFIED FOR CULTURE MEDIUM USED DURING IVF/ICSI TREATMENT AND NATURALLY CONCEIVED PREGNANCIES

Outcome	IVF/ICSI Vitrolife G-1 PLUS *n* = 153	IVF/ICSI SAGE 1-Step *n* = 252	Naturally conceived pregnancies *n* = 430	*P*-value	Missing
Fetal sex, male	77 (51.3)	122 (50.2)	211 (50.0)	0.961	20
Birthweight percentile	42 (19–74)	41 (20–70)	48 (23–76)	0.168	65
Birthweight, g	3350 (3046–3650)	3337 (2995–3675)	3350 (2963–3705)	0.913	40
Gestational age, days	276 (269–283)	276 (267–283)	272 (265–279)	0.001[a,b]	51
Small for gestational age	22 (15.0)	33 (15.7)	56 (13.6)	0.772	65
Large for gestational age	14 (9.5)	16 (7.6)	50 (12.1)	0.197	65
Preterm birth	8 (5.3)	23 (10.5)	42 (10.1)	0.176	50
Pregnancy-induced hypertension	7 (4.8)	12 (4.9)	39 (9.2)	0.052	21
Pre-eclampsia	3 (2.1)	9 (3.7)	21 (5.0)	0.253	21
Gestational diabetes mellitus	9 (6.2)	24 (9.8)	30 (7.1)	0.342	21

All data are given as *n* (%) or mean (interquartile range). *P*-values <0.05 indicate statistical significance.

[a] Significantly different between pregnancies after culture in Vitrolife G-1 PLUS and naturally conceived pregnancies.

[b] Significantly different between pregnancies after culture in SAGE 1-Step and naturally conceived pregnancies.

ICSI = intracytoplasmic sperm injection; IQR = interquartile range; IVF = in vitro fertilisation.

TABLE 5 FIRST-TRIMESTER EMBRYONIC GROWTH AND MORPHOLOGICAL DEVELOPMENT AFTER CULTURE IN VITROLIFE G-1 PLUS AND SAGE-1 STEP, COMPARED WITH NATURALLY CONCEIVED PREGNANCIES, IN THE TOTAL POPULATION AND STRATIFIED FOR FETAL SEX

Population	Parameter	Model 1					Model 2				
		Vitrolife G-1 PLUS		SAGE 1-Step		Naturally conceived pregnancies	Vitrolife G-1 PLUS		SAGE 1-Step		Naturally conceived pregnancies
		Beta (95% CI)	*P*-value	Beta (95% CI)	*P*-value		Beta (95% CI)	*P*-value	Beta (95% CI)	*P*-value	
Total population *n* = 835	CRL, √mm	0.035 (–0.021, 0.091)	0.216	0.053 (0.005, 0.101)	0.031	Reference	0.013 (–0.050, 0.076)	0.690	0.029 (–0.027, 0.084)	0.308	Reference
	EV, $\sqrt[3]{}$ml	0.021 (–0.008, 0.049)	0.153	0.043 (0.018, 0.068)	0.001	Reference	0.020 (–0.012, 0.052)	0.220	0.040 (0.012, 0.069)	0.005	Reference
	Carnegie stage	0.202 (0.019, 0.384)	0.030	0.214 (0.049, 0.379)	0.011	Reference	0.138 (–0.068, 0.344)	0.188	0.171 (–0.019, 0.360)	0.077	Reference
Females *n* = 405	CRL, √mm	0.008 (–0.075, 0.090)	0.855	0.026 (–0.046, 0.099)	0.473	Reference	–0.007 (–0.103, 0.089)	0.889	0.031 (–0.054, 0.131)	0.471	Reference
	EV, $\sqrt[3]{}$ml	0.000 (–0.041, 0.042)	0.987	0.012 (–0.025, 0.048)	0.525	Reference	0.009 (–0.039, 0.056)	0.725	0.021 (–0.022, 0.063)	0.342	Reference
	Carnegie stage	0.135 (–0.134, 0.405)	0.323	0.239 (–0.001, 0.479)	0.051	Reference	0.103 (–0.214, 0.419)	0.524	0.292 (0.001, 0.584)	0.049	Reference
Males *n* = 410	CRL, √mm	0.060 (–0.019, 0.140)	0.138	0.083 (0.011, 0.155)	0.024	Reference	0.039 (–0.053, 0.130)	0.407	0.058 (–0.025, 0.140)	0.170	Reference
	EV, $\sqrt[3]{}$ml	0.047 (0.006, 0.088)	0.025	0.078 (0.041, 0.115)	<0.001	Reference	0.049 (0.002, 0.095)	0.040	0.078 (0.035, 0.120)	<0.001	Reference
	Carnegie stage	0.284 (0.018, 0.551)	0.036	0.272 (0.017, 0.526)	0.036	Reference	0.295 (–0.004, 0.594)	0.053	0.229 (–0.055, 0.513)	0.114	Reference

P-values <0.05 indicate statistical significance.

Model 1: Adjusted for gestational age.

Model 2: Adjusted for gestational age, maternal age, BMI, parity, geographical background, level of education, periconceptional use of alcohol, cigarettes and folic acid supplements.

Fetal sex was unknown in 20 pregnancies.

BMI = body mass index; CRL = crown rump length; EV = embryonic volume.

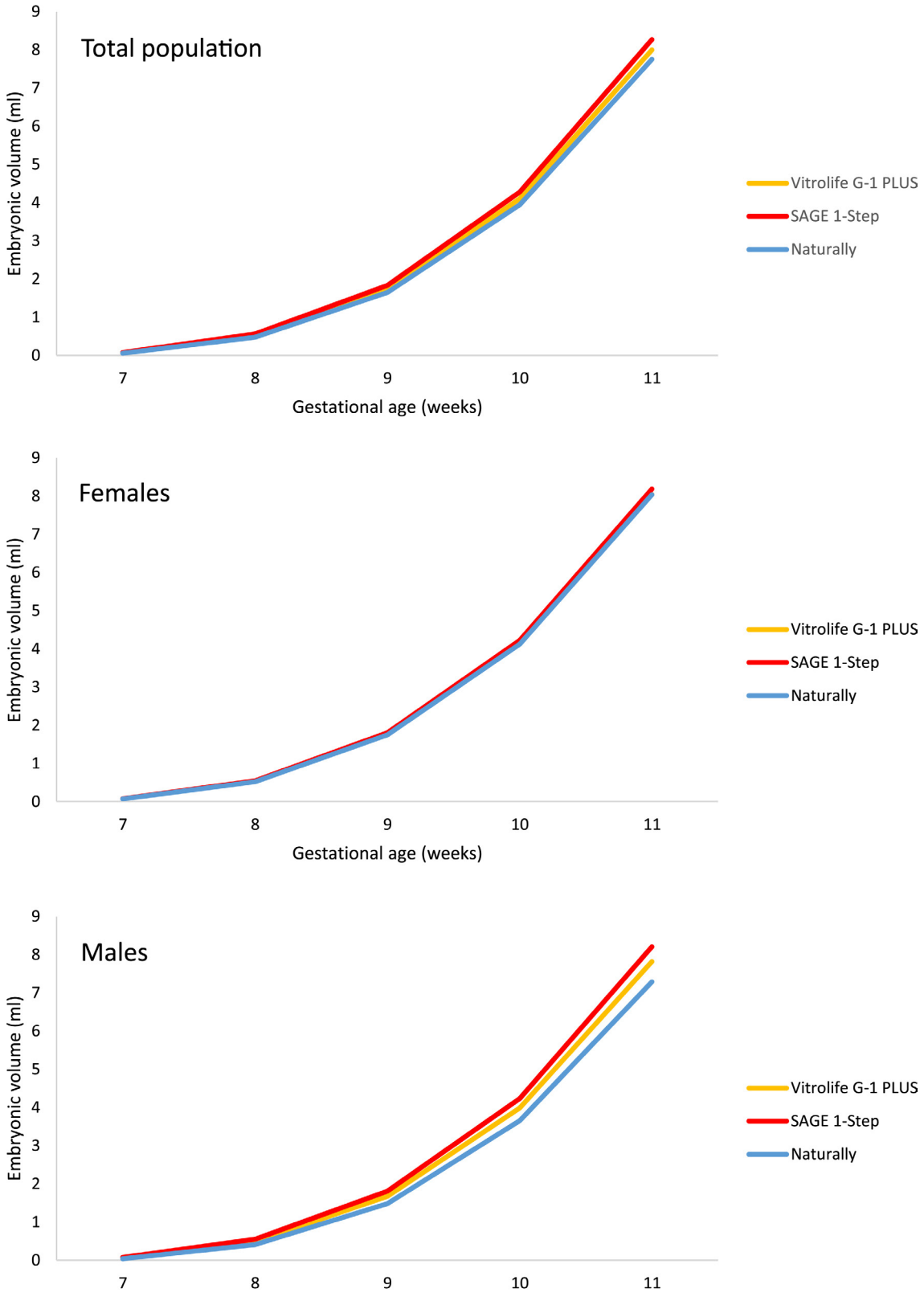

FIGURE 2 Trajectories of embryonic growth presented by embryonic volumes after culture in Vitrolife G-1 PLUS (orange, *n* = 153), SAGE 1-Step (red, *n* = 252) and naturally conceived pregnancies (blue, *n* = 430), in the total population and stratified for fetal sex. Boxes show the relative and absolute differences at 7 and 11 weeks of gestational age compared with naturally conceived pregnancies. To obtain the data for this graph, embryonic volume was estimated per week and retransformed to original values.

complications between the three groups (TABLE 4).

DISCUSSION

This study demonstrates a faster first-trimester embryonic growth after culture in SAGE 1-Step medium compared with culture in Vitrolife G-1 PLUS. A sex-specific effect was observed after culture in SAGE 1-Step medium, meaning a faster embryonic growth in male embryos. Regarding second-trimester fetal growth and perinatal outcomes, no significant differences were observed between the culture media in the total population and in males and females separately.

Finally, when embryos cultured in SAGE 1-Step were compared with naturally conceived pregnancies, the following were observed: (i) faster growth in male embryos, (ii) faster morphological development in female embryos, and (iii) a larger estimated fetal weight, head circumference and biparietal diameter in male fetuses when second-trimester growth parameters were studied.

The impact of culture medium on first-trimester growth has only been investigated in one other study. No differences were observed in first-trimester CRL between embryos cultured in Vitrolife G-1.3 PLUS and Cook K-SICM (*Nelissen et al., 2013*). However, the same study did show a difference in second-trimester biparietal diameter and head circumference between pregnancies after culture in Vitrolife G-1.3 PLUS and Cook K-SICM, which is also seen in the current study when SAGE 1-Step was compared with naturally conceived pregnancies. Yet when the current study method is compared with that of *Nelissen et al. (2013)*, there are fundamental differences. Besides the difference in studied culture media, they only used a single CRL measurement performed at 7–8 weeks of gestational age as a parameter for first-trimester growth. In a small subset an additional transabdominal CRL measurement was performed at 12 weeks of gestational age. They also applied other statistical methods. These differences might explain the different results between the two studies.

An explanation for the observed differences in first-trimester growth and morphological development of pregnancies after culture in different media is that during the first half of pregnancy, the primary determinant is the embryo's own (epi-)genetic blueprint (*Boron and Boulpaep, 2012*). During the second half of pregnancy, however, external factors (i.e. maternal, placental or environmental) have a stronger effect on fetal growth, which might mitigate or correct the effect of different culture media (*Gaillard et al., 2011*; *Mongelli and Gardosi, 1995*; *Osgerby et al., 2002*; *Rosenberg et al., 2005*). Interestingly, in another study performed by the current study group, preimplantation development was several hours faster in embryos cultured in SAGE 1-Step than in embryos cultured in Vitrolife G-1 PLUS (data not published). Although embryonic growth was already deviant during culture, this study indicates that growth diverged after implantation. Additionally, the differences in first-trimester embryonic growth were primarily observed in embryonic volume, but not in CRL. Although CRL measurements are the gold standard for embryonic growth and relatively easy to perform, embryonic volume measurements have added value. It has been described in structurally or chromosomally abnormal embryos that CRL was comparable to normal embryos while embryonic volume was smaller, indicating a higher sensitivity (*Rousian et al., 2018*).

It is possible that the embryonic epigenome is affected by different aspects of IVF/ICSI treatment (*Bergsjo et al., 2007*; *Kleijkers et al., 2015*). One of these aspects is the possibility of cryopreserving surplus embryos and transferring them in a later cycle. However, pregnancies after frozen–thawed embryo transfer are associated with increased post-implantation growth (*Maheshwari et al., 2012*). Therefore, in this study stratified analyses were performed in pregnancies after fresh embryo transfer and after frozen–thawed embryo transfer. Although the proportion of pregnancies after frozen–thawed embryo transfer was smaller in both media (Vitrolife 30.1% and SAGE 32.9%), the results of these analyses indicate that the effect of culture medium is more pronounced in freshly transferred embryos. A possible explanation might be that the cryopreservation process moderates the impact of culture media.

Another aspect of IVF/ICSI treatment is the diversity in culture media and its ingredients. Culture media vary notably in concentrations of energy substrates and nutrients (*Morbeck et al., 2014*, *2017*). For example, methionine, an essential amino acid, is present in SAGE 1-Step but not in Vitrolife G-1 PLUS. Methionine is one of the methyl donors in one-carbon metabolism, which is essential for molecular biological processes involved in growth and morphological development, i.e. cell replication, differentiation, apoptosis and epigenetic programming (*Steegers-Theunissen et al., 2013*; *Xu and Sinclair, 2015*). Furthermore, the glucose concentration in Vitrolife G-1 PLUS is approximately 2.5-fold higher than in SAGE 1-Step (*Morbeck et al., 2014*, *2017*). Yet, preimplantation embryos have a limited capacity to utilize glucose prior to compaction and high concentrations of glucose might even be detrimental for early development (*Conaghan et al., 1993*). Little is known about the effects of high concentrations of glucose during in-vitro and post-implantation development in humans. Several reports show that prenatal maternal hyperglycaemia acts as a primary teratogen (*Eriksson et al., 2003*). Additionally, extensive research has demonstrated that women with poorly regulated diabetes have an increased risk of severe pregnancy complications, such as miscarriages, congenital malformations and LGA babies (*Miller et al., 1981*; *Tennant et al., 2014*). Interestingly, the increased risk for congenital malformations appears to be restricted to male infants (*Evers et al., 2009*).

The current study also found the impact of culture medium to be more pronounced in male embryos, suggesting a sex specificity in the response or susceptibility to environmental stressors. It is likely that differences in chromosomal content between the sexes, i.e. X/Y chromosomes, contribute to the observed discrepancies between male and female embryos in the associations between culture medium and first-trimester growth trajectories. Sex chromosome-encoded genes are expressed at different levels, because Y-linked genes are exclusively expressed in males and X-linked genes have a higher expression in females during early development (*Bermejo-Alvarez et al., 2010*; *Fiddler et al., 1995*). Differences in the expression of these genes can lead to sex-related differences directly by interfering in molecular pathways and indirectly through the regulation of

autosomal genes, thereby affecting the susceptibility to environmental stressors such as culture media (*Bermejo-Alvarez et al., 2011*). This is supported by mouse studies showing that even optimized IVF conditions can reprogramme post-implantation growth, fat deposition and glucose homeostasis in a sexually dimorphic fashion (*Donjacour et al., 2014*; *Feuer et al., 2014*).

Second-trimester growth parameters and perinatal outcomes were largely comparable between culture media in this study. Several studies have investigated the impact on birthweight of culture medium used during IVF/ICSI treatment. A clear overview of these studies shows that only five out of eleven studies demonstrate significant differences in birthweight between culture media (*Zandstra et al., 2015*). These studies vary greatly in design, statistical methods and culture media used. An explanation for the fact that the current study did not find any differences in absolute and relative birthweight might be due to differences in these factors, e.g. culture medium and sample size. Moreover, it does not eliminate the possibility that specific culture medium components alter the susceptibility to developing diseases later in life. For example, the observed effects of culture medium on first-trimester growth and morphological development are comparable to those of smoking ($beta_{CRL}$ –0.055) (*Van Dijk et al., 2018*; *van Uitert et al., 2013*). The association between maternal prenatal smoking and susceptibility to diseases, even into adulthood, is indisputable and many efforts have been made to stop maternal preconceptional smoking (*Hylkema and Blacquiere, 2009*; *Power et al., 2010*). Although little is known about the associations between susceptibility to diseases in adulthood and IVF/ICSI treatment variables such as culture medium, it has been demonstrated that first-trimester growth is inversely associated with cardiovascular outcomes in childhood, independent of birthweight (*Jaddoe et al., 2014*).

To our knowledge, there are no studies that have compared post-implantation growth and development in pregnancies after culture in different media with post-implantation growth and development after natural conception, which complicates comparison and interpretation. However, numerous studies have demonstrated that pregnancies after IVF/ICSI treatment are at increased risk for several complications (*Pandey et al., 2012*). Because this study found an impact of culture medium on first-trimester growth, it might be that culture media may also play a role in the pathogenesis of pregnancy complications, as these often originate in the periconception period (*Steegers-Theunissen et al., 2013*).

The unique and extensive collection of longitudinal first-trimester three-dimensional ultrasound data using state-of-the-art imaging techniques enables precise and innovative measurements that provide sufficient power to accurately model growth. By taking both growth and morphological development into account it was possible to meticulously investigate the impact of the different culture media. Moreover, because data on second-trimester and pregnancy outcomes were also included, the impact of culture medium across the complete course of pregnancy could be studied.

However, the two culture media studied were used during consecutive periods. Although there were no changes in laboratory variables such as oxygen concentration and incubators, a possible bias cannot be excluded. Furthermore, because the participants were recruited from a tertiary university hospital, the external validity of this explorative study is limited. To minimize the effects of potential confounders, adjusted analyses were performed. Also, patients were allowed to participate more than once in the current study. However, this is only a small proportion of the study population, and sensitivity analyses, in which second-time participation was excluded, demonstrated similar results (data not shown). Finally, residual confounding, due to unmeasured conditions, cannot be excluded, as the study has an observational cohort design.

In conclusion, this study shows that culture media significantly impact post-implantation embryonic growth trajectories and morphological development, which are associated with health and the development of non-communicable diseases later in life. Therefore, it is of utmost importance to optimize this environment (*Heindel et al., 2015*). Moreover, differences in childhood phenotype between culture media have already been observed (*Zandstra et al., 2018*). So, as a final statement, it is recommended that culture media manufacturers extend their focus towards offspring health when optimizing culture media, as 2–4% of children born today are the result of IVF/ICSI treatment (European IVF Monitoring Consortium *et al.*, 2022).

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SUPPLEMENTARY MATERIALS

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