The periconception environment of the human embryo and the impact on reproductive and pregnancy outcomes

With emphasis on the impact of maternal obesity and *in vitro* fertilisation treatment

Linette van Duijn

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The periconception environment of the human embryo and the impact on reproductive and pregnancy outcomes
With emphasis on maternal obesity and in vitro fertilisation treatment

De periconceptionele omgeving van het menselijk embryo en de impact op reproductieve en zwangerschapsuitkomsten
Met een focus op maternale obesitas en in vitro fertilisatie behandeling

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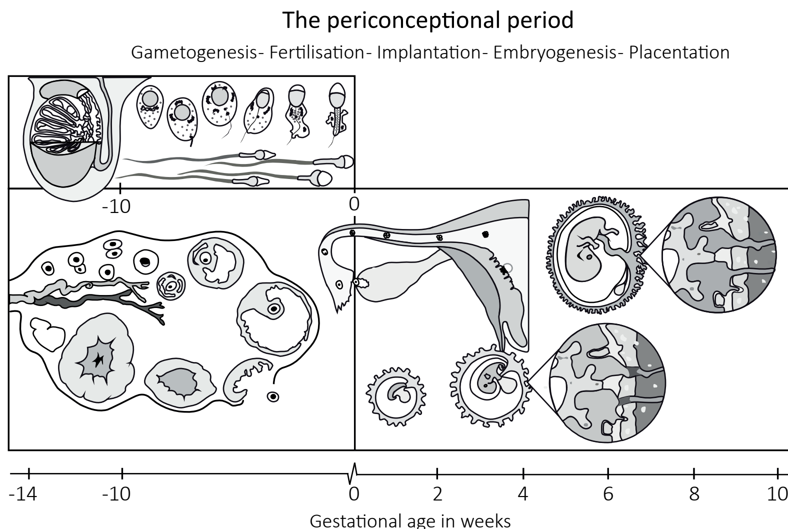
1

General introduction and aims

Developmental Origins of Health and Disease

A series of studies by professor David Barker published in the early 1990s, demonstrating associations between birthweight, early life mortality and adult death from ischemic heart disease, laid the foundation for the Barker hypothesis, now known as the paradigm of the Developmental Origins of Health and Disease (DOHaD) (1-5). The main concept of this paradigm is that, in order to increase extra-uterine survival, the developing fetus adapts to the intra-uterine environment. This may result in altered growth, structure, and function of several tissues. However, as a result of a mismatch between intra-uterine and extra-uterine conditions, these prenatal adaptations can induce an increased risk for postnatal adverse health outcomes, such as adiposity. Mechanisms of epigenetic programming, which are heritable and acquire changes in the genetic material that do not affect the underlying DNA sequence, have been proposed to underlie embryonic and fetal programming (6). Initially, studies involving the DOHaD paradigm focused on environmental factors during the second half of pregnancy and birth outcomes. As evidence emerged that also environmental factors during the first half of pregnancy and even during the preconception period can be crucial for offspring health, the DOHaD society recognised the importance of the periconception period in 2016. As standard obstetric care generally starts around 10 weeks of gestation, a significant part of this period has unfortunately already passed, together with the opportunity to optimise health.

Figure 1. An overview of developmental processes in the periconception period (7).



From Steegers-Theunissen et al. The periconception period, reproduction and long-term health of offspring: The importance of the one-carbon metabolism. Hum Reprod Update, Volume 19, Issue 6. November/December 2013, Pages 640-655. Reprinted with permission from: Oxford University Press.

Developmental processes in the periconception period

The periconception period comprises 14 weeks prior to fertilisation up until 10 weeks thereafter and can therefore be divided into two periods; the preconception period and the postconception period (7). The preconception period lasts 14 weeks and starts with differentiation of pre-antral follicles (8). During this period, the male and female gametes and the female endometrium are prepared for fertilisation and subsequent implantation. During the postconception period, the zygote undergoes major alterations to facilitate implantation and embryonic development, whereas the endometrium interacts with the embryo to initiate placental development (**Figure 1**).

Gametogenesis

The first part of gametogenesis starts early in embryonic development, in the embryonic gonadal ridge. In female embryos, primordial germ cells (PGCs) undergo several rounds of mitosis and differentiate into oogonia. Next, meiosis I is initiated and the primary oocytes are enclosed by a layer of pre-granulosa cells. This leads to the formation of primordial follicles, which remain in this resting state until recruitment in postnatal reproductive life (9). Although over 7 million primordial follicles are formed during fetal life, only approximately 2 million are present at birth. Until menarche another 1.5 million of these primordial follicles are lost and at the time of the first menstrual period approximately 400.000 to 600.000 will remain in the ovaries. Every month approximately 1000 follicles will be recruited out of the primordial pool and only one will eventually be selected to become the dominant follicle. Hence, only 400-600 will reach the ovulatory stage during reproductive life (10). It takes approximately 85 days from primordial follicle activation to the pre-ovulatory stage (11). During the early follicular phase of the menstrual cycle, oocyte recruitment is initiated by follicle stimulating hormone (FSH). The rising oestrogen concentrations during the late follicular phase trigger the surge of luteinizing hormone (LH), which induces final maturation of the dominant follicle. During this surge, meiosis I is completed, resulting in a large haploid oocyte. Immediately thereafter, meiosis II is initiated, which is completed upon fertilisation (12, 13). This specific regulation of meiotic divisions allows the fertilised oocyte to maintain maximal cytoplasmic volume. This cytoplasm contains essential factors and structures for the first cleavage divisions, such as mitochondria, proteins and mRNA. In males, gametogenesis is resumed at puberty and continues until death. The complete process of spermatogenesis takes approximately 78-120 days. During this period, germ cells undergo major morphological and biomedical alterations, such as chromatin remodelling and formation of the acrosomal cap, to gain full motility and fertilising capacity (14). It is of utmost importance to optimise the *in vivo* environment in which these processes take place, as it can potentially affect future embryonic development.

Fertilisation and preimplantation embryo development

Successful fertilisation is the first important step in embryogenesis. After ovulation, the oocyte migrates through the female reproductive tract, where it reaches the sperm in the ampulla of the fallopian tube. After binding to the zona pellucida, the individual sperm releases its acrosome, which contains enzymes to promote penetration (15). After fusion of the oocyte and sperm membranes, the nucleus of the sperm enters the cytoplasm of the oocyte. This fusion triggers a cortical reaction in the oocyte, which prevents entry of other sperm by making the zona pellucida impermeable (16). Simultaneously, the oocyte completes meiosis II.

After fertilisation, the so-called zygote contains the haploid female and male pronuclei. Before the parental genetic material can be paired, the highly condensed chromatin of the sperm has to undergo major transformations. Protamines are replaced by maternal histones and the majority of the paternal genome is actively demethylated (17). Approximately 18-20 hours after entry of the sperm, the pronuclear membranes disintegrate and the maternal and paternal chromosomes align on the equatorial plate, after which the first cleavage division takes place (18). After 3 successful cleavage divisions, approximately 3 days after fertilisation, the embryo consists of 8 cells. From this moment onwards, the cell-to-cell adherence increases by the spreading of tight junctions. After several more cleavage divisions, the embryo consists of approximately 16-32 cells and appears as a lump of indistinguishable cells named a morula (19). In contrast to the paternal genome, the maternal genome passively demethylates over the course of these cleavage stages, with the exception of imprinted regions (20-23).

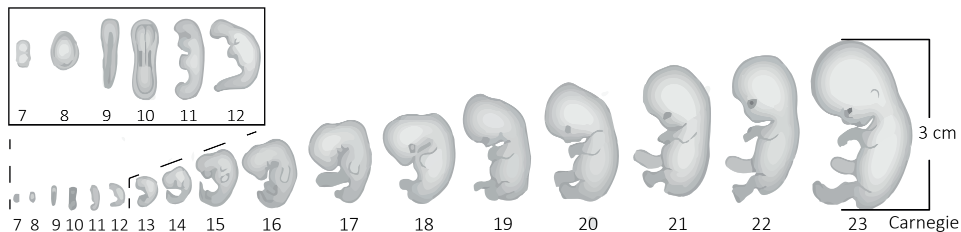
With the formation of a cavity within the morula at day 5 after fertilisation, the embryo enters the blastocyst stage (24). At this stage, two types of cells can be distinguished, the trophectoderm and the inner cell mass (ICM) (25). The ICM further develops into the epiblast and the hypoblast. The epiblast is the tissue layer that will form the future embryo, whereas the trophoblast and hypoblast will give rise to extra-embryonic tissues, such as the placenta and yolk sac (26). After reaching the uterine cavity, the hatching blastocyst adheres to the endometrial epithelium and the process of implantation is initiated.

Post-implantation embryo development

Embryonic growth and development after implantation can be divided into two periods. In the first period, lasting from embryonic day 7 until day 20 (3-5 weeks of gestation), the amniotic cavity and bilaminar embryonic disc, which consists of the epiblast and hypoblast, are formed (27). Next, the epiblast undergoes gastrulation. During gastrulation, cells of the epiblast differentiate into three germ layers, the ectoderm, mesoderm and endoderm, each of which will give rise to specific tissues, and the axes of the body (i.e. dorsal-ventral and anterior-posterior) are established (28, 29). The second period, lasting from embryonic

day 20 to approximately embryonic day 60 (5-10 weeks of gestation), primarily consists of organogenesis and growth. Organogenesis starts with the process of neurulation, during which the early nervous system is formed (30). Other essential structures that are formed include the circulatory system and somites, which will give rise to the vertebral column (31, 32). With the closure of the secondary palate at the 10th week of gestation, the embryonic period is finished. As the basis for all organs and structures is formed, this is also the period in which congenital malformations originate (33). Embryonic development is very well documented and classified by the Carnegie Institute in the early 1900s. The Carnegie classification system comprises the period from fertilisation up until the fetal period and is based on pathological research of internal and external morphological development, irrespective of age and size (34) (**Figure 2**).

Figure 2. Embryonic development, classified by the Carnegie stages (35).



From Bakker et al. An interactive three-dimensional digital atlas and quantitative database of human development. Science. 2016 Nov 25;354(6315). Reprinted with permission from AAAS.

Placental development

Placental development is initiated after successful hatching of the blastocyst onto the endometrium (36). The aforementioned trophoblast cells of the blastocyst interact with the surface epithelium to invade in the underlying endometrium, which transforms during pregnancy into specialised tissue called the decidua. Next, the trophoblast cells differentiate into two distinct layers: the inner mononucleated cytotrophoblast and outer multinucleated syncytiotrophoblast (37). In the syncytiotrophoblast lacunae appear, which enlarge and merge, resulting in a trabecular system. Meanwhile, on the maternal side, the syncytiotrophoblast erodes maternal tissue, allowing blood from the spiral arteries to flow into the lacular network (38). In the underlying cytotrophoblast layer, chorionic villi are formed and expand into the surrounding syncytiotrophoblast. Some of these villi will connect to the basal layer of the decidua, the so-called anchoring villi, to ensure a firm connection to the uterine wall, whereas the villi growing outward into the intervillous space will enlarge the surface area for maternal-fetal nutrient exchange (39). On the maternal side of the placenta, spiral arteries are invaded by extravillous trophoblast cells, which replace the vascular endothelium and smooth muscle cells (40). Moreover, invasion of extravillous trophoblast cells also causes temporary arterial plugs, which may protect the

developing embryo from excessive oxidative stress by decreasing maternal blood flow and gradually dissolve between week 10 and 11 of gestation (38, 41, 42). This process of vascular remodelling allows maternal blood to flow through the intervillous space with low pressure, so that gasses and nutrients can optimally be exchanged between mother and fetus. We and others hypothesise that suboptimal vascular remodelling plays an important role in the pathophysiology of placenta-related pregnancy complications such as pregnancy-induced hypertension and preeclampsia (43-45).

Epigenetic (re)programming during the periconception period

During fertilisation, the haploid oocyte and sperm fuse and their genetic information is combined, resulting in a diploid zygote. Since the 19th century, it has been recognised that inheritable information is transmitted from parents to offspring through genetic material inside the gametes (46). However, over the last century it has become evident that transgenerational transmission of inhereditary information is not fully explained by the DNA-sequence alone (47). This has led to the introduction of the term 'epigenetics', defined as the study of changes in the function of genes that are inheritable through mitosis and/or meiosis, that do not involve a modification of DNA sequencing and can be reversible (48). There are several mechanisms by which gene expression is regulated, including DNA methylation, histone modifications and through noncoding RNAs (49). Of these mechanisms, DNA methylation is the most widely studied. During mammalian reproduction, two major epigenetic reprogramming events take place. The first is during gametogenesis, when PGCs are demethylated genome-wide, and sex-specific methylation patterns, i.e. imprints, are established during the final stages of gametogenesis. The second event is during early embryo development. After fertilisation, the parental genomes undergo genome-wide demethylation followed by the establishment of unique *de novo* methylation patterns post-implantation (50). Despite these extensive reprogramming events, numerous epigenetic modifications resist these erasures and replacements. As a result, non-genomic inhereditary information can be transmitted to the offspring and affect its phenotype. Although these inherited epigenetic marks are relatively stable throughout life, environmental forces can result in adaptive epigenetic modifications. Through the gametes, these environmental forces have the potential to impact next generations.

Periconception environment: Obesity

An example of such an environmental force is the availability of food. Historically, the availability of food, i.e. energy, depended greatly on the environment and climate changes, and shortages of food and starvation were relatively common. The ability to accumulate excessive energy in times of abundance provided great evolutionary advantages, especially during times of famine. By storing excessive energy as fat, primary functions such as energy metabolism, immune function, and reproduction could be maintained. During this period, obesity was even considered as attractive; since it was associated with fertility and prosperity (51).

With the agricultural revolution, food became more abundant and obesity more common. However, adipose tissue is more than simply an energy depot; it secretes various cytokines, hormones and growth factors, and when its quantity exceeds physiological levels, numerous processes are chronically disrupted on immune, endocrine and vascular levels (52, 53). Already in the Roman era, obesity was recognised by Hippocrates as a disease of imbalance (54).

Over the past decades, the global prevalence of obesity has risen to epidemic proportions, i.e. 20%, thereby affecting millions of people across every age, sex, race and income level (55-57). It is believed that the increased availability to inexpensive calorie-dense food and decreased energy expenditure, e.g., sedentary lifestyle, have attributed significantly (58). The social consequences of this increased prevalence are immense. Obesity is associated with many non-communicable diseases such as diabetes mellitus type 2, hypertension and cancer (55, 57). As a result, obese people have a lower quality adjusted life expectancy, as morbidity and mortality are higher (59, 60).

However, the detrimental impact of obesity extends beyond the current generation (61). Since adiposity also affects women of reproductive age, it has the potential to impact growth, development and long-term health of the next generation. Although adipose tissue is important for physiological reproductive processes such as oogenesis, excessive levels can have adverse effects on reproduction (56). This is substantiated by several studies showing that pregnancies in women with an elevated body mass index (BMI) are more often complicated with a congenitally malformed fetus or preeclampsia (62). Furthermore, maternal nutrition during pregnancy, especially in the first-trimester, is not only associated with childhood obesity, but more importantly, also with increased risks of cardiovascular and metabolic morbidity and mortality in adulthood (63, 64).

Obesity has also been described to impact other crucial processes in the periconception period, such as gametogenesis, fertilisation and implantation. For example, elevated levels of adiposity are associated with increased oestrogen production, which inhibits secretion of gonadotrophins (65). As a result, overweight and obese women have a higher incidence of anovulation and disturbances in follicle maturation (66). The follicular microenvironment itself is also altered, i.e. higher levels of triglycerides, insulin, and lactate, which detrimentally affects oocyte function and embryo developmental potential (67, 68). This is further underlined by research demonstrating reduced conception rates in overweight and obese women who conceive naturally (69). Although the exact mechanisms are not yet fully understood, studies suggest that mitochondrial dysfunction, DNA damage and epigenetic programming, caused by increased oxidative stress and altered glucose and oestrogen levels, may play a role in the poorer reproductive performance of obese women (70-72).

Periconception environment: Assisted reproductive technologies

For most of history, involuntary childlessness was considered divine intervention. Throughout the ages, various remedies for infertility have been proposed; couples consulted an oracle or priest, used sexual organs of animals or underwent spa treatment, all to enhance their fertility (73). It is only since the Scientific Revolution that reproduction is acknowledged as a matter of biology. With the discovery of spermatozoa by Antoni van Leeuwenhoek in 1677, the first step towards assisted reproduction was made (74). Three centuries and numerous technological advancements later, different assisted reproductive technologies can be used to improve the chances of achieving a pregnancy. These modalities include ovulation induction to stimulate regular ovulation in women with ovulatory dysfunction, intrauterine insemination and *in vitro* fertilisation (IVF) with or without intracytoplasmic sperm injection (ICSI) to facilitate fertilisation of the oocyte by the sperm. The latter two modalities are relatively complicated, as fertilisation takes place *ex-vivo* and *in-vitro* which requires meticulous handling of the gametes. It is only a few decades since the first IVF-baby (1978) and ICSI-baby (1991) were born (75, 76).

During these treatments, the ovaries are hyperstimulated with exogenous FSH. To prevent premature ovulation, stimulation treatment is often preceded by a gonadotrophin releasing hormone (GnRH) agonist to induce downregulation of LH production or accompanied by a GnRH-antagonist to prevent a premature LH-surge (77). After sufficient follicle growth, oocyte maturation is triggered 35 hours prior to retrieval with human gonadotrophin (hCG), which resembles endogenous LH. Oocytes are retrieved through ultrasound-guided aspiration and transported to the laboratory where they are prepared for fertilisation by the sperm. Usually this is ejaculated sperm, either fresh or frozen, but also surgically retrieved testicular or epididymal sperm can be used, depending on the male factor underlying the subfertility. In IVF treatment, the oocyte and sperm are brought together in a petri dish to promote spontaneous fertilisation. In ICSI treatment, a single sperm cell is directly injected into the cytoplasm of the oocyte. After successful fertilisation and pre-implantation development, the best embryo is selected for transfer based on morphological criteria (78, 79). Since 1984, it is also possible to cryopreserve surplus embryos of adequate quality for an optional later transfer (80).

During IVF/ICSI, there are several alterations to the physiological processes in the periconception period. For example, the environment in which the preimplantation embryo is cultured is considerably different from the female reproductive tract. Moreover, the process of cryopreservation of embryos is unparalleled in the physiological periconception period. Even after transferring the embryo back into the physiological environment of the uterus, the consequences of *in-vitro* fertilisation can be noticed. Pregnancies after IVF/ICSI treatment with either fresh or frozen-thawed embryo transfer (ET) have increased risks for several complications, such as congenital malformations and preterm birth, when compared

to naturally conceived pregnancies (81, 82). However, the majority of studies investigating short-term health in IVF/ICSI offspring demonstrate reassuring results (83, 84). Yet, there are some studies showing that children born after IVF/ICSI treatment have a higher prevalence of precursors of impaired cardio-metabolic health, such as obesity and elevated blood pressure, than naturally conceived offspring (85). These observations could be worse in adult life and may ultimately result in chronic cardio-metabolic disease. It has been suggested that epigenetic reprogramming is one of the underlying mechanisms and that other factors such as chronic excessive oxidative stress and mitochondrial dysfunction are also involved (86). This has been supported by animal studies showing that IVF alters methylation and gene expression in blastocysts. When these alterations persist into adulthood, they can result in vascular and metabolic dysfunction and may even be transmitted to the next generation (87). Also in humans, a link between IVF/ICSI and epigenetic modifications has been suggested, as some epidemiological studies indicate a higher incidence of imprinting disorders in children conceived via IVF/ICSI (88, 89). As IVF and ICSI are relatively new and complicated treatments, it remains unclear during which processes the gametes and embryos are most vulnerable for these perturbations.

Hypothesis of this thesis

In summary, it is evident that a disruption of the biological environment in which periconceptional processes take place, can have a lifelong effect on offspring health (90, 91). For example, an abundance of adipose tissue, i.e. obesity, results in chronically elevated levels of oxidative stress and an altered follicular microenvironment. Also, during IVF treatment, either with or without ICSI, the gametes and embryos are exposed to additional *in vitro* stressors. From this background, we hypothesise that early embryonic environmental factors, with an emphasis on maternal obesity and features of assisted reproductive technologies, impair embryo growth and development during the preimplantation and post-implantation period, as well as the development of the placenta.

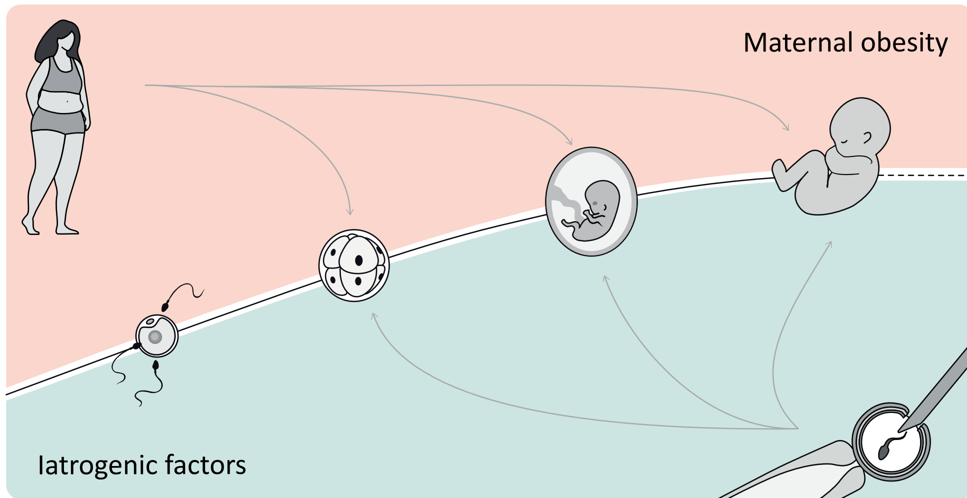
Aims of this thesis

The main aim of this thesis is to investigate periconceptional maternal obesity and features of artificial reproductive technology as exposures, in association with preimplantation and post-implantation embryo and placental outcome (**Figure 3**).

The main objectives of this thesis are to investigate:

1. The impact of maternal obesity on periconceptional outcomes, including preimplantation and post-implantation embryo growth and development (Part I).
2. Associations between features of *in vitro* fertilisation and preimplantation and post-implantation embryo growth and development and placental development (Part II).

Figure 3. Illustration of the objectives of this thesis.



Methodology

All studies described in this thesis were conducted at the Department of Obstetrics and Gynaecology of the Erasmus University MC, Rotterdam, the Netherlands (**Figure 4**).

Rotterdam Periconception Cohort

The Rotterdam Periconception Cohort (Predict study) is an ongoing prospective cohort study, conducted from 2009 onwards and embedded in the patient-care of the Erasmus University MC (92). This cohort study is designed as a life course approach of research and care and aims to identify risk, predictive and preventative factors for a healthy pregnancy in the critical periconception period. Women and their male partners are enrolled during this period, with follow-up until 12 months after delivery. Women of at least 18 years of age, less than 10 weeks pregnant with a singleton pregnancy and able to speak and read the Dutch language, were eligible for participation. Throughout participation, data on parental characteristics, general health, medical (obstetric) history, diet and lifestyle are collected through self-administered extensive questionnaires, which are verified by a researcher. Furthermore, women undergo 3D transvaginal ultrasound examinations at weeks 7, 9 and 11 of gestation, at which data is collected to perform precise embryonic and placental measurements offline.

Virtual Placenta study

Between January 2017 and March 2018, the Virtual Placenta study was performed as a sub-cohort of the Predict study (93). Similar inclusion and exclusion criteria as for the Predict study were applied and participating couples received the same questionnaires. As the Virtual Placenta study focused on imaging placental development, additional data

such as Doppler waveforms of the uterine artery, were collected at the regular study visits. Moreover, women underwent extra ultrasound examinations at 13, 22 and 32 weeks of gestation.

Virtual EmbryoScope study

The Virtual EmbryoScope study is embedded as a sub-cohort in the Predict study and conducted from 2017 onwards. In this study, couples are included prior to undergoing IVF treatment, with or without ICSI. After enrolment, participating couples fill out an adapted version of the questionnaire that Predict participants receive in the first trimester of pregnancy. Thereafter, women undergo ovarian stimulation and oocyte retrieval conform routine care. After fertilisation, the resulting zygotes are cultured in the EmbryoScope to obtain time-lapse information. Furthermore, both men and women have their blood drawn at the moment of fresh ET, to validate diet and lifestyle information. Follow-up information on clinical treatment outcomes is retrieved from medical records. Moreover, Virtual EmbryoScope participants with an ongoing singleton pregnancy after either fresh or frozen-thawed ET, are eligible to continue participation in the Predict study.

Figure 4. Overview of the studies and data used for this thesis.

Preconceptional	Conception	First trimester	Second trimester	Third trimester	Delivery	1-year postpartum
Virtual EmbryoScope		**Predict**				
Questionnaire	Timelapse Blood withdrawel	Questionnaire (3D) Ultrasounds blood withdrawal	Questionnaire		Questionnaire Cord blood	Questionnaire
		Virtual Placenta				
			(3D) Ultrasounds	(3D) Ultrasounds		

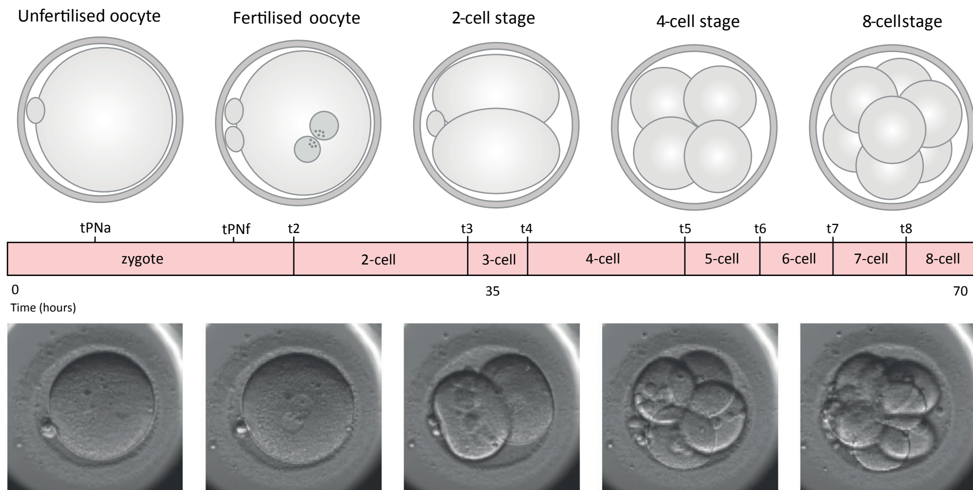
Visualisation techniques

To study associations between maternal and laboratory exposures and (preimplantation) embryo development, fetal development and placental development in detail, it is of utmost importance to adequately and precisely visualise this period.

Preimplantation embryo development

Preimplantation embryo development is traditionally studied by morphological evaluation at standardised time points, thereby disregarding the majority of development (78, 79). The introduction of time-lapse monitoring has allowed to continuously monitor preimplantation development and has provided the opportunity to improve embryo selection (94, 95). The EmbryoScope™ is an incubator with a specialised built-in microscope, which automatically records the embryo in seven focal planes every 10 minutes. Moreover, the EmbryoScope™ is a controlled environment for undisturbed embryo culture, as there is no need to handle or move the embryo for assessment (**Figure 5**).

Figure 5. Preimplantation embryo development until day 3 after fertilisation.

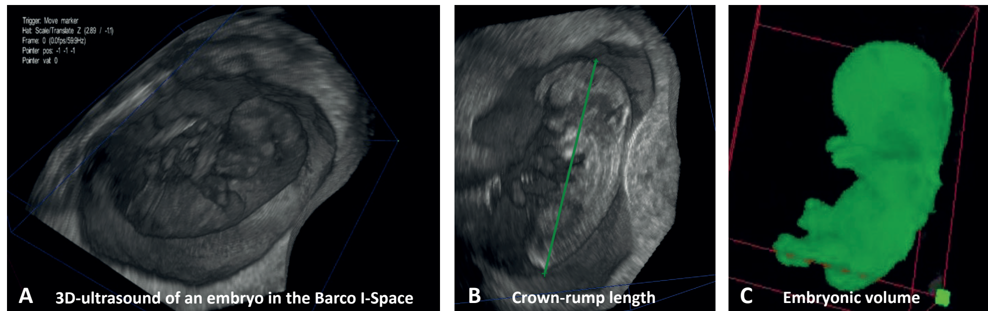


Post-implantation embryonic development

It is possible to study post-implantation embryonic development from 5 weeks of gestation onwards by using two-dimensional (2D) transvaginal ultrasound. Clinically, first-trimester ultrasound is applied as a diagnostic tool for pregnancy localisation and viability (96). By adding a third dimension (3D) to these images, visualisation improves and more structures can be measured. However, these 3D images are usually depicted on a 2D-screen, thereby not optimally using the depth that the third dimension provides. To better use this third dimension, images can be visualised using virtual reality (VR) techniques. An example of a VR system is the Barco I-Space. This system transforms the 3D-ultrasound dataset to an interactive VR hologram, which allows adequate depth perception and manipulation of the projected images (97, 98).

By projecting the first-trimester 3D-ultrasound images, gathered using high-resolution transvaginal ultrasound probes, into the Barco I-Space, besides measurements of simple structures like the crown-rump length (CRL), more complex structures such as the length of the limbs and umbilical cord can be measured more accurately (99, 100). The latter two examples are both measurements that can only be measured using real depth perception, as provided by VR. Moreover, the combination of first-trimester transvaginal 3D-ultrasound with VR techniques also provides the opportunity to measure the volume of (extra-) embryonic structures as well as to assess features of morphological development for the classification of the Carnegie stages (101, 102) (**Figure 6**).

Figure 6. 3D-ultrasound images of embryonic development in the Barco I-Space.



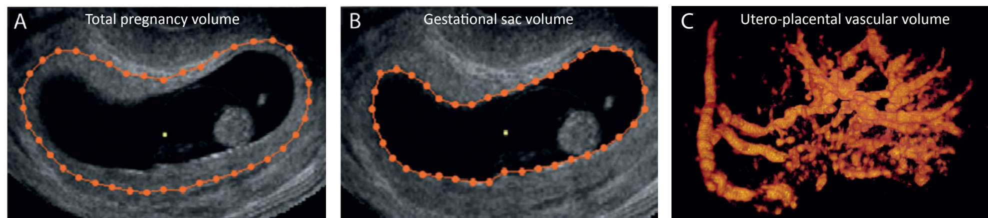
Legend: A) 3D ultrasound dataset projected in the I-Space. B) Measurement of the crown-rump length. C) Measurement of the embryonic volume.

Placental development

In clinical practice, placental function and development is measured during the second half of pregnancy, by studying fetal growth and (uterine) blood flow. In early pregnancy, placental development and functioning can also be studied using 3D ultrasound techniques for placental volume measurements (PV; including trophoblast and vasculature). These measurements can be performed offline with the rotational method of Virtual Organ Computer-aided AnaLysis (VOCAL) tool (103). The volume is rotated around its vertical axis in 12 steps of 15°. In each 2D-image, the total pregnancy and gestational sac is delineated, thereby creating volumes of these structures (**Figure 7a, 7b**). By subtracting the volume of the gestational sac from the total pregnancy volume, the placental volume can be determined.

The combination of 3D ultrasound with power Doppler (PD) allows visualisation of the vessels, or the so-called utero-placental vasculature volume (uPVV, including mainly maternal decidual vessels). When combined with a VR system, 3D-PD ultrasound images can be projected as a hologram, which allows precise measurements of the uPVV (93)(**Figure 7c**).

Figure 7. 3D-ultrasound images of placental development.



Legend: A) Delineation of the total pregnancy volume using VOCAL. B) Delineation of the gestational sac volume using VOCAL. C) Measurement of the utero-placental vasculature volume in the Barco I-Space using VR.

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Maternal obesity and embryo development

2

Maternal Body Mass Index and periconception growth and development: A systematic review

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Submitted.

ABSTRACT

Background: Over the past decades, the prevalence of obesity has increased worldwide, which has significant consequences for reproduction. Although most studies regarding obesity and reproduction focus on pregnancy outcome and complications, it is generally assumed that these complications originate in the relatively understudied periconception period. This study aims to provide an overview of the evidence on the impact of maternal body mass index (BMI) on oocyte quantity and maturity and preimplantation embryo quality during *in vitro* fertilisation treatment, first-trimester growth and development, and miscarriage.

Methods: A systematic search was conducted on Embase, Medline, PubMed, Web of Science, Cochrane and Google Scholar databases for relevant studies published up to January 2021. Eligible studies were selected after full-text assessment by three authors and consensus was reached through discussion. The ErasmusAGE tool was used to score methodological quality.

Results: The initial search identified 13,906 articles, of which 101 human and animal studies were retrieved for full-text assessment, resulting in 75 articles for analyses. Maternal BMI was negatively associated with the number of (mature) oocytes in 7 (n=25) human studies and 1 (n=3) of the animal studies and with preimplantation embryo quality in 13 (n=35) human studies and 8 (n=10) animal studies. First-trimester growth was significantly associated with maternal BMI in 4 (n=9) studies. Nineteen of the 44 articles described a significant association between a higher maternal BMI and miscarriage.

Conclusion: This review demonstrates a small impact of maternal BMI on periconceptional outcomes in humans. In animals, this association is more evident, as conditions are standardised and confounding is minimised. As numerous studies show a negative association between maternal BMI and reproductive success, future research should focus on causal pathways, early and adequate recognition of complications and preventive strategies.

INTRODUCTION

Over the past 50 years, the global prevalence of obesity has increased from 4.5% to 19.5%, reaching pandemic proportions (1, 2). Leader in this emerging global health problem are the United States of America, with over 40% of the adult population classifying as obese (3). However, the prevalence of obesity has also started to rise in developing countries, which is likely due to improved economy and wealth (4, 5). This pandemic of obesity does not exclude women in the reproductive period, which has far reaching consequences. For example, children of obese women have an increased risk of being obese themselves or developing cardiovascular diseases (6, 7). Expanding our knowledge on how adiposity impacts reproduction may improve the health of future generations and halt the increasing prevalence of obesity.

It is generally believed that excess energy consumption, limited energy expenditure and genetic predisposition are involved in the pathophysiology of overweight and obesity (8). The abundance of stored fats leads to increased levels of proinflammatory cytokines and free fatty acids, secreted by adipocytes. These increased levels dysregulate lipid and glucose metabolism, which contribute to disruptions on endocrine, immune and vascular levels (9). As a result, overweight and obesity are positively associated with numerous non-communicable diseases, which greatly attributes to the impact of this emerging health problem (10). For example, an extensive meta-analysis has demonstrated that obesity in females is associated with a more than 12-fold increased risk of diabetes type 2 (10).

Interestingly, several non-communicable diseases find their origin early in life. Already in 1986, Barker et al. have shown that prenatal and early life nutrition is linked to ischaemic heart disease in adult life (11). This, together with many similar findings, has led to the paradigm of Developmental Origins of Health and Disease (DOHaD), which postulates that environmental exposures in utero may have serious consequences for an individual's short-term and long-term health (12).

Moreover, it is stated that the periconception period, defined as 14 weeks prior to and 10 weeks after conception, is critical for a healthy pregnancy (13). In this period, several crucial processes take place. During gametogenesis, spermatozoa and oocytes are prepared for fertilisation by major morphological and molecular alterations. After fertilisation, chromatin in the maternal and paternal genome undergoes extensive reorganisation to the embryonic configuration, which is crucial for developmental potency (14, 15). As limited genome transcription occurs during the first two cell cycles, it is considered likely that maternal factors stored in the oocyte are important for these transitions. After the zygote has undergone cleavage divisions, the embryo undergoes compaction and then the first lineage specification event occurs, resulting in formation of the blastocyst that is ready to implant. After implantation, the blastocyst will initiate placentation and gastrulation, which is the onset for the development of major organ

systems. Finally, with the closure of the secondary palate of the embryo in week 10 after fertilisation, the periconception period ends.

Most studies regarding maternal BMI and pregnancy so far have focussed on complications, birthweight and neonatal outcomes. The impact of BMI on relevant periconceptional outcome measures are less well studied, although most of them underlie perinatal outcomes. Normally, the periconception period is a challenging period to study. This is because pregnancy is often not recognised until several weeks after conception, expecting couples often announce pregnancy only at the end of the first-trimester and even more couples keep their wish to conceive private (16). These barriers are minimised during assisted reproduction techniques (ART), thereby giving physicians and researchers the opportunity to provide adequate care and study processes normally not visible in naturally conceived pregnancies.

Since an elevated BMI has a profound impact on health and susceptibility to non-communicable diseases, it is likely that it also has a detrimental impact on the processes involved in human reproduction, such as gametogenesis, fertilisation, and embryonic growth and development. With this systematic review, we aim to give an overview of the evidence on associations between periconceptional maternal BMI and oocyte quantity and maturity, and preimplantation embryo quality during *in vitro* fertilisation treatment, first-trimester growth and development, and miscarriage.

METHODS

Search strategy

Searches were performed using the databases of Embase, Medline, PubMed, Web of Science, Google Scholar and the Cochrane databases. The search strategies consisted of MeSH terms and keywords including, but not limited to, BMI, obesity, overweight, anthropometric, preconception, pregnancy, crown-rump length, growth, miscarriage, preimplantation, development, quality and embryo. These were combined using the Boolean operator 'or'. We searched reference lists from included studies and systematic reviews to include relevant remaining articles. The search protocol was designed and registered a priori with the PROSPERO registry (Prospero 2018: CRD42018106519).

Systematic review eligibility criteria

Main outcomes are divided into preimplantation and post-implantation outcomes. Preimplantation outcomes comprised of oocyte parameters (total number, metaphase II oocytes), embryo morphology, and embryonic quality. First-trimester embryonic growth (crown-rump length, estimated fetal weight and other parameters of growth) and miscarriage were considered as post-implantation outcomes. Databases were searched

until January 2021. The results of all the outcome searches were combined with 'or'. The results of maternal BMI and outcome searches were then combined with 'and'.

Inclusion and exclusion criteria

Human observational cohort studies, cross-sectional studies and case control studies published prior to January 2021 were eligible for inclusion in the review. Animal studies were eligible for inclusion in the review only if they reported on preimplantation outcomes. We excluded letters, conference abstracts, editorials, case reports, reviews and meta-analyses. Articles that only reported on second-trimester, third-trimester growth or birth outcomes, the impact of oocyte donor BMI, and articles not published in English were also excluded.

Study selection, full text review and data extraction

LD, MR and MR selected papers for the full-text review independently from each other, based on title and abstract revision. Full-text review and data extraction were also independently carried out by LD, MR and MR. Data were entered into a template specifically designed for this review and differences were resolved by discussion between these three authors. Extracted data included the country of origin, year of publication, study design, study population (including human or animal), exposures of interest, outcome data, exclusion criteria, statistical analysis, potential confounders, results and conclusion. If possible, absolute numbers and estimated effects were recalculated to odds ratios (OR) to facilitate comparison of studies.

Quality of study and risk of bias

To assess the quality of the studies included in this review, we used the ErasmusAGE quality score for systematic reviews (**Table 1**). This tool is based on previously published scoring systems and consists of five items, covering study design, study size, method of measuring exposure and outcome and analysis (17, 18). The parameters for these items can be adapted, based on literature and expert discussion, as relevant for each review. Each of the five items can be assigned zero, one or two points, generating a total score between zero and ten, with ten representing the highest quality.

RESULTS

Study selection

The flowchart depicted in **Figure 1** summarises the process of literature search and study selection. The initial search yielded 24,676 records, of which 10,413 were duplicates. Of the remaining 14,263 records, a total of 14,162 were excluded because they did not fulfil the selection criteria. The full texts of 101 articles were read, of which 30 were excluded. Cross-reference checking led to an additional inclusion of 4 articles, resulting in 75 remaining articles for analysis.

Table 1. ErasmusAGE quality score form for systematic reviews adjusted for: Maternal BMI and periconception growth and development: A systematic review.

Original: ErasmusAGE, 24 June 2013.

This quality score can be used to assess the quality of studies included in systematic reviews and meta-analyses and is applicable to both interventional and observational studies. The score was designed based on previously published scoring systems (PMID 20724400, https://www.nccmt.ca/knowledge-repositories/search/14). The quality score is composed of five items, and each item is allocated 0, 1 or 2 points. This allows a total score between 0 and 10 points, of which 10 represents the highest quality.

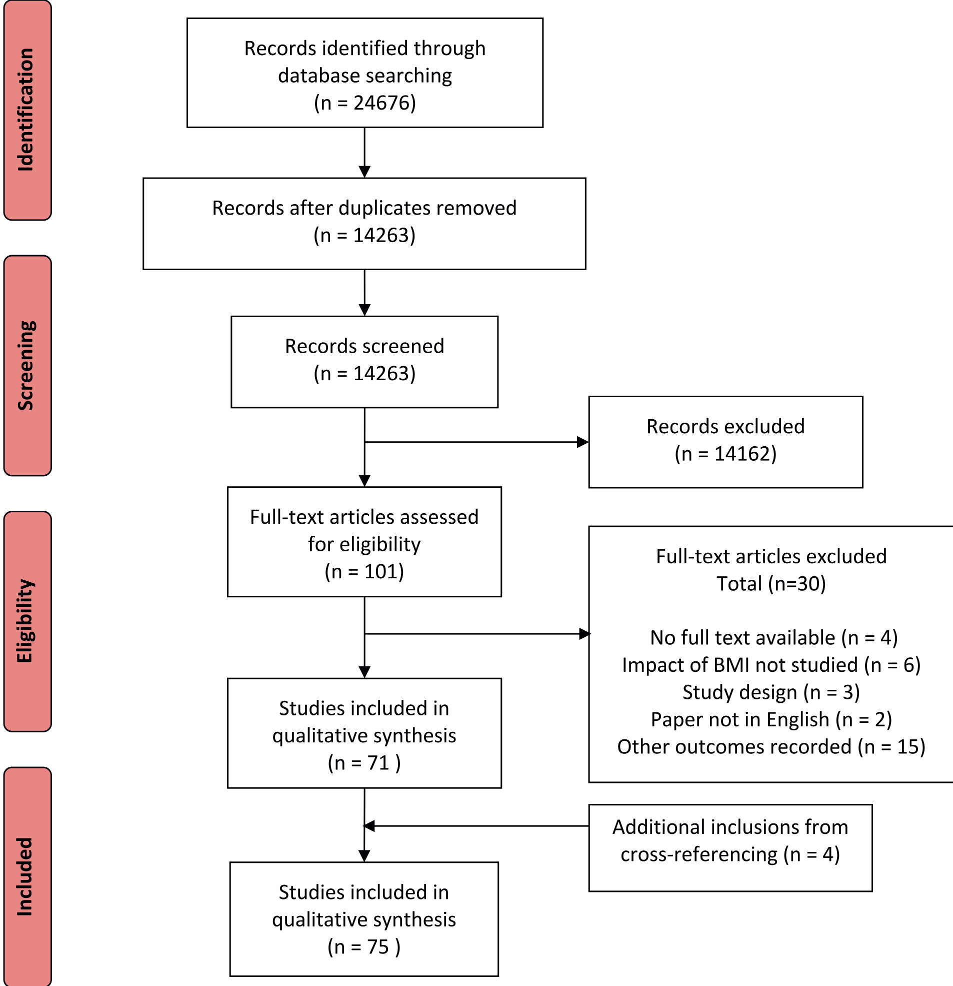
The version presented below is a general version and requires adaptation for each review separately, e.g. what is a small or large study size within the study field, what exposure and outcome measurement methods are adequate, and what the key confounders are. Decisions on these detailed criteria should be based on literature, guidelines and/or discussions with experts. The criteria should be defined prior to the review process.

1	**Study design**
0	for studies with cross-sectional data collection
1	for studies with longitudinal data collection (both retrospective and prospective)
2	for intervention studies
2	**Study size (predefined)***
0	small population for analysis (preimplantation: <160; post-implantation: <1000 patients)
1	intermediate population for analysis (preimplantation: 160-400; post-implantation: 1000-10,000 patients)
2	large population for analysis (preimplantation: >400; post-implantation: >10,000 patients
3	**Exposure** *Observational studies*
0	0 if the study used no appropriate exposure measurement method or if not reported
1	1 if the study used moderate quality exposure measurement methods
2	2 if the study used adequate exposure measurement method
4	**Outcome**
0	if the study used no appropriate outcome measurement method or if not reported
1	if the study used moderate quality outcome measurement methods
2	if the study used adequate outcome measurement methods (Sperm quality according to WHO, sperm epigenetics, miscarriage, preterm birth, small for gestational age, fetal abnormalities)
5	**Adjustments †***
0	if findings are not controlled for at least key confounders
1	if findings are controlled for key confounders
2	if findings are additionally controlled for additional covariates or when an intervention is adequately randomized

* Needs to be specified for each review, based on literature, guidelines and/or expert opinions in the field.

† Either adjusted for in the statistical analyses; stratified for in the analyses; or not applicable (e.g. a study in women only does not require controlling for sex

Figure 1. Flowchart of in- and excluded studies.



The characteristics of the included studies are demonstrated in **Table 2**. Of these 75 articles, 65 are human studies, of which 29 are identified as prospective, and 24 as retrospective cohort studies, 10 as cross-sectional studies and 2 as case control study, and 10 are animal studies, respectively.

Table 2. Main characteristics of the 75 included studies, stratified by study design and sample size.

Author	Year	Country	Study population	Sample size	Exposure	Outcome	Quality
Human studies							
Prospective cohort studies							
Pan et al.	2016	China	Women pregnant after receiving preconception health care	536,098	Pre-pregnancy BMI into categories	Miscarriage	8
Bak et al.	2016	Denmark	Women 11-14 weeks pregnant	187,486	Pre-pregnancy BMI into categories	CRL	4
Syngelaki et al.	2011	UK	Women 11-13 weeks pregnant	41,577	First-trimester BMI continuously and into categories	Miscarriage	9
Zhou et al.	2019	China	Nulliparous women <20 weeks pregnant	18,481	First-trimester BMI into categories	Miscarriage	7
Zhong et al.	2017	China	Couples undergoing IVF/ICSI treatment	10,139	Pre-pregnancy BMI into categories	Miscarriage	5
Thagaard et al.	2017	Denmark	Women 11-14 weeks pregnant	9,055	Pre-pregnancy BMI continuous	CRL, BPD	5
Hahn et al.	2014	Denmark	Women aged 18-40 years, contemplating pregnancy	5,132	Pre-conception BMI into categories	Miscarriage	6
Zhou et al.	2016	China	Women aged 18-40 years, contemplating pregnancy	2,940	Pre-pregnancy BMI into categories	Miscarriage	5
Zhang et al.	2018	USA	Women 8-14 weeks pregnant	2,585	First-trimester BMI into categories	BPD, EFW	8
Kim et al.	2021	USA	Couples undergoing IVF	1,889	Pre-treatment BMI into categories	Miscarriage, blastocyst formation rate	6
Mook-Kanamori et al.	2010	The Netherlands	Women 10-14 weeks pregnant with a regular menstrual cycle	1,631	First-trimester BMI in standard deviations	CRL	8
Cantonwine et al.	2016	USA	Women <15 weeks pregnant	868	First-trimester BMI continuously	CRL	7
Schliep et al.	2015	USA	Couples undergoing their first fresh IFV/ICSI cycle	721	Pre-treatment BMI into categories	Embryo morphology at day 5	9
Al-Hakmani et al.	2016	Oman	Women <12 weeks pregnant	700	First-trimester BMI into categories	Miscarriage	4
Reyes et al.	2012	Mexico	Women <13 weeks pregnant	546	Pre-pregnancy BMI into categories	Miscarriage	6
Deshmukh et al.	2016	India	Women <12 weeks pregnant	500	First-trimester BMI into categories	Miscarriage	3
Vellamkondu et al.	2017	India	Pregnant women undergoing first- trimester screening	440	First-trimester BMI into categories	Miscarriage	2
Zhang et al.	2015	USA	Ovulatory women undergoing their first IVF/ICSI treatment, aged 18-38 years	439	Pre-treatment BMI into categories	No. (mature) oocytes, no. embryos at cleavage stage and at blastocyst stage	7
Selovic et al.	2020	Croatia	Women <13 weeks pregnant	400	First-trimester BMI into categories	CRL	3
Rehman et al.	2012	Pakistan	Ovulatory women undergoing IVF/ICSI treatment, aged 18-40 years and >2 years infertile	323	Pre-treatment BMI into categories	Miscarriage	6
Kirkegaard et al.	2016	Denmark	Women undergoing IVF/ICSI treatment, aged <38 years, without endometriosis and with ≥8 oocytes retrieved	243	Pre-treatment BMI continuously	Embyro morphokinetics	5
Ramezanzadeh et al.	2012	Iran	Infertile women undergoing IVF/ICSI treatment, aged 18-40 years	236	Pre-treatment BMI and physical activity	No. oocytes retrieved, embryo quality and cleavage rate	7
Farhi et al.	2010	Israel	Women undergoing IVF/ICSI treatment, aged <38 years, <3 previous attempts and ≥2 fresh high quality ET	233	Pre-treatment BMI into categories	Miscarriage , no. (mature) oocytes retrieved	6

Lin et al.	2017	China	Ovulatory women undergoing their first fresh IVF treatment, aged 21-35 years	189	Pre-treatment BMI into categories	Miscarriage, no. oocytes retrieved, embryo cleavage rate, available embryos rate, good quality embryo rate	5
Chavarro et al.	2012	USA	Women undergoing IUI or IVF/ICSI treatment	170	Pre-treatment BMI into categories	No. (mature) oocytes, embryo quality and cleavage rate	7
Loveland et al.	2001	USA	Women undergoing fresh IVF treatment, aged <40 years	139	Pre-treatment BMI into categories	Miscarriage, no. oocytes retrieved	4
Mirabi et al.	2017	Iran	Women undergoing IVF/ICSI treatment, aged <38 years	105	Pre-treatment BMI into categories	No. oocytes, oocyte morphology, embryo morphology	4
Van Uitert et al.	2013	The Netherlands	Women 6-8 weeks pregnant after natural conception or IUI	87	First-trimester BMI continuously	CRL	6
Burnik Papler et al.	2019	Slovenia	Women undergoing 1st or 2nd IVF treatment for tubal occlusion	60	Pre-treatment BMI into categories	No. mature oocytes retrieved, embryo morphology	2
Retrospective cohort studies							
Kawwass et al.	2016	USA	Women undergoing fresh IVF/ICSI treatment	180,855	Pre-treatment BMI continuously and into categories	Miscarriage	8
Chaemsaithong et al.	2018	China	Women <14 weeks pregnant	61,807	First-trimester BMI continuously	Miscarriage	9
Cowans et al.	2011	UK	Pregnant women undergoing first- trimester screening	41,779	BMI continuously	Nuchal translucency	5
Van Ravenswaaij et al.	2011	The Netherlands	Pregnant women undergoing first-trimester screening	28,566	First-trimester weight	Miscarriage	6
Zhang et al.	2019	China	Women undergoing first FET after freeze-all	22,043	Pre-treatment BMI into categories	Miscarriage, embryo morphology	7
Oliva et al.	2020	USA	Women undergoing IVF treatment	12,618	Pre-treatment BMI into categories	Miscarriage, no. mature oocytes retrieved, blastocyst formation rate	5
Wang et al.	2016	China	Couples undergoing their first IVF/ICSI treatment	12,061	Pre-treatment parental BMI into categories	Miscarriage, no. oocytes retrieved, no. embryos cleaved, no. available embryos	8
Tang et al.	2021	China	Women undergoing FET after freeze-all	8,755	Pre-treatment BMI into categories	Miscarriage, embryo morphology	6
Romanski et al.	2021	USA	Women undergoing their first fresh IVF/ICSI treatment	7,370	Pre-treatment BMI into categories	Miscarriage	4
Moragianni et al.	2012	USA	Women undergoing their first fresh IVF/ICSI treatment	4,609	Pre-treatment BMI into categories	Miscarriage, embryo morphology	8
Cozzolino et al.	2021	Spain	Women undergoing IVF with preimplantation genetic testing for aneuploidy	3,480	Pre-treatment BMI into categories	Miscarriage, no. mature oocytes retrieved	6
Sun et al.	2020	China	Women undergoing their first fresh IVF treatment	2,531	Pre-treatment BMI continuously	Miscarriage	6
Zander-Fox et al.	2012	Australia	Women undergoing IVF/ICSI treatment, aged <38 years	2,089	Pre-treatment BMI into categories	Miscarriage , embryo quality at day 2-6	7
Shah et al.	2011	USA	Women undergoing their first fresh IVF/ICSI treatment cycle	1,721	Pre-treatment BMI into categories	Miscarriage, no. oocytes, embryo morphology	8

Table 2. Continued.

Sarais et al.	2016	Italy	Women undergoing their first IVF/ICSI treatment	1,602	Pre-treatment BMI into categories	Miscarriage, no. (mature) oocytes, embryo quality of transferred embryos	8
Bartolacci et al.	2019	Italy	Women undergoing their first fresh IVF treatment	1,528	Pre-treatment BMI into categories	Miscarriage, no. (mature) oocytes retrieved, embryo morphokinetics and morphology	8
Ben-Haroush et al.	2018	Israel	Women undergoing their first fresh IVF treatment	1,345	Pre-treatment BMI into categories	Miscarriage	4
Barrie et al.	2021	UK	Women undergoing IVF/ICSI treatment	639	BMI continuously	Embryo morphokinetics	5
Russo et al.	2017	Canada	Women undergoing a fresh single-blastocyst ET, aged <40 years	520	Pre-treatment BMI into categories	Miscarriage	9
Vilarino et al.	2011	Brazil	Women undergoing IVF/ICSI treatment	191	Pre-treatment BMI into groups	Miscarriage, no. (mature) oocytes, embryo quality	5
Garcia-Ferreyra et al.	2021	Ecuador	Women undergoing IVF/ICSI treatment	191	Pre-treatment BMI into categories	Miscarriage, no. oocytes, embryo quality, blastocyst formation rate	3
Comstock et al.	2015	USA	Women undergoing IVF treatment	120	Overweight with metabolic dysfunction and obesity	Miscarriage, no. oocytes retrieved, embryo quality, blastocyst formation rate	4
Di Gregorio et al.	2018	Italy	Women undergoing ICSI treatment	114	Pre-treatment BMI into categories	Miscarriage, no. (mature) oocytes retrieved, embryo morphology	2
Gordon et al.	2018	USA	Pregnant women undergoing first-trimester screening	100	First-trimester BMI into categories	CRL	5
Case-control studies							
Prost et al.	2020	France	Women undergoing FET	1,415	Pre-treatment BMI into categories	Miscarriage, embryo quality	3
Zargar et al.	2018	Iran	Women pregnant after ART	318	19 factors, e.g. BMI>30	Miscarriage	5
Cross-sectional studies							
Bellver et al.	2010	Spain	Women undergoing IVF/ICSI treatment	6,500	Pre-treatment maternal BMI into categories	Miscarriage, embryo morphology (fragmentation and no. cells)	6
Lin et al.	2019	China	Women with PCOS undergoing FET, aged 20-35 years	1,556	Pre-treatment BMI into categories	Miscarriage, embryo quality	5
Braga et al.	2012	Brazil	Couples undergoing IVF/ICSI treatment	420	Pre-treatment BMI continuously	Blastocyst expansion, ICM quality and TE quality	6
Rittenberg et al.	2011	United Kingdom	Women pregnant after blastocyst transfer resulting from IVF/ICSI treatment, aged <40 years	413	Pre-treatment BMI into categories	Miscarriage, no. oocytes retrieved, no. 8-cell day 3 embryos, no. day 5 blastocysts	8
Setti et al.	2019	Brazil	Women undergoing their first ICSI treatment	402	Pre-treatment BMI continuously and into categories	Miscarriage, no. (mature) oocytes, embryo quality, blastocyst formation rate	8
Sathya et al.	2010	India	Women undergoing IVF/ICSI treatment, aged <40 years	308	Pre-treatment BMI into categories	Miscarriage, oocyte and embryo quality at day 3	3

Rehman et al.	2018	Pakistan	Women undergoing ICSI treatment, aged 20-40 years	282	Pre-treatment BMI continuously	Embryo morphology	4
Depalo et al.	2011	Italy	Women undergoing their first IVF/ICSI treatment, aged <40 years	268	Pre-treatment BMI into categories	Oocyte morphology, embryo morphology	4
Garalejic et al.	2017	Serbia	Non-obese women with endometriosis undergoing their first fresh IVF treatment	156	Pre-treatment BMI into categories	No. (good quality) oocytes, no. (good quality) embryos	5
Trenkic et al.	2016	Serbia	Women with PCOS undergoing IVF treatment	123	Pre-treatment BMI into categories	Miscarriage, no. (mature) oocytes, embryo morphology	1
Animal studies							
Sirotkin et al.	2018	Slovakia	ICR female mice, second generation	209	First generation high-energy diet or standard pellet diet	Embryo morphology	5
McPherson et al.	2015	Australia	C57BL/6 female mice	162	High fat diet or isocaloric control diet	Embryo morphology and ICM and TE cell number	9
Kubandova et al.	2014	Slovakia	ICR female mice, second generation	111	First generation high-energy diet or standard pellet diet	No. embryos that reached blastocyst, cleavage or lower stages of development and blastocyst quality	6
Ma et al.	2012	China	C57BL/6 female mice	90	High fat diet or standard rodent chow	Zygote cleavage rate on day 2, 6-8-cell embryo ratio on day 3. Degeneration rate and developmental potential of fresh and thawed embryos	6
Binder et al.	2012	Australia	C57BL/6 female mice	56	High fat diet or control diet	Cell number in blastocysts, embryo developmental kinetics	5
Fabian et al.	2017	Slovakia	ICR female mice, second generation	49	First generation high-energy diet or standard pellet diet	No. (mature) oocytes, embryo morphology	4
Finger et al.	2015	Australia	C57BL/6 female mice	48	High fat diet or control diet	No. oocytes, blastocyst morphology and embryo morphokinetics	7
Luzzo et al.	2012	USA	ICR female mice	30	High fat diet or isocaloric control diet	Embryo development up to the 2-cell stage (2-cell, 1-cell or degraded) and % of 2-cell embryos which developed into blastocysts	7
Bermejo-Alvarez et al.	2012	USA	NIH Swiss female mice	21	High fat diet or control diet	No. oocytes ovulated and no. blastocysts	6
Igosheva et al.	2010	UK	C57BL/6 female mice	20	Obesogenic diet or standard chow diet	No. zygotes and blastocysts, ICM and TE cell number and blastocyst total cell number	6

ART, artificial reproductive techniques. BMI, body mass index. FET, frozen embryo transfer. ICM, inner cell mass. ICSI, intracytoplasmic sperm injection. IVF, in vitro fertilisation. IUI, intra-uterine insemination. PCOS; polycystic ovarian syndrome. TE, trophectoderm

Preimplantation outcomes: Human studies

Thirty-eight studies investigated differences in preimplantation outcomes for maternal BMI. Of these, 23 reported on oocytes retrieved during IVF/ICSI treatment (19-41) (**Table 3**). The majority (n=19), found no impact of maternal BMI on the number of oocytes retrieved. One study, by Wang et al. reported more oocytes retrieved during IVF treatment in women with a BMI ≥25 kg/m^2 than in women with a BMI <25 kg/m^2, but not during ICSI treatment (39). Three studies reported a lower oocyte yield for higher BMI, although Zhang et al. only observed this after conventional stimulation as opposed to minimal stimulation (Clomiphene citrate in conjunction with daily injection of 75-150 IU gonadotrophin) (24, 40, 41).

Nineteen studies reported on oocyte maturity, of which the majority (n=14) found no impact of maternal BMI (19-24, 26, 29, 30, 34-38, 41-45). Mirabi et al. described an inverse relation between the percentage of mature oocytes and maternal BMI (30). One reported a lower percentage and three a lower number of mature oocytes for a higher maternal BMI (24, 34, 41, 44).

Eight studies investigated the percentage or total number of fertilised oocytes that reached the cleavage stage (23, 24, 27, 32, 35, 37, 39, 41), of which five observed no impact of maternal BMI. Wang et al. reported more embryos reaching the cleavage-stage and more usable embryos for maternal BMI ≥25 kg/m2 in the IVF group, whereas Setti et al. and Di Gregorio et al. reported a lower number of cleavage embryos for a higher maternal BMI (24, 35, 39). Six studies reported on embryo morphology in the cleavage stage, of which only Garcia-Ferreyra et al. described fewer cells per embryo for women with a BMI >25 kg/m^2 than for women with a normal BMI (46). Three articles studied morphokinetic development (19, 47, 48). Although the majority of associations was non-significant, all studies reported at least one significant association between a higher maternal BMI and slower preimplantation embryo development.

Furthermore, 19 studies investigated preimplantation embryo quality (19, 21-23, 26, 27, 29, 31, 32, 34, 35, 37, 38, 40, 45, 46, 49-51). Albeit several scoring systems were applied, most studies found no significant association (n=15). Four studies described lower quality of cleavage embryos with increasing BMI, although Metwally et al. only observed this in women aged <35 years (27, 29, 35, 37).

Fourteen studies investigated blastocyst outcomes (21, 30, 33-35, 41, 42, 44, 46, 52-56), of which 8 investigated the total number or rate of embryos that reached the blastocyst stage. The majority (n=6) found no significant impact of maternal BMI. Two studies showed a lower blastocyst formation rate in women with a BMI ≥25 kg/m2 (42, 46). Seven of the 14 studies reported on blastocyst quality. Of these, Braga et al. reported decreased expansion and hatching for increasing BMI, while the inner cell mass (ICM) and trophectoderm (TE) quality was not affected by BMI (52). Mirabi et al. reported better blastocyst quality for normal weight women than for obese women, whereas the remaining articles found no differences (21, 30, 46, 54-56).

Table 3. Description and summary of data for 38 studies that investigated associations and correlations between maternal obesity and preimplantation outcomes.

Author	Exposure	Oocyte		Cleavage embryo			Blastocyst	
		Total number	Mature	Rate or total number (availability)	Morphology / morphokinetics	Quality	Rate or total number	Quality
Prospective cohort studies								
Kim et al. 2021 N=1,889	<18.5 kg/m^2						56%	
	18.5-24.9 kg/m^2						51%	
	25-29.9 kg/m^2						51%	
	≥30.0 kg/m^2						55%	
Schliep et al. 2015 N=721								Aioki et al. score
	<18.5 kg/m^2							15.5 (14.1-17.0)
	18.5-24.9 kg/m^2							14.6 (14.2-15.0)
	25-29.9 kg/m^2							15.3 (14.6-15.9)
	30-34.9 kg/m^2							13.7 (12.8-14.6)
	≥35 kg/m^2							15.0 (13.9-16.0)
Zhang et al. 2015 N=439	<18.5 kg/m^2	**Conv.: 14.5±0.8** Min.: 4.8±+0.3	**Conv.: 11.2±0.7** Min.: 4.1±+0.3	Conv.: 9.5±0.6 Min.: 3.6±0.2			Conv.: 6.2±0.4 Min.: 2.7±+0.2	
	18.5-24.9 kg/m^2	**Conv.: 11.7±0.8** Min.:4.2±0.3	**Conv.: 9.2±0.7** Min.: 3.5±0.3	Conv.: 8.5±0.7 Min.: 3.2±0.3			Conv.: 5.8±0.5 Min.: 2.9±0.3	
	25-29.9 kg/m^2	**Conv.: 8.8±1.3** Min.: 3.9±0.8	**Conv.: 7.1±1.1** Min.: 3.6±0.6	Conv.: 6.7±1.1 Min.: 3.3±0.5			Conv.: 5.1±0.8 Min.: 2.6±0.4	
Kirkegaard et al. 2016 N=243	BMI continuous				t2 (%): 0.42 (-6.6, 8.0) t3 (%): 5.1 (-22.0, 13.0) t4 (%): -1.1 (-7.9, 6.2) t5 (%): 0.83 (-6.5, 8.7) t3-t2 (%): -2.9 (-9.9, 4.5) **t4-t3 (%): 32 (-48, -12)** tEB (h): 0.36 (-3.9, 4.7) tFB (h): -0.31 (-4.8, 4.2) DC (OR): 0.13 (0.04, 0.45)			
Ramezanzadeh et al. 2012 N=236						Veeck's criteria, grade I&II		
	<25 kg/m^2	13.49±10.45		71.97%±33.48		5.34±5.04		
	25-30 kg/m^2	10.01±8.80		68.96%±37.06		4.73±4.80		
	>30 kg/m^2	9.65±6.96		75.52%±41.57		4.29±3.80		
Farhi et al. 2010 N=233	<25 kg/m^2	12.3±6.0						
	≥25 kg/m^2	12.9 ±6.7						
Lin et al. 2017 N=189	18.5-23.9 kg/m^2	8.30±3.46		Total: 96.84%±6.94 Usable:79.71%±16.76		**In-house grade, good** **65.04%±18.78**		
	≥24 kg/m^2	7.85±3.09		Total: 96.62%±8.52 Usable: 77.11%±18.63		**57.99%±22.22**		

Table 3. Continued.

Chavarro et al. 2012 N=170	<20 kg/m²	9.1 (8.0-10.2)	7.3 (6.3-8.4)		Day 3, 6-8 cells	In-house grade, poor (%)		
	20–22.4 kg/m²	9.2 (8.0-10.6)	8.2 (7.1-9.4)		Comparable*	Comparable*		
	22.5–24.9 kg/m²	9.9 (8.6-11.2)	8.7 (7.6-9.9)					
	25–29.9 kg/m²	8.5 (7.3-9.9)	7.6 (6.6-8.9)					
	≥30 kg/m²	10.8 (9.0-10.7)	9.0 (7.6-10.7)					
Loveland et al. 2001 N=139	<25 kg/m²	14.1±6.1						
	≥25 kg/m²	15.1±6.6						
Mirabi et al. 2017 N=105								**Gardner criteria (%)**
	18.5-24.9 kg/m²	8.24±5.5	**84%±31**					**4A: 0.23±0.3**
								2B: 0.29±0.07
	25-29.9 kg/m²	9.02±6.0	**86%±24**					4A: 0.15±23
								2B: 0.15±0.25
	≥30 kg/m²	8.68±4.5	**65%±36**					**4A: 0.05±0.14**
								2B: 0.29±0.07
Burnik Papler et al. 2019 N=60						In-house grade, good		In-house grade, good
	19.0-24.9 kg/m²	12.4±7.4	73%±22			73.2%		62.7%
	≥30 kg/m²	11.3±7.5	70%±26			71.2%		50.0%
Retrospective cohort studies								
Zhang et al. 2019 N=22,043						Cummins criteria, top (%)		
	<18.5 kg/m²					21.9		
	18.5-22.9 kg/m²					23.0		
	23-27.4 kg/m²					23.4		
	>27.5 kg/m²					21.3		
Oliva et al. 2020 N=12,618	<18.5 kg/m²		**11.3±8.1**				**5.3±5.5**	
	18.5-24.9 kg/m²		**10.4±7.4**				**4.8±5.0**	
Wang et al. 2016 N=12,061	Both parents <25 kg/m²	**IVF: 10.57±5.93**		**IVF, total: 8.65±5.01**				
				IVF, usable: 5.42±3.45				
		ICSI: 10.21±5.08		ICSI, total: 7.58±4.23				
	Maternal ≥25 kg/m² and paternal <25 kg/m²			ICSI, usable 5.29±3.21				
		IVF: 10.43±5.52		**IVF, total: 8.93±5.39**				
				IVF, usable: 5.66±3.53				
		ICSI: 10.59±5.48		ICSI, total: 7.90±4.51				
				ICSI, usable: 5.56±3.46				
Tang et al. 2021 N=8,755						Cummins criteria, high		
	<18.5 kg/m²					97.11%		
	18.5-24.9 kg/m²					97.33%		
	>25 kg/m²					96.95%		
Moragianni et al. 2012 N=4,609						In-house grade, mean		
	<18.5 kg/m²	11.46±6.62				7.62±5.15		
	18.5-24.9 kg/m²	10.90±7.23				9.00±4.96		
	25-29.9 kg/m²	10.80±6.96				9.09±5.00		
	30-34.9 kg/m²	9.98±5.89				9.06±5.37		
	35-39.9 kg/m²	10.21±7.58				8.53±4.79		
	≥40 kg/m²	10.48±6.99				9.29±5.03		

Cozzolino et al.	<18. kg/m²		12.2 (11.3–13.1)			
2021	18.5-24.9 kg/m²		11.4 (11.2–11.7)			
N=3,480	25-29.9 kg/m²		11.9 (11.3–12.5)			
	>30 kg/m²		11.7 (10.8–12.5)			
Zander-Fox et al.	18.5-24.9 kg/m²	**10.5±0.2**			Veeck's criteria	
2012	25-29.9 kg/m²	**10.7±0.3**			Comparable*	
N=2,089	30-34.9 kg/m²	**9.2±0.4**				
	35-39.9 kg/m²	**9.8±0.5**				
	≥40 kg/m²	**9.2±0.5**				
Shah et al. 2011				Day 3, 8-cells (RR)		
N=1,721	<18.5 kg/m²	15.1 (12.7-18.0)	11.9 (9.9-14.3)	1.11 (0.82-1.48)		
	18.5-24.9 kg/m²	15.4 (13.0-18.2)	12.7 (10.8-15.1)	Ref		
	25-29.9 kg/m²	16.3 (15.1-17.6)	13.5 (12.5-14.6)	1.16 (1.02-1.32)		
	30-34.9 kg/m²	15.4 (13.8-17.1)	13.0 (11.6-14.6)	1.03 (0.84-1.26)		
	35-39.9 kg/m²	13.3 (11.4-15.4)	11.3 (9.7-13.2)	1.07 (0.84-1.37)		
	≥40 kg/m²	14.2 (12.3-16.4)	11.1 (9.5-13.0)	0.93 (0.69-1.24)		
Sarais et al. 2016					In-house grade, mean	
N=1,602	<18.5 kg/m²	8.26±5.87	**74.56%±21.93**		1.90±0.66	53.57%±23.09
	18.5-24.9 kg/m²	8.17±6.09	**76.93%±24.75**		2.03±0.71	56.57%±22.46
	25-29.9 kg/m²	7.88±5.83	**73.15%±24.96**		1.96±0.73	53.07±%20.75
	≥30 kg/m²	6.22±4.73	**67.24%±30.05**		1.92±0.77	52.65%±29.47
Bartolacci et al.				t8 (hours)	Sarais et al., score	
2019	<18.5 kg/m²	10.20±5.19	67.26%±1.98	55.49 (51.37-63.48)	1.92±0.68	
N=1,528	18.5-24.99 kg/m²	10.10±5.10	67.18%±1.85	55.66 (50.89-62.89)	1.97±0.73	
	25.0-29.99 kg/m²	9.90±4.71	67.31%±2.01	**56.72 (51.83-63.92)**	1.89±0.75	
	≥30 kg/m²	9.38±4.26	67.33%±2.15	**57.89 (51.60-65.94)**	1.87±0.77	
Barrie et al. 2021	BMI continuous			**β_{t2} -0.009 h**		
N=639				β_{t3} -0.008 h		
				β_{t4} -0.012 h		
				β_{t5} -0.010 h		
				β_{t6} -0.037 h		
				β_{t7} -0.033 h		
				β_{t8} -0.049 h		
				β_{t9} -0.036 h		
				β_{tM} -0.043 h		
				β_{tSB} -0.060 h		
				β_{tB} -0.052 h		
Metwally et al.					In-house grade, mean	
2007	18.5-24.9 kg/m²	<35y: 8.1±0.41	<35y: 6.9±0.35		**<35y: 2.0±0.60**	
N=426		>35y: 6.4±0.53	>35y: 6.9±0.46		>35y: 2.1±0.90	
	25-29.9 kg/m²	<35y: 8.1±0.54	<35y: 6.6±0.50		**<35y: 1.9±0.09**	
		>35y: 6.6±0.85	>35y: 6.6±0.71		>35y: 2.0±0.11	
	≥30 kg/m²	<35y: 9.0±6.0	<35y: 7.2±0.54		**<35y: 2.3±1.40**	
		>35y: 5.2±0.7	>35y: 7.2±0.65		>35y: 1.9±0.13	

Table 3. Continued.

Vilarino et al. 2011 N=191	<25 kg/m² ≥25 kg/m²	7±4.68 7±5.13	5.50±3.86 5.08±3.81			In-house grade, good 1.26±1.52 1.33±1.50		
Garcia-Ferreyra et al. 2021 N=191	18.5-24.9 kg/m² 25.0-29.9 kg/m² ≥30 kg/m²				Day 3, cells per embryo 7.3±0.79 **6.7±1.73** **6.8±1.04**	In-house grade, good 84.0% 82.3% 79.9%	48.2% **40.0%** **40.1%**	Gardner criteria, good 92.9% 91.5% 88.7%
Comstock et al. 2015 N=120	18.5-24.9 kg/m² 25-29.9 with metabolic dysfunction or ≥30 kg/m²		13.7±6.2 14.1±6.7		Day 3, ≥5-cells 6.4±4 5.7±4		**Of >5 cells on day 3** **57.2%** **43.6%**	
Di Gregorio et al. 2018 N=114	18.5-24.9 kg/m² 25.0-29.9 kg/m² ≥30 kg/m²	7.88±5.99 6.25±4.15 **3.0±3.16**	2.97±1.36 **2.19±1.32** **1.25±1.50**	1.81±1.15 1.94±1.06 **1.00±1.15**				
Case-control studies								
Prost et al. 2020 N=1,415	18.5-24.9 kg/m² ≥30 kg/m²							Gardner criteria, top 54.1% 50.0%
Cross-sectional studies								
Bellver et al. 2010 N=6,500	<20 kg/m² 20-24.9 kg/m² 25-29.9 kg/m² ≥30 kg/m² Continuous	12.6±7.7 12.6±8.0 12.3±7.9 13.0±8.8	8.8±5.5 8.9±5.9 8.5±5.6 9.1±6.2		Day 2 r_{blast}=0.012, r_{frag}=0.006 Day 3 r_{blast}=0.011, r_{frag}=0.007			
Lin et al. 2019 N=1,556	18.5-24.9 kg/m² 25.0-29.9 kg/m² ≥30 kg/m²							Gardner criteria, high 97.26% 96.92% 96.81%
Braga et al. 2012 N=420	BMI continuous							In-house morphological score **Total: r=-0.038 p=0.046** ICM: r= 0.011, p=0.520 TE: r=-0.014, p=0.401
Rittenberg et al. 2011 N=413	18.5-24.9 kg/m² ≥25 kg/m²	16.0±6.7 16.1±7.2			Day 3, 8-cells 4.3±2.6 4.0±2.6		Total number 6.2±3.2 6.3±3.1	

Setti et al. 2019 N=402	BMI continuous <24.9 kg/m² ≥25.0 kg/m²	β 0.007	β 0.010	 **6.3 ± 0.2** **5.0 ± 0.2**	In-house grade, high **Day 2, 23.3%±0.5** Day 3, 24.3%±0.6 **Day 2, 15.3%±0.5** Day 3, 22.5%±0.7	 47.8%±0.7 46.4%±0.9
Sathya et al. 2010 N=308	 <25 kg/m² 25-30 kg/m² >30 kg/m²		 64.7% 75.5% 64.4%		Gardner criteria, good 3.3±0.8 3.6±1.1 3.5±0.7	
Rehman et al. 2018 N=282	 BMI continuous				Racowsky criteria β 0.06	
Depalo et al. 2011 N=268	 <25 kg/m² ≥25 kg/m²	 5.80±2.09 5.78±2.10	 79.2% 79.4%	Of fertilised oocytes 83.12% 86.08%	Veeck's criteria, grades (%) I: 82.3 II: 15.3 III: 2.3 I: 81.7 II: 14.5 III: 3.8	
Garalejic et al. 2017 N=156	 <18.5 kg/m² 18.5-24.9 kg/m² 25-29.9 kg/m²	 6.13±1.13 6.26±3.36 6.25±3.60	 3.50±2.22 3.97±2.44 4.25±2.22		Top quality (Istanbul consensus) 2.38±1.56 2.90±1.92 2.50±1.54	
Trenkic et al. 2016 N=123	 ≤25 kg/m² >25 kg/m²	 14.06±7.89 11.48±6.52	 10.07±6.33 7.44±4.86	 8.42±4.90 6.40±3.96	In-house grade, class I **3.04±3.10** 1.24±1.74	

tEB, time to start formation of a blastocoel. tFB, time to full blastocyst formation. DC, direct cleaving. tM, time to start of compaction. tSB, time to start of blastulation. tB, time to full blastocyst; R_{blast}, Correlation with blastomere number. R_{frag}, Correlation with fragmentation. RR, relative risk. Significant outcomes are in bold.

In summary, only 7 studies showed associations between maternal BMI and number of (mature) oocytes and 12 reported that a higher maternal BMI is associated with reduced embryo quality.

Preimplantation outcomes: Animal studies

Ten animal studies investigated the impact of a high fat diet on preimplantation outcomes, all in mice (**Table 4**). Three articles investigated *in vivo* embryo development (57-59). Fabian et al. showed no impact of body fat on the number of oocytes, although the number of immature oocytes was higher in obese mice (57). Kubandova et al. found a significantly higher number of embryos per dam in mice with 8-11% body fat than in mice with a physiological amount of body fat (7-8%), whereas both Sirotkin et al. and Kubandova et al. reported reduced embryo development in dams with <7% or >11% body fat, compared to dams with 7-8% or 8-11% body fat.

Two studies investigated the number of oocytes ovulated after 12 weeks of either high fat or control diet, of which only Finger et al. reported more ovulated oocytes in the control group (60, 61).

Five studies investigated cleavage stage embryos (60, 62-65), of which 3 compared morphology. Both Luzzo et al. and McPherson et al. reported better *in vivo* embryo morphology at 46 and 44 hours post-hCG, respectively, for mice fed a control diet (63, 65). In addition, McPherson et al. also reported poorer *in vitro* embryo development after 43 hours of culture for obese female mice. Ma et al. reported no impact of maternal diet on the number of zygotes that developed to the 2-cell stage *in vivo* (64). However, for mice fed a control diet, a higher proportion of the embryos developed into six-to-eight-cell embryos. Two other studies reported on morphokinetic embryo development after 12 weeks of high fat diet. Binder et al. showed a delay in reaching the 2- to 8-cell stage in obese mice, whereas Finger et al. only observed a 1 hour delay in pronuclear breakdown (60, 62). Yet, when developmental timings were based on embryo pronuclear breakdown as t=0, significant differences were observed from the 5-cell division onwards.

All studies investigating the direct impact of maternal high fat diet, reported on blastocyst outcomes (n=7) (60-66). Two showed no impact of maternal diet on the number of blastocysts (61, 66). Finger et al. observed an 11% decrease in the percentage of fertilised zygotes reaching the blastocyst stage after high fat diet (60). Luzzo et al. also observed that more of the embryos that reached the 2-cell stage developed to blastocysts in the control group than in the high fat diet group (63). Two studies reported on embryo morphology after *in vitro* fertilisation. Of these, Ma et al. demonstrated better developmental potential 97 and 114 hours post-hCG for embryos of normal weight mice than embryos of obese mice (64). McPherson et al. observed no impact of maternal diet on *in vitro* embryo development after 74 and 91 hours of culture, but did observe reduced *in vivo* blastocyst development after maternal high fat diet (65).

Table 4. Description and summary of data for 10 studies that investigated associations between maternal obesity and preconceptional outcomes in animals.

Author	Animal	Exposure	Oocyte	Cleavage embryo			Blastocyst					
			Total number	Total number	morphology	morphokinetics (h)	Total number	Morphology	Total cell number	ICM cell number	TE cell number	ICM: TE ratio
Sirotkin et al. 2018 N=209	ICR female mice, second generation	Body fat: <7% 7-8% 8-11% >11%		Similar*	Retarded**: **18%** 12% 10% **14%**							
McPherson et al. 2015 N=162	C57BL/6 female mice	High fat diet Isocaloric control diet			**44 hours:** 2-cell: 70.3% **>3-cell: 0.0%** 2-cell: 55.8% **>3-cell: 19.4%**			**90 hours:** **<Morula: 51.6%** **Exp. blastocyst: 1.7%** **<Morula: 33.2%** **Exp. blastocyst: 39.1%**	30.6±2.2 34.5±1.9	**7.1±0.8** **10.2±0.8**	**23.9±2.7** **25.1±1.8**	
Kubandova et al. 2014 N=111	ICR female mice, second generation	Body fat: <7% 7-8% 8-11% >11%		11.12±0.52 10.00±0.63 **12.27±0.51** 11.59±0.54	**<16 cells**:** **18%** 10% 7% **15%**				54.18±0.93 56.34±0.83 54.95±1.00 55.29±0.75			
Ma et al. 2012[1] N=90	C57BL/6 female mice	High fat diet Standard rodent chow			**6-8 cell ratio:** **62.4%±4.2** **75.1% ±5.3**			**Day 5 (%):** Early blastocyst: 49.4 **Exp. blastocyst: 24.6** Early blastocyst: 39.1 **Exp. blastocyst: 48.3**				
Binder et al. 2012 N=56	C57BL/6 female mice	High fat diet Control diet				**t2: 31.1±0.1** **t3: 50.9±0.1** **t5: 61.8±0.1** **t8: 63.4±0.1** **t2: 30.2±0.1** **t3: 49.5±0.1** **t5: 60.8±0.1** **t8: 62.2±0.1**			**48.2±1.0** **54.2±0.8**			
Fabian et al. 2017 N=49	ICR female mice, second generation	Body fat: 7-8% >11%	23.53±2.56 24.88±2.43	18.55±3.18 22.81±2.72	Degenerated 17.64% 22.20%		**57.35%** **47.95%**		47.91±0.92 50.87±1.09			
Finger et al. 2015 N=48	C57BL/6 female mice	High fat diet Control diet	**12.6 ± 1.95** **16.6 ± 1.10**			**tPNB: -1**	**-11%**		**Reduced***	Similar*	**Reduced***	Similar*
Luzzo et al. 2012 N=30	ICR female mice	High fat diet Isocaloric control diet			**46 hours:** **2-cell: 35%** **1-cell: 35%** **Degraded: 30%** **2-cell: 80%** **1-cell: 10%** **Degraded: 10%**		**of 2-cell embryos:** **50%** **65%**					
Bermejo-Alvarez et al. 2012 N=21	NIH Swiss female mice	High fat diet Control diet	13.4 ± 0.9 12.0 ± 0.9				13.4 ±0.9 12.0 ±0.9					
Igosheva et al. 2010 N=20	C57BL/6 female mice	Obesogenic diet Standard chow diet					7.7±1.6 6.7±1.2		44.8±3.5 45.8±2.1	14.1±0.6 14.3±1.2	30.6±0.8 31.7±1.6	22.2±1.3 24.1±5.2

Only outcomes after fresh ET demonstrated. *exact numbers not given in article.** estimated from figure. Significant outcomes are in bold.

Four studies investigated total blastocyst cell number, three of which also investigated ICM and TE cell number (60, 62, 65, 66). Igosheva et al. reported no impact of maternal diet on blastocyst cell number after *in vivo* fertilisation, whereas McPherson et al. reported lower cell numbers for maternal high fat diet (65, 66). In addition, the latter study also reported comparable blastocyst numbers after *in vitro* fertilisation, which is in contrast to the studies of Binder et al. and Finger et al., who both reported reduced blastocyst numbers for maternal high fat diet after *in vitro* fertilisation (60, 62). Regarding total ICM cell counts, Igosheva et al. and Finger et al. reported no differences, whereas McPherson et al. observed a reduced total ICM cell count after both *in vivo* and *in vitro* development for maternal high fat diet (60, 65, 66). Igosheva et al. reported a comparable TE cell count after *in vivo* fertilisation, whereas McPherson et al observed a reduced TE cell count for maternal high fat diet. Finger et al. reported a lower TE cell count after *in vitro* fertilisation. No impact was found on the ratio of ICM:TE cell numbers.

In 8 out of 10 animal studies the maternal BMI was negatively associated with number of (mature) oocytes and preimplantation embryo quality.

Miscarriage

The association between maternal BMI and miscarriage was evaluated in 44 studies (**Table 5**). Of these, 33 were performed in women pregnant after IVF/ICSI treatment and 11 in women who conceived naturally. Of this latter group, 3 articles studied preconceptional BMI and 8 first-trimester BMI. The definition of miscarriage varied considerably between the studies, ranging from embryonic loss prior to 5 weeks of gestation to no live birth. Although the majority divided maternal BMI into categories according to the WHO classification, 11 articles applied alternative cut-off values for the normal weight maternal BMI category.

Of the 33 studies that were performed in pregnancies after IVF/ICSI treatment (19, 20, 24, 25, 27, 28, 31, 33-40, 42-46, 49-51, 53-55, 67-73), one investigated BMI as a continuous variable and showed a positive association (71). Six studies applied cut-off values not described by the WHO. Of these, two studies applied a cut-off value of ≥24 kg/m2 for being overweight, of which Zhong et al. demonstrated higher odds of miscarriage for overweight women (27, 73). Two applied a cut-off value of ≥23 kg/m2, which was associated with an increased risk of miscarriage in both (49, 51). Furthermore, two studies defined a BMI <20 kg/m2 as being underweight, of which Bellver et al. showed higher odds of a miscarriage for underweight women (20, 70).

Table 5. Description and summary of data for 44 studies that investigated associations between maternal BMI and risk of miscarriage.

Author	Sample size	BMI classification	Definition of miscarriage	Total number of miscarriages	OR (95%CI)
Prospective cohort studies					
Pan et al. 2016	536,098	<18.5 kg/m² 18.5-23.9 kg/m² 24.0-27.9 kg/m² ≥28 kg/m²	<28 weeks GA	15,437	**1.11 (1.06-1.17)** Ref 1.02 (0.97-1.08) **1.13 (1.02-1.26)**
Syngelaki et al. 2011	41,577	BMI continuous <25 kg/m² 25.0-29.9 kg/m² 30.0-34.9 kg/m² ≥35 kg/m²	<24 weeks GA	Not described	**1.03 (1.01-1.05)** Ref 1.01 (0.79-1.28) **1.63 (1.24-2.13)** **1.44 (1.02-2.04)**
Zhou et al. 2019	18,481	<18.5 kg/m² 18.5-22.9 kg/m² 23.0-27.5 kg/m² ≥27.5 kg/m²	<20 weeks GA	549	1.01 (0.69-1.47) Ref 1.17 (0.97-1.41) **1.69 (1.21-2.35)**
Zhong et al. 2017	10,139	<24 kg/m² ≥24 kg/m²	<28 weeks GA	1,574	Ref **1.27 (1.11-1.46)**
Hahn et al. 2014	5,132	<20 kg/m² 20.0-24.9 kg/m² 25.0-29.9 kg/m² ≥30 kg/m²	<22 weeks GA	732	1.00 (0.81-1.24) Ref 0.90 (0.73-1.09 1.23 (0.89-1.54)
Zhou et al. 2016	2,940	<18.5 kg/m² 18.5-23.9 kg/m² 24.0-27.9 kg/m² ≥28 kg/m²	<20 weeks GA	229	**2.05 (1.30-3.23)** **Ref** **1.71 (1.04-2.81)** **2.01 (1.10-3.68)**
Kim et al. 2021	1,889	<18.5 kg/m² 18.5-24.9 kg/m² 25.0-29.9 kg/m² ≥30 kg/m²	Not defined	Not described	Not available
Al-Hakmani et al. 2016	700	18.5-24.9 kg/m² 25.0-29.9 kg/m² ≥30 kg/m²	Not defined	63	Ref 1.45 (0.30-2.88) 1.89 (0.99-3.61)
Reyes et al. 2012	546	18.5-24.9 kg/m² 25.0-29.9 kg/m² ≥30 kg/m²	<20 weeks GA	26	Ref 0.56 (0.19-1.60) 0.90 (0.35-2.30)
Deshmukh et al. 2016	500	18.5-24.9 kg/m² 25.0-29.9 kg/m² 30.0-39.9 kg/m² ≥40 kg/m²	Not defined	2	Ref 2.21 (0.04-112.2) 8.22 (0.33-203.6) **40.69 (1.60-1032)**

Table 5. Continued.

Vellamkondu et al. 2017	440	≤23.0 kg/m² >23.0 kg/m²	Not defined	22	Ref 1.04 (0.43-2.49)
Rehman et al. 2012	323	<18 kg/m² 18.0-22.9 kg/m² 23.0-25.9 kg/m² ≥26.0 kg/m²	<5 weeks GA	61	0.73 (0.22-2.44) Ref **5.18 (1.95-13.72)** 0.69 (0.35-1.36)
Farhi et al. 2010	233	<25.0 kg/m² ≥25.0 kg/m²	Not defined	29	Ref 0.81 (0.34-1.94)
Lin et al. 2017	189	18.5-23.9 kg/m² ≥24.0 kg/m²	<20 weeks GA	12	Ref 1.24 (0.30–5.18)
Loveland et al. 2001	139	<25.0 kg/m² ≥25.0 kg/m²	<20 weeks GA	8	Ref 3.42 (0.74-15.84)
Retrospective cohort studies					
Kawwass et al. 2016	180,855	<18.5 kg/m² 18.5-24.9 kg/m² 25-29.9 kg/m² ≥30 kg/m²	<20 weeks GA	22,088	0.99 (0.91-1.07) Ref N/A **1.39 (1.34-1.43)**
Chaemsaithong et al. 2018	61,807	BMI continuous	<24 weeks GA	159	**1.058 (1.016-1.103)**
Van Ravenswaaij et al. 2011	28,566	Weight continuous, kg	<16 weeks GA	150	1.01 (0.99-1.02)
Zhang et al. 2019	22,043	<18.5 kg/m² 18.5-22.9 kg/m² 23.0-27.4 kg/m² ≥27.5 kg/m²	<24 weeks GA	1,483	0.94 (0.78-1.14) Ref **1.27 (1.12-1.45)** **1.75 (1.41-2.17)**
Oliva et al. 2020	12,618	<18.5 kg/m² 18.5-24.9 kg/m²	<13 weeks GA	107	1.11 (0.51-2.43) Ref
Wang et al. 2016	12,061	Both parents <25 kg/m² Maternal ≥25 kg/m² and paternal <25 kg/m²	Not defined	978	Ref **IVF: 1.64 (1.26-2.13)** ICSI: 1.13 (0.68-1.87)
Tang et al. 2021	8,755	<18.5 kg/m² 18.5-24.9 kg/m² >25 kg/m²	<24 weeks GA	523	1.20 (0.93-1.56) Ref 1.28 (0.99-1.66)
Romanski et al. 2021	7,370	18.5-24.9 kg/m² 25-29.9 kg/m² 30-34.9 kg/m² 35-39.9 kg/m² >40 kg/m²	Failed pregnancy after observed gestational sac	518	Ref **1.35 (1.09-1.68)** **1.71 (1.27-2.32)** **1.69 (1.07-2.67)** 1.67 (0.85-4.25)

Moragianni et al. 2012	4,609	<18.5 kg/m² 18.5-24.9 kg/m² 25-29.9 kg/m² 30-34.9 kg/m² 35-39.9 kg/m² ≥40 kg/m²	<20 weeks GA	Not described	0.93 (0.43-2.00) Ref 1.04 (0.83-1.30) 1.22 (0.89-1.66) 1.26 (0.85-1.26) **1.92 (1.14-3.22)**
Cozzolino et al. 2021	3,480	<18.5 kg/m² 18.5-24.9 kg/m² 25.0-29.9 kg/m² ≥30 kg/m²	<20 weeks GA	Not described	Not available
Sun et al. 2020	2,531	BMI continuous	Not defined	114	**1.07 (0.99-1.15)**
Zander-Fox et al. 2012	2,089	18.5-24.9 kg/m² 25-29.9 kg/m² 30-34.9 kg/m² 35-39.9 kg/m² ≥40 kg/m²	No fetal heartbeat	71	Ref 1.09 (0.63-1.91) 0.50 (0.19-1.30) 0.34 (0.08-1.46) 0.56 (0.13-2.43)
Shah et al. 2011	1,721	<18.5 kg/m² 18.5-24.9 kg/m² 25-29.9 kg/m² 30-34.9 kg/m² 35-39.9 kg/m² ≥40 kg/m²	No live birth	Not described	0.29 (0.04-2.30) Ref 0.63 (0.37-1.08) 0.97 (0.46-2.08) 0.75 (0.24-2.31) 1.44 (0.55-3.80)
Sarais et al. 2016	1,602	<18.5 kg/m² 18.5-24.9 kg/m² 25-29.9 kg/m² ≥30 kg/m²	<20 weeks GA	Not described	1.37 (0.42-4.47) Ref 2.24 (0.86-5.84) 4.75 (0.70-32.37)
Bartolacci et al. 2019	1,528	<18.5 kg/m² 18.5-24.99 kg/m² 25.0-29.99 kg/m² ≥30 kg/m²	<20 weeks GA	Not described	**2.10 (1.12-3.95)** Ref 0.94 (0.47-1.90) 1.64 (0.62-4.02)
Ben-Haroush et al. 2018	1,345	<25.0 kg/m² 25.0-30.0 kg/m² ≥30.0 kg/m²	Not defined	81	Ref 1.42 (0.74-2.70) 1.04 (0.51-2.09)
Russo et al. 2017	520	<20 kg/m² 20-24.9 kg/m² 25-29.9 kg/m² 30-39.9 kg/m² ≥40 kg/m²	<20 weeks GA	Not described	Not available
Vilarino et al. 2011	191	<25 kg/m² ≥25 kg/m²	<22 weeks GA	23	Ref 1.20 (0.42-3.40)

Table 5. Continued.

Garcia- Ferreyra et al. 2021	191	18.5-24.9 kg/m^2 25.0-29.9 kg/m^2 >30.0 kg/m^2	Not defined	3	Ref 1.26 (0.08-20.82) 3.14 (0.18-53.60)
Comstock et al. 2015	120	18.5-24.9 kg/m^2 25-29.9 kg/m^2 with metabolic dysfunction or ≥30 kg/m^2	<24 weeks GA	12	Ref 3.00 (0.77-11.69)
Di Gregorio et al. 2018	114	18.5-24.9 kg/m^2 25.0-29.9 kg/m^2 >30.0 kg/m^2	Not defined	9	Ref 0.62 (0.11-3.36) 1.35 (0.24-7.63)
Case-control studies					
Prost et al. 2020	1,415	18.5-24.9 kg/m^2 >30.0 kg/m^2	Not defined	126	Ref 1.63 (0.96-2.76)
Zargar et al. 2018	318	≤30 kg/m^2 >30 kg/m^2	No live birth	119	Ref **2.74 (1.68-4.45)**
Cross-sectional studies					
Bellver et al. 2010	6,500	BMI continuous <20 kg/m^2 20-24.9 kg/m^2 25-29.9 kg/m^2 ≥30 kg/m^2	<22 weeks GA	172	1.01 (0.99-1.03) **1.22 (1.00-1.49)** Ref 1.09 (0.89-1.33) **1.64 (1.22-2.22)**
Lin et al. 2019	1,556	18.5-24.9 kg/m^2 25.0-29.9 kg/m^2 >30.0 kg/m^2	No live birth	130	Ref **1.57 (1.04-2.36)** 1.40 (0.80-2.42)
Rittenberg et al. 2011	413	18.5-24.9 kg/m^2 ≥25 kg/m^2	<23 weeks GA	Not described	Ref **Fresh ET: 2.4 (1.4-3.8)** **Frozen ET: 3.0 (1.2-7.5)**
Setti et al. 2019	402	≤24.9 kg/m^2 >25.0 kg/m^2	<20 weeks GA	17	Not available
Sathya et al. 2010	308	<25 kg/m^2 25-29.9 kg/m^2 ≥30 kg/m^2	Missed abortion	22	Ref 4.00 (0.84-18.95) **9.00 (1.72-47.22)**
Trenkic et al. 2016	123	≤25.0 kg/m^2 >25.0 kg/m^2	Not defined	8	Ref 2.03 (0.25-18.29)

BMI, body mass index. ET, embryo transfer. OR, odds ratio. Significant outcomes are in bold.

Four articles studied the association between maternal BMI and miscarriage by applying an alternative classification that divides obesity into three sub-classes, resulting in 6 groups of maternal BMI (31, 36, 40, 69). Of these, Romanski et al. described increased odds for a miscarriage in women with a BMI 25.0-39.9 kg/m2, whereas Moragianni et al. showed this for women with a BMI >40 kg/m2 (31, 69). Shah et al. and Zander-Fox et al. demonstrated no significant associations (36, 40).

Five studies applied the classical WHO-classification (19, 34, 43, 53, 68). Of these, two reported a positive association between obesity and miscarriage, one between underweight and miscarriage and two demonstrated no significant associations.

Nonetheless, the majority subdivided maternal BMI in two groups: <25 and ≥25 kg/m^2. Six of them reported no association with miscarriage (25, 28, 35, 37, 38, 42). Rittenberg et al. reported increased odds of miscarriage for women with an elevated BMI after both fresh embryo transfer (ET) as well as after frozen-thawed ET (33). In line with this, Wang et al. demonstrated increased odds for miscarriage only after IVF, but not after ICSI for women with a BMI ≥25 kg/m^2 (39).

Of the remaining studies, 4 demonstrated no significant association between maternal BMI and miscarriage (24, 44, 46, 50, 55, 67). Two reported a positive association between obesity and miscarriage and one between overweight and miscarriage (54, 72, 74).

Of the 11 studies that were performed in women who conceived naturally (75-85), three studied the independent variable, i.e. weight and BMI, on a continuous scale (76, 81, 82). Chaemsaithong et al. and Syngelaki et al. reported higher odds of a miscarriage for an increased BMI, whereas van Ravenswaaij et al. reported no association between weight and miscarriage. Syngelaki et al. also reported a higher risk of miscarriage in women with a BMI of 30-34.9 kg/m^2 and ≥35 kg/m^2.

Three studies applied the classical WHO-classification, of which 2 showed no significant associations (75, 80). One study divided the obesity category in 30-39.9 kg/m^2 and ≥40 kg/m^2 and found a positive association in the latter group (77).

Five studies applied cut-off values not described by the WHO, of which 2 reported no significant associations (78, 79, 83-85). Two defined a normal BMI as 18.5-23.9 kg/m^2, which both found that a BMI <18.5 kg/m^2 and ≥28 kg/m^2 was associated with higher odds of miscarriage (79, 84). One reported higher odds of a miscarriage in women with a BMI ≥27.5 kg/m^2 (85).

To summarise, 19 of the 44 articles described a significant association between maternal BMI and miscarriage.

First-trimester growth and development

Nine articles evaluated the association between maternal BMI and first-trimester growth and development (86-94) (**Table 6**), of which six studied CRL. Of these, three investigated maternal BMI on a continuous scale (87, 92, 93). Only Thagaard et al. reported a smaller CRL for a higher BMI. Of those that that investigated BMI in categories, only Selovic et al. reported a significant association, i.e. a larger CRL for overweight and obese women (89-91).

Cowans et al. reported a thicker nuchal translucency for a higher maternal BMI (88). Biparietal diameter (BPD) was studied by Thagaard et al. and Zhang et al., of which only the first showed a smaller BPD for a higher BMI (92, 94). Zhang et al. reported impact of maternal BMI on estimated fetal weight, femur length and abdominal circumference (94). Bak et al. studied the impact of BMI on the discrepancy between estimated due dates based on last menstrual period versus CRL measurements, and reported higher odds for discrepancy in both underweight as well as overweight and obese women (86).

In summary, there is no irrefutable association between maternal BMI and first-trimester growth and development.

DISCUSSION

Oocyte and preimplantation embryo quality

Maternal BMI was associated with lower numbers of (mature) oocytes in only a few studies. In all of the human studies, selection of ovarian stimulation protocols and/or dosage was based on patient characteristics such as age, antral follicle count and previous response. Although individualised ovarian stimulation treatment likely confounds the association between BMI and oocyte yield, standardising treatment should be avoided as this may result in a higher incidence of poor ovarian response or ovarian hyperstimulation syndrome (95). Better indicators of oocyte developmental competence might be morphological factors, molecular markers or intracellular temperature (96). Yet, research on these indicators in association with maternal BMI or diet remains scarce, especially in humans.

We did not find clear evidence that maternal BMI affects human preimplantation embryo quality. A possible explanation is that the majority of study populations were relatively small, especially when compared to studies investigating post-implantation outcomes. Another explanation is that most clinics set a minimum and maximum BMI prior to treatment, which limits the possibilities to study the impact of extremities in maternal BMI. Moreover, there are many factors that affect preimplantation embryo quality, such as culture conditions, maternal age and folic acid supplementation (52, 74, 97-99). Thus, more sensitive markers of preimplantation embryo quality are needed.

Table 6. Description and summary of data for 9 studies that investigated associations between maternal BMI and first-trimester growth and development.

Author	Sample size	Exposure	Outcome definition	Effect size
Prospective cohort studies				
Bak et al. 2016	187,486	<18.5 kg/m² 18.5-24.9 kg/m² 25.0-29.9 kg/m² 30.0-34.9 kg/m² 35.0-39.9 kg/m² >40.0 kg/m²	OR for discrepancy EDD-LMP minus EDD-US ≤7 days	**1.11 (1.01-1.22)** Ref 0.97 (0.93-1.02) **1.14 (1.07-1.23)** **1.28 (1.15-1.42)** **1.73 (1.50-1.98)**
Thagaard et al. 2017	9,055	BMI continuous	CRL BPD	**-0.08 mm (SE 0.02) *p*<0.001** **-0.03 mm (SE 0.01) *p*<0.001**
Zhang et al. 2018	2,585	19.0-29.9 kg/m² >30 kg/m²	at 11 weeks GA: EFW BPD FL AC	43.9 g (95%CI 43.6-44.2) 43.9 g (95%CI 43.0-44.7) 15.4 mm (95%CI 15.3-15.5) 15.4 mm (95%CI 15.2-15.6) 4.1 mm (95%CI 4.1-4.1) 4.2 mm (95%CI 4.1-4.3) 45.4 mm (95%CI 45.1-45.6) 45.4 mm (95%CI 44.8-45.9)
Mook-Kanamori et al. 2010	1,631	BMI in standard deviation (=4.08 units)	CRL	-0.01 mm (95%CI -0.35, 0.33)
Cantonwine et al. 2016	868	BMI continuous	CRL	-0.156 mm (SE 0.110)
Selovic et al. 2020	400	18.5-24.9 kg/m² 25.0-29.9 kg/m² ≥30.0 kg/m²	CRL	53.03 mm **54.05 mm** **54.84 mm**
Gordon et al. 2018	100	<30 kg/m² >35 kg/m²	CRL	65.99 ± 7.75 mm 65.19 ± 7.06 mm
van Uitert et al. 2013	87	BMI continuous	CRL	0.095 mm (95%CI -0.11, 0.17)
Retrospective cohort studies				
Cowans et al. 2011	41,779	BMI continuous	Correlation with log_{10} NT MoM ΔNT	**0.0277 *p*<0.001** **0.0208 *p*<0.001**

EDD, estimated due date. LMP, last menstrual period. US, ultrasound. Significant outcomes are in bold.

The majority of included animal studies reported negative associations between high fat diet and *in vivo* preimplantation embryo outcomes. A possible explanation is that the embryos are longer exposed to the maternal diet, thereby it is a more accurate reflection of natural development. A suboptimal maternal diet affects the composition of fluids in the female reproductive tract. For example, excess triglycerides and lactate accumulate in the maturing oocyte, thereby affecting its metabolism and subsequently reducing embryo developmental potential (100). Similar mechanisms have been described for uterine luminal fluids, i.e. the direct embryo environment. Proper embryo-maternal interaction is critical for programming and development of (extra-)embryonic cell lineages, thus critical for fetal growth trajectories (101). It is likely that a suboptimal maternal diet has a detrimental effect on this interaction, thereby negatively impacting *in vivo* embryo development. As embryos for *in vitro* development are cultured in synthetic medium, development is impacted to a lesser extent by maternal factors, which is also suggested by an animal study in this review (65).

Miscarriage

A high maternal BMI was associated with an increased risk of miscarriage in 19 out of the 44 studies (20, 31, 33, 39, 45, 49, 51, 54, 68, 69, 71, 72, 76, 77, 79, 81, 84, 85). Variations in the moment or method of BMI measurement may underlie the inconsistent results.

Although the exact underlying mechanisms are not completely elucidated, it has been suggested that increased insulin resistance alters the endometrium by a reduced production of adhesion factors and lower serum levels of immunosuppressive proteins (102). Oocyte donations are an elegant model to study the distinct role of the endometrium in miscarriages. Interestingly, these studies report inconclusive results on the association between recipient BMI and miscarriage rate (103, 104). So in conclusion, it is likely that other factors, such as immunological dysregulation, are also involved in the aetiology of miscarriages (105).

First-trimester growth and development

The majority of studies did not show an irrefutable association between maternal BMI and first-trimester growth and development. A possible explanation is that during the first 10 to 11 weeks of gestation, the embryo is supported by histotrophic nutrition trough the yolk sac, i.e. maternal endometrial gland secretions (106, 107). Little is known of the impact of maternal obesity on histotrophic nutrition, although some studies show alterations in endometrial function (108, 109). Another explanation is that the effect of maternal BMI is relatively small during the first-trimester but increases as pregnancy progresses, which also explains the numerous studies demonstrating associations between maternal BMI and pregnancy outcomes (110, 111).

Strengths and limitations

This is the first study to systematically review the evidence currently available on the impact of maternal BMI on periconceptional outcomes. By including studies from different continents, the risk of inclusion bias was reduced and the generalizability of our results increased. Another strength is that the majority of studies had a prospective cohort design, which reduces the risk of reverse causality, recall and selection bias. This review also included animal studies using rodent models of high-fat diet-induced obesity. Although it has been suggested that these models do not directly translate to human obesity, they facilitate to study the impact of overnutrition independent of confounders (112). Moreover, it is possible to study preimplantation development after *in vivo* fertilisation. As embryos are longer exposed to the maternal diet, it is likely a more accurate reflection of natural reproduction.

Nonetheless, the overall amount and quality of evidence was relatively low. Furthermore, maternal BMI was categorised according to different classifications, embryo quality was studied using multiple scoring systems and the definition of miscarriage ranged from embryonic loss prior to 5 weeks of gestation to no live birth. This considerable variation in exposures and outcomes hampered comparison of results and performing extensive meta-analyses. Also, the included studies were at risk for several biases. Firstly, some were based on self-reported BMI, allowing social desirability bias. Secondly, couples with an early miscarriage or unplanned pregnancy were generally not included, thereby inducing selection bias. Finally, the majority of studies were performed in couples undergoing IVF/ICSI treatment, which hampers extrapolation to couples who conceive naturally and investigation of the full range of BMI, since extremities are often excluded from treatment.

Conclusion

This systematic review shows that in humans, the evidence supporting our hypothesis that maternal obesity has a detrimental impact on periconceptional outcomes, is relatively scarce. In animal studies, however, there is more evidence supporting this hypothesis, which further underlines the difficulty of reliable BMI assessment and the multifactorial aetiology of periconceptional outcomes in humans. Standardisation of outcome definition and measurement in future studies, together with data sharing, will facilitate researchers to perform robust meta-analyses and calculate risk ratios, which will improve our understanding of the true impact of maternal BMI on periconceptional outcomes (113).

The alarming increase in the prevalence of obesity over the past decades is of major concern (1). It is associated with an increased risk of pregnancy complications and reduced fertility, thereby affecting reproductive success (114). Therefore, further research should focus on causal pathways, early and adequate recognition and preventive strategies. For the latter, the periconception period can be a critical window of opportunity, as people are more open

to behavioural change (115). Proper diet and lifestyle adaptations would not only increase current health and offspring health, but also reduce subsequent diseases later in life and perinatal morbidity and mortality.

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Higher preconceptional maternal body mass index is associated with faster early preimplantation embryonic development: The Rotterdam Periconception Cohort

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ABSTRACT

Background: Overweight and obesity affect millions of people globally, which has also serious implications for reproduction. For example, treatment outcomes after *in vitro* fertilisation (IVF) are worse in women with a high body mass index (BMI). However, the impact of maternal BMI on embryo quality is inconclusive. Our main aim is to study associations between preconceptional maternal BMI and morphokinetic parameters of preimplantation embryos and predicted implantation potential. In addition, associations with clinical IVF outcomes are investigated.

Methods: From a tertiary hospital, 268 women undergoing IVF or IVF with intracytoplasmic sperm injection (ICSI) were included; 143 normal weight, 79 overweight and 46 obese women. The embryos of these women were cultured in the EmbryoScope, a time-lapse incubator. The morphokinetic parameters of preimplantation embryos and predicted implantation potential, assessed by the KIDScore algorithm were longitudinally evaluated as primary and secondary outcomes, respectively. The tertiary outcomes included clinical outcomes, i.e. fertilisation, implantation and live birth rate.

Results: After adjustment for patient- and treatment-related factors, we demonstrated in 938 embryos that maternal BMI is negatively associated with the moment of pronuclear appearance (β_{tPNa} -0.070 hours (95%CI -0.139, -0.001), p=0.048), pronuclear fading (β_{tPNf} -0.091 hours (95%CI -0.180, -0.003), p=0.043 and the first cell cleavage (β_{t2} -0.111 hours (95%CI -0.205, -0.016), p=0.022). Maternal BMI was not significantly associated with the KIDScore and tertiary clinical treatment outcomes. In embryos from couples with female or combined factor subfertility, the impact of maternal BMI was even larger (β_{tPNf} -0.170 hours (95%CI -0.293, -0.047), p=0.007; β_{t2} -0.199 hours (95%CI -0.330, -0.067), p=0.003). Additionally, a detrimental impact of BMI per point increase was observed on the KIDScore (β -0.073 (se 0.028), p=0.010).

Conclusions: Higher maternal BMI is associated with faster early preimplantation development. In couples with female or combined factor subfertility, a higher BMI is associated with a lower implantation potential as predicted by the KIDScore. Likely due to power issues, we did not observe an impact on clinical treatment outcomes. However, an effect of faster preimplantation development on post-implantation development is conceivable, especially since the impact of maternal BMI on pregnancy outcomes has been widely demonstrated.

INTRODUCTION

Overweight and obesity affect millions of people of all ages, genders, ethnicities and income levels (1). Although the pathophysiology of adiposity is highly complex and multifactorial, it is fundamentally caused by a positive energy imbalance and influenced by genetic and numerous environmental factors (2, 3). Surplus energy is stored as fat, which leads to a disruption of numerous physiological processes on endocrine, immune and vascular levels (4). This explains why an elevated body mass index (BMI) is associated with various non-communicable diseases, such as diabetes type 2 and cancer (5, 6).

Obesity and overweight not only increase the risk of non-communicable diseases, but can also impact reproduction (7). As almost half of the women in the reproductive period are overweight or obese, this has serious consequences. Adiposity affects metabolic and endocrine processes involved in fertility, which leads to an increased risk of miscarriages, reduced conception rate and anovulation (8-10). Therefore, overweight and obesity are likely overrepresented in women receiving fertility treatment, such as *in vitro* fertilisation (IVF) (11).

Outcomes after IVF treatment are poorer in women with a high BMI compared to normal weight women (12-14). The mechanisms by which adiposity affects reproduction are not yet fully understood. It is suggested that obesity interferes with biological processes and pathways at endocrine, follicular, uterine and embryonic levels (15-18). For example, obesity increases follicular fluid concentrations of lipids, metabolites and inflammatory markers, and impacts gene expression of cumulus cells, which can impair oocyte development (19, 20). Interestingly, the impact of maternal BMI on embryo quality is inconclusive (18, 21-23).

Since three decades, preimplantation embryo development can be closely observed with time-lapse imaging (24). This technique is increasingly used to study associations between embryo development and implantation, and to improve embryo selection by algorithms such as the KIDScore (25). Prospective randomised trials report conflicting results on the improvement of success rates after embryo selection based on time-lapse parameters, and also indicate that these parameters are subject to patient-related factors (26, 27). Moreover, previous studies investigating the impact of BMI on these parameters report conflicting results and are exclusively performed in cycles with intracytoplasmic sperm injection (ICSI) (28, 29).

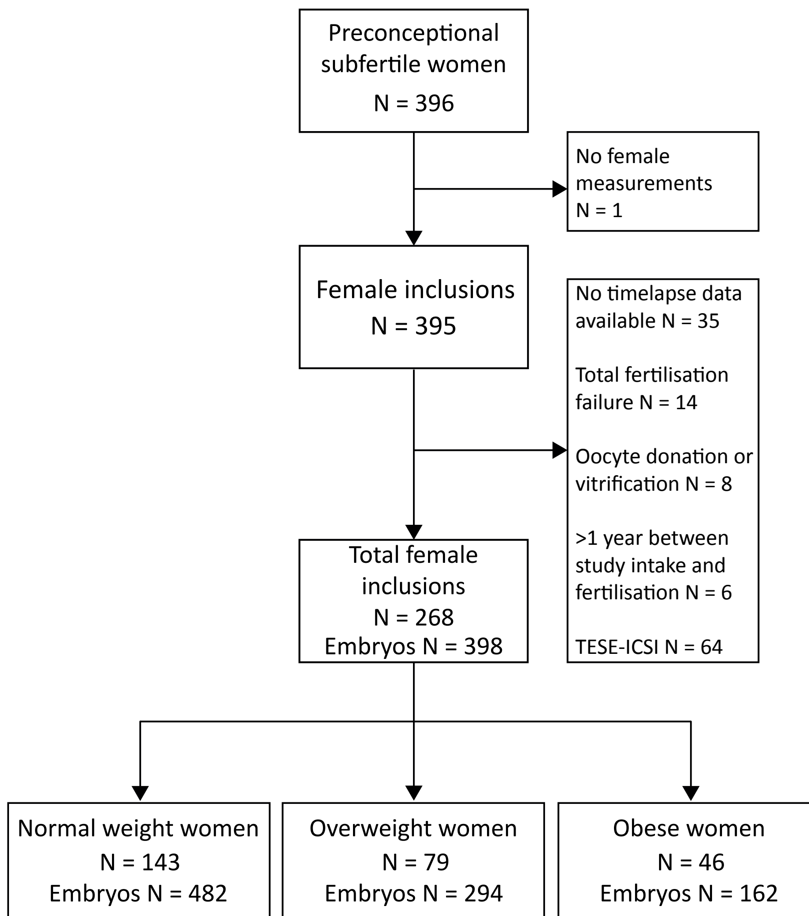
From this background, the main aim of this study is to investigate the hypothesis that a high maternal BMI is detrimentally associated with preimplantation embryo quality, as assessed by developmental time-lapse parameters and predicted implantation potential (KIDScore). In addition, we also investigated associations with clinical treatment outcomes after IVF/ICSI treatment.

METHODS

Study design

The data used for this study was collected between May 2017 and December 2019 as part of the Virtual EmbryoScope study. This is an ongoing prospective sub-study of the Rotterdam Periconception Cohort, an observational open prospective tertiary hospital-based cohort, embedded in the outpatient clinic of the Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Center, Rotterdam, The Netherlands (30). The Rotterdam Periconception Cohort focuses on periconceptional influences on reproductive success and adverse pregnancy outcomes and health of the offspring up to 1 year of age. For this study, subfertile couples with an indication for IVF/ICSI treatment, aged 18 years or older with adequate understanding of the Dutch language were eligible for participation.

Figure 1. Flowchart of the VIRTUAL EmbryoScope study population embedded in the Rotterdam Periconception Cohort.



Abbreviations: TESE, testicular sperm extraction. ICSI, intracytoplasmic sperm injection.

For this study, couples were excluded if: 1) no fertilisation occurred and no embryos were available (n=14); 2) the embryos were not cultured in the EmbryoScope (n=35); 3) IVF/ICSI treatment was performed with donated or vitrified oocytes (n=8); 4) treatment was performed after >1 year following inclusion in the study (n=6) or 5) ICSI was performed with testicular extracted sperm (n=64) (**Figure 1**).

In vitro fertilisation procedures and time-lapse imaging

Ovarian stimulation, oocyte retrieval and IVF/ICSI procedures were performed as previously described (31, 32). Women underwent ovarian stimulation with either recombinant follicle stimulating hormone (rFSH) or urinary FSH, with gonadotrophin-releasing hormone (GnRH) agonist or GnRH-antagonist co-treatment. Ovarian stimulation protocols were standardised; the distribution of GnRH-agonist or -antagonist protocol reflects policy changes over time and not patient selection. FSH dosage was based on maternal age, BMI, antral follicle count, anti-mullerian hormone (AMH) level and prior response to gonadotrophins (if applicable). Final follicular maturation was triggered with human chorionic gonadotrophin (hCG) or a GnRH-agonist. Oocytes were collected 35 hours later and cultured in SAGE human tubal fluid medium (HTF, CooperSurgical, Trumbull, CT, USA); supplemented with 5% human serum albumin (CooperSurgical) under an oil overlay (CooperSurgical).

After insemination, IVF oocytes were cultured overnight in drops of 100 µl HTF medium in universal GPS dishes (CooperSurgical) under oil. The next morning, only fertilised dipronucleate oocytes were transferred to an EmbryoSlide (Vitrolife, Goteborg, Sweden) for culture in the EmbryoScope™ time-lapse incubator (Vitrolife). ICSI oocytes were denuded and transferred to an EmbryoSlide directly after sperm injection. Injected oocytes or embryos were cultured individually in 25 µl of SAGE 1-Step medium (Cooper Surgical) under 1.4 ml oil. Culture in the EmbryoScope™ was conform conventional culture, performed using customised settings, with a temperature setting of 36.8 °C and in an atmosphere containing 7% O_2 and 4.5% CO_2. This atmosphere was validated to result in a pH of the SAGE 1-Step culture medium of 7.2-7.3.

Images were automatically recorded every 10 minutes after exposure to a single red LED (635 nm, <0.1 second per image) with a monochrome CCD camera. On day 3 after fertilisation, embryo evaluation and selection for embryo transfer (ET) was based on conventional morphology, i.e. number of blastomeres, fragmentation, size equality, and signs of early compaction, without the support of time-lapse information. Selection of embryos for cryopreservation was performed on day 4, based on the degree of compaction and fragmentation.

Two weeks after ET, implantation was biochemically confirmed by a positive β-hCG test. Pregnancy was confirmed by ultrasound at 7 and 12 weeks of gestation by the presence of a gestational sac and fetal heartbeat

Study parameters

Participants completed a preconceptional questionnaire covering demographic and lifestyle details. A researcher verified all data at study entry and measured anthropometrics. Subfertility diagnoses were retrieved from medical records and, when applicable, stratified according to the WHO classification of anovulation (33).

Time-lapse parameters were annotated manually according to the definitions of the ESHRE consensus for dynamic monitoring of human preimplantation development (34). All freshly transferred and cryopreserved embryos were annotated for the following morphokinetic parameters: pronuclear appearance (tPNa), pronuclear fading (tPNf), t2, t3, t4, t5, t6, t7 and t8. tPNa was defined as the first frame in which both pronuclei had appeared, and tPNf as the first frame in which both had faded. Timing of reaching the 2-, 3-, 4-, 5-, 6-, 7-, and 8-cell stage was defined as t2, t3, t4, t5, t6, t7 and t8, respectively. Some of these parameters were used to assign each embryo a KIDScore (**Supplemental table 1**). This is a generally applicable embryo deselection tool based on 6 parameters, of which the lowest score (=1) corresponds with a chance of implantation of 5%, whereas the highest score (=5) corresponds with a 36% chance of implantation (25). Validation of the KIDScore in our clinic showed that KIDScore 1 embryos implant in 23% of cases, increasing to 52% for KIDScore 5 embryos after SET. Internal validation of inter-observer reproducibility demonstrated extremely close agreement for the timings of tPNf until t5 (intraclass correlation coefficient (ICC) >0.95). A moderate agreement was found for t6, t7 and t8 (ICC 0.23-0.40).

Clinical treatment outcomes were retrieved from medical records. Pre-transfer clinical treatment outcomes included: fertilisation rate, which was calculated by dividing the number of fertilised oocytes by the number of metaphase II oocytes retrieved and embryo usage rate, which was calculated by dividing the number of usable embryos, i.e. all freshly transferred and cryopreserved, by the number of fertilised oocytes. Post-transfer clinical treatment outcomes included: a positive β-hCG-test, fetal heartbeat, live birth and cumulative pregnancy rate, which was defined as an ongoing pregnancy resulting from either fresh ET or frozen-thawed ET from the studied treatment cycle within a two year follow-up period.

Statistical analyses

Based on initial reports in human embryos, preimplantation embryos from obese women are expected to be developmentally delayed (35). Based on culture of the first 900 embryos in our EmbryoScope™, average time needed from pronuclear disappearance to the 4-cell stage was 16.7 hours (SD +/-5). The intra-cluster correlation coefficient (ICC) was 0.16. To show a delay of at least 2 hour (0.4SD) in reaching the 4-cell stage, while correcting for statistical clustering of embryos derived from the same patient (1+([average no. of embryos/

patient]-1)*ICC = 1+4*0.1591= 1.64), we needed 100*1.64 = 164 embryos in each group to achieve an 80% power to detect this 2 hour difference at α= 0.05.

Continuous baseline data were compared between women with normal weight, overweight and obesity, using Kruskall-Wallis tests for continuous data and chi-square tests for categorical data.

Analyses of morphokinetic parameters were performed on transferred and cryopreserved embryos. Since couples often have multiple embryos and embryos from a couple are likely to exhibit comparable developmental patterns, linear regression analyses are not appropriate. Therefore, we applied linear mixed models with time-lapse parameters as response variables and BMI as independent variable.

Proportional odds models were used to study the association between maternal BMI and the KIDScore, using the ordinal package in R (Rune Haubo B Christensen). This model is for ordinal outcomes like the KIDScore, with patient-specific intercepts to account for the correlation between sibling embryos.

Associations between BMI and continuous treatment outcomes, such as fertilisation rate, were analyzed using linear regression. For associations between BMI and dichotomous outcomes, such as a positive β-hCG-test, logistic regression was applied.

All associations were studied with maternal BMI as a continuous variable. Two models were constructed for analyses on morphokinetic parameters and the KIDScore; a crude model without adjustments and the adjusted model with adjustments for maternal age, fertilisation method, type of ovarian stimulation and paternal BMI and age. Analyses on treatment outcomes were adjusted for maternal age and type of ovarian stimulation. Post-hoc analyses were performed with maternal BMI divided into categories of normal weight (BMI ≤25 kg/m^2), overweight (BMI>25kg/m^2) and obese (BMI>30 kg/m^2), with normal weight as reference category. Furthermore, we stratified analyses of morphokinetic parameters for fertilisation method, as a 2-hour delay between insemination and fertilisation has been suggested for IVF embryos (36). In addition, we performed sub-analyses of embryos of couples with only a female subfertility diagnosis (e.g. endometriosis or PCOS) and a male partner with normal semen parameters, or combined female and male factor subfertility. All analyses were performed in SPSS statistics 25.0 (IBM, Armonk, USA) and R (R: A language and Environment for Statistical Computing, version 3.1.3, 2015 for Windows, R Core Team, Vienna, Austria). Two-sided P-values <0.05 were considered significant.

RESULTS

Baseline characteristics

A total of 268 women (n=938 embryos) were included, of whom 143 were of normal weight (n=482 embryos), 79 overweight (n=294 embryos) and 46 obese (n=162 embryos) (**Figure 1**). Baseline characteristics were comparable between the three groups, except that normal weight women were more often highly educated than overweight and obese women (62.7%, 51.9% and 30.4%, respectively, *p*=0.004) (**Table 1**). Additionally, types of female type subfertility are not significantly different between the three groups (**Supplemental table 2**).

Table 1. Baseline characteristics of the VIRTUAL EmbryoScope study population (n=268).

	Normal weight women N=143		**Overweight women N=79**		**Obese women N=43**			
	Median/N	**IQR/%**	**Median/N**	**IQR/%**	**Median/N**	**IQR/%**	**P-value**	**Missing**
Maternal factors								
Age, years	34.3	30.5-38.3	32.9	29.2-36.6	36.3	30.3-39.6	0.110	0
Geographic origin, Western	119	83.3	68	86.1	37	80.4	0.709	1
Educational level							**0.004**	1
Low	6	4.2	6	7.6	5	10.9		
Intermediate	47	33.1	27	58.7	32	40.5		
High	89	62.7	41	51.9	14	30.4		
Folic acid supplements, yes	135	95.1	75	94.9	42	91.3	0.632	1
Vitamins, yes	80	60.6	37	50.7	17	41.5	0.073	22
Alcohol, yes	63	44.4	27	34.2	18	39.1	0.328	1
Cigarettes, yes	19	13.4	12	15.2	7	15.2	0.914	1
Treatment factors								
Cause of subfertility							0.987	0
Female factor	44	30.8	22	27.8	12	26.1		
Male factor	51	35.7	30	38.0	17	37.0		
Combined	28	19.6	17	21.5	9	19.6		
Unexplained	20	14.0	10	12.7	8	17.4		
ICSI, yes	79	55.2	50	63.3	28	60.9	0.478	0
Oocytes aspirated	8	5-12	9	6-14	8	5-12	0.278	2
Ovarian stimulation, GnRH-agonist	26	18.2	18	22.8	12	26.1	0.459	0

IQR, interquartile range. ICSI, intracytoplasmic sperm injection. Significant differences are depicted in bold.

Morphokinetic parameters

Linear mixed model analyses showed negative betas for all morphokinetic parameters, indicating a faster development for every increase in BMI point (**Table 2**). However, this was only significant for tPNf (β_{crude} -0.119 hours (95%CI -0.206, -0.031), *p*=0.008; $\beta_{adjusted}$ -0.091 hours (95%CI -0.180, -0.003), *p*=0.043) and t2 (β_{crude} -0.142 hours (95%CI -0.235, -0.049), *p*=0.003; $\beta_{adjusted}$ -0.111 hours (95%CI -0.205, -0.016), *p*=0.022). Stratification for fertilisation method demonstrated that the negative beta for tPNa is almost exclusively based on ICSI embryos (**Table 3**). Interestingly, sub-analyses of embryos from couples with female or combined factor subfertility showed an even larger impact of BMI on tPNf (β_{crude} -0.164 hours (95%CI -0.286, -0.042), *p*=0.009; $\beta_{adjusted}$ -0.170 hours (95%CI -0.293, -0.047), *p*=0.007)

and t2 (β_{crude} -0.194 hours (95%CI -0.323, -0.064), p=0.004; $\beta_{adjusted}$ -0.199 hours (95%CI -0.330, -0.067), p=0.003) (**Table 4**).

Table 2. The impact of maternal BMI on morphokinetic parameters.

Morphokinetic parameter	Crude		Adjusted		Missing
	Beta (95%CI) hours	P-value	Beta (95%CI) hours	P-value	
tPNa	-0.074 (-0.163, 0.015)	0.102	-0.070 (-0.139, -0.001)	**0.048**	448*
tPNf	-0.119 (-0.206, -0.031)	**0.008**	-0.091 (-0.180, -0.003)	**0.043**	23
t2	-0.142 (-0.235, -0.049)	**0.003**	-0.111 (-0.205, -0.016)	**0.022**	3
t3	-0.100 (-0.223, 0.023)	0.109	-0.039 (-0.168, 0.089)	0.548	5
t4	-0.122 (-0.246, 0.001)	0.053	-0.087 (-0.220, 0.047)	0.201	8
t5	-0.102 (-0.266, 0.061)	0.220	-0.053 (-0.229, 0.122)	0.549	13
t6	-0.100 (-0.263, 0.063)	0.229	-0.073 (-0.251, 0.105)	0.418	33
t7	-0.069 (-0.242. 0.103)	0.429	-0.014 (-0.204, 0.175)	0.881	65
t8	-0.002 (-0.178, 0.174)	0.982	0.067 (-0.125, 0.259)	0.492	151

CI, confidence interval. Adjusted for maternal age, fertilisation method, type of ovarian stimulation and paternal BMI and age.
* In cases of regular IVF, embryos are only transferred to the EmbryoScope after PN inspection, thus tPNa cannot be observed.

Post-hoc analyses demonstrated a significantly positive beta for t8 in embryos of overweight women, when compared to embryos of normal weight women, (β_{crude} 1.744 hours (95%CI 0.087, 3.401), p=0.039; $\beta_{adjusted}$ 2.541 hours (95%CI 0.774, 4.308), p=0.005) (**Table 5**). Embryos of obese women reached tPNf and t2 faster than embryos of normal weight women (tPNf: β_{crude} -1.065 hours (95%CI -2.082, -0.047) , p=0.040; t2: β_{crude} -1.311 hours (95%CI -2.934, -0.227), p=0.018; t2: $\beta_{adjusted}$ -1.101 hours (95%CI -2.195, -0.008), p=0.048).

Implantation potential, predicted by the KIDScore

The association between maternal BMI and predicted implantation potential, assessed by the KIDScore, was studied by a proportional odds model. The crude model showed a non-significant effect estimate of -0.019 (se 0.015, p=0.206), indicating a lower KIDScore for a higher BMI. The adjusted model demonstrated a comparable estimate (β -0.020 (se 0.017), p=0.218).

Interestingly, sub-analyses of embryos from couples with either a female or a combined factor subfertility diagnosis demonstrated a significant impact of BMI on the KIDScore (β_{crude} -0.049 (se 0.025), p=0.052; $\beta_{adjusted}$ -0.073 (se 0.028), p=0.010). These observations indicate that a higher maternal BMI has a detrimental impact on the predicted implantation potential of embryos of women with an underlying cause for their subfertility.

Post-hoc analyses of maternal BMI into categories also demonstrated non-significant associations between either overweight or obesity and predicted implantation potential ($\beta_{overweight}$ 0.008 (se 0.014, p=0.547); $\beta_{obesity}$ -0.253 (se 0.174, p=0.178)). Similar results were found in the adjusted model ($\beta_{overweight}$ 0.126 (se 0.192, p=0.403); $\beta_{obesity}$ -0.260 (se 0.192, p=0.177)).

Table 3. The impact of maternal BMI on morphokinetic parameters, stratified for fertilisation method.

Morphokinetic parameter	IVF n=111 women				ICSI n=157 women			
	Crude		Adjusted		Crude		Adjusted	
	Beta (95%CI) hours	P-value	Beta (95%CI) hours	P-value	Beta (95%CI) hours	P-value	Beta (95%CI) hours	P-value
tPNa*	0.429 (-1.647, 2.508)	0.232	n/a		-0.070 (-0.131, -0.010)	**0.024**	-0.071 (-0.140, -0.001)	**0.046**
tPNf	-0.103 (-0.247, 0.041)	0.158	-0.115 (-0.270, 0.040)	0.143	-0.091 (-0.187, 0.005)	0.064	-0.080 (-0.186, 0.026)	0.136
t2	-0.152 (-0.306, 0.002)	0.053	-0.146 (-0.311, 0.019)	0.083	-0.095 (-0.196, 0.007)	0.067	-0.146 (-0.311, 0.019)	0.083
t3	-0.085 (-0.290, 0.119)	0.409	-0.046 (-0.273, 0.180)	0.685	-0.071 (-0.214, 0.073)	0.333	-0.031 (-0.183, 0.121)	0.690
t4	-0.101 (-0.312, 0.109)	0.342	-0.086 (-0.315, 0.143)	0.459	-0.105 (-0.251, 0.042)	0.161	0.081 (-0.242, 0.080)	0.320
t5	-0.081 (-0.354, 0.192)	0.557	-0.036 (-0.337, 0.265)	0.812	-0.075 (-0.273, 0.123)	0.456	-0.052 (-0.265, 0.161)	0.630
t6	-0.024 (-0.293, 0.245)	0.858	-0.018 (-0.319, 0.283)	0.906	-0.120 (-0.323, 0.085)	0.251	-0.103 (-0.326, 0.120)	0.362
t7	-0.080 (-0.363, 0.203)	0.577	-0.027 (-0.348, 0.295)	0.870	-0.022 (-0.239, 0.193)	0.836	-0.002 (-0.239, 0.235)	0.984
t8	-0.078 (-0.373, 0.217)	0.601	-0.038 (-0.371, 0.294)	0.820	0.084 (-0.132, 0.301)	0.443	0.122 (-0.116, 0.360)	0.310

Adjusted for maternal age, type of ovarian stimulation and paternal BMI and age. CI, confidence interval. *n=5 for tPNa in IVF-population. Significant differences are depicted in bold.

Table 4. The impact of maternal BMI on morphokinetic parameters of embryos of couples with either a female factor or combined factor subfertility diagnosis (n=476).

Morphokinetic parameter	Crude		Adjusted		Missing
	Beta (95%CI) hours	P-value	Beta (95%CI) hours	P-value	
tPNa	-0.300 (-0.256, 0.196)	0.790	-0.011 (-0.120, 0.098)	0.841	290
tPNf	-0.164 (-0.286, -0.042)	**0.009**	-0.170 (-0.293, -0.047)	**0.007**	0
t2	-0.194 (-0.323, -0.064)	**0.004**	-0.199 (-0.330, -0.067)	**0.003**	0
t3	-0.169 (-0.353, 0.015)	0.072	-0.097 (-0.287, 0.092)	0.311	0
t4	-0.214 (-0.397, -0.031)	0.022	-0.203 (-0.399, -0.008)	**0.042**	0
t5	-0.127 (-0.358, 0.105)	0.280	-0.060 (-0.308, 0.187)	0.629	3
t6	-0.138 (-0.371, 0.095)	0.243	-0.148 (-0.401, 0.104)	0.247	10
t7	-0.148 (-0.394, 0.099)	0.237	-0.097 (-0.366, 0.173)	0.478	27
t8	-0.096 (-0.358, 0.167)	0.472	-0.021 (-0.316, 0.273)	0.887	68

Adjusted for maternal age, fertilisation method, type of ovarian stimulation and paternal BMI and age. CI, confidence interval. Significant differences are depicted in bold.

Table 5. Differences in morphokinetic parameters of embryos from overweight and obese women, compared to embryos of normal weight women.

Morphokinetic parameter	Crude				Adjusted				Missing
	Overweight		Obese		Overweight		Obese		
	Beta (95%CI) hours	P-value	Beta (95%CI) hours	P-value	Beta (95%CI) hours	P-value	Beta (95%CI) hours	P-value	
tPNa	-0.593 (-1.438, 0.252)	0.167	-0.404 (-1.422, 0.614)	0.433	-0.316 (-0.981, 0.349)	0.348	-0.628 (-1.401, 0.144)	0.110	448
tPNf	-0.737 (-1.588, 0.114)	0.089	-1.065 (-2.082, -0.047)	**0.040**	-0.127 (-0.984, 0.730)	0.770	-0.914 (-1.940, 0.111)	0.080	23
t2	-0.630 (-1.533, 0.273)	0.171	-1.311 (-2.394, -0.227)	**0.018**	-0.050 (-0.955, 0.856)	0.914	-1.101 (-2.195, -0.008)	**0.048**	3
t3	-0.395 (-1.583, 0.794)	0.514	-0.852 (-2.278, 0.573)	0.240	0.352 (-0.876, 1.580)	0.573	-0.228 (-1.711, 1.254)	0.762	5
t4	-0.768 (-1.969, 0.434)	0.210	-0.825 (-2.267, 0.617)	0.261	-0.239 (-1.523, 1.044)	0.713	-0.418 (-1.966, 1.131)	0.596	8
t5	0.571 (-0.989, 2.131)	0.472	-0.626 (-3.501, 0.249)	0.089	1.331 (-0.319, 2.982)	0.113	-1.080 (-3.079, 0.918)	0.288	13
t6	0.510 (-1.039, 2.059)	0.517	-1.644 (-3.526, 0.237)	0.086	1.191 (-0.470, 2.852)	0.159	-1.446 (-3.479, 0.587)	0.162	33
t7	0.942 (-0.683, 2.566)	0.542	-0.378 (-3.353, 0.598)	0.171	1.672 (-2.996, 1.264)	0.060	-0.866 (-2.996, 1.264)	0.424	65
t8	1.744 (0.087, 3.401)	**0.039**	-0.994 (-2.977, 0.989)	0.324	2.541 (0.774, 4.308)	**0.005**	-0.235 (-2.367, 1.896)	0.828	151

Adjusted for maternal age, fertilisation method, type of ovarian stimulation and paternal BMI and age. CI, confidence interval. Significant differences are depicted in bold.

Pre- and post-transfer clinical treatment outcomes

Crude linear regression analysis was applied to investigate associations between maternal BMI and the tertiary outcomes. The association between maternal BMI and total number of fertilised oocytes showed that for every point increase in BMI, the total number of fertilised oocytes per patient increased 0.024 (95%CI -0.075, 0.124, p=0.630), yet this was not significant (**Table 6**). Maternal BMI was also not significantly associated with other pre- or post-transfer clinical treatment outcomes, such as implantation rate (odds ratio 0.994 (95%CI 0.936, 1.054), p=0.994).

Table 6. The impact of maternal BMI on IVF/ICSI treatment outcome parameters.

	Crude		Adjusted	
Pre-transfer	**Beta (95%CI)**	**P-value**	**Beta (95%CI)**	**P-value**
Total fertilised oocytes	0.024 (-0.075, 0.124)	0.630	0.031 (-0.067, 0.129)	0.532
Fertilisation rate	-0.003 (-0.009, 0.003)	0.329	-0.003 (-0.009, 0.003)	0.303
Total usable embryos	0.018 (-0.049, 0.085)	0.605	0.021 (-0.046, 0.088)	0.536
Usage rate	0.000 (-0.007, 0.007)	0.913	0.000 (-0.007, 0.007)	0.928
Post-transfer	**OR (95%CI)**	**P-value**	**OR (95%CI)**	**P-value**
Positive β-hCG-test n=106	0.994 (0.936, 1.054)	0.994	0.997 (0.938, 1.060)	0.930
Gestational sac n=97	0.997 (0.939, 1.059)	0.923	1.000 (0.940, 1.064)	0.998
Fetal heartbeat n=90	0.985 (0.927, 1.047)	0.630	0.986 (0.926, 1.050)	0.663
Live born[1] n=61	0.998 (0.92, 1.069)	0.949	1.000 (0.932, 1.073)	0.992
Cumulative pregnancy[2] n=132	1.036 (0.977, 1.097)	0.238	1.044 (0.983, 1.109)	0.163

Adjusted for maternal age and type of ovarian stimulation. CI, confidence interval. OR, odds ratio. 1) missing n=22 2) missing n=8.

Post-hoc analyses of pre- and post-transfer clinical treatment outcomes of overweight and obese women as separate groups demonstrated no significant associations, when compared to normal weight women (**Table 7**).

Table 7A. Post-transfer treatment outcome parameters per BMI category.

	Normal weight women n=143		Overweight women n=79		Obese women n=46		
	N	%	N	%	N	%	P-value
Positive β-hCG-test	70	43.8%	43	49.4%	20	35.1%	0.237
Gestational sac	62	38.8%	41	47.1%	18	31.6%	0.163
Fetal heartbeat	58	36.3%	36	41.4%	17	29.8%	0.369
Live born	36	25.2%	28	34.1%	12	23.1%	0.257

Table 7B. Differences in treatment outcome parameters for overweight and obese women, compared to normal weight women.

	Crude				Adjusted			
	Overweight		Obese		Overweight		Obese	
Pre-transfer	**Beta (95%CI)**	**P-value**	**Beta (95%CI)**	**P-value**	**Beta (95%CI)**	**P-value**	**Beta (95%CI)**	**P-value**
Total fertilised oocytes	0.541 (-0.420, 1.501)	0.269	0.183 (-0.969, 1.335)	0.755	0.428 (-0.524, 1.379)	0.377	0.315 (-0.824, 1.455)	0.586
Fertilisation rate	-0.019 (-0.079, 0.041)	0.542	-0.025 (-0.097, 0.047)	0.491	-0.015 (-0.076, 0.045)	0.617	-0.029 (-0.101, 0.044)	0.434
Total usable embryos	0.351 (-0.298, 1.000)	0.288	0.097 (-0.681, 0.876)	0.805	0.292 (-0.357, 0.940)	0.377	0.164 (-0.612, 0.941)	0.677
Usage rate	-0.029 (-0.097, 0.038)	0.394	0.012 (-0.070, 0.093)	0.777	-0.026 (-0.095, 0.042)	0.446	0.010 (-0.072, 0.092)	0.815
Post-transfer	**OR (95%CI)**	**P-value**	**OR (95%CI)**	**P-value**	**OR (95%CI)**	**P-value**	**OR (95%CI)**	**P-value**
Positive β-hCG-test	1.349 (0.750, 2.428)	0.318	0.843 (0.420, 1.693)	0.632	1.296 (0.705, 2.380)	0.404	0.895 (0.435, 1.842)	0.763
Gestational sac	1.459 (0.807, 2.640)	0.211	0.931 (0.459, 1.888)	0.843	1.392 (0.754, 2.752)	0.291	0.986 (0.473, 2.053)	0.970
Fetal heartbeat	1.191 (0.654, 2.170)	0.567	0.872 (0.426, 1.785)	0.709	1.114 (0.600, 2.068)	0.733	0.908 (0.433, 1.902)	0.798
Live born	1.303 (0.665, 2.552)	0.440	1.051 (0.469, 2.354)	0.904	1.254 (0.632, 2.491)	0.517	1.125 (0.494, 2.560)	0.780
Cumulative pregnancy[2]	1.446 (0.823, 2.540)	0.199	1.229 (0.663, 2.545)	0.446	1.304 (0.723, 2.350)	0.378	1.510 (0.742, 3.074)	0.256

Adjusted for maternal age and type of ovarian stimulation. CI, confidence interval. OR, odds ratio. 1) missing n=22 2) missing n=8.

DISCUSSION

Summary of findings

This study aimed to investigate the hypothesis that an elevated BMI in women undergoing IVF/ICSI treatment has a detrimental impact on 1) preimplantation morphokinetic parameters until day 3 of development, 2) predicted implantation potential and 3) pre- and post-transfer clinical treatment outcomes. We observed that a higher maternal BMI is associated with a faster progression through the cleavage stages. No significant association of maternal BMI with predicted implantation potential, as assessed by the KIDScore algorithm, was shown. However, in embryos of couples with female or combined factor subfertility, maternal BMI was associated with faster early embryonic development and lower predicted implantation potential. In addition, no significant associations were shown between maternal BMI and the tertiary clinical treatment outcomes.

When maternal BMI was divided into categories, we observed delayed reaching of the 8-cell stage in embryos of overweight women, whereas embryos of obese women reach the 2-cell stage faster than embryos of normal weight women. Morphokinetic embryonic quality and clinical treatment outcomes were comparable between the three groups.

Interpretation

In the first study investigating the impact of maternal BMI on morphokinetic parameters, embryos of normal weight and obese infertile donors developed comparably (29). However, recently a delay in late cleavage divisions (t5, t8) was shown for embryos of overweight and obese women, which is (partially) in contrast to our findings (28). This study is not directly comparable to ours, as ICSI cycles were studied exclusively, whereas we studied both IVF and ICSI cycles. This may have direct implications, by differences in fertilisation techniques, as well as indirect, by differences in study population. In contrast to the study of Bartolacci et al., Leary et al. reported that embryos of overweight and obese women reach the morula stage, and subsequently the blastocyst stage, faster than embryos of normal weight women, although these embryos also have a higher rate of cleavage-stage arrest (35). Although it is beyond the scope of the current study, the impact of maternal BMI on blastocyst formation rate, an important predictor for implantation, remains inconclusive. Some report no impact or negative impact of high maternal BMI on blastocyst formation, whereas a recent large study reports a higher blastocyst formation rate for obese women (35, 37-41).

It is hypothesised that maternal adiposity may have an effect prior to fertilisation. The altered metabolic environment, as a result of an imbalanced diet and chronic excessive oxidative stress, contributes to an abnormal follicular microenvironment (20, 42). This aberrant microenvironment can derange several pathways including the one-carbon metabolism, which is important for numerous processes involved in reproduction, such as protein and DNA synthesis and redox regulation (43). This hypothesis is supported by mouse studies

showing an effect of obesity on oocyte polarisation, reactive oxygen species levels and DNA methylation, including methylation of metabolism-related genes, such as the leptin promotor region (44, 45). In humans, it has been demonstrated that rising BMI affects regulation of oocyte RNA expression and oocyte metabolism (35, 46, 47). Furthermore, the maternally-inherited genome passively demethylates with each cell-division, reaching the lowest level at the blastocyst stage, whereas the paternally-inherited genome actively demethylates within eight hours after fertilisation (48-50). Similarly, studies of human preimplantation embryos show that oocyte molecular programs are gradually degraded during the first three days after fertilisation and those of the embryo genome are activated, culminating between the 4- and 8-cell stage (51-53). This suggests that early preimplantation embryonic development is primarily driven by the maternal (epi-)genome.

A common cause of female subfertility is polycystic ovarian syndrome (PCOS), which is associated with obesity. PCOS is characterised by a combination of polycystic ovaries, hyperandrogenism and anovulation (54, 55). Research in women with PCOS undergoing IVF/ICSI shows impaired developmental competence of oocytes, yet preimplantation embryonic development is unaffected (56-59). In our study, sub-analyses of embryos of women with PCOS demonstrated no significant impact of BMI on morphokinetic parameters (data not shown).

Interestingly, the differences in individual morphokinetic parameters did not translate into differences in the KIDScore distribution. Although the KIDScore is based on only a limited number of parameters, the impact of maternal BMI on these parameters may be too small to induce a shift in the distribution of KIDScores. In embryos of couples with female or combined factor subfertility, however, we found a larger impact of BMI on individual morphokinetic parameters, which may explain the significant negative impact of maternal BMI on the KIDScore. As this is the first study to investigate the impact of maternal BMI on a morphokinetic quality score, comparison to other studies is limited. The KIDScore is a widely applicable morphokinetic (de)selection tool, as it ranks embryo's according to their implantation potential, regardless of the fertilisation technique used and culture conditions applied (25). Furthermore, it has a high blastulation predictability and performs superior to conventional morphology evaluation for predicting live births, when applied to day 3 embryos (60). However, morphokinetic based embryo selection may not be accessible for all fertility clinics, as time-lapse imaging is a relatively expensive technique when compared to conventional culture. In line with this, there are several studies that have investigated the impact of maternal BMI on conventional morphological quality. Although these studies differ in terms of parameters of morphological quality and statistical methods, only one demonstrated an impact of maternal BMI on embryo morphology, suggesting that the impact of maternal BMI on embryonic quality is relatively small (18, 21, 28, 61, 62).

As a tertiary outcome we have addressed the impact of maternal BMI on clinical treatment outcomes in our dataset and found no significant associations. It is very likely that the absence of significant findings can be explained by a lack of power. Yet, the detrimental impact of maternal BMI on success rates of IVF/ICSI treatment has been widely shown in other studies. A recent meta-analysis of over 600,000 women reported a 15% smaller chance of a live birth after IVF/ICSI treatment for obese women compared to normal weight women (63). Moreover, an additional factor in post-transfer outcomes is the uterine environment. A large retrospective study of over 9,500 normal weight oocyte donors reported lower success rates for obese recipients than for normal weight recipients (64). Although the exact mechanisms by which obesity alters endometrial receptivity are poorly understood, it is suggested that decidualisation is impaired by genetic dysregulation (65-67).

Strengths and limitations

By applying a standardised method to measure BMI prior to IVF/ICSI treatment, instead of relying on self-reported data, we reduced the risk of response bias. Moreover, BMI was also categorised according to the World Health Organization classification to facilitate comparison between studies. Statistical strengths are the application of linear mixed model analyses, which takes the clustering-effect of multiple embryos from one women into account, and adjustments for important treatment factors and paternal factors such as age and BMI, so that maternal effects could be studied independently. Another strength is the use of the KIDScore to evaluate embryonic morphokinetic quality at day 3 after fertilisation. This deselection tool is universally applicable and has area under the curve of 0.65 for prediction of implantation, which can be considered as a fair predictor (25).

The main limitation of our study is that we have only data until day 3 of development and not until the blastocyst stage, as this is associated with higher rates of pregnancy and live birth (68). This study was conducted in a time in which fresh transfer of cleavage embryos was routine care in most IVF clinics, including ours, but future research should include embryonic development until day 5. Also, due to the inclusion of IVF treatments, the moment of pronuclear appearance could not be observed in these cases. Although the diverse study population increases the generalizability of our results, it can also be considered a weakness. As it included both IVF and ICSI treatments and different stimulation protocols, it is a source of possible bias and may elicit divergent results. Furthermore, it is standard care at our clinic to only perform IVF/ICSI treatment in women with a BMI <34 kg/m^2, as IVF/ICSI treatment in women with a higher BMI is rarely feasible and associated with increased pregnancy complications (69). Nonetheless, this practice induces a selection bias for this study. Also, our study population did not comprise any women with underweight. This limited the possibilities to investigate the impact of the full range of maternal BMI. Finally, this study was performed at a tertiary university based hospital. Although not all subfertile couples were in need of tertiary referral or care, our results cannot be automatically extrapolated

to the general subfertile population, which may have consequences for the external validity of this study.

Conclusions

In this study we show that maternal BMI is positively associated with faster progression through the pronuclear and early cleavage stages and negatively with embryo implantation potential. So far, and very likely due to lack of power, no associations between maternal BMI and clinical treatment outcomes were observed.

Overweight and obesity are complex diseases and often the result of the interplay between nutrition, lifestyle and genetics. Future research is needed to elucidate the pathophysiological processes involved in the effects of maternal BMI on preimplantation development. Possible explanations might be found in alterations in oocyte quality, DNA damage and decreased cytoplasmic quality, as we observed an impact of maternal BMI on embryo quality in couples with female or combined factor subfertility. In addition, potential metabolic alterations underlying the observed differences in preimplantation development may also have consequences for post-implantation development. Although not demonstrated in this study, the negative effect of increased BMI on ART treatment outcomes has been widely reported. Moreover, maternal overweight and obesity have serious implications for pregnancy outcome and offspring health. Therefore, it is recommended to optimise lifestyle to achieve a healthy weight prior to IVF/ICSI treatment, for example by effective eHealth coaching programs (70, 71). A healthy weight maximises the general efficiency of the treatment and minimises alterations in the (early) development of the future generation.

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Supplemental material

Supplemental table 1. Morpokinetic parameters of the KIDScore algorithm.

Morphokinetic parameter
Number pronuclei equals 2 at the 1-cell stage
Time from insemination to pronuclear fading (tPNf)
Time from insemination to the 2-cell stage
Time from insemination to the 3-cell stage
Time from insemination to the 5-cell stage
Number of cells 66 hours after fertilisation

Supplemental table 2. Female type of subfertility, stratified for maternal BMI.

	Normal weight N = 72	**Overweight N = 39**	**Obese N = 21**	
	N (%)	**N (%)**	**N (%)**	**P-value**
Uterine factor	4 (5.6)	0 (0.0)	0 (0.0)	0.179
Tubal factor	14 (19.4)	7 (17.9)	4 (19.0)	0.982
Endometriosis	22 (30.6)	9 (23.1)	8 (38.1)	0.459
PCOS	26 (36.1)	18 (46.2)	9 (42.9)	0.566
WHO-2 non-PCOS[a]	9 (12.5)	3 (7.7)	0 (0.0)	0.201
WHO-3[a]	2 (2.8)	1 (2.6)	1 (4.8)	0.895
Other[b]	0 (0.0)	2 (5.3)	0 (0.0)	NA

The sum of diagnoses is higher than the sum of participants, as some women received multiple diagnoses. PCOS. Polycystic Ovarian Syndrome. WHO, World Health Organisation.
[a] Stratified according to the WHO classification of anovulation (72).
[b] Cases included hyperprolactinemia, and ovulation disorder, not specified according to the WHO classification.

4

Periconceptional maternal body mass index and the impact on post-implantation (sex-specific) embryonic growth and morphological development

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ABSTRACT

Objective: Women with obesity have an increased risk of pregnancy complications. Although complications generally present in the second and third trimester of pregnancy, most of them develop in the periconception period. Moreover, fetal sex also impacts pregnancy course and outcome. Therefore, our aim is to study (sex-specific) associations between periconceptional maternal body mass index (BMI) and embryonic growth and morphological development.

Methods: A total of 884 women with singleton pregnancies were selected from the Rotterdam Periconception Cohort, comprising 15 women with underweight, 483 with normal weight, 231 with overweight and 155 with obesity. Longitudinal three-dimensional ultrasound examinations were performed at 7, 9 and 11 weeks of gestation for offline measurements of crown-rump length (CRL), embryonic volume (EV), and Carnegie stages. Analyses were adjusted for maternal age, parity, ethnicity, education and periconceptional lifestyle.

Results: A negative trend was observed for embryos of women with obesity (β_{EV} -0.03, p=0.086), whereas embryonic growth and developmental trajectories in women with overweight were comparable to those with normal weight. Maternal underweight was associated with faster morphological development ($\beta_{Carnegie}$ 0.78, p=0.004). After stratification for fetal sex, it was demonstrated that female embryos of underweight women grow and morphologically develop faster than those of normal weight women (β_{EV} 0.13, p=0.008; $\beta_{Carnegie}$ 1.39, p<0.001), whereas female embryos of women with obesity grow slower (β_{EV} -0.05, p=0.027).

Conclusion: We found that periconceptional maternal underweight is associated with faster embryonic growth, especially in females. In contrast, female embryos of women with obesity grow slower than female embryos of women with normal weight. This may be the result of altered female adaptation to the postnatal environment. Future research should focus on strategies for optimizing preconceptional maternal weight, to reduce BMI-related pregnancy complications and improve the health of future generations.

INTRODUCTION

Over the past few decades the prevalence of obesity has increased rapidly across every age, sex, race and income level (1). Although the etiology of obesity is highly complex and multifactorial, excessive energy consumption and a sedentary lifestyle play important roles in the pathogenesis (2). The abundance of stored fat leads to a dysregulation of lipid and glucose metabolism, as well as to increased levels of cytokines, secreted by excess adipocytes (3). As a result, the majority of physiological processes is chronically disrupted on endocrine, immune and vascular levels, which explains why obesity is associated with numerous non-communicable diseases, such as diabetes mellitus type 2, hypertension and cancer. According to the World Health Organization (WHO), obesity is a major emerging preventable public health issue of a similar proportion as undernutrition (4).

This epidemic of obesity also includes women of reproductive age. Women with obesity of reproductive age have an additional burden, as obesity can also disrupt physiological processes involved in fertility and pregnancy (5, 6). Altered gonadotrophin and steroid hormone levels contribute to anovulation, but even regularly ovulating women with an elevated body mass index (BMI) have reduced conception rates (7). Once women with obesity do conceive, pregnancies are at increased risk for both complications affecting the mother, such as pregnancy induced hypertension, pre-eclampsia and gestational diabetes, as well as complications affecting the fetus, such as macrosomia, birth defects and stillbirth (5, 8). Interestingly, also fetal sex has an impact on pregnancy course and outcome. Male fetuses are larger at birth and may be at higher risk for developing pregnancy complications (9, 10).

However, knowledge is scarce about when in pregnancy the pathophysiological mechanisms underlying these increased risks arise. We hypothesise that maternal obesity-related pregnancy complications have a strong origin in the periconception period, defined as 14 weeks prior until 10 weeks after conception (11). It is during this period, that crucial processes such as fertilisation, embryogenesis, placentation and adaptation to pregnancy take place. Moreover, these processes take place in the maternal body serving as the direct environment. Studies have shown that a suboptimal maternal environment can have serious consequences for both pregnancy course as well as offspring health later in life (12, 13).

The majority of studies regarding maternal periconceptional BMI and first-trimester outcomes focused on the risk of miscarriage and congenital malformations, and show that this is increased in women with overweight or obesity (14, 15). Less is known about the effect of maternal BMI on first-trimester growth and development. The few studies that have been performed were based on measurements at the end of the first-trimester, thereby neglecting a large part of the periconception period (16, 17).

The introduction of three-dimensional (3D) ultrasound techniques, in combination with virtual reality (VR), allows in-depth perception and assessment of novel volumetric measurements of the embryo in the first-trimester as early as 7 weeks of gestation. This approach provides the opportunity to study embryonic growth and development by precise measurements of CRL, embryonic volume (EV) and even morphological development, defined by the Carnegie stages (18-20).

From this background, our main aim is to investigate associations between periconceptional maternal BMI and (sex-specific) first-trimester embryonic growth and morphological developmental trajectories using state-of-the-art imaging techniques. The secondary aim is to study the impact of maternal BMI on second-trimester fetal size and neonatal outcomes.

METHODS

Study population

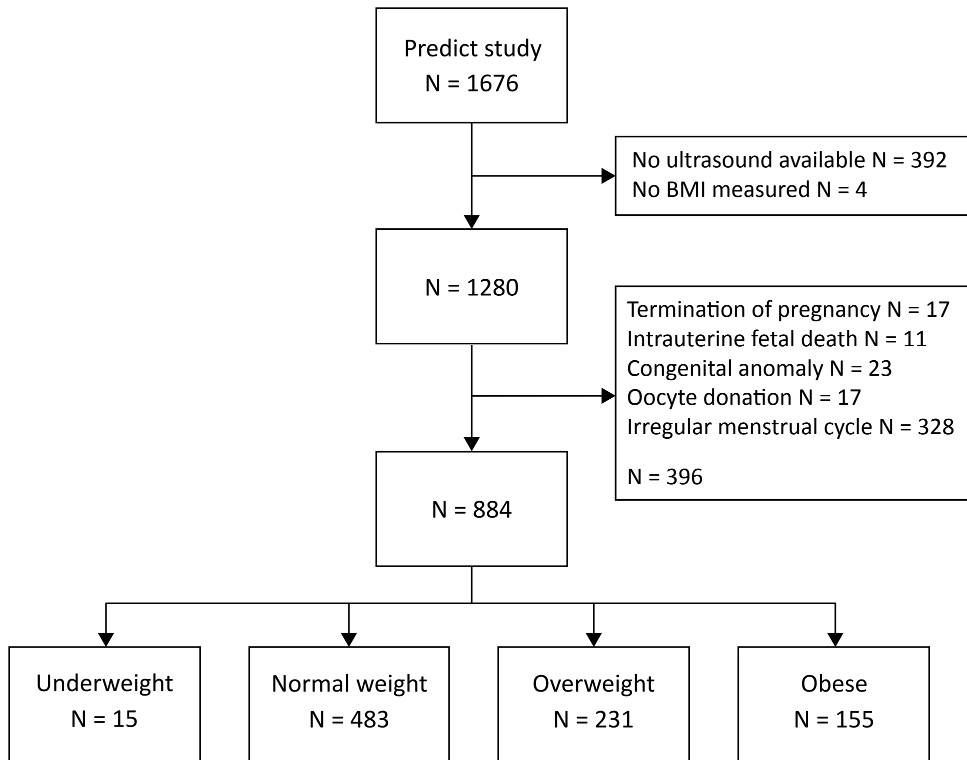
The data used for this study was collected as part of the Rotterdam Periconception Cohort. This ongoing prospective tertiary hospital-based cohort is conducted since November 2010 at the outpatient clinic of the Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Center, Rotterdam, The Netherlands (21). Women who were at least 18 years of age, with an ongoing singleton pregnancy <10 weeks of gestational age (GA) were eligible for participation.

Women with a strict regular cycle between 25-31 days, who conceived naturally and women who conceived after *in vitro* fertilisation (IVF) treatment were selected for this study. Women pregnant after oocyte donation, pregnancies resulting in a miscarriage, fetal death, fetuses or neonates with congenital malformations, and terminated pregnancies were excluded (**Figure 1**).

Ethical approval

This study was approved by the local Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center, Rotterdam, The Netherlands (MEC-2004-277). Prior to participation, all participants provided written informed consent.

Figure 1. Flowchart of the study population.



4

First-trimester ultrasound data and measurements

In the pilot study, between November 2010 and December 2012, women received weekly transvaginal 3D ultrasound scans from enrollment up to the 13th week of pregnancy. Thereafter, ultrasound scans were performed only in week 7, 9, and 11 of gestation, as this was sufficient to accurately model growth (21, 22). Trained sonographers performed ultrasound examinations with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D-View software (General Electrics Medical Systems, Zipf, Austria). To optimally use the third dimension, 3D ultrasound data were transferred afterwards to the Barco I-Space (a Cave Automatic Virtual Environment–like virtual reality (VR) system) (23). Next, an interactive VR hologram was created using V-scope volume rendering software, which allowed true depth perception.

The CRL was measured three times per ultrasound examination by using a tracing application, and the mean of these measurements was used for analyses (19). Embryonic volume (EV) was measured (semi-)automatically, using differences in gray scales. EV was measured once per ultrasound examination conform the technique previously described (18). Stages of morphological development were assessed by applying the Carnegie criteria

for internal and external morphological characteristics (24). The accuracy and reliability of these measurements and classification have been previously described as excellent (18-20).

Study parameters

At enrollment, standardised anthropometric measurements were performed, including height with 0.1 cm accuracy and weight with 0.1 kg accuracy (anthropometric rod and weighing scale; SECA, Hamburg, Germany). Women completed questionnaires regarding general characteristics and periconceptional nutrition and lifestyle. Extracted data included age, obstetric history, geographical background, educational level and use of cigarettes, alcohol and folic acid supplements. All data were verified by research administration at study entry. Geographical background was categorised into Western or non-Western and educational level into low, medium and high, according to the definition of Statistic Netherlands. Use of cigarettes and alcohol was defined as any use during the periconception period, defined as 14 prior to 8 weeks after conception (11). Periconceptional daily use of folic acid supplements was defined as adequate, whereas no or post-conception initiation was defined as inadequate.

GA was based on the reported last menstrual period for naturally conceived pregnancies. For pregnancies conceived after IVF, GA was based on the conception date. This is the oocyte retrieval date minus 14 days for pregnancies after fresh embryo transfer (ET) and the ET date minus 19 days for pregnancies after frozen-thawed ET.

Second-trimester ultrasound data were retrieved from the anomaly scan, which is performed between 18 and 22 weeks GA. Extracted data included estimated fetal weight (EFW), head circumference (HC), biparietal diameters (BPD), abdominal circumference (AC) and femur length (FL).

Neonatal outcomes were retrieved from questionnaires and verified by medical records. Extracted data included sex, GA, and weight at birth. Small for gestational age (SGA) was classified as birthweight <10th percentile and large for gestational age (LGA) was classified as birthweight >90th percentile. Preterm birth (PTB) was defined as delivery <37+0 GA.

Statistical analyses

The study population was stratified based on the BMI classification of the WHO, resulting in four groups of women; 1) underweight (BMI <18.5 kg/m^2), 2) normal weight (BMI 18.5-24.9 kg/m^2), 3) overweight (BMI 25-29.9 kg/m^2) and 4) obese (≥30 kg/m^2) . Baseline characteristics were compared using Kruskall-Wallis tests for continuous variables and X^2 for categorical variables.

Associations between maternal BMI categories and embryonic growth and morphological development were studied using linear mixed models, for which pregnancies of women with a normal BMI were used as reference category. To obtain linearity, CRL was root-transformed and EV was cube root-transformed. In model 1 we adjusted for GA only. Model 2 was additionally adjusted for maternal age, parity, conception method, geographical background, educational level and periconceptional use of cigarettes, alcohol and folic acid supplements.

Second-trimester EFW and individual growth parameters were expressed as percentiles, since GA and fetal size are highly correlated and based on population-based fetal growth charts (25, 26). Differences in percentiles between the four groups were studied using Kruskall-Wallis tests.

Birthweight was also expressed in percentiles based on Dutch reference curves, which take fetal sex and GA at birth into account (27). Differences in birthweight, birthweight percentiles and GA at birth between the four groups were studied using Kruskall-Wallis tests. Differences in the prevalence of SGA, LGA and PTB were assessed by X^2. Sub-analyses were performed after stratification for fetal sex. All analyses were performed using SPSS 25.0 (IBM SPSS Statistics, Armonk, NY). P-values ≤0.05 were considered significant.

RESULTS

Study population

The study population comprised 884 pregnant women; 15 with underweight, 483 of normal weight, 231 with overweight and 155 with obesity (**Figure 1**). Underweight women were significantly younger than those with normal weight and overweight (31.0, 32.6 and 32.6 years, respectively, p=0.030) and had a lower waist-hip ratio than those with overweight and obesity (0.77, 0.87, 0.88, respectively, p<0.001) (**Table 1**). Normal weight women were slightly higher educated when compared to women with underweight, overweight and obesity (p<0.001). Women with obesity were less often from a Western background than normal weight women (74.2% vs. 87.2%, p=0.002), and reported a lower periconceptional use of folic acid supplements (76.2% vs. 86.9% p=0.013) and alcohol (21.7% vs. 33.8%, p=0.031). These women also conceived less often after IVF/ICSI treatment than women with underweight and overweight (39.4% vs. 73.3% and 49.8%, p=0.036).

Table 1. Baseline characteristics of the Rotterdam Periconception cohort population, stratified for maternal body mass index categories.

	Underweight N=15	Normal weight N=483	Overweight N=231	Obesity N=155		
	Median / N (IQR /%)	Median / N (IQR /%)	Median / N (IQR /%)	Median / N (IQR /%)	P-value	Missing
Age, years	31.0 (25.5-34.0)	32.6 (29.9-35.5)	32.6 (29.4-36.1)	31.8 (27.7-35.5)	**0.030**[c,d,f,h]	
Waist hip ratio	0.77 (0.76-0.84)	0.83 (0.79-0.88)	0.87 (0.80-0.92)	0.88 (0.83-0.94)	**<0.001**[d,e,f,g]	37
Nulliparous	11 (73.3)	271 (56.1)	111 (48.1)	76 (49.0)	0.055	
IVF/ICSI, yes	11 (73.3)	222 (46.0)	115 (49.8)	61 (39.4)	**0.036**[e,h]	
Western ethnicity	12 (80.0)	421 (87.2)	188 (81.4)	115 (74.2)	**0.002**[f,g]	
Education					**<0.001**[c,f,g]	21
Low	3 (20.0)	23 (4.8)	19 (8.6)	18 (12.1)		
Middle	4 (26.7)	139 (29.1)	85 (38.3)	68 (45.6)		
High	8 (53.3)	315 (66.0)	118 (53.2)	63 (42.3)		
Alcohol, yes[a]	3 (20.0)	162 (33.8)	62 (30.0)	33 (21.7)	**0.031**[g]	11
Smoking, yes[a]	3 (20.0)	62 (12.9)	41 (18.1)	21 (13.8)	0.299	11
Folic acid, yes[b]	13 (86.7)	417 (86.9)	185 (81.5)	115 (76.2)	**0.013**[g]	11
Fetal sex, male	5 (33.3)	248 (52.1)	116 (51.1)	73 (47.4)	0.412	12

IQR, interquartile range.
a. Any use during the 14 weeks prior to 10 weeks after conception.
b. Daily use during the 14 weeks prior to 10 weeks after conception.
c. Significantly different between women with underweight and normal weight.
d. Significantly different between women with underweight and overweight.
e. Significantly different between women with underweight and obesity.
f. Significantly different between women with normal weight and overweight.
g. Significantly different between women with normal weight and obesity.
h. Significantly different between women with overweight and obesity.

First-trimester embryonic growth and morphological development

In the total study population it was found that embryos of women with obesity are significantly smaller regarding EV trajectories than embryos of women of normal weight in model 1 (β -0.04 (95%CI -0.07, -0.01) *p*=0.017) (**Table 2**). In model 2, we observed a similar trend (β -0.03 (95%CI -0.06, 0.00) *p*=0.086).

After retransformation, the EV trajectories of embryos of underweight women is on average 0.62 cm^3 larger (7.7%) at 11 weeks GA than embryos of normal weight women, whereas for embryos of women with overweight this is 0.01 cm^3 smaller (-0.2%) and for embryo of women with obesity this is 0.38 cm^3 (-4.7%) (**Figure 2**). Embryos of underweight women proceeded faster through the Carnegie stages than embryos of normal weight women in both models ($\beta_{model\ 1}$ 0.73 (95%CI 0.20, 1.25) *p*=0.006; $\beta_{model\ 2}$ 0.78 (95%CI 0.25, 1.31) *p*=0.004). CRL trajectories were comparable between the four groups.

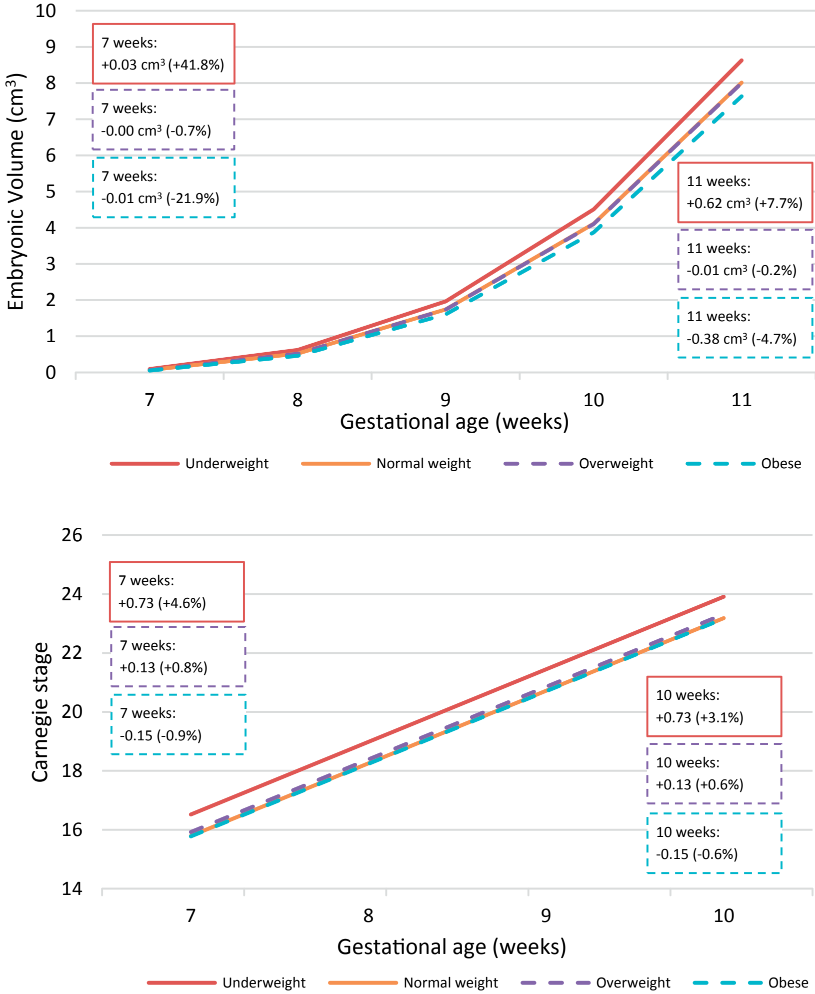
Table 2. First-trimester embryonic growth and morphological development stratified for maternal body mass index categories, in the total population and after stratification for fetal sex.

		Model 1						Model 2					
		CRL		EV		Carnegie		CRL		EV		Carnegie	
		Beta (95%CI) √mm)	P-value	Beta (95%CI) ∛cm3	P-value	Beta (95%CI)	P-value	Beta (95%CI) √mm)	P-value	Beta (95%CI) ∛cm3	P-value	Beta (95%CI)	P- value
Total population	Underweight n=15	0.02 (-0.14, 0.18)	0.797	0.05 (-0.03, 0.13)	0.196	0.73 (0.20, 1.25)	**0.006**	0.04 (-0.12, 0.20)	0.632	0.06 (-0.02, 0.14)	0.109	0.79 (0.25, 1.31)	**0.004**
	Normal weight n=483	ref		ref		ref		ref		ref		ref	
	Overweight n=231	0.00 (-0.04, 0.05)	0.988	-0.00 (-0.03, 0.02)	0.792	0.13 (-0.04, 0.30)	0.121	0.00 (-0.05, 0.05)	0.984	-0.00 (-0.03, -0.02)	0.725	0.11 (-0.06, 0.28)	0.198
	Obesity n=155	-0.05 (-0.11, 0.01)	0.092	-0.04 (-0.07, -0.01)	**0.017**	-0.02 (-0.21, 0.18)	0.877	-0.04 (-0.09, 0.03)	0.268	-0.03 (-0.06, 0.00)	0.086	0.00 (-0.21, 0.21)	0.995
Female embryos	Underweight n=10	0.09 (-0.11, 0.28)	0.394	0.12 (0.02, 0.21)	**0.020**	1.31 (0.66, 1.95)	**<0.001**	0.12 (-0.08, 0.32)	0.232	0.13 (0.03, 0.23)	**0.008**	1.39 (0.74, 2.05)	**<0.001**
	Normal weight n=228	ref		ref		ref		ref		ref		ref	
	Overweight n=111	-0.03 (-0.10, 0.04)	0.405	-0.01 (-0.05, 0.02)	0.494	0.07 (-0.17, 0.31)	0.562	-0.03 (-0.10, 0.04)	0.425	-0.01 (-0.05, 0.02)	0.443	0.05 (-0.19, 0.30)	0.690
	Obesity n=81	-0.11 (-0.19, -0.03)	**0.010**	-0.06 (-0.10, -0.02)	**0.003**	-0.19 (-0.47, 0.09)	0.173	-0.08 (-0.16, 0.01)	0.088	-0.05 (-0.09, -0.01)	**0.027**	-0.13 (-0.42, 0.15)	0.359
Male embryos	Underweight n=5	-0.05 (-0.34, 0.25)	0.749	-0.03 (-0.18, 0.12)	0.715	0.02 (-0.94, 0.98)	0.966	-0.10 (-0.40, 0.19)	0.493	-0.07 (0.22, 0.08)	0.354	-0.08 (-1.06, 0.89)	0.867
	Normal weight n=248	ref		ref		ref		ref		ref		ref	
	Overweight n=116	0.03 (-0.04, 0.10)	0.436	0.01 (-0.03, 0.04)	0.703	0.17 (-0.09, 0.42)	0.192	0.02 (-0.05, 0.10)	0.551	0.00 (-0.04, 0.04)	0.971	0.12 (-0.14, 0.38)	0.376
	Obesity n=73	0.01 (-0.07, 0.10)	0.746	-0.01 (-0.05, 0.04)	0.769	0.16 (-0.15, 0.47)	0.320	0.02 (-0.7, 0.12)	0.630	-0.00 (-0.05, 0.04)	0.872	0.13 (-0.20, 0.45)	0.446

CRL, crown rump length. EV, embryonic volume.
Model 1: Adjusted for GA.
Model 2: Adjusted for GA, maternal age, parity, conception method, geographical background, level of education, periconceptional use of alcohol, cigarettes and folic acid supplements

4

Figure 2. Embryonic trajectories of embryonic volumes (EV) and Carnegie stages for maternal underweight (red), normal weight (yellow), overweight (purple) and obesity (blue).



Boxes show the relative and absolute differences at 7 and 10 or 11 weeks GA compared to pregnancies with maternal normal weight.

After stratification for fetal sex, it was observed in model 1 that female embryos of women with obesity have a smaller CRL and EV than those of women with normal weight (β_{CRL} -0.11 (95%CI -0.19, -0.03) *p*=0.010; β_{EV} -0.06 (95%CI -0.10, -0.02) *p*=0.003). For EV this association was significant in model 2 (β_{EV} -0.05 (95%CI -0.09, -0.01) *p*=0.027). In contrast, female embryos of underweight women were larger regarding EV ($\beta_{model\ 1}$ 0.12 (95%CI 0.02, 0.21) *p*=0.020; $\beta_{model\ 2}$ 0.13 (95%CI 0.03, 0.23) *p*=0.008) and proceeded faster through the Carnegie

stages ($\beta_{model\ 1}$ 1.31 (95%CI 0.66, 1.95) p<0.001; $\beta_{model\ 2}$ 1.39 (95%CI 0.74, 2.05) p<0.001). For male embryos, CRL, EV and Carnegie stages were comparable between the four groups.

Sub-analyses of first-trimester growth and morphological development after exclusion of pregnancies resulting in SGA, PTB or pre-eclampsia demonstrated an attenuated impact of BMI in the adjusted model (**Supplemental table 1**).

Second-trimester fetal size

In the total population, percentiles of EFW, HC, BPD, AC and FL were not significantly different between the fetuses of women in the four BMI categories (**Table 3, supplemental table 2**). Comparable results were observed after stratification for fetal sex (**Supplemental table 2**).

4

Neonatal outcomes

Neonates of underweight women showed a lower relative birthweight than neonates of women with overweight or obesity (26th, 49th and 52th percentile, respectively, p=0.003) and lower absolute birthweight than neonates of women with normal weight, overweight or obesity (3000, 3350, 3338 and 3400 grams, respectively, p=0.001) (**Table 3**). The prevalence of PTB was also higher in this group compared to neonates of women with normal weight, overweight or obesity (26.7%, 6.6%, 8.6% and 12.0%, respectively, p=0.012).

Table 3. Second trimester and neonatal outcomes for maternal body mass index categories.

	Underweight N=15	**Normal weight N=483**	**Overweight N=231**	**Obesity N=155**		
	Median / N (IQR /%)	**Median / N (IQR /%)**	**Median / N (IQR /%)**	**Median / N (IQR /%)**	**P-value**	**Missing**
EFW, percentiles	74.5 (56.0-83.9)	68.3 (45.4-86.2)	71.8 (45.6-89.0)	72.3 (44.1-90.6)	0.610	54
Birthweight, percentiles	26 (7-46)	44 (22-69)	49 (24-78)	52 (24-85)	**0.003**[b,c]	63
Birthweight, grams	3000 (2645-3310)	3350 (3050-3650)	3338 (2969-3716)	3400 (2950-3780)	**0.026**[c]	27
Gestational age, days	275 (258-279)	275 (268-282)	274 (266-281)	271 (264-278)	**0.001**[a,b,c]	30
SGA	5 (33.3)	66 (14.6)	28 (13.3)	17 (11.9)	0.147	63
LGA	0 (0)	33 (7.3)	23 (10.9)	32 (22.4)	**<0.001**[c,d,e]	63
Preterm birth	4 (26.7)	31 (6.6)	19 (8.6)	18 (12.0)	**0.012**[a,b,d]	30

IQR, interquartile range. EFW, estimated fetal weight. SGA, small for gestational age. LGA, large for gestational age.
a. Significantly different between women with underweight and normal weight.
b. Significantly different between women with underweight and overweight.
c. Significantly different between women with underweight and obesity.
d. Significantly different between women with normal weight and obesity.
e. Significantly different between women with overweight and obesity.

Neonates of women with obesity were born earlier and more often preterm than neonates of normal weight women (271 vs. 275 days, p=0.001, 12.0% vs. 6.6%, p=0.012, respectively) and were more often LGA compared to women with underweight, normal weight and overweight (22.4%, 0.0%, 7.3% and 10.9%, respectively, p<0.001).

DISCUSSION

In this study we investigated associations between periconceptional maternal BMI, divided into categories, and embryonic growth and morphological development, fetal size and neonatal outcomes. We show that embryos of underweight women grow and morphologically develop faster than embryos of normal weight women. A trend was observed for a slower growth in embryos of women with obesity. After stratification for fetal sex, we observed that female embryos of underweight women grow and develop faster than those of normal weight women, whereas female embryos of women with obesity grow slower. Interestingly, these effects were not observed in male embryos.

No impact of periconceptional maternal BMI was observed on second-trimester growth. Neonates of underweight women had a lower relative and absolute birthweight compared to neonates of normal weight women. The prevalence of PTB was higher in neonates of both women with underweight as well as those with obesity than in neonates of normal weight women, yet neonates of women were with obesity more often LGA whereas neonates of underweight women were more often SGA.

To our knowledge, we are the first to investigate the impact of periconceptional maternal BMI categories on first-trimester embryonic growth and morphological development in a longitudinal fashion. Previous studies have investigated this association based on a single measurement of CRL. Thagaard et al. reported a negative association with obesity, whereas Gordon et al. showed no association (16, 17). In line with the results of Thagaard et al., we observed a trend towards a negative association between maternal obesity and embryonic growth. A possible explanation for the fact that we were unable to demonstrate a significant impact, is the lack of power, as the group of Thagaard et al. consisted of 1,400 women with obesity. A post-hoc power analysis demonstrated that we needed to include 261 women with obesity to demonstrate a significant difference (data not shown).

Slower first-trimester growth in pregnancies of women with obesity is not in line with perinatal outcomes, as these pregnancies have an increased risk of macrosomia and LGA (28). However, during the first-trimester, maternal-embryonic nutrient exchange is relatively limited, as it is prevented by trophoblast plugs until 10 to 11 weeks of gestation (29). It is only after gradual dissolution of these plugs that nutrient exchange is enabled. Therefore, it is hypothesise that obesity has an impact prior to maternal-fetal nutrient exchange, for example on a follicular level or on early maternal-embryo interaction in the oviduct. Animal studies show that obesity induces mitochondrial dysfunction and affects preimplantation embryo metabolism (30). Furthermore, it also reduces the levels of regulatory molecules in the reproductive tract involved in embryonic development, such as colony stimulating factor 2 (CSF2) (31).

Moreover, we also observed a positive association between maternal underweight and first-trimester morphological development. Underweight women often consume an insufficient amount of nutrients to optimally perform physiological processes, such as maintaining the one-carbon metabolism. This metabolism is essential for cellular physiology, as it is involved in biosynthesis, methylation and redox reactions (32). Lack of important nutrients, such as proteins and folate, can derange the one-carbon metabolism, with serious consequences. For example, genome-scale analyses of DNA methylation in adults, exposed to periconceptional famine six decades earlier, showed differentially methylated regions involved in positive regulation of growth and early developmental processes (33). This might also explain why prenatal exposure to undernutrition is associated with numerous non-communicable diseases, such as metabolic syndrome and vascular diseases (34).

Furthermore, the associations between maternal periconceptional BMI and first-trimester growth and morphological development was only demonstrated in female embryos. Sex differences in the rate of fetal growth and birthweight have long been recognised (35). However, recent findings show an impact of sex on embryonic development already prior to implantation, as male preimplantation embryos develop faster than female embryos (36, 37). A dominant concept in current literature is that differences in survival and pregnancy outcomes for fetal sex are the result of different growth strategies. It is hypothesised that females are more sensitive to signals from the maternal environment, such as obesity or underweight, leading to moderate changes in growth and a better adaptation to postnatal environment. Males on the other hand, have a more divergent growth curve, as they adapt poorly by inducing minimal changes in gene expression and biological processes when exposed to an adverse environment (38, 39). This hypothesis is supported by mouse studies demonstrating that maternal prenatal diet has a larger metabolic effect in male offspring than in female offspring, whereas in placentas prenatal maternal diet has an opposite effect (40, 41). Another mouse study also showed that changes in secretions of maternally derived CSF2 alter female development differently than male development in terms of growth and methylation (42). This may explain our observations, as CSF2 levels are affected by adiposity (31).

Our study did not demonstrate a significant association between maternal BMI categories and second-trimester fetal size. Research regarding this subject remains inconclusive, as some report a positive association and others report no association (43-47). It is suggested that differences in study design, population and statistical methods underlie these inconsistent results. However, it has been demonstrated that maternal obesity does not hamper accurate estimation of fetal weight (48).

The impact of maternal BMI on neonatal outcomes has been widely studied, with a focus on birthweight and preterm birth. An extensive meta-analysis of 45 studies shows that women with overweight or obesity have increased odds of a neonate born LGA ($OR_{overweight}$ 1.53;

$OR_{obesity}$ 2.08), whereas underweight women have an OR of 1.81 for a SGA neonate (28). Our results regarding neonatal outcomes are in line with these large studies. It is likely that the increased odds for SGA neonates in underweight women and LGA in women with overweight or obesity can be attributed to malnutrition or overnutrition in these women. Moreover, we found that pregnancies in women with underweight or obesity result more often in PTB. Although the increased risk for preterm birth in underweight women has been frequently reported in other large studies, the underlying mechanisms remain largely unknown (49-52). The increased risk for preterm birth in women with obesity is also observed in two large meta-analyses, both including over 1 million pregnancies (53, 54). Regarding preterm birth, it is suggested that elective preterm deliveries due to maternal or fetal risks, such as preeclampsia and gestational diabetes, underlie this increased risk (55, 56). This is confirmed in our data, as the incidence of preterm birth is no longer significantly different between the four BMI groups after exclusion of iatrogenic preterm births (data not shown). Moreover, our sub-analyses of first-trimester growth and morphological development after exclusion of pregnancies resulting in SGA, PTB or pre-eclampsia also suggest that especially these pregnancies are associated with a detrimental impact of maternal BMI. Adequate growth and development in the first-trimester is crucial for fetal, neonatal and postnatal health (57, 58). Our study is in line with the paradigm of Developmental Origins of Health and Disease (DOHaD), which posits that the environment during gestation, and potentially even preconceptionally, can have lifelong effects on offspring health (59).

A major strength of our study is the extensive collection of first-trimester longitudinal 3D ultrasound data, which enables innovative and precise measurements to accurately model growth. Also, by taking both growth as well as morphological development into account, we were able to thoroughly study the impact of maternal BMI. Furthermore, weight and length to calculate BMI were measured at enrolment by a trained research nurse, thereby reducing the risk of reporting bias. Moreover, by including data on second-trimester growth and pregnancy outcome we were able to study the impact of periconceptional maternal BMI over the complete course of pregnancy.

However, this study also has some limitations. Firstly, patients were recruited from a tertiary university hospital, which reduces the external validity of our results to the general population. Secondly, despite the adjustments for potential confounders in our analyses, residual confounding cannot be excluded, as this is inherent to the observational cohort design. Moreover, the possibility of overadjustment in the underweight group cannot be excluded. Finally, although we were able to demonstrate significant associations between maternal underweight and first-trimester growth and morphological development, it is recommended to interpret our results with caution, as it is based on a limited number of participants in this subgroup.

In conclusion, we demonstrate a trend towards a negative association of maternal obesity and first-trimester growth trajectories. We also show a positive association between maternal underweight and first-trimester embryonic growth and morphological development, especially in female embryos.

Despite the global epidemic of obesity, a considerable amount of people are still malnourished, in low and middle incomes countries but also in Western society. Our observations further underline the need to recognise and monitor these people, as the found effect estimates for underweight are more than twice as large as those for smoking (β_{EV} -0.047)(60). As a final statement, future research should focus on strategies for optimisation of maternal weight prior to pregnancy, so that the prevalence of BMI-related pregnancy complications reduces and the health of future generations improves.

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Supplemental material

Supplemental table 1. First-trimester embryonic growth and morphological development after excluding pregnancies resulting in SGA, PTB or pre-eclampsia for maternal body mass index categories; of the total population and after stratification for fetal sex.

		Model 1						Model 2					
		CRL		EV		Carnegie		CRL		EV		Carnegie	
		Beta (95%CI) √mm)	**P-value**	**Beta (95%CI) ∛cm3**	**P-value**	**Beta (95%CI)**	**P-value**	**Beta (95%CI) √mm)**	**P-value**	**Beta (95%CI) ∛cm3**	**P-value**	**Beta (95%CI)**	**P- value**
Total population	Underweight n=8	0.06 (-0.16, 0.28)	0.570	0.02 (-0.09, 0.13)	0.729	0.44 (-0.24, 1.12)	0.205	0.06 (-0.16, 0.28)	0.576	0.02 (-0.10, 0.13)	0.776	0.50 (-0.19, 1.19)	0.152
	Normal weight n=390	ref		ref		ref		ref		ref		ref	
	Overweight n=187	0.02 (-0.03, 0.07)	0.454	0.00 (-0.03, 0.03)	0.963	0.13 (-0.06, 0.32)	0.173	0.02 (-0.03. 0.08)	0.476	-0.00 (-0.03, -0.03)	0.944	0.10 (-0.09, 0.29)	0.295
	Obesity n=126	-0.04 (-0.10, 0.03)	0.244	-0.03 (-0.07, -0.00)	**0.042**	0.04 (-0.18, 0.26)	0.732	-0.02 (-0.09, 0.04)	0.486	-0.03 (-0.06, 0.01)	0.142	0.06 (-0.17, 0.28)	0.618
Female embryos	Underweight n=6	0.10 (-0.15, 0.35)	0.427	0.05 (-0.08, 0.18)	0.431	0.71 (-0.06, 1.48)	0.071	0.14 (-0.12, 0.40)	0.285	0.07 (-0.06, 0.21)	0.260	0.93 (0.13, 1.73)	**0.023**
	Normal weight n=187	ref		ref		ref		ref		ref		ref	
	Overweight n=84	-0.01 (-0.09, 0.07)	0.848	-0.01 (-0.05, 0.03)	0.701	0.05 (-0.21, 0.31)	0.708	-0.00 (-0.08, 0.08)	0.969	-0.00 (-0.05, 0.04)	0.853	0.03 (-0.24, 0.30)	0.826
	Obesity n=64	-0.09 (-0.18, -0.00)	**0.045**	-0.06 (-0.10, -0.01)	**0.015**	-0.15 (-0.45, 0.14)	0.312	-0.06 (-0.16, 0.03)	0.184	-0.05 (-0.09, 0.00)	0.069	-0.06 (-0.37, 0.25)	0.715
Male embryos	Underweight n=2	-0.04 (-0.47, 0.38)	0.836	-0.05 (-0.27, 0.16)	0.624	-0.19 (-1.52, 1.14)	0.781	-0.10 (-0.52, 0.32)	0.644	-0.10 (-0.31, 0.11)	0.349	-0.19 (-1.53, -1.16)	0.785
	Normal weight n=196	ref		ref		ref		ref		ref		ref	
	Overweight n=99	0.05 (0.03, 0.13)	0.248	0.01 (-0.03, 0.05)	0.652	0.18 (-0.10, 0.45)	0.216	-0.03 (-0.06, 0.10)	0.533	-0.00 (-0.04, 0.04)	0.926	0.08 (-0.20, 0.37)	0.559
	Obesity n=61	0.01 (-0.08, 0.11)	0.781	-0.01 (-0.06, 0.04)	0.719	0.22 (-0.13, 0.56)	0.213	0.01 (-0.09, 0.12)	0.789	-0.01 (-0.06, 0.05)	0.749	0.16 (-0.19, 0.52)	0.364

CRL, crown rump length. EV, embryonic volume.
Model 1: Adjusted for GA.
Model 2: Adjusted for GA, maternal age, parity, conception method, geographical background, level of education, periconceptional use of alcohol, cigarettes and folic acid supplements.

Supplemental table 2. Second trimester growth parameters stratified for maternal body mass index categories, in the total population and stratified for fetal sex.

		Underweight N=15	Normal weight N=483	Overweight N=231	Obesity N=155		
		Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	P-value	Missing
Total population	EFW	74.5 (56.0-83.9)	68.3 (45.4-86.2)	71.8 (45.6-89.0)	72.3 (44.1-90.6)	0.610	54
	HC	60.9 (35.9-76.5)	58.0 (35.2-76.3)	62.0 (35.9-77.5)	57.5 (33.1-76.1)	0.875	52
	BPD	57.0 (12.6-76.5)	46.7 (21.7-75.7)	50.4 (19.5-74.3)	43.4 (18.8-68.3)	0.707	64
	AC	71.5 (63.8-85.4)	76.9 (58.5-91.1)	78.1 (56.6-92.4)	78.9 (52.8-91.1)	0.979	53
	FL	79.3 (53.5-81.6)	61.3 (39.1-81.7)	67.5 (45.7-81.9)	69.6 (38.7-87.8)	0.124	53
Female fetuses	EFW	74.1 (55.7-77.0)	65.4 (41.7-84.1)	61.4 (40.0-84.4)	60.4 (36.7-84.5)	0.831	28
	HC	73.2 (76.7-23.7)	48.5 (29.1-68.2)	49.1 (26.3-67.8)	47.7 (22.8-68.0)	0.520	28
	BPD	67.9 (93.9-63.2)	36.7 (16.5-63.8)	35.6 (11.6-60.7)	28.2 (12.1-61.4)	0.284	35
	AC	71.0 (63.2-86.1)	71.8 (55.3-87.6)	70.5 (52.0-89.5)	69.6 (41.2-87.4)	0.610	28
	FL	67.6 (50.2-81.7)	61.4 (39.8-81.9)	61.1 (41.8-81.4)	68.6 (31.1-87.1)	0.861	28
Male fetuses	EFW	75.6 (52.6-88.3)	70.1 (49.1-90.4)	80.4 (54.6-93.1)	82.8 (54.1-92.8)	0.179	22
	HC	54.7 (25.4-79.8)	65.8 (45.3-82.6)	71.7 (47.3-84.1)	65.2 (81.0-33.7)	0.663	20
	BPD	49.8 (5.4-79.8)	60.1 (30.1-82.9)	65.0 (31.1-85.1)	58.6 (33.7-74.0)	0.533	25
	AC	72.0 (58.5-90.6)	79.9 (62.0-93.0)	83.9 (67.0-94.0)	87.4 (66.3-94.3)	0.482	21
	FL	80.1 (52.9-86.2)	63.4 (37.2-81.7)	68.0 (46.3-82.3)	71.6 (49.6-88.1)	0.106	21

IQR, interquartile range. EFW, estimated fetal weight. HC, head circumference. BPD, biparietal diameter. AC, abdominal circumference. FL, femur length.
EFW was calculated using the Hadlock formula: $\log(EFW) = 1.326 - 0.00326 \times AC \times FL + 0.0107 \times HC + 0.0438 \times AC + 0.158 \times FL$.

4

In vitro fertilisation treatment and development of the embryo and placenta

5

The impact of culture medium on morphokinetics of cleavage stage embryos: An observational study

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ABSTRACT

Purpose: To study the impact of culture media on preimplantation morphokinetics used for predicting clinical outcomes.

Methods: All IVF- and ICSI-cycles performed between 2012 and 2017 with time-lapse information available, were included. In November 2014, culture medium was changed from Vitrolife G-1 PLUS to SAGE 1-Step. Each embryo was retrospectively assigned a morphokinetic-based KIDScore for prediction of implantation. Clinical outcomes were retrieved from medical records. Linear mixed models were used to study differences in morphokinetic parameters, a proportional odds model for KIDScore ranking and logistic regression for differences in clinical outcomes. All analyses were adjusted for patient and treatment characteristics.

Results: In 253 (63.1%) cycles, embryos (n=671) were cultured in Vitrolife and in 148 (36.9%) cycles, embryos (n=517) were cultured in SAGE. All cleavage divisions occurred earlier for SAGE embryos than for Vitrolife embryos (2-cell: -2.28 (95%CI -3.66, -0.89), 3-cell: -2.34 (95%CI -4.00, -0.64), 4-cell: -2.41 (95%CI -4.11, -0.71), 5-cell: -2.54 (95%CI -4.90, -0.18), 6-cell: -3.58 (95%CI -6.08, -1.08), 7-cell: -5.62 (95%CI -8.80, -2.45) and 8-cell: -5.32 (95%CI -9.21, -1.42) hours, respectively). Significantly more embryos cultured in SAGE classified for the highest KIDScore compared to embryos cultured in Vitrolife ($p<0.001$). No differences were observed in clinical outcomes.

Conclusion: Our results demonstrate an impact of culture medium on preimplantation embryo developmental kinetics, which affects classification within the KIDScore algorithm, while pregnancy outcomes were comparable between the groups. This study underscores the need to include the type of culture medium in the development of morphokinetic-based embryo selection tools.

INTRODUCTION

Since the first successful *in vitro* fertilisation (IVF) treatment in 1978, delivery rates have increased to over 30% per oocyte pick up (1-4). This increase can be attributed to several advances, which have led to improvements in culture conditions (5-7). However, *in vitro* culture conditions still poorly mimic the human *in vivo* environment and it has been hypothesised that the *in vitro* environment during IVF exposes the preimplantation embryo to additional stressors. To minimise the detrimental effects of the *in vitro* culture environment on human embryo development, past research aimed to improve culture media for IVF. Two competing approaches regarding IVF media have been advocated, often paraphrased as: '*back to nature*' and '*let the embryo choose*' (8).

The first approach is based on the knowledge of embryo metabolism and compositions of fluids in the female reproductive tract, resulting in sequential media for fertilisation, the cleavage stages and blastocyst development (9). For the second approach, culture media components were systematically adjusted using mouse preimplantation embryos, resulting in a single step formulation that enabled culture of human embryos from fertilisation up to the blastocyst stage (10-12).

Although studies suggest that culture in single step media results in the development of more blastocysts than culture in sequential culture media, clinical outcomes are similar (13-16). This may be attributed to either differences in the composition of media or to the fact that single step culture media allow undisturbed embryo culture, as there is no need to transfer the embryo to the next culture medium on day 3 of development (17). Despite comparable implantation rates between media, some studies report differences in fetal size, birthweight and even postnatal weight at 2 years (18-20). Recently we have demonstrated that these differences can arise as early as the first-trimester, as embryos cultured in SAGE 1-Step exhibit faster growth and development than embryos cultured in Vitrolife G-1 PLUS (21).

Little is known if these differences are also observed prior to implantation. Since 1997, preimplantation embryo development can be closely observed with time-lapse imaging (22). This technique is increasingly used to investigate the association between preimplantation development and implantation, and to improve embryo selection for transfer. However, prospective randomised trials on time-lapse based embryo selection report conflicting results on the improvement of success rates, and indicate that embryo developmental kinetics are subject to patient related factors and local laboratory variables (23-25). To prevent that these variables affect embryo selection, an universally applicable algorithm for embryo selection has been developed (26). This algorithm, the Known Implantation Data (KID) Score, is a (de)selection tool that ranks embryos according to their implantation potential, regardless of patient characteristics, the fertilisation technique used and culture conditions applied. Moreover, it has a high blastulation predictability and performs superior

to conventional morphology evaluation for predicting live births, when applied to day 3 embryos (27).

Reports on the impact of the type of culture medium on morphokinetics are conflicting, and the impact on the performance of morphokinetic selection algorithms remains poorly investigated (28-31). In this study, we retrospectively compare morphokinetics between embryos cultured in Vitrolife G-1 PLUS™ and SAGE 1-Step™. The primary aim of this study is to investigate associations between these two culture media and preimplantation embryo morphokinetic parameters in both IVF and intracytoplasmic sperm injection (ICSI) treatments. The secondary aim is to investigate the impact of culture medium on the predicted implantation potential as assessed by the KIDScore algorithm. This is compared to the observed implantation potential by studying the associations between culture media and clinical outcomes. Expanding our knowledge will not only help in understanding the role of culture medium in preimplantation embryonic development, but can also help in improving embryo selection.

METHODS

Study design and participants

This patient registry cohort study was conducted between 31 January 2012 and 26 July 2017 at the outpatient Fertility Clinic of the Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Center Rotterdam, The Netherlands. Couples undergoing IVF or ICSI treatment with ejaculated sperm, and who had their embryos cultured in the EmbryoScope™ time-lapse incubator (Vitrolife, Goteborg, Sweden) were included. If a couple underwent multiple cycles during the study period, only the first treatment cycle was included. This is defined as the first cycle for the wish to conceive during the study period. The morphokinetic parameters were extracted from the EmbryoViewer™ database, while data on patient characteristics and clinical outcomes were retrieved from medical records. A (post-hoc) sample size calculation revealed that a sample of at least 132 embryos per culture medium had 80% power to detect a 1-hour difference with a significance level (α) of 5% (two-tailed).

Ovarian stimulation, oocyte retrieval and IVF procedures

Ovarian stimulation, oocyte retrieval and IVF and ICSI procedures were performed as described previously, with the following modifications: women underwent ovarian stimulation with either a GnRH-agonist or GnRH-antagonist combined with recombinant-follicle stimulating hormone (rFSH) or urinary-FSH administration (32, 33). Ovarian stimulation protocols are standardised at our centre and the distribution of GnRH-agonist or GnRH–antagonist protocols reflects policy changes over time and not patient selection. FSH or rFSH dosage was based on female age, antral follicle count and prior response to gonadotrophins (if applicable). Final follicular maturation, i.e. ovulation, was triggered

with human chorionic gonadotrophin (hCG) or a GnRH-agonist. Oocytes were collected 35 hours later and cultured in G-IVF PLUS (Vitrolife, Goteborg, Sweden) during the period from January 31st, 2012 to November 17th, 2014. From November 17th, 2014 onwards, oocytes were cultured in SAGE human tubal fluid medium (HTF, CooperSurgical, Trumbull, CT, United States of America); supplemented with 5% human serum albumin (CooperSurgical) under an oil overlay (CooperSurgical). Oocytes were subsequently fertilised conform routine IVF or ICSI procedures. For manipulation of the oocytes for ICSI outside the incubator, either G-MOPS plus (Vitrolife) or SAGE HEPES buffered HTF (CooperSurgical) was used.

Embryo culture, annotations and transfer

IVF oocytes were cultured overnight in drops of 100µl fertilisation medium in universal GPS dishes (CooperSurgical) under oil after insemination. The next morning, only fertilised oocytes with two pronuclei were transferred to an EmbryoSlide (Vitrolife). Denuded ICSI oocytes were transferred to an EmbryoSlide directly after injection. Oocytes or embryos were individually cultured in the EmbryoScope in 25 µl of culture medium under 1.4 ml oil.

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Between January 31st, 2012 and November 17th, 2014, embryos were cultured using G-1 PLUS medium (Vitrolife). After embryo selection for transfer on day 3, remaining embryos were transferred to a fresh EmbryoSlide containing G-2 PLUS medium. From November 17th, 2014 onwards, SAGE 1-Step medium (Cooper Surgical) was used for culture from day 0/1 until day 4, primarily for practical reasons. During the complete study period, fresh embryo transfer (ET) was performed on day 3 after ovum pick-up. Embryos were evaluated on a single image acquired 66-68 hours after fertilisation without support of time-lapse information and selection was based on number of blastomeres, fragmentation, size equality, and signs of early compaction. Remaining embryos with more than 12 blastomeres or 30% of compaction were selected for cryopreservation on day 4.

Embryos were cultured at 36.8 °C in an atmosphere containing 7% oxygen. To achieve a stable pH-level of 7.2-7.3, the level of CO_2 was adjusted to 5.5% for the Vitrolife media and 4.5% CO_2 for the SAGE media in all incubators. In our clinic it is standard care to transfer a single embryo. Only women aged ≥38 years without medical contra-indications or women who underwent 2 or more fresh IVF or ICSI cycles could opt for double embryo transfer. Pregnancy was confirmed biochemically by a positive hCG test (two weeks after ET) and by ultrasound (5 weeks after ET). The cumulative pregnancy rate was defined as a pregnancy resulting from either fresh ET or any frozen-thawed ET of embryos from the treatment cycle included in the study cohort within a two year follow-up period.

Time-lapse imaging and assessment

Every 10 minutes, images were recorded automatically in seven focal planes (15 µm intervals) with a monochrome CCD camera after exposure to a single red LED (635 nm, <0.1 second per

image, total light exposure time <50 sec/day per embryo). For IVF embryos, t=0 was defined as the moment of insemination. For ICSI embryos, t=0 was defined as the moment of injection of the last oocyte. Depending on the number of oocytes, the total procedure takes between 20-50 minutes. Manual annotations were performed according to the definitions and guidelines of the ESHRE consensus for dynamic monitoring of human preimplantation development (34). All freshly transferred and cryopreserved embryos were individually annotated for the following developmental time points: tPNf, t2, t3, t4, t5, t6, t7 and t8. tPNF was defined as the first frame on which both pronuclei had faded. The exact timings of reaching the 2-, 3-, 4-, 5-, 6-, 7-, and 8-cell stage of each embryo were defined as t2, t3, t4, t5, t6, t7 and t8, respectively. Furthermore, intervals between developmental timings were calculated (t3-t2 and t5-t4). If the interval t3-t2 was 5 hours or less, the embryo was registered as direct unequal cleaving (DUC). These parameters were used by the Vitrolife embryo-viewer software to assign each embryo a KIDScore. This score is based on the KIDScore algorithm (Vitrolife), a generally applicable morphokinetic algorithm for implantation based on six parameters (**Supplemental table 1**) (26). In this algorithm a KIDScore of 1 corresponds with a low predicted implantation potential, whereas embryos classified as score 5 have a high predicted implantation potential (5% vs. 36% in the dataset based on which the model was developed). The inter-observer and intra-observer agreement for developmental events observed by time-lapse images is reported to be high (35-37). Internal validation of inter-observer reproducibility demonstrated extremely close agreement for the timings of tPNf until t5 (intraclass correlation coefficient (ICC) >0.95). A moderate agreement was found for t6, t7 and t8 (ICC 0.23-0.40).

Statistical analyses

Continuous baseline data were tested using Mann-Whitney U test and categorical baseline data were tested using chi-square test. Time-lapse data were analysed using linear mixed models, which offer the possibility to analyse repeated measurements, multiple embryos from the same woman and are able to deal with missing values. In these models, time-lapse parameters were the response variables and culture medium was the independent variable. To adjust for potential confounders, two models were constructed. Model 1 only took similarities between embryos from the same women, also known as clustering, into account. Model 2 was additionally adjusted for female age, fertilisation method (IVF or ICSI), type of ovarian stimulation protocol (agonist or antagonist), culture under low oxygen conditions and the passage of time. The latter variable is based on the assumption that overall embryo quality gradually improves over time due to minor and unknown improvements in practice (38-40). Two sub-analyses regarding morphokinetic development were performed. The first was performed in ICSI treatments only, as t=0 corresponds to the actual moment of fertilisation in ICSI embryos, whereas in IVF embryos there is an approximately 2-hour delay between insemination and fertilisation (41). For this analysis, similar models were constructed but not adjusted for fertilisation method. The second sub-analysis was performed in freshly transferred embryos that implanted successfully, using linear regression, as only one embryo

per patient is considered and there is no effect of clustering.

The effect of the culture medium on the KIDScore was investigated using a proportional odds model, a model for ordinal outcomes with patient specific intercepts to account for correlation between embryos (42). Here, we also performed a sub-analysis that considered only the group of freshly transferred embryos that implanted successfully, by using a chi-square test.

Clinical outcomes were analysed using logistic regression and adjusted for female age, fertilisation method and stimulation protocol, as other laboratory procedures were similar between the two media.

Embryos cultured in Vitrolife were used as reference in all analyses. All statistical analyses were performed in SPSS statistics 24.0 (IBM, Armonk, USA) and R (R: A language and Environment for Statistical Computing, version 3.1.3, 2015 for Windows, R Core Team, Vienna, Austria). Two-sided *P*-values <0.05 were considered significant.

RESULTS

Four-hundred and one treatment cycles were included in this study population. In 253 (63.1%) cycles, the embryos (n=671) were cultured in Vitrolife medium; in the remaining cycles (n=148, 36.9%), embryos (n=517) were cultured in SAGE medium. This number of embryos is sufficient to demonstrate a 1-hour differences, according to our (post-hoc) power analysis. Patient and treatment characteristics are shown in **Table 1**. Female age, body mass index (BMI) and number of aspirated oocytes were comparable between the two groups. Ovarian stimulation using GnRH- agonist co-treatment was more common in the Vitrolife group than in the SAGE group (30.0% and 77.1%, respectively, $p<0.001$). The Vitrolife group included less ICSI treatment cycles than the SAGE group (41.1% and 98.0%, respectively, $p<0.001$).

Table 1. Baseline characteristics of study population.

	Vitrolife G-1 PLUS		SAGE 1-Step		
	Median/N	IQR / %	Median/N	IQR / %	P-value
Women (embryos)	253 (671)		148 (517)		
Age, years	34.5	30.6-38.8	34.0	29.8-38.1	0.458
BMI, kg/m2*	23.4	21.3-26.8	24.0	21.8-26.4	0.233
Fertilisation, ICSI	104	41.1	145	98.0	<0.001
Stimulation protocol, agonist**	27	30.0	108	77.1	<0.001
Oocytes aspirated per patient, n	7	4-10	6	4-9	0.572

BMI, body mass index. ICSI, intracytoplasmic sperm injection. IQR, interquartile range.
* missing n=201. ** missing n=171

Morphokinetic parameters

After adjustment for clustering, female age, fertilisation method, type of ovarian stimulation co-treatment, oxygen levels at culture and overall improvement in embryo development over time, linear mixed model analysis shows that embryos cultured using SAGE medium reached the pronuclear fading moment 2.13 (95%CI -3.41, -0.84, p=0.001) hours faster than embryos cultured in Vitrolife medium (**Table 2**). The cleavage divisions to the 2-, 3-, 4-, 5-, 6-, 7, and 8-cell stage occurred 2.28 (95%CI -3.66, -0.89, p=0.001), 2.34 (95%CI -4.00, -0.64, p=0.07), 2.41 (95%CI -4.11, -0.71, p=0.006), 2.54 (95%CI -4.90, -0.18, p=0.035), 3.58 (95%CI -6.08, -1.08, p=0.005), 5.62 (95%CI -8.80, -2.45, p=0.001) and 5.32 (95%CI -9.21, -1.42, p=0.008) hours faster, respectively. SAGE embryos reach the 2-cell stage 7.4% faster and the 8-cell stage 6.0% faster than Vitrolife embryos (**Figure 1**).

Table 2. Differences in morphokinetic parameters of IVF and ICSI embryos cultured in SAGE 1-step compared to Vitrolife G-1 PLUS.

Morphokinetic parameter	Model 1		Model 2*			
	Beta (95% CI), hours	P-value	Beta (95% CI), hours	P-value	Missing
tPNf	-2.10 (-2.68, -1.51)	<0.001	-2.13 (-3.41, -0.84)	0.001	31
t2	-2.09 (-2.71, -1.46)	<0.001	-2.28 (-3.66, -0.89)	0.001	21
t3	-2.98 (-3.74, -2.22)	<0.001	-2.34 (-4.00, -0.64)	0.007	34
t4	-2.10 (-2.86, -1.34)	<0.001	-2.41 (-4.11, -0.71)	0.006	50
t5	-3.39 (-4.43, -2.34)	<0.001	-2.54 (-4.90, -0.18)	0.035	97
t6	-3.12 (-4.29, -1.94)	<0.001	-3.58 (-6.08, -1.08)	0.005	452
t7	-3.43 (-4.92, -1.95)	<0.001	-5.62 (-8.80, -2.45)	0.001	550
t8	-3.27 (-5.17, -1.38)	0.001	-5.32 (-9.21, -1.42)	0.008	626
t3-t2	-0.82 (-1.36, -0.28)	0.003	0.18 (-1.03, 1.39)	0.767	34
t5-t4	-1.49 (-2.25, -0.73)	<0.001	-0.18 (-1.88, 1.53)	0.839	97
t3-tPNf	-0.93 (-1.48, -0.39)	0.001	-0.15 (-1.37, 1.07)	0.807	63
(t5-t3)/(t5-t2)	0.00 (-0.02, 0.03)	0.716	-0.03 (-0.09, 0.02)	0.205	97

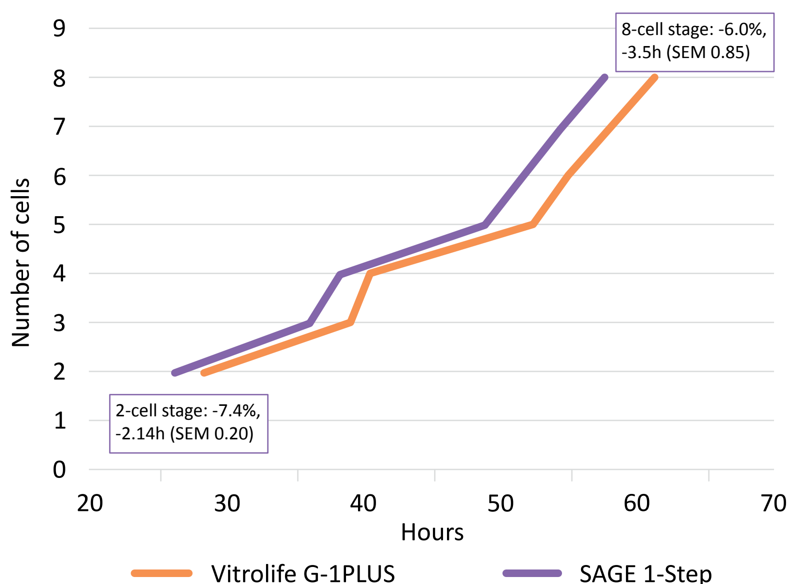
*Adjustments were made for female age, fertilisation method, type of ovarian stimulation, lowered oxygen culture and overall improvement in embryo development over time. CI, confidence interval.

The SAGE group contained more ICSI treatment cycles and to investigate the possibility that this is the underlying cause for the faster developmental kinetics observed in this group, we also performed a sub-analyses of ICSI embryos only (n=757). These analyses showed that ICSI embryos cultured in SAGE (n=513) reach the pronuclear fading moment faster than ICSI embryos cultured in Vitrolife medium (n=244). These analyses confirmed that ICSI embryos cultured in SAGE reach the 2- (-3.09h (95%CI -5.14, -1.03) p=0.003), 5- (-4.67h (95%CI -9.47, -0.05) p=0.047), 6- (-7.25h (95%CI -12.96, -1.55) p=0.013) and 7-cell stage (-9.21h (95%CI -17.75, -0.67) p=0.035) faster than ICSI embryos cultured in Vitrolife (**Table 3**).

DUC was more prevalent in SAGE embryos than in Vitrolife embryos (n=79 (15.4%) vs. n=57 (8.9%) respectively, p=0.001). Exclusion of DUC embryos did not change the observation of faster embryo development in the SAGE group (data not shown). It is likely that the observation of significantly more DUC embryos in SAGE medium is related to the difference

in IVF:ICSI treatment ratio between the two culture media, as comparable DUC rates are observed in ICSI-embryos only (SAGE: 15.5%; Vitrolife: 12.2%, *p*=0.235).

Figure 1. Morphokinetic developmental trajectories of preimplantation embryos cultured in Vitrolife G-1 PLUS (light blue) and SAGE 1-step medium (dark blue).



SEM, standard error of the mean.

Table 3. Differences in morphokinetic parameters of ICSI embryos cultured in SAGE 1-Step compared to Vitrolife G-1 PLUS culture medium.

Morphokinetic parameter	Model 1		Model 2*	
	Beta (95% CI), hours	P-value	Beta (95% CI), hours	P-value
tPNf	-1.91 (-2.60, -1.21)	<0.001	-2.69 (-4.73, -0.65)	0.010
t2	-1.93 (-2.67, -1.18)	<0.001	-3.09 (-5.14, -1.03)	0.003
t3	-2.95 (-3.96, -1.94)	<0.001	-2.22 (-5.58, 1.13)	0.193
t4	-2.19 (-3.21, -1.17)	<0.001	-1.63 (-5.13, 1.87)	0.360
t5	-2.28 (-4.14, -1.41)	<0.001	-4.76 (-9.47, -0.05)	0.047
t6	-3.34 (-4.77, -1.90)	<0.001	-7.25 (-12.96, -1.55)	0.013
t7	-3.86 (-5.62, -2.10)	<0.001	-9.21 (-17.75, -0.67)	0.035
t8	-4.65 (-6.90, -2.39)	<0.001	-9.13 (-19.30, 1.05)	0.078
t3-t2	-0.81 (-1.57, -0.05)	0.037	1.13 (-1.52, 3.79)	0.400
t5-t4	-0.70 (-1.66, 0.25)	0.148	-2.92 (-6.44, 0.60)	0.104
t3-tPNf	-1.07 (-1.85, -0.29)	0.008	0.38 (-2.38, 3.14)	0.786
(t5-t3)/(t5-t2)	0.01 (-0.02, 0.05)	0.472	-0.09 (-0.21, 0.04)	0.178

*Adjustments were made for female age, type of ovarian stimulation, lowered oxygen culture and overall improvement in embryo development over time. CI, confidence interval.

To investigate if the faster development in SAGE medium is related to embryo quality, we performed sub-analyses of only IVF- and ICSI-embryos that successfully implanted

after fresh ET (n=143). These also demonstrated that embryos cultured in SAGE reached the moment of pronuclear fading, t2, t4 and t6 faster than embryos cultured in Vitrolife, suggesting faster development in SAGE medium for embryos with similar implantation potential (**Supplemental table 2**).

Retrospective assignment of KIDScores

The distribution of KIDScores was significantly different between embryos cultured in SAGE and embryos cultured in Vitrolife (proportional odds model: $p<0.001$) (**Figure 2**). Embryos cultured in SAGE fell significantly more often within the optimal time ranges corresponding to a KIDScore of 5 than embryos cultured in Vitrolife (n=240 (46.4%) and n=52 (7.7%), respectively). At the same time, more embryos cultured in SAGE had a KIDScore of 1 than embryos cultured in Vitrolife (n=91 (17.6%) and n=58 (8.6%), respectively). In a small number of embryos, a KIDScore could not be assigned (Vitrolife n=58 (8.6%), SAGE n=10 (1.9%)) due to the fact that some developmental stages could not be assessed on the available images.

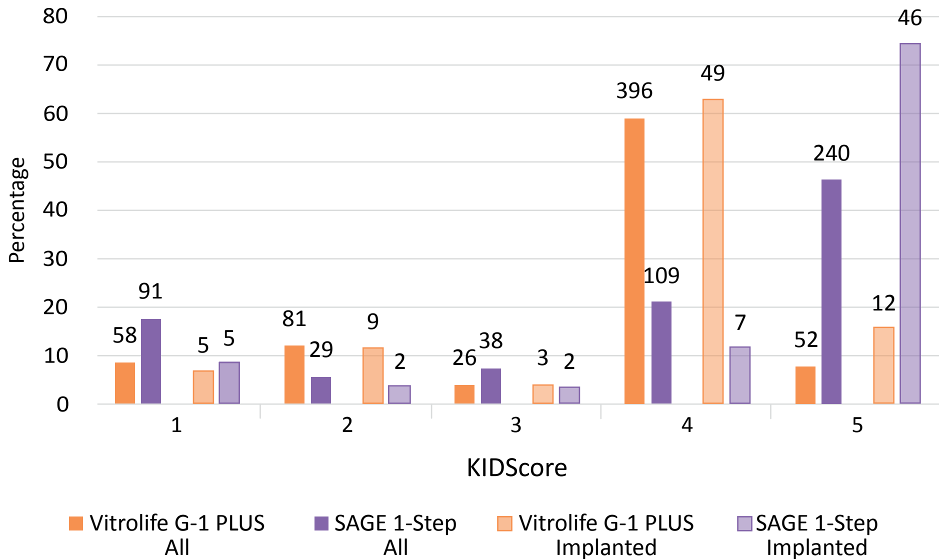
Sub-analysis of the freshly transferred embryos that implanted successfully, demonstrated a similar distribution across the KIDScores (**Figure 2**). It was found that the majority of successfully implanted embryos in the Vitrolife group classified for a KIDScore of 4, whereas for successfully implanted SAGE embryos the majority classified for the optimal KIDScore of 5.

Clinical treatment outcomes

Table 3 demonstrates the clinical outcomes per patient after culture in SAGE and Vitrolife. After fresh ET (n=372), 80 (33.6%) women in the Vitrolife group and 63 (47.0%) women in the SAGE group had a positive hCG-test. Crude logistic regression analysis, with Vitrolife as reference, showed an odds ratio (OR) of 1.81 (95%CI 1.21, 2.71, p=0.004) for positive hCG-test, 1.93 (95%CI 1.29, 2.88, p=0.001) for presence of a gestational sac, 1.75 (95%CI 1.14, 2.69, p=0.010) for presence of fetal heartbeat and 1.45 (95%CI 0.92, 2.28, p=0.110) for live birth for embryos cultured in SAGE (**Table 4**). However, after adjustment for female age, fertilisation method and type of ovarian stimulation, none of the clinical outcomes after fresh ET were significantly different.

The crude OR for cumulative pregnancy rate, i.e. a pregnancy resulting either after fresh ET or after frozen-thawed ET within a two-year follow-up period after oocyte retrieval, is 1.70 (95%CI 1.12, 2.57, p=0.013) for embryos cultured in SAGE. However, after adjustments, we found no differences for this clinical treatment outcome between the culture media.

Figure 2. Distribution of KIDScores of embryos cultured in Vitrolife G-1 PLUS (light blue) and SAGE 1-Step medium (dark blue).



All embryos: p<0.001 (proportional odds model). Implanted embryos: p<0.001 (chi-square). Numbers depicted above the bars represent absolute numbers.

Table 4. Clinical outcomes per patient after culture in SAGE 1-step compared to culture in Vitrolife G-1 PLUS.

	Vitrolife G-1 PLUS	SAGE 1-Step	Crude		Adjusted*		
	N (%)	N (%)	OR (95% CI)	P-value	OR (95% CI)	P-value	Missing
After fresh ET	N=238	N=134					
Positive hCG-test	80 (33.6)	63 (47.0)	1.81 (1.21, 2.71)	0.004	1.74 (0.82, 3.69)	0.150	0
Gestational sac	76 (31.9)	62 (46.3)	1.93 (1.29, 2.88)	0.001	1.80 (0.85, 3.85)	0.128	0
Fetal heartbeat	61 (25.6)	48 (44.0)	1.75 (1.14, 2.69)	0.010	1.58 (0.71, 3.52)	0.256	0
Live born	54 (23.2)	37 (28.7)	1.45 (0.92, 2.28)	0.110	0.99 (0.43, 2.31)	0.986	10
After fresh and frozen ETs	N=253	N=148					
Cumulative ongoing pregnancy	86 (34.0)	69 (46.6)	1.70 (1.12, 2.57)	0.013	1.80 (0.84, 3.90)	0.134	0

*Adjustments were made for female age, fertilisation method and type of ovarian stimulation.
ET, embryo transfer. OR, odds ratio. CI, confidence interval.

DISCUSSION

The aims of this study were to compare embryo morphokinetics and clinical outcomes between two different embryo culture media used during conventional IVF and during ICSI treatment. We observed that embryos cultured in SAGE culture medium reached the pronuclear fading moment earlier and proceeded faster through all the cleavage divisions than embryos cultured in Vitrolife, regardless of the fertilisation method. These embryos also qualified significantly more often for the highest KIDScore, suggesting a higher implantation potential. However, when only successfully implanted embryos were considered, those cultured in SAGE still showed faster development. Also, we found no significant differences in clinical outcomes, such as implantation and cumulative pregnancy rate, after correction

for confounders. This suggests that culture medium has a direct impact on embryo developmental kinetics, but this does not necessarily reflect in implantation potential.

Although the timing of cleavage divisions is different between embryos cultured in Vitrolife and embryos cultured in SAGE, it remains unknown which specific components drive these differences. It has been suggested that variations in pyruvate, lactate, amino acid and glucose concentrations between culture media may affect embryo morphokinetics and metabolism (43-45). In mice, it has been shown that fast-cleaving embryos have a higher glucose consumption rate than slow-cleaving embryos (46). This is seemingly in contrast with our results, as we observe faster development of embryos cultured in the medium with lower glucose content (SAGE). Yet, during the first stages of human preimplantation embryo development pyruvate and lactate are the primary source of energy, and high levels of glucose during the cleavage stages might be counterproductive for preimplantation development (47, 48).

Other components in culture media, such as amino acid concentrations, are also likely to have a significant effect on early embryo development. Amino acids are important during the cleavage stages, as they regulate embryo physiology (49-52). It has been demonstrated that embryos with a lower amino acid turnover develop more often to the blastocyst stage and have less DNA damage (53-55). This is in line with the 'quiet embryo hypothesis' of Leese (56), which postulates that embryos with a low metabolism have a higher viability as they endure environmental stress relatively well. However, the presence of amino acids in culture medium is ambivalent, as they also contribute to ammonium build-up. High levels of ammonium can have a detrimental effect on embryo development and pregnancy rates (57, 58). Minimizing ammonium build-up is especially critical for single step media, as not refreshing the media might otherwise result in detrimental ammonium levels during 5 days of culture.

In addition to the unknown mechanisms underlying the differences in morphokinetics, the clinical impact of these differences is also largely unknown. Although several studies indicate that fast-developing embryos have a higher potential for implantation, the added clinical value of numerous (de)selection tools has yet to be conclusively established (26, 46, 59-64). For example, after undisturbed culture in a self-contained incubator, clinical outcomes are comparable between morphokinetic-based and morphology-based embryo selection [61]. It is likely that the differences we observed in individual morphokinetic parameters between culture media, translated to differences in our studied deselection tool, the KIDScore. We showed that a larger proportion of SAGE embryos qualified for the highest score, compared to Vitrolife embryos, which might be explained by more embryos reaching the 8-cell stage before 66 hours after fertilisation. Although the predicted implantation potential, i.e. the KIDScore, was higher in the SAGE group, the observed implantation potential, as well as

cumulative pregnancy rate, were comparable between the two culture media. Interestingly, after implantation, SAGE embryos also exhibit faster growth and development than Vitrolife embryos (21).

Since the KIDScore was developed for general applicability in different treatment and culture conditions, its test characteristics were not studied in specific culture media. Yet, our results suggest that the type of culture medium may impact the discriminatory value of the KIDScore. For example, even in embryos that implanted successfully, the KIDScore was significantly different between culture media. Moreover, the KIDScore distribution of transferred and cryopreserved Vitrolife embryos is considerably different from the distribution described by Petersen et al (26). However, it should be noted that the KIDScore algorithm and its distribution are based on embryos that had been selected for transfer, whereas we included all embryos that had been selected for transfer and cryopreservation.

Since we show that type of culture medium affects multiple individual morphokinetic parameters, our findings may have consequences for the discriminatory value and applicability of other (de)selection tools. Therefore, it is recommended that IVF-clinics should either validate morphokinetic-based selection tools prior to implementation or develop their own clinic-specific selection tool, since the ideal developmental kinetics of human embryos under different culture conditions remains largely unknown.

Previous research regarding the effect of culture medium on morphokinetic parameters in human embryos is scarce and reports conflicting results. Some studies report no effect of culture medium on morphokinetic parameters, whereas others demonstrate faster development for single step culture medium (28-31). Despite the fact that randomisation was applied in all of these studies, sample sizes were small, statistical analyses were mainly based on t-testing and none of the studies corrected for clustering of multiple embryos from one woman. Without considering clustering, it is possible that observed effects are essentially based on a large number of embryos from only a few patients. Therefore, we applied linear mixed models, which enable precise modelling of embryo development by taking into account both clustering as well as confounders such as age and type of ovarian stimulation. Also, a post-hoc power-analysis confirmed that the size of our study population is sufficient to demonstrate a 1-hour difference in preimplantation development. Another benefit of this patient registry cohort is the diverse study population. Our study population consisted of IVF as well as ICSI treatments, and different ovarian stimulation approaches have been used, which increases the generalizability of our results. However, the ratio of IVF:ICSI treatments was not comparable within the groups of studied culture media. It is essential to correct for fertilisation method when studying time-lapse parameters, as differences between IVF- and ICSI-embryos have been suggested (65). We approached this in two ways: we adjusted for fertilisation method in our linear mixed model and also performed

a sensitivity analysis in ICSI embryos only. By doing so, we demonstrate an impact of the culture medium as early as the moment of pronuclear fading in analyses both including all embryos as well as including ICSI embryos only. However, others have suggested to use tPNf as a starting point (41). With this method, a potential impact on the moment of pronuclear fading is missed, and observations in subsequent cleavage stages might also be affected.

The main limitation of our study is that we only data have data available from the cleavage stage development and not up to the blastocyst stage. As blastocyst transfer is associated with higher pregnancy and live birth rates (66), assessment of this stage is likely to be more informative. This study was performed in a time in which fresh transfer of cleavage embryos was routine care in most IVF clinics, including ours. Future research should include blastocyst development as it may provide a more extensive understanding of the impact of culture medium. Although our patient data for BMI and stimulation regimen is incomplete, the sensitivity analysis showed similar results for patients with complete data (n=230) and patients with incomplete data (n=171), suggesting a low risk of selection bias (data not shown). The studied culture media were used during consecutive time periods. To minimise this effect of confounding due to a secular trend, we also corrected for laboratory-specific changes and the moment of culture. Finally, despite the adjustments for potential confounders in our analyses, residual confounding cannot be excluded, as this is inherent to the observational study design.

Conclusion

Our results demonstrate an impact of culture medium on preimplantation embryo developmental kinetics and this affects classification within the KIDScore algorithm. Other studies show that culture medium can alter post-implantation growth as early as the second trimester of pregnancy and even impact offspring health at nine years of age (18, 67). However, it remains unknown which specific component(s) in culture medium drive these differences. Since over half a million babies are born after IVF and ICSI treatment each year, more studies should shift the focus from the effects of IVF culture conditions on implantation to their effect on (preimplantation) embryo development (68). Moreover, early embryonic development has been linked to postnatal health and development (69). This, in combination with the results of Dumoulin et al., further warrants the need to elucidate the association between preimplantation embryo kinetics, post-implantation growth and offspring health.

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Supplemental material

Supplemental table 1. Morpokinetic parameters of the KIDScore algorithm.

Morphokinetic parameter
Number pronuclei equals 2 at the 1-cell stage
Time from insemination to pronuclear fading (tPNf)
Time from insemination to the 2-cell stage (t2)
Time from insemination to the 3-cell stage (t3)
Time from insemination to the 5-cell stage (t5)
Number of cells 66 hours after fertilisation

Supplemental table 2. Differences in morphokinetic parameters of implanted embryos (n=143) cultured in SAGE 1-Step compared to Vitrolife G-1 PLUS culture medium after fresh embryo transfer.

Morphokinetic parameter	Model 1		Model 2*		Missing
	Beta (95% CI), hours	P-value	Beta (95% CI), hours	P-value	
tPNf	-2.51 (-3.38, -1.64)	<0.001	-2.44 (-4.42, -0.45)	0.017	1
t2	-2.65 (-3.62, -1.68)	<0.001	-2.80 (-4.99, -0.61)	0.013	0
t3	-2.79 (-4.06, -1.52)	<0.001	-2.66 (-5.50, 0.18)	0.066	2
t4	-2.82 (-3.98, -1.65)	<0.001	-3.45 (-6.01, -0.80)	0.011	2
t5	-2.88 (-4.71, -1.04)	0.002	-3.70 (-7.99, 0.59)	0.090	5
t6	-4.12 (-6.04, -2.21)	<0.001	-4.34 (-8.53, -0.14)	0.043	45
t7	-3.78 (-6.03, -1.53)	0.001	-3.81 (-8.81, 1.78)	0.133	58
t8	-2.75 (-5.29, -0.21)	0.034	-2.46 (-7.91, 3.00)	0.372	75
t3-t2	-0.04 (-1.07, 0.98)	0.935	0.36 (-1.95, 2.66)	0.760	2
t5-t4	-0.26 (-1.70, 1.18)	0.724	-0.09 (-3.40, 3.23)	0.959	5
t3-tPNf	-0.21 (-1.22, 0.79)	0.675	-0.07 (-2.31, 2.17)	0.950	3
(t5-t3)/(t5-t2)	-0.01 (-0.06, 0.04)	0.651	-0.05 (-0.15, 0.06)	0.351	5

*Adjustments were made for female age, fertilisation method, type of ovarian stimulation, lowered oxygen culture and overall improvement in embryo development over time. CI, confidence interval.

5

The impact of culture medium used in IVF-treatment on post-implantation embryonic growth and development with emphasis on sex-specificity: The Rotterdam Periconception cohort

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ABSTRACT

Research question: Are there (sex-specific) differences in first-trimester embryonic growth and morphological development between two culture media used for *in vitro* fertilisation (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 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, EV), and morphological (Carnegie stage) measurements.

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

Conclusions: Here we show that SAGE 1-Step culture medium accelerates embryonic growth trajectories compared to 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.

INTRODUCTION

Globally, over ten million children have been conceived through *in vitro* fertilisation (IVF) treatment since the birth of the first IVF baby in 1978 (1, 2). Success rates have increased to a livebirth rate of 28% per oocyte aspiration, which can be attributed to several advancements, such as intra cytoplasmic sperm injection (ICSI) and improved culture conditions (3, 4). 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 optimise culture conditions (5). Interestingly, it has also been suggested that these complications are associated with fetal sex (6). 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 physiologic *in vivo* environment, as the *in vitro* milieu exposes the preimplantation embryo to stressors not present in the *in vivo* milieu (4, 7, 8). Regarding culture media formulations, two main protocols can be distinguished. In the sequential protocol, different media are used for fertilisation, 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 (9-11). Interestingly, clinical outcomes such as pregnancy rate per oocyte aspiration are similar for the two protocols (12).

A second important issue is that variations in IVF/ICSI-procedures can impact not only success rates, but also post-implantation development. For example, culture medium used during the first 3 days post-fertilisation can affect fetal size, birthweight and even postnatal weight at 2 years of age (13-15). 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 take place (16). Appropriate growth and development in the first-trimester is 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 lenght (CRL), birthweight and cardiovascular outcomes in childhood (17-20). First-trimester embryonic growth and morphological development can be meticulously studied by combining three-dimensional (3D) ultrasound techniques with virtual reality (VR). This allows in-depth perception and precise assessment of novel volumetric and morphologic measurements [21].

Moreover, there is also some evidence that IVF/ICSI-procedures affect the sex ratio in favour of males (22, 23). 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 (24, 25). In humans, less is known if exposure to culture medium impacts post-implantation embryonic growth and development also in a sex-specific manner.

In this study, the primary aim is 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 is to compare second-trimester size and perinatal outcomes. Finally, we aim to compare post-implantation growth and development after culture in these two media with natural conception.

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 Gynaecology of the Erasmus MC, University Medical Center, Rotterdam, The Netherlands (26). The data used for this study was obtained between November 2009 and August 2018. Women of ≥18 years with an ongoing intra-uterine singleton pregnancy less than 10+0 weeks pregnant were eligible for participation. Women pregnant after IVF/ICSI were recruited from the outpatient clinic of Reproductive Medicine. Women pregnant after natural conception were recruited from the outpatient clinic of Obstetrics. Reasons for referral to this outpatient clinic varied from complications in a previous pregnancy (e.g. severe postpartum hemorrhage) 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, intra-uterine 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 (**Supplemental table 1**).

Ethical approval

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

In vitro fertilisation and culture procedures

Ovarian stimulation, oocyte retrieval, subsequent IVF/ICSI procedures, and embryo morphology assessment were performed as described previously (27, 28). 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 (G5-series, Vitrolife, Goteborg, Sweden) overnight for fertilisation. After fertilisation, embryos were transferred to G-1 PLUS cleavage stage medium (Vitrolife, Goteborg, Sweden). 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, Connecticut, United States of America) was used for culture from day 0 until day 4. During the study period, embryo transfer (ET) was performed on day 3 after fertilisation and supernumerary embryos of adequate quality, based on number of blastomeres and amount of compaction, were cryopreserved on day 4 by slow freezing (29). During the study period, it was routine care in most IVF clinics, including ours, to perform fresh transfer of cleavage embryos. No other modifications to the procedures than the change in culture medium have been made during the study period.

Study parameters

At enrolment, standardised anthropometric measurements were performed. Women filled out questionnaires regarding general characteristics and periconceptional nutrition and lifestyle. Geographic origin was categorised into Western or Non-Western and educational level was categorised according to the definition of Statistics Netherlands (30). For pregnancies conceived after IVF/ICSI, gestational age (GA) was based on the conception date, which is the oocyte retrieval date minus 14 days for pregnancies after fresh ET and the ET date minus 19 days for pregnancies after frozen-thawed ET. For naturally conceived pregnancies, GA was based on the reported last menstrual period.

First-trimester growth was expressed by serial CRL and embryonic volume (EV) measurements, and morphological development was assessed by Carnegie stages, based on internal and external characteristics (31). 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 (32). Second-trimester estimated fetal weight (EFW), head circumference (HC), biparietal diameter (BPD), abdominal circumference (AC) and femur length (FL) were retrieved from the second-trimester anomaly scan. Birth outcomes such as sex, GA, 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 GA.

Ultrasound data

Ultrasound examinations were performed in gestational week 7, 9 and 11 by trained sonographers with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D-View software (General Electrics Medical Systems, Chicago, IL, United States of America,). Afterwards, 3D ultrasound data were transferred to the Barco I-Space (a Cave Automatic Virtual Environment–like virtual reality (VR) system) to optimally use the third dimension (33). By using the V-scope volume rendering software, an interactive VR hologram was created, allowing true depth perception. The technique, accuracy and reliability of CRL and EV measurements and Carnegie classification have been previously described (31, 32, 34).

Statistical analyses

The study population was stratified in three groups of pregnancies; 1) after IVF/ICSI treatment with G-1 PLUS cleavage stage medium, 2) after IVF/ICSI treatment with SAGE 1-Step medium and 3) naturally conceived. Baseline characteristics were compared by Kruskall-Wallis tests for continuous variables (since not all variables were parametrically distributed) and by chi-square tests for categorical variables. If these tests yielded significant results, post-hoc tests for pairwise comparisons, i.e. chi-square 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 analyses, CRL was root-transformed and EV was cube root-transformed to approximate linearity. Analyses were performed using Vitrolife G-1 PLUS as reference category. In model 1 we adjusted for GA only. Model 2 was additionally adjusted for maternal age, BMI, parity, geographical background, level of education and periconceptional use of alcohol, cigarettes and folic acid supplements. Additionally, these analyses were also performed using naturally conceived pregnancies as reference category. To create graphs of embryonic growth trajectories, the EV was estimated per week of GA based on model 1, and retransformed to the original values by cubing.

Percentiles of EFW, HC, BPD, AC and FL were calculated from population-based fetal growth charts (35, 36). Differences were studied using Kruskall-Wallis tests. Birthweight was expressed in percentiles based on Dutch reference curves, which take GA at birth and fetal sex into account (37). Differences in birthweight percentiles, birthweight and GA at delivery were analyzed by the Kruskall-Wallis test as well and differences in prevalence of PTB, SGA and LGA by X^2. Sub-analyses were performed for each fetal sex separately for first- and second-trimester growth and development and for type of ET, i.e. fresh or frozen-thawed. P-values ≤0.05 were considered significant. All analyses were performed using SPSS 25.0 (IBM SPSS Statistics, Armonk, NY).

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 6 patients participated with both a naturally conceived and an IVF/ICSI pregnancy, and 3 with pregnancies after IVF/ICSI treatment with Vitrolife G-1 PLUS culture medium as well as after treatment 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.

Figure 1. Flowchart of the study population.

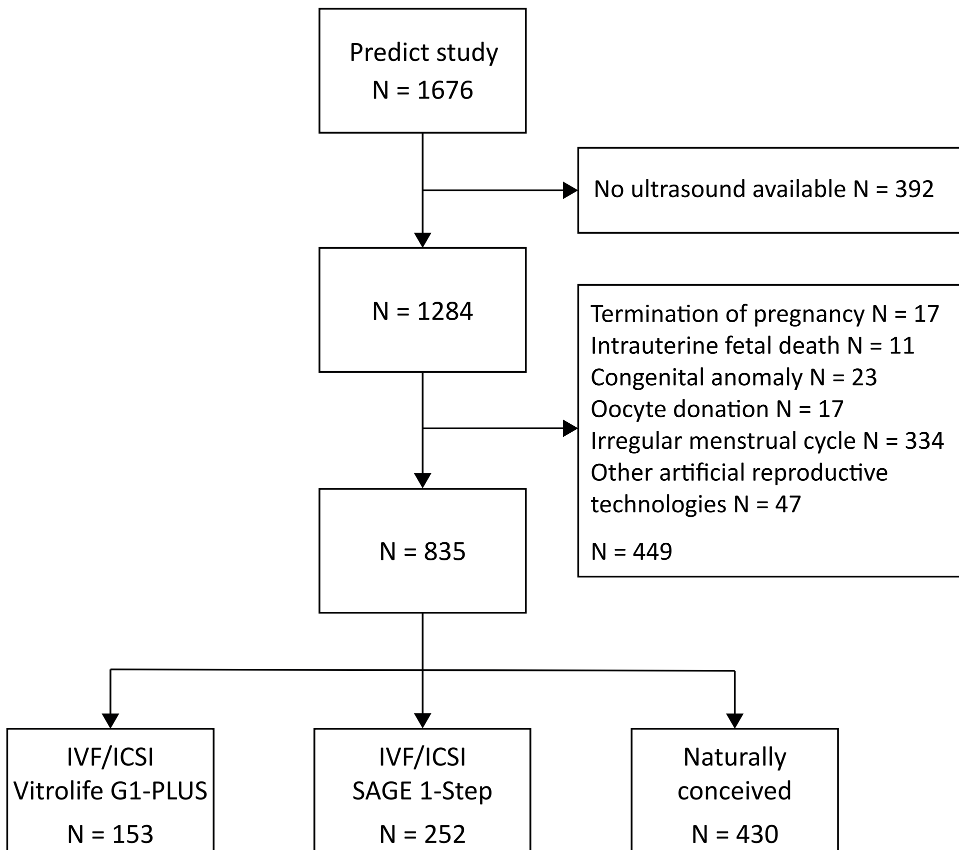


Table 1. Baseline characteristics of the study population, stratified for culture medium used during IVF/ICSI treatment and naturally conceived pregnancies.

	IVF/ICSI Vitrolife G1-PLUS N=153	IVF/ICSI SAGE 1-Step N=252	Naturally conceived pregnancies N=430		
	Median / N (IQR /%)	**Median / N (IQR /%)**	**Median / N (IQR /%)**	**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**[d,e]	
Body mass index, 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**[c,d,e]	
Western geographic origin	125 (81.7)	207 (82.1)	360 (83.7)	0.794	
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[a]	31 (20.5)	57 (22.9)	163 (38.3)	**<0.001**[d,e]	9
Smoking, yes[a]	15 (9.9)	34 (13.7)	73 (17.1)	0.084	9
Folic acid supplements, yes[b]	145 (96.7)	240 (96.4)	302 (70.7)	**<0.001**[d,e]	9
Embryo transfer, frozen-thawed	46 (30.1)	83 (32.9)	n/a	0.548	

IQR, interquartile range. ET, embryo transfer

a. Any use during the 14 weeks prior to 10 weeks after conception.
b. Daily use during the 14 weeks prior to 10 weeks after conception.
c. Significantly different between pregnancies after culture in Vitrolife G-1 PLUS and in SAGE 1-Step after pairwise comparisons.
d. Significantly different between pregnancies after culture in Vitrolife G-1 PLUS and naturally conceived pregnancies after pairwise comparisons.
e. Significantly different between pregnancies after culture in SAGE 1-Step and naturally conceived pregnancies after pairwise comparisons.

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

Embryos cultured in SAGE 1-Step grow significantly faster regarding EV than embryos cultured in Vitrolife G-1 PLUS in both models (model 1 β 0.025 (95%CI 0.004-0.045), *p*=0.017; model 2 β 0.030 (95%CI 0.008-0.052), *p*=0.007 (**Table 2**)). Interestingly, after stratification, this was only observed in embryos after fresh ET (model 1 β 0.036 (95%CI 0.010-0.062), *p*=0.007; model 2 β 0.031 (95%CI 0.004-0.059), *p*=0.024 (**Supplemental table 2**)). Furthermore, growth patterns of embryos after IVF and embryos after ICSI are comparable (**Supplemental figure 1**). After stratification for fetal sex, this finding was only observed in male embryos (model 1 β 0.044 (95%CI 0.014-0.074) *p*=0.004; model 2 β 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 EFW, HC, BPD, AC and FL were comparable between the two culture media (**Table 3**). After stratification for fetal sex, similar results were observed for both female and male fetuses.

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 .

		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	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, ∛cm³	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, ∛cm³	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, ∛cm³	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

CRL, crown rump length. EV, embryonic volume.
Model 1: Adjusted for GA.
Model 2: Adjusted for GA, 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.

Table 3. Percentiles of second-trimester fetal growth parameters of pregnancies after IVF/ICSI with Vitrolife G1-PLUS or SAGE 1-Step and naturally conceived pregnancies, in the total population and stratified for fetal sex.

		IVF/ICSI Vitrolife G1-PLUS N=153	IVF/ICSI SAGE 1-Step N=252	Naturally conceived pregnancies N=430		
		Median (IQR)	Median (IQR)	Median (IQR)	P-value	Missing
Total population	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	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	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

IQR, interquartile range. EFW, estimated fetal weight. HC, head circumference. BPD, biparietal diameter. AC, abdominal circumference. FL, femur length. 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.

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, preeclampsia and gestational diabetes mellitus were comparable between both media.

Table 4. Perinatal outcomes stratified for culture medium used during IVF/ICSI treatment and naturally conceived pregnancies.

	IVF/ICSI Vitrolife G-1 PLUS N=153	IVF/ICSI SAGE 1-Step N=252	Naturally conceived N=430		
	Median / N (IQR /%)	Median / N (IQR /%)	Median / N (IQR /%)	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, grams	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
Preeclampsia	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

IQR, interquartile range.

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.

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

Embryos cultured in SAGE 1-Step show faster embryonic growth than naturally conceived pregnancies, depicted by CRL, EV and morphological development in model 1 (β_{CRL} 0.053 (95%CI 0.005-0.101), *p*=0.031; β_{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 EV (β 0.040 (95%CI 0.012-0.069, *p*=0.005).

After stratification, it was observed in female embryos cultured in SAGE-1 Step medium showed faster morphological development than naturally conceived female embryos in model 2 (β 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 to naturally conceived embryos (β_{CRL} 0.083 (95%CI 0.011-0.155), *p*=0.024; β_{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 EV in model 2 (β_{EV} 0.078 (95%CI 0.035-0.120), *p*<0.001). Male embryos cultured in Vitrolife G-1 PLUS also show faster embryonic growth depicted by EV and morphological development than those naturally conceived (β_{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 EV in the model 2 (β_{EV} 0.049 (95%CI 0.002-0.095), *p*=0.040).

Table 5. First-trimester embryonic growth and morphological development after culture in Vitrolife G-1 PLUS and SAGE-1 Step, compared to naturally conceived pregnancies, in the total population and stratified for fetal sex.

		Model 1						Model 2					
		Vitrolife G-1 PLUS		SAGE 1-Step		Naturally		Vitrolife G-1 PLUS		SAGE 1-Step		Naturally	
		Beta (95%CI)	P-value	Beta (95%CI)	P-value	Beta (95%CI)	P-value	Beta (95%CI)	P-value	Beta (95%CI)	P-value	Beta (95%CI)	P-value
Total population N=853	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, $\sqrt[3]{cm^3}$	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, $\sqrt[3]{cm^3}$	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, $\sqrt[3]{cm^3}$	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	

CRL, crown rump length. EV, embryonic volume.
Model 1: Adjusted for GA.
Model 2: Adjusted for GA, 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.

After retransformation, the EV of embryos cultured in SAGE 1-Step is on average 0.52 cm^3 larger (6.7%) at 11 weeks GA than naturally conceived embryos, whereas for embryos cultured in Vitrolife G-1 PLUS this is 0.25 cm^3 (3.2%) (**Figure 2**). In female embryos these estimates are smaller for SAGE 1-Step: 0.14 cm^3 (1.8%) and there was no difference for Vitrolife G-1 PLUS: 0.00 cm^3 (0.0%), whereas for males these were larger (SAGE 1-Step: 0.92 cm^3 (12.6%); Vitrolife G-1 PLUS: 0.53 cm^3 (7.3%)).

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 larger EFW (83.4 and 71.1, respectively, p=0.033), HC (72.0 and 64.6, respectively, p=0.047) and BPD (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 complications between the three groups (**Table 4**).

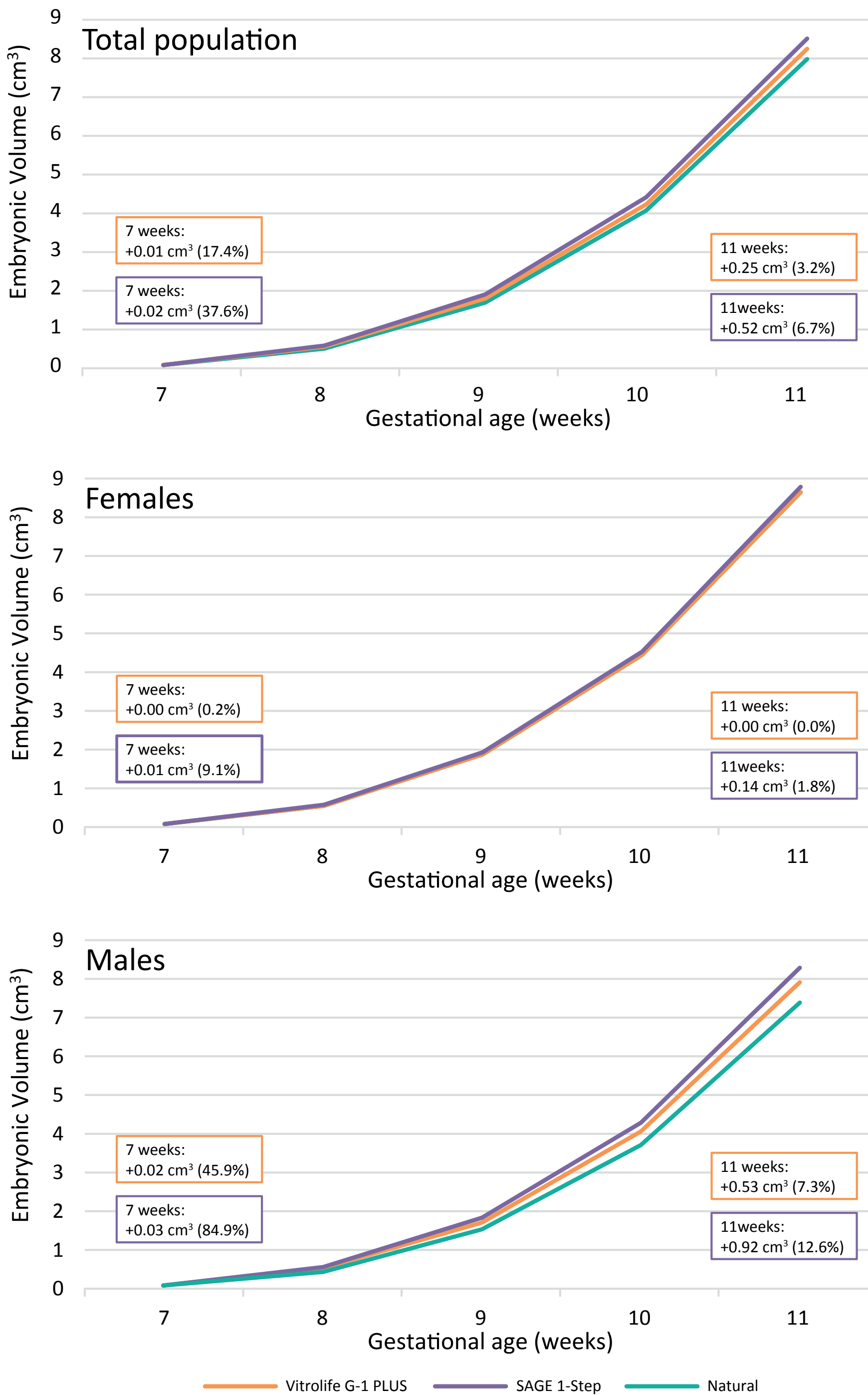
DISCUSSION

This study demonstrates a faster first-trimester embryonic growth after culture in SAGE 1-Step medium compared to 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 to naturally conceived pregnancies we observed the following: 1) faster growth in male embryos, 2) faster morphological development in female embryos, and 3) a larger EFW, HC and BPD in male fetuses when second-trimester growth parameters were studied.

In only one other study the impact of culture medium on first-trimester growth was investigated. No differences were observed in first-trimester CRL between embryos cultured in Vitrolife G1.3 PLUS and Cook K-SICM (15). However, the same study did show a difference in second-trimester BPD and HC between pregnancies after culture in Vitrolife G1.3 PLUS and Cook K-SICM, which is also seen in our study when SAGE 1-Step was compared to naturally conceived pregnancies. Yet, when the current study method is compared to those of Nelissen et al, there are fundamental differences. Besides the difference in studied culture media, they only used a single CRL measurement performed at 7-8 weeks GA as a parameter

Figure 2. Trajectories of embryonic growth presented by embryonic volumes (EV) 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 GA compared to naturally conceived pregnancies.

for first-trimester growth. In a small subset an additional transabdominal CRL measurement at 12 weeks GA was performed. Finally, they applied other statistical methods. These differences might explain the different results between both 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 embryos' own (epi-)genetic blueprint (38). 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 (35, 39-41). Interestingly, in another study performed by our group, preimplantation development was several hours faster in embryos cultured in SAGE 1-Step than in embryos cultured in Vitrolife G-1 PLUS (42). 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 EV, but not in CRL. Although CRL measurements are the gold standard for embryonic growth and relatively easy to perform, EV measurements have added value. It has been described in structurally or chromosomally abnormal embryos that CRL was comparable to normal embryos while EV was smaller, indicating a higher sensitivity (43).

It is possible that the embryonic epigenome is affected by different aspects of IVF/ICSI treatment (44, 45). One of these aspects is the possibility to cryopreserve surplus embryos and transfer them in a following cycle. However, pregnancies after frozen-thawed ET are associated with increased post-implantation growth (46). Therefore, we performed stratified analyses in pregnancies after fresh ET and after frozen-thawed ET. Although the proportion of pregnancies after frozen-ET 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 (10, 11). 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 the 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 (16, 47). Furthermore, the glucose concentration in Vitrolife G-1 PLUS is approximately 2.5 fold higher than in SAGE 1-Step (10, 11). Yet, preimplantation embryos have a limited capacity to utilise glucose prior to compaction and high levels of glucose might even be detrimental for early development (48). Little is known about the effects of high levels of glucose during *in vitro* and post-implantation development in humans. Several reports show that

prenatal maternal hyperglycaemia acts as a primary teratogen (49). Additionally, extensive research has demonstrated that women with poor-regulated diabetes have an increased risk of severe pregnancy complications, such as miscarriages, congenital malformations and babies born large for gestational age (50, 51). Interestingly, the increased risk for congenital malformations appears to be restricted to male infants (52).

We 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, since Y-linked genes are exclusively expressed in males and X-linked genes have a higher expression in females during early development (53, 54). 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 (55). This is supported by mouse studies showing that even optimised IVF conditions can reprogram post-implantation growth, fat deposition and glucose homeostasis in a sexually dimorphic fashion (56, 57).

Second-trimester growth parameters and perinatal outcomes were largely comparable between culture media in our study. There are several studies that investigated the impact of culture medium used during IVF/ICSI treatment on birthweight. A clear overview of these studies shows that only five out of eleven studies demonstrate significant differences in birthweight between culture media (58). These studies vary greatly in design, statistical methods and studied culture media. An explanation for the fact that we 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 develop 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 (β_{CRL} -0.055) (59, 60). The association between maternal prenatal smoking and susceptibility to diseases, even into adulthood, is indisputable and many efforts to stop maternal preconceptional smoking have been made (61, 62). 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 (17).

To our knowledge, there are no studies that 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 (5). Since we observe 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 (16).

Strengths and limitations

The unique and extensive collection of longitudinal first-trimester 3D ultrasound data using state-of-the-art imaging techniques, enables precise and innovative measurements that provided sufficient power to accurately model growth. By taking both growth as well as morphological development into account we were able to meticulously investigate the impact of the different culture media. Moreover, since data on second-trimester and pregnancy outcome were also included, the impact of culture medium across the complete course of pregnancy could be studied.

However, the two studied culture media were used during consecutive periods. Although there were no changes in laboratory variables such as oxygen level and incubators, a possible bias cannot be excluded. Furthermore, since the participants were recruited from a tertiary university hospital, the external validity of this explorative study is limited. To minimise the effects of potential confounders, adjusted analyses were performed. Also, patients were allowed to participate more than once in our study. However, this is only a small proportion of our 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, we show 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 optimise this environment (63). Moreover, differences in childhood phenotype between culture media have already been observed (64). So, as a final statement, we recommend culture media manufacturers to extend the focus towards offspring health when optimizing culture media, as 2-4% of the children born today are the result of an IVF/ICSI treatment (65).

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Supplemental material

Supplemental table 1. Comparison of exclusions between pregnancies after culture in Vitrolife G-1 PLUS, SAGE 1-Step, and naturally conceived pregnancies.

	IVF/ICSI Vitrolife G-1 PLUS N=168		**IVF/ICSI SAGE 1-Step N=265**		**Naturally conceived N=814**		**P-value**
	N	**%**	**N**	**%**	**N**	**%**	
Termination of pregnancy	1	0.6	1	0.4	13	1.6	0.212
Intra-uterine fetal death	1	0.6	3	1.1	7	0.9	0.839
Congenital anomaly	5	3	7	2.6	11	1.4	0.201

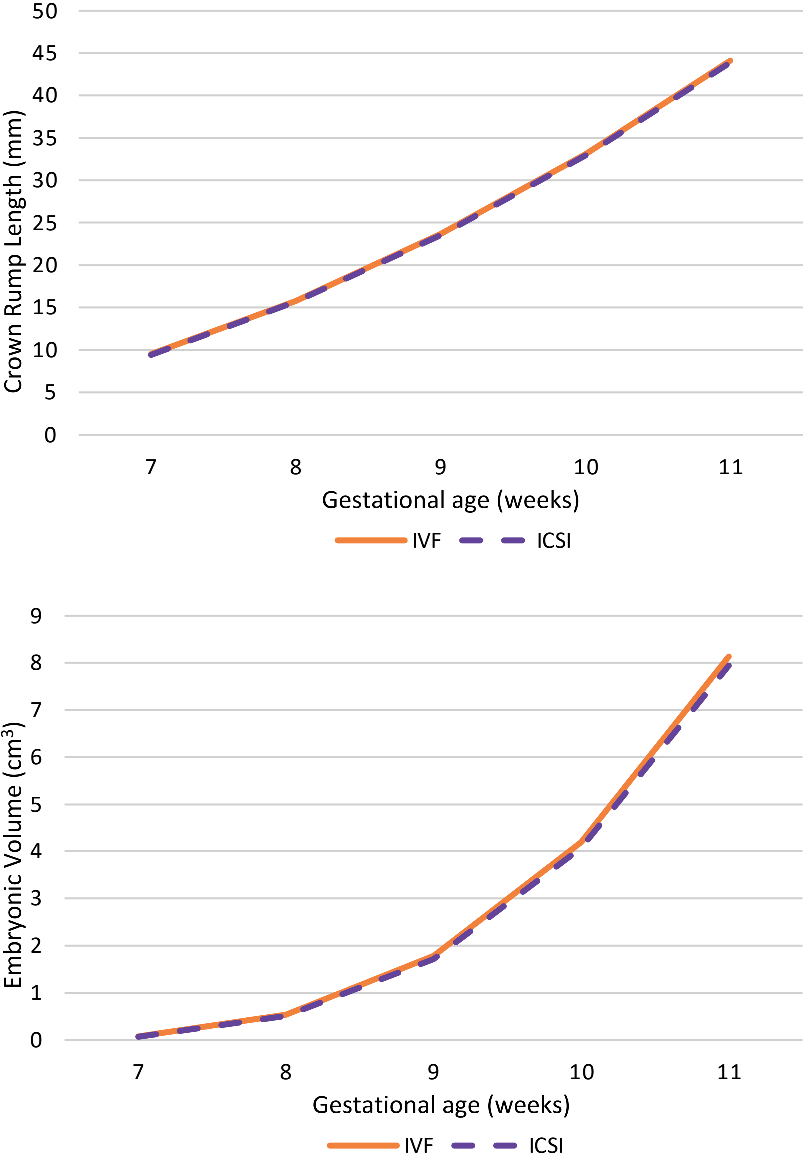
*These numbers deviate from the numbers depicted in figure 1. This can be explained by the fact that some of the pregnancies were excluded during a later stage of the selection process.

Supplemental table 2. First-trimester embryonic growth and morphological development after culture in SAGE 1-Step, relative to culture in Vitrolife G-1 PLUS, after fresh ET and after frozen-thawed ET.

		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**	**Beta (95%CI)**	**P-value**	**Beta (95%CI)**	**P-value**
Fresh ET	CRL, √mm	Reference		0.025 (-0.020, 0.071)	0.276	Reference		0.017 (-0.029, 0.063)	0.467
	EV, ∛cm³	Reference		0.036 (0.010, 0.062)	**0.007**	Reference		0.031 (0.004, 0.059)	**0.024**
	Carnegie stage	Reference		0.085 (-0.113, 0.284)	0.397	Reference		0.106 (-0.108, 0.321)	0.327
Frozen-thawed ET	CRL, √mm	Reference		-0.00 (-0.076, 0.076)	0.997	Reference		0.030 (-0.054, 0.114)	0.482
	EV, ∛cm³	Reference		-0.008 (-0.056, 0.041)	0.754	Reference		0.007 (-0.049, 0.061)	0.807
	Carnegie stage	Reference		-0.054 (-0.359, 0.250)	0.772	Reference		0.063 (-0.263, 0.389)	0.702

Model 1: Adjusted for GA.
Model 2: Adjusted for GA, maternal age, BMI, parity, geographical background, level of education, periconceptional use of alcohol, cigarettes and folic acid supplements.

Supplemental figure I. Trajectories of embryonic growth presented by crown rump length (CRL) and embryonic volumes (EV) after in vitro fertilisation (IVF, orange) or intracytoplasmic sperm injection (ICSI, purple).



Model 1: Adjusted for GA.
Model 2: Adjusted for GA, maternal age, BMI, parity, geographical background, level of education, periconceptional use of alcohol, cigarettes and folic acid supplements.

Prenatal growth trajectories and birth outcomes after frozen-thawed extended culture embryo transfer and fresh embryo transfer: The Rotterdam Periconception cohort

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ABSTRACT

Research question: Are there differences in prenatal growth trajectories and birth outcomes between singleton pregnancies conceived after in vitro fertilisation (IVF) treatment with either frozen-thawed extended culture embryo transfer (ET) at day 5, fresh ET at day 3 or naturally conceived pregnancies?

Design: From a prospective hospital-based cohort, a total of 859 singleton pregnancies were selected, including 133 conceived after IVF with frozen-thawed ET, 276 after fresh ET, and 450 naturally conceived pregnancies. Longitudinal three-dimensional (3D) ultrasound scans were performed at 7, 9 and 11 weeks of gestation for offline crown-rump length (CRL) and embryonic volume (EV) measurements. Second trimester estimated fetal weight was based on growth parameters obtained during the routine fetal anomaly scan at 20 weeks of gestation. Birth outcome data were collected from medical records.

Results: No differences regarding embryonic growth trajectories were observed between frozen-thawed ET and fresh ET. Birthweight percentiles after fresh ET were lower than after frozen-thawed ET (38.0 vs 48.0 (p=0.046) respectively). The prevalence of non-iatrogenic preterm birth was significantly lower in pregnancies resulting from fresh ET as compared to frozen-thawed ET (4.7% vs 10.9% (p=0.026) respectively). Compared to naturally conceived pregnancies, birthweight percentiles and percentage of non-iatrogenic PTB were significantly lower in pregnancies after fresh ET and gestational age at birth was significantly higher.

Conclusion: Here we show that embryonic growth is comparable between singleton pregnancies conceived after fresh and frozen-thawed ET. The lower relative birthweight and PTB rate in pregnancies after fresh ET than after frozen-thawed ET and naturally conceived pregnancies warrants further investigation.

INTRODUCTION

Since the birth of the first *in vitro* fertilisation (IVF) baby in 1978, worldwide more than eight million children are conceived via assisted reproductive techniques (ART) (1, 2). Success rates in terms of ongoing pregnancy rates per cycle have increased to 30% (3). There are numerous factors underlying the increasing success rates, such as improvements in embryo culture conditions and the ability to cryopreserve surplus embryos for later transfer (4-6). Improvements in cryopreservation techniques have attributed to a live birth rate after frozen-thawed ET of up to 40% per transfer (7, 8). Since pregnancy and live birth rates are similar for fresh and frozen embryos, an increasing number of fertility clinics are applying the 'freeze-all strategy' as standard treatment. A great advantage of the 'freeze-all strategy' is a reduction in ART-related maternal morbidity. The incidence of the ovarian hyperstimulation syndrome (OHSS) is significantly decreased since ET takes place in either a natural cycle or a hormonal replacement treatment (HRT) cycle (9). However, the increasing use of frozen-thawed embryos may not be without concerns. Recent studies show that pregnancies resulting from frozen-thawed ET are associated with an increased risk of maternal hypertensive disorders in pregnancy, babies large for gestational age (LGA) and high birthweight, when compared to pregnancies resulting from fresh ET (10, 11).

Various explanations for the reported differences between the pregnancy outcomes of fresh and frozen ET have been postulated. One is the detrimental effect of ovarian stimulation on the receptiveness of the endometrium and subsequent embryo implantation for fresh ET. During a menstrual cycle, the human endometrium is receptive to implantation for about 4-6 days; and synchronisation of embryo and endometrium is crucial for successful implantation (12-14). It has been suggested that outcomes after fresh ET are affected by supraphysiologic hormone levels, as a consequence of ovarian stimulation treatment, which may affect synchronisation by altering the level and timing of endometrial receptivity, with subsequent suboptimal implantation, embryonic growth and development (15-17). Another explanation is that the freeze-thaw procedure of cryopreservation directly affects embryo cell and DNA quality and programming of CpGs and histone methylation profiles (18).

Based on current literature, it remains unclear which periods and mechanisms during pregnancy are most vulnerable for insults that affect embryonic and fetal growth trajectories. Our hypothesis is that the origin of these differences in prenatal growth trajectories lies in the periconception window, defined as 14 weeks before until 10 weeks after conception. This is the period of oogenesis, spermatogenesis, embryogenesis, placentation, and early maternal adaptation to pregnancy. During this period, embryonic structures proliferate and differentiate and embryonic cells have the highest developmental plasticity (19).

Nowadays, in early pregnancy, embryonic growth and development can be measured by two-dimensional (2D) ultrasound techniques. The introduction of 3D ultrasound in

combination with virtual reality (VR) techniques allows in-depth perception and assessment of novel volumetric measurements. This approach provides the great opportunity to study embryonic growth in early pregnancy by measuring more precisely crown-rump length (CRL), but also embryonic volume (EV) (20, 21).

The primary aim of this study is to compare prenatal growth trajectories and birth outcomes between singleton pregnancies conceived after in vitro fertilisation (IVF) treatment with either frozen-thawed extended culture embryo transfer (ET) at day 5 and fresh ET at day 3. Secondary, we compared these two groups of pregnancies with pregnancies after natural conception.

MATERIAL AND METHODS

Ethical approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving patients were approved by the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Centre, Rotterdam, the Netherlands on 15th October 2004. Written informed consent was obtained from all women and their male partner at enrolment.

Study population

The data for this study were collected in the Rotterdam Periconception Cohort (Predict study), an ongoing prospective tertiary hospital-based cohort embedded in the outpatient clinic of the Department of Obstetrics and Gynaecology of the Erasmus, University Medical Center Rotterdam, the Netherlands (22, 23). From November 2010 onwards, women of at least 18 years of age with an ongoing intrauterine singleton pregnancy, who are less than 8 weeks pregnant are eligible for participation in this ongoing cohort. During the inclusion period, patients were allowed to participate more than once.

For the current analysis we selected women from the total study group recruited up until August 2018. We only included women who conceived through IVF treatment, with or without ICSI, either after frozen-thawed day 5 ET or fresh day 3 ET and women who conceived naturally with a regular cycle between 21-35 days and known first day of last menstrual period. Pregnancies conceived in women with a regular menstrual cycle are dated based on the first day of their last menstrual period, and for women with a cycle of <25 or 31>days, also on cycle length. Women with an irregular cycle are excluded because pregnancy dating is based on CRL by ultrasound measurements. Since, in our study CRL is one of the main outcome parameters, inclusion of pregnancies dated based on CRL would be statistically incorrect.

In vitro fertilisation and cryopreservation procedures

Ovarian stimulation, oocyte retrieval, IVF procedures and assessment of embryo morphology were performed as described previously (24). Embryos were cultured in Vitrolife G5 series (Vitrolife, Goteborg, Sweden) until 17 November 2014; and from this moment onwards in SAGE 1-Step™ (Origo/Cooper Surgical™, Denmark). Embryo evaluation and selection for transfer were carried out on day 3 after oocyte retrieval. Selection was based on developmental stage and morphology, per standard procedures. Cell number, regularity of blastomeres, fragmentation and morphological aspects such as signs of early compaction were recorded. Supernumerary embryos were cultured until day 4, when selection for cryopreservation was performed based on the degree of embryo compaction and the presence of fragmentation as described previously (25). Only embryos with at least 30% compaction, or embryos with more than 12 blastomeres were selected for cryopreservation. First, embryos were incubated in culture medium containing 1.0M dimethyl sulfoxide (DMSO) for 10 minutes, transferred to 1.5M DMSO in culture medium and loaded into straws (CBS High Security embryo straw, CryoBioSystem, Saint-Ouen-Sur-Iton, France). Next, cryopreservation was performed by slow freezing in a controlled-rate freezer (Kryo 360, Planer, Sunbury-on-Thames, United Kingdom). Straws were cooled to -6 ˚C before seeding and subsequently cooled to -40 ˚C at 0.3 ˚$Cmin^{-1}$. Finally, the straws were cooled rapidly at -25 ˚$Cmin^{-1}$ to -140 ˚C, before immersion in liquid nitrogen and storage in nitrogen vapor. Duration of storage varied between 1 month and up to five years.

Thawing of embryos was performed typically in the afternoon, four days after the detection of a spontaneous ovulatory surge by urinary LH testing performed in a natural cycle. This procedure occurred at room temperature by consecutive washes in decreasing concentrations of DMSO in buffered culture medium until March 2016. After that, thawing was performed using the Quinn’s Advantage™ Thaw Kit (CooperSurgical, Trumbull, United States of America) according to manufacturer’s instructions. After thawing, the embryos were checked 1 hour later for survival and then cultured overnight in 1 ml Sage 1-Step culture medium at 37˚C in an atmosphere of 5% CO_2 and 7% O_2. The next afternoon, that is 5 days after the spontaneous LH surge, embryo morphology was assessed and embryos were transferred only if they showed post-thaw progression in development.

Study parameters

Standardised anthropometric measurements were carried out, including maternal height with 0.1 cm accuracy and weight with 0.1 kg accuracy (anthropometric rod and weighing scale; SECA, Hamburg, Germany). Women completed self-administered questionnaires regarding general characteristics as well as periconceptional lifestyle behaviors. Extracted data included maternal age, parity, geographic origin, educational level, periconceptional use of cigarettes, alcohol and folic acid (FA) supplements.

The gestational age of pregnancies after frozen-thawed ET was calculated based on the time of fertilisation and the moment of ET (corresponding to a day 5 embryo or GA of 19 days). The GA of pregnancies after fresh ET was calculated based on the conception date (=oocyte retrieval date corresponding with GA of 17 days). For naturally conceived pregnancies, the GA was calculated based on the reported last menstrual period. Furthermore, to calculate estimated fetal weight (EFW), second trimester fetal growth parameters (head circumference (HC), abdominal circumference (AC) and femur length (FL)) were obtained during the fetal anomaly scan around 20 weeks of gestation, and retrieved from medical records. Pregnancy outcomes such as GA, weight and fetal sex at birth as well as pregnancy induced hypertension (PIH), preeclampsia (PE) and gestational diabetes mellitus (GDM) were also retrieved from medical records and delivery reports. For the IVF/ICSI population medical records were searched to determine whether pregnancy was conceived after a fresh ET or frozen-thawed ET.

Geographic origin was categorised as Western (both parents born in a country in North America, Oceania or Europe, Turkey excluded) or Non-Western (one of the parents born in Turkey or a country in Africa, Asia or South America). Educational level was categorised according to the definition of Statistics Netherlands in low (primary/lower vocational/ intermediate secondary), intermediate (higher secondary/intermediate vocational) or high (higher vocational/university). Smoking and alcohol were defined as any nicotine or alcohol use in the periconception period. Folic acid (FA) supplement use was defined as daily periconceptional use of a FA supplement (0.4mg, 0.5mg or 5 mg). Small for gestational age (SGA) was defined as birthweight below the 10th percentile and LGA was defined as birthweight above the 90th percentile. Preterm birth (PTB) was defined as GA at delivery <37+0 weeks. Iatrogenic causes of preterm birth were removed for subgroup analysis. All inductions of labor were defined as iatrogenic. However, in our study population induction of labour was only indicated where there was a suspicion of macrosomia.

Ultrasound data

From November 2010 untill December 2012 women received weekly transvaginal 3D ultrasound scans from enrolment up to the 13th week of pregnancy. From December 2012 onwards, the number of ultrasound scans was reduced to three scans at the 7th, 9th and 11th week of gestation, as this was sufficient to accurately model growth. Ultrasound scans were performed with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D View software (General Electrics Medical Systems, Zipf, Austria).

Subsequently, images were transferred to the Barco I-Space (a Cave Automatic Virtual Environment–like virtual reality system). The V-Scope volume rendering application creates an interactive hologram, allowing real depth perception. Using a tracing application, the CRL was measured three times per embryo, and the mean of these measurements was

used for analysis (21). Besides allowing depth perception, V-Scope also offers the possibility to measure the EV (semi-) automatically. For this purpose, gray scale differences are used. The EV measurements are performed once, as previously described (20). All measurements were performed by trained research staff.

Statistical analysis

Baseline characteristics were compared using the Kruskall-Wallis test for continuous variables and the Chi-squared test for categorical variables.

To consider the correlation between the ultrasound measurements of the same pregnancy, we used linear mixed models to assess the associations of embryonic growth between pregnancies conceived after frozen-thawed ET and fresh ET. Embryonic growth trajectories of pregnancies after frozen-thawed ET were compared to pregnancies after fresh ET as reference. Additionally, embryonic growth trajectories of both groups were also compared with natural pregnancies. For analysis, a square root transformation was applied to the CRL and a third root transformation to EV, which led to linearity with GA.

To adjust for potential confounders, two different models were constructed with pregnancies after fresh ET as reference. Model 1 was adjusted for GA only. Model 2 was additionally adjusted for fetal sex and the maternal covariates age, body mass index (BMI), geographic origin, parity, education, periconceptional use of cigarettes, alcohol and FA supplement use. Second trimester EFW was calculated using the Hadlock formula with HC, AC and FL in centimeter: $\log(EFW) = 1.326 - 0.00326 \times AC \times FL + 0.0107 \times HC + 0.0438 \times AC + 0.158 \times FL$ (26). Percentiles of EFW were calculated from population-based fetal growth charts (27).
To adjust for fetal sex and GA at delivery, weight at birth was expressed in percentiles based on Dutch reference curves for birthweight (28). Differences in birthweight percentiles, birthweight and GA at birth were studied using the Kruskall-Wallis test. Differences in PTB, SGA and LGA were studied using the Chi-squared test.

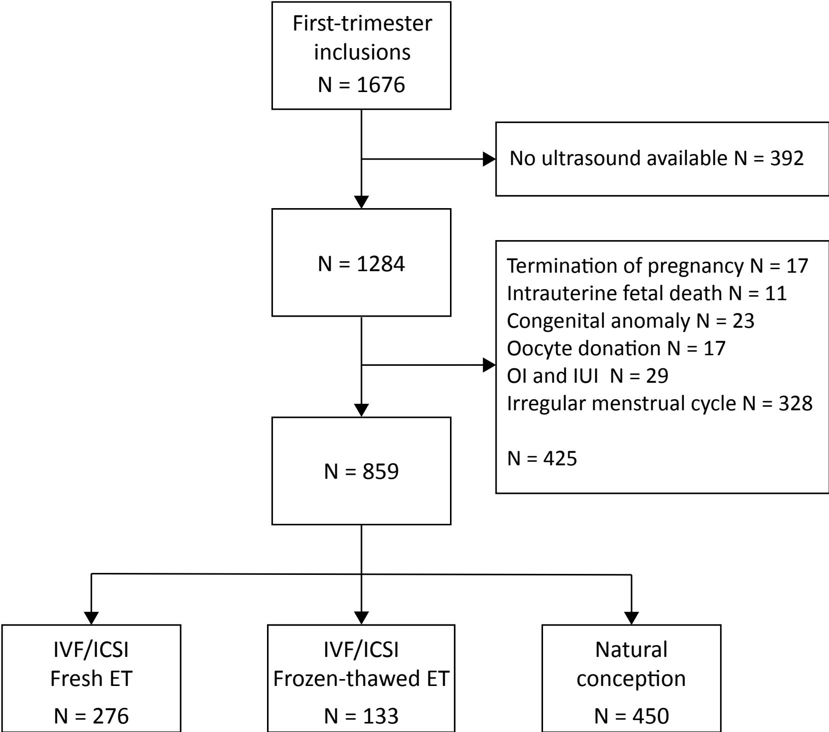
P-values <0.05 were considered statistically significant. All analyses were performed using SPSS package 25.0 (IBM SPSS Statistics, Armonk, NY) and R (R: A language and Environment for Statistical Computing, version 3.1.3, 2015 for Windows, R Core Team, Vienna, Austria).

RESULTS

1,676 pregnant women were included in the Rotterdam Periconception Cohort. Patients who did not receive first-trimester ultrasound examinations were excluded (n=392). Furthermore, 425 pregnancies were excluded because of termination of pregnancy (n=17), intrauterine fetal death (n=11), congenital anomalies (n=23), oocyte donation (n=17), pregnancies after other artificial reproductive technologies (n=29) and women with an irregular cycle (n=328) (**Figure 1**). There are no significant differences between the groups

of pregnancies complicated by congenital anomalies and pregnancies without congenital anomalies between fresh ET, frozen ET and naturally conceived pregnancies (*p*=0.211) (data not shown).

Figure 1. Flowchart of inclusion and exclusion of the study population.



OI, ovulation induction. IUI, intrauterine insemination. IVF, *in vitro* fertilisation. ICSI, intracytoplasmic sperm injection. ET, embryo transfer.

From the 859 included pregnancies, 133 were conceived after frozen-thawed ET, 276 after fresh ET, and 450 after natural conception. Of the 133 pregnancies after frozen-thawed ET, 13 were after a freeze-all strategy. A total of 42 women participated twice, of which 5 patients participated with both a naturally conceived and an IVF/ICSI pregnancy, and 9 with both a pregnancy after fresh ET and after frozen-thawed ET. Included pregnancies are significantly different regarding maternal age (32.4 vs. 31.5 years, *p*=0.002), parity (52.3% vs. 45.1% nulliparous, *p*=0.014), geographic origin (83.1% vs. 75.2% Western, *p*=0.001) and periconceptional use of folic acid supplements (83.2% vs. 75.3% adequate use, *p*=0.001) as compared to excluded pregnancies (data not shown).

Baseline characteristics:

At baseline, women pregnant after frozen-thawed ET were significantly less often nulliparous than women pregnant after fresh ET (60.9% and 72.5%, *p*=0.018) and used alcohol significantly more (28.6% and 19.2%, *p*=0.033) (**Table 1**). Pregnancies after frozen-thawed ET were less often the result of a double embryo transfer (DET) than pregnancies after fresh ET (3.8% vs. 13.4%, *p*=0.003), however the incidence of a vanishing twin syndrome was comparable (0.8% vs. 1.1%, *p*=0.747). Other baseline characteristics were not significantly different between the two groups.

Table 1. Baseline characteristics of study population of pregnant women.

	IVF/ICSI Frozen-thawed ET N = 133		IVF/ICSI Fresh ET N = 276			
	Median / N	**IQR / %**	**Median / N**	**IQR /N**	**P-value**	**Missing**
Maternal characteristics						
Age, years	32.8	29.5-36.0	33.0	30.2-36.1	0.456	0
Body mass index, kg/m^2	24.3	21.7-27.7	24.4	21.7-28.0	0.778	17
Nulliparous	81	60.9	200	72.5	**0.018**	0
Geographic origin: Western	103	77.4	233	84.4	0.084	0
Education					0.650	14
Low	7	5.4	20	7.5		
Middle	53	41.1	100	37.6		
High	69	53.5	146	54.9		
Periconceptional lifestyle						
Alcohol, yes[a]	38	28.6	52	19.2	**0.033**	5
Smoking, yes[a]	15	11.3	34	12.5	0.714	5
Folic acid, yes[b]	128	96.2	261	96.7	0.826	6
Treatment characteristics						
Double embryo transfer	5	3.8	37	13.4	**0.003**	0
Vanishing twin syndrome	1	0.8	3	1.1	0.747	0

IQR, interquartile range. IVF, in vitro fertilisation. ICSI, intracytoplasmic sperm injection. ET, embryo transfer.

a. Any use during the 14 weeks prior to 10 weeks after conception.

b. Daily use during the 14 weeks prior to 10 weeks after conception.

When compared with naturally conceived pregnancies, it was demonstrated that women pregnant after fresh ET were significantly older than women who conceived naturally (33.0 and 31.8 years, *p*=0.001) (**Supplemental table 1**). Furthermore, there were significant differences between women in the frozen-thawed, fresh ET and naturally conceived group regarding maternal parity (nulliparous: 60.9%, 72.5% and 37.3%, respectively, *p*<0.00)) and alcohol consumption (28.6%, 19.2% and 37.9%, respectively, *p*<0.001). Women who conceived naturally also used folic acid significantly less adequate than women pregnant after fresh ET or after frozen thawed ET (71.1%, 96.7% and 96.2%, respectively, *p*<0.001).

First-trimester:

The linear mixed model regression analysis (model 1) of pregnancies following frozen-thawed ET compared to pregnancies after fresh ET, showed no significant differences for

CRL (β -0.003 (95%CI -0.045, 0.037)) and EV measurements (β 0.006 (95%CI -0.019, 0.030)) (**Table 2**). After adjusting for the confounders (model 2, see Statistical analysis section of the Methods and Materials), similar results were observed regarding CRL (β -0.010 (95%CI -0.055, 0.035)) and EV measurements (β -0.001 (95%CI -0.028, 0.027)). The crude comparison with naturally conceived pregnancies revealed a positive association for pregnancies after fresh ET and CRL (β 0.060 (95%CI 0.015, 0.105)) and EV (β 0.039 (95%CI 0.016, 0.062)), and pregnancies after frozen-thawed ET and EV (β 0.044 (95%CI 0.015, 0.074)). In model 2, significant associations remained for fresh ET and frozen-thawed ET and EV (fresh: β 0.047 (95%CI 0.014, 0.081); frozen-thawed ET: β 0.046 (95%CI 0.018, 0.075)) (**Table 2**).

Table 2. Beta's from the models for the different modes of conception with respect to estimates of embryonic growth trajectories of serial crown-rump length (CRL) and embryonic volume (EV) measurements.

	Model 1				**Model 2**			
	CRL		**EV**		**CRL**		**EV**	
	Beta (95%CI) √mm	**P-value**	**Beta (95%CI) ∛cm³**	**P-value**	**Beta (95%CI) √mm**	**P-value**	**Beta (95%CI) ∛cm³**	**P-value**
Fresh ET	reference		reference		reference		reference	
Frozen-thawed ET	-0.003 (-0.045, 0.037)	0.853	0.006 (-0.019, 0.030)	0.644	-0.010 (-0.055, 0.035)	0.662	-0.001 (-0.028, 0.027)	0.969
Naturally conceived	reference		reference		reference		reference	
Fresh ET	0.060 (0.015, 0.105)	**0.008**	0.039 (0.016, 0.062)	**0.001**	0.051 (-0.004, 0.106)	0.071	0.047 (0.014, 0.081)	**0.006**
Frozen-thawed ET	0.057 (-0.001, 0.115)	0.055	0.044 (0.015, 0.074)	**0.003**	0.044 (-0.022, 0.111)	0.191	0.046 (0.018, 0.075)	**0.001**

ET, embryo transfer. CRL, crown rump length. EV, embryonic volume. GA, gestation age.
Model 1: Adjusted for GA.
Model 2: Model 1 + maternal covariates (age, body mass index, parity, education, geographic origin, smoking, alcohol, folic acid, fetal sex).

Second trimester:

The z-scores of mid-pregnancy EFW were comparable in pregnancies after frozen-thawed ET and after fresh ET (median z-scores 74.5 and 66.9, respectively (*p*=0.068)). When compared to naturally conceived pregnancies, it was demonstrated that fetuses after frozen-thawed ET have a higher mid-pregnancy EFW than naturally conceived fetuses (median z-score: 74.5 and 68.1, *p*=0.020) (**Table 3**).

Birth outcomes:

The median birthweight percentile of pregnancies after frozen-thawed ET was significantly higher than of pregnancies after fresh ET (48 and 38 (*p*=0.046)), whereas the gestational age at birth was lower (275 and 276 days (*p*=0.044)) (**Table 3**). There were no significant differences regarding the frequencies of PIH, PE and GDM between the two groups. However, when iatrogenic causes of PTB (induction or suspicion of macrosomia) were excluded, the prevalence of PTB was significantly lower in the fresh ET group than in the frozen-thawed ET group (4.7% vs. 10.9%, *p*=0.026).

Table 3. Second trimester estimated fetal weight and birth outcomes for the different modes of conception

	IVF/ICSI pregnancies frozen-thawed ET N = 133		IVF/ICSI pregnancies fresh ET N = 276			Naturally conceived pregnancies N = 450			
Second trimester	**Median**	**IQR**	**Median**	**IQR**	**P-value**	**Median**	**IQR**	**P-value**	**Missing**
Estimated fetal weight, z-score	74.5	55.8-89.1	66.9	45.9-87.9	0.068	68.1	41.6-87.4	0.072[a]	42
Third trimester	**Median**	**IQR**	**Median**	**IQR**	**P-value**	**Median**	**IQR**	**P-value**	**Missing**
Birthweight percentile	48	24-79	38	18-69	**0.046**	48	24-76	**0.021[b]**	65
Gestational age, days	275	266-281	276	270-283	**0.044**	272	256-280	**<0.001[b]**	53
Birthweight, grams	3373	3045-3680	3333	3000-3649	0.607	3355	2965-3713	0.869	40
Third trimester	**N**	**%**	**N**	**%**	**P-value**	**N**	**%**	**P-value**	**Missing**
Small for gestational age	13	11.8	43	17.1	0.204	59	13.7	0.329	65
Large for gestational age	12	10.9	19	7.5	0.292	53	12.3	0.151	65
Preterm birth, total	14	11.8	15	5.9	0.050	41	9.4	0.125	53
Preterm birth, excluding iatrogenic	13	10.9	12	4.7	**0.026**	40	9.2	0.054[b]	53
Pregnancy induced hypertension	10	7.8	12	4.4	0.116	40	9.1	0.072	23
Preeclampsia	5	3.9	7	2.6	0.470	21	4.7	0.354	23
Gestational diabetes mellitus	12	9.4	21	7.8	0.581	33	7.5	0.771	23

IQR, interquartile range. IVF, in vitro fertilisation. ICSI, intracytoplasmic sperm injection. ET, embryo transfer.
Significantly different between a) frozen-thawed ET and naturally conceived pregnancies, and b) fresh ET and naturally conceived pregnancies.
Data are presented as Median (IQR) or as numbers (%).

When compared to naturally conceived neonates, it was found that neonates after fresh ET have a lower relative birthweight (birthweight percentile: 38 and 48, p=0.021) and were born a few days earlier (gestational age: 272 and 276 days, p<0.001). There were no significant differences regarding the frequencies of PIH, PE and GDM between the three groups. In addition, no significant differences in absolute birthweight and the frequencies of SGA and LGA were observed. However, the prevalence of non-iatrogenic PTB was lower in pregnancies after fresh ET compared to naturally conceived pregnancies (4.7% and 9.2% p=0.033).

DISCUSSION

We observed that pregnancies after fresh ET compared with pregnancies after frozen-thawed ET exhibit similar first-trimester growth trajectories and mid-pregnancy EFW, but a lower relative birthweight and overall and non-iatrogenic PTB rates. Furthermore, our data showed that transfer of a fresh day 3 embryo resulted less often in PTB compared to extended cultured frozen-thawed ET. The latter group of pregnancies show similar pregnancy outcomes as compared to natural conceived pregnancies, suggesting that application of a freeze-all strategy could be an acceptable option.

Despite using a highly sensitive technique, no differences in embryonic growth trajectories between pregnancies after fresh and frozen-thawed ET were observed. We did however find differences between embryonic growth of frozen-thawed and fresh ET as compared to natural conceived embryos, suggesting an effect of artificial reproductive techniques. Our

findings are in agreement with a recent study which showed that the size of the embryo in the first-trimester was not significantly different between pregnancies after fresh or frozen-thawed ET and IUI (29). We revealed that pregnancies both after frozen-thawed and fresh ET exhibit larger embryonic growth in the first-trimester of pregnancy than natural pregnancies, which is line with our previous study in a subset of the same cohort showing embryos after IVF/ICSI pregnancies to be larger (30).

We found that the relative birthweight was lower in pregnancies after fresh ET than in pregnancies after frozen-thawed ET. This is in line with previous reports suggesting lower birthweights of babies born after fresh ET as compared to frozen-thawed ET (29, 31, 32). Although birthweight percentiles were significantly lower after fresh ET, it is a crude birth outcome, influenced by many maternal conditions and exposures. For example, studies in babies born during the Dutch hunger winter showed comparable birthweights, however later in life these babies were at increased risk for cardiovascular disease and cancer (33, 34). Hence, subtle epigenetic effects of the cryopreservation procedure might only be seen after years of childhood development. So comparable pregnancy outcomes do not necessarily mean comparable postpartum cognitive and behavioral development. Therefore, our results are supportive of longitudinal follow-up of children conceived via diverse assisted reproduction techniques to provide insight into these associations.

We find a lower risk of PTB in pregnancies after fresh ET as compared to pregnancies conceived after frozen-thawed ET. This is in contrast with recent studies; Maheshwari et al. report a significant decrease in the prevalence of PTB after frozen-thawed ET as compared to fresh ET (10, 11). Our study was conducted with fresh embryos transfers on day 3 in a time period that this was routine clinical practice in most IVF clinics and also in our clinic. Surplus embryos were cultured for one extra day before freezing and one extra day after thawing to investigate viability before transfer. In our study it is difficult to distinguish the effect of cryopreservation on PTB versus the effect of extended embryo culture, as all cryopreserved embryos are cultured for a longer time than freshly transferred embryos.

Studies investigating the effect of extended culture on risk of PTB report conflicting results. Two large studies show no enhanced risk of PTB after cleavage-stage ET compared to blastocyst-stage ET (35, 36), whereas one larger and two smaller studies report a greater risk of PTB after blastocyst-stage ET compared to cleavage-stage ET (37-39). Our study in which frozen-thawed day 5 or blastocysts stage embryos were transferred shows no increased risk of PTB. No previous studies investigated the effect of cryopreservation and extended culture on first-trimester embryonic growth and the incidence of PTB, therefore a power analysis was not possible on beforehand. The posthoc sample size calculation using an α-level of 0.05 and power of 80%, revealed that at least 190 participants are needed to accurately show significance, which are present in our current study. Our study was not powered to

investigate the effect of cryopreservation in subgroups of IVF and ICSI. Previous studies have shown no differences in first-trimester embryonic growth and development (40) and live birth rate (41, 42) between conventional IVF and ICSI pregnancies. However, the potential additional effect of the process of cryopreservation has not yet been investigated and therefore, future larger studies should take this technique into account.

Various explanations for the reported differences have been postulated. One of these is the effect of ovarian stimulation on the endometrium quality and receptivity for subsequent embryo implantation. Supraphysiologic hormone levels of follicle stimulating hormone and human chorionic gonadotropin during ovarian stimulation may affect synchronisation of embryo and endometrium by altering the level and timing of endometrial receptivity, with subsequent altered implantation, embryonic and placental growth and development (15-17).

Moreover, in the study period, cleavage stage embryos were cryopreserved using a slow-freeze method with (DMSO) as cryoprotectant agent. The addition of DMSO to the culture medium of mouse embryos can cause changes in RNA, protein expression of DNA methyltransferases and histone modifying enzymes, resulting in differences in the overall epigenome and structurally abnormal embryos (43). Moreover, the whole process of freezing and thawing can also affect general cell structure and DNA stability. In bovine embryos, expression and methylation of imprinted and developmentally important genes is significantly altered after the freeze-thawing process (44, 45). Embryonic growth and pregnancy outcomes are dependent on trophoblast and placental functioning, where placental dysfunction is strongly associated with vascular related pregnancy complications, such as PTB and preeclampsia. Pregnancies resulting after frozen-thawed ET are associated with more anatomic and vascular placental pathology (46, 47). Furthermore, epigenetic differences in placentae are described, with different microRNA expression profiles in term placentae after frozen-thawed ET and fresh ET (48). The influence of the cryopreservation process on trophoblast and placental functioning needs further elucidation.

Strengths and limitations

A strength of this study is the collection of multiple prospective longitudinal (3D) ultrasound examinations during the first and second trimester of pregnancy and the resulting pregnancy outcome data. We also included naturally conceived pregnancies as reference group, allowing us to compare outcomes between fresh and frozen-thawed ET in a wider perspective. Furthermore, we used innovative methods and standardised protocols to precisely measure the outcome variables. We corrected for multiple confounders to minimise residual confounding, yet due to the observational character of the study this can never be completely excluded. The analyses were adjusted to minimise the effects of baseline differences between the three groups. Additionally, the embryo with the best

morphological appearance will generally be used for fresh ET, which means that embryos used for frozen-thawed ET are automatically of lesser morphological quality. This might have affected our results. Our findings cannot be automatically extrapolated to other IVF clinics, as our study population is from a tertiary hospital which has consequences for the external validation of the results.

The majority of pregnancies resulting from frozen-thawed ET occurred after transfer in a natural cycle (n=108). Previous studies, however, showed no differences regarding live birth rate, preterm birth and birthweight after frozen-thawed ET in natural vs. hormonal cycles (49-51). Furthermore, our study consisted of only frozen-thawed ET after slow-freezing, while newer techniques like vitrification were not included in our study. There is some evidence that the clinical pregnancy rate after vitrification is higher over slow-freezing (52). However, clinical outcomes, such as preterm birth and birthweight, are comparable between vitrified and slow-frozen embryos (53). Patients were allowed to participate more than once in our study, however as this is a small proportion of our study population, it is unlikely to have affected our results. Therefore, we did not make additional adjustments for this.

Conclusion

Due to the increasing success of IVF/ICSI treatment and supernumerary good quality embryos, a rising number of children are being born after a frozen-thawed ET. Also, the freeze-all strategy is increasingly applied to optimise success rates, to circumvent OHSS risk and to prevent a possible detrimental effect of the ovarian stimulation on endometrial receptivity and implantation rates (9).

It is reassuring that pregnancy outcome and PTB rates in pregnancies after frozen-thawed ET are comparable to pregnancies after natural conception. The lower risk of PTB after fresh ET as compared to frozen-thawed and natural conceptions, suggest that fresh ET may provide some advantages to extended culture of frozen-thawed ET. Furthermore, the impact of extended culture of these cryopreserved embryos after thawing warrants further investigation. Overall, our findings for pregnancies after frozen/thawed ET are reassuring with regard to safety. However, little is known about offspring health later in life when conceived through IVF/ICSI. So, ideally, before adopting a general policy of elective freezing of all embryos after IVF/ICSI, more research is needed to assess the impact of the cryopreservation procedure on embryo development and offspring health later in life.

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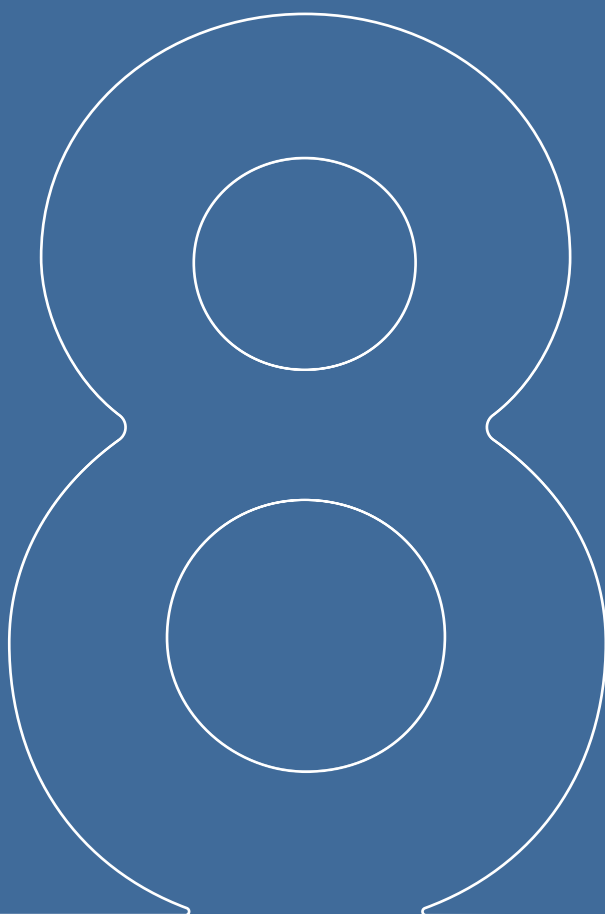
Supplemental material

Supplemental table 1. Baseline characteristics of study population of pregnant women.

	IVF/ICSI Frozen-thawed ET N = 133		IVF/ICSI Fresh ET N = 276		Naturally conceived pregnancies N = 450			
	Median / N	IQR /%	Median / N	IQR /%	Median / N	IQR /%	P-value	Missing
Maternal characteristics								
Age, years	32.8	29.5-36.0	33.0	30.2-36.1	31.8	28.9-35.0	**<0.01**[e]	0
Body mass index, kg/m^2	24.3	21.7-27.7	24.4	21.7-28.0	24.4	22.1-28.7	0.16	36
Nulliparous	81	60.9	200	72.5	168	37.3	**<0.01**[c,d,e]	0
Geographic origin: Western	103	77.4	233	84.4	378	84.0	0.16	0
Education							0.16	20
Low	7	5.4	20	7.7	34	7.7		
Middle	53	41.4	100	37.6	137	30.9		
High	69	53.5	146	54.9	273	61.5		
Periconceptional lifestyle								
Alcohol, yes[a]	38	28.6	52	19.2	169	37.9	**<0.01**[c,d,e]	9
Smoking, yes[a]	15	11.3	34	12.5	77	17.3	0.10	9
Folic acid, yes[b]	128	96.2	261	96.7	318	71.1	**<0.01**[d,e]	9

IQR, interquartile range.

a. Any use during the 14 weeks prior to 10 weeks after conception.
b. Daily use during the 14 weeks prior to 10 weeks after conception.
c. Significantly different between pregnancies after frozen-thawed ET and after fresh ET.
d. Significantly different between pregnancies after frozen-thawed ET and naturally conceived pregnancies.
e. Significantly different between pregnancies after fresh ET and naturally conceived pregnancies.



The influence of frozen-thawed and fresh embryo transfer on utero-placental (vascular) development: The Rotterdam Periconception cohort

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ABSTRACT

Study question: Does frozen-thawed or fresh embryo transfer (ET) influence utero-placental (vascular) development, when studied using three-dimensional (3D) ultrasound and virtual reality imaging techniques?

Summary answer: In the first-trimester, placental developmental parameters, i.e. placental volume (PV) and utero-placental vascular volume (uPVV), were comparable between pregnancies resulting from frozen-thawed ET, fresh ET and natural conception; in the second and third trimester, uterine artery Doppler indices were lower in pregnancies after frozen-thawed ET compared to pregnancies after fresh ET and natural conception.

What is known already: Pregnancies after frozen-thawed ET are at risk of developing placenta-related pregnancy complications. There is strong evidence that impaired first-trimester spiral artery remodelling is involved in the pathophysiology of these complications. Studies on longitudinal placental development in pregnancies with different modes of conception, i.e. after frozen-thawed ET, fresh ET or natural conception, are lacking.

Study, design, size, duration: Women with singleton pregnancies were included before 10 weeks of gestation, between January 2017 and July 2018, as a subcohort of the ongoing Rotterdam Periconception cohort. Results were partially validated in 722 women from the total cohort, which was conducted from November 2010 onwards.

Participants/materials, setting, methods: A total of 214 women, of whom 32 conceived after frozen-thawed ET, 56 conceived after fresh ET and 126 conceived naturally, were selected**.** PV and uPVV measurements were obtained at 7, 9 and 11 weeks of gestation by transvaginal three-dimensional (3D) (power Doppler) ultrasound. The uterine artery pulsatility index (UtA-PI) and resistance index (UtA-RI) were measured transvaginally at 7, 9, 11 and 13 weeks and abdominally at 22 and 32 weeks of gestation by pulsed wave Doppler ultrasound. In the validation cohort, the PV was measured in 722 women. Associations between mode of conception and placental development were studied using linear mixed models.

Main results and the role of chance: First-trimester parameters of placental development, i.e. PV, uPVV, UtA-PI and UtA-RI, were comparable between pregnancies after frozen-thawed and fresh ET and naturally conceived pregnancies. In our validation cohort, comparable results were found for PV. However, second- and third-trimester UtA-PI and UtA-RI in pregnancies after frozen-thawed ET were significantly lower than in pregnancies after fresh ET ($\beta_{UtA\text{-}PI}$ -0.158 (95%CI -0.268, -0.048), *p*=0.005; $\beta_{UtA\text{-}RI}$ -0.052 (95%CI -0.089, -0.015), p=0.006). Second- and third- trimester uterine artery indices in pregnancies after fresh ET were comparable to those in pregnancies after natural conception.

Limitations and reasons for caution: The main limitation of this study is the lack of power to optimally detect differences in placental development and placenta-related pregnancy outcomes between pregnancies after different modes of conception. Moreover, our population was selected from a tertiary hospital and included a relatively limited number of pregnancies. Therefore, external validity of the results should be confirmed in a larger sample size.

Wider implications of the findings: These findings indicate no significant impact of conception mode on early placental development and a beneficial impact for frozen-thawed ET on second- and third-trimester Doppler indices. This suggests that frozen-thawed ET may not be as detrimental for placental perfusion as previous research has demonstrated. As the number of clinics applying the ‘freeze-all strategy’ increases, future research should focus on establishing the optimal uterine environment, with regards to hormonal preparation, prior to ET to reduce placental-related pregnancy complications after frozen-thawed ET.

INTRODUCTION

In vitro fertilisation treatment (IVF) is a relatively successful fertility treatment to achieve pregnancy. However, these pregnancies are associated with increased risks of placenta-related pregnancy complications, such as preeclampsia and fetal growth restriction (1). These complications also have consequences for long-term maternal and offspring health (2, 3). Moreover, recent studies have demonstrated that some complications are more prevalent in pregnancies after frozen-thawed embryo transfer (ET) than after fresh ET (4, 5). However, a potential life-threatening complication of IVF treatment is the ovarian hyperstimulation syndrome (OHSS), which is caused by pharmacologic ovarian stimulation (6). To lower the risks of this complication, an increasing number of fertility clinics transfer only frozen-thawed embryos as the standard procedure (7). Although this strategy does not seem to reduce IVF success rates in terms of implantation, there may be serious consequences for the prevalence of preeclampsia after IVF-treatment (8).

A well-developed placenta is essential for successful pregnancy. It is crucial that the placenta is able to exchange gasses and supply nutrients to facilitate the embryo in reaching its full growth potential (9). Placental development starts 6-10 days after conception, when embryonic trophoblast cells invade the decidua (10). The maternal decidua is supplied with blood from the spiral arteries, which extensively remodel from the first-trimester onwards after invasion of the extravillous trophoblast (11). Deficiencies in remodelling have been associated with severe pregnancy complications, such as miscarriage, fetal growth restriction and preeclampsia (12, 13).

IVF-treatment procedures can have several consequences for embryo implantation and placental development. For example, it has been suggested that the supraphysiological levels of hormones used for ovarian stimulation during IVF-treatment impact endometrial development (14-16). Also, studies show that the hormonal preparation of the endometrium prior to frozen-thawed ET may have a detrimental effect on maternal adaptation to pregnancy (17).

It is challenging to assess *in vivo* placental development during pregnancy. To measure features of placental development, two-dimensional (2D) and three-dimensional (3D) ultrasound techniques, as well as 3D power Doppler (PD) can be applied. Such features include first-trimester placental volume (PV), utero-placental vascular volume (uPVV) and uterine vascular parameters such as pulsatility and resistance indices of the uterine arteries (UtA-PI and UtA-RI). Some of these features have been associated with birthweight, placental weight and even placenta-related pregnancy complications (18-22).

Less is known about the impact of IVF treatment with fresh or frozen-thawed ET on these features of first-trimester placental development. The only two studies that distinguished

between pregnancies after fresh and frozen-thawed ET have demonstrated a larger first-trimester PV in pregnancies after frozen-thawed ET than in pregnancies after fresh ET (23, 24). However, both studies had a cross-sectional design and PV was obtained at the end of the first-trimester. Although this may provide insight in the pathophysiology underlying the increased risk of placenta-related pregnancy complications in pregnancies after frozen-thawed ET, longitudinal data of several features of placental development are lacking. It is likely that variations in IVF-treatment have already had an impact on placental development earlier in gestation, as implantation initiates placental development by inducing alterations in the decidua and spiral arteries (25). Therefore, our aim is to study differences in placental development, measured by longitudinal 2D and 3D (including virtual reality (VR)) ultrasound measurements in the first, second and third trimester, between pregnancies resulting from fresh ET, frozen-thawed ET and after natural conception.

METHODS

Study population

The data used for this study was collected as part of the VIRTUAL Placenta study (Dutch Trial Register number: 6684), a subcohort of the Rotterdam Periconception Cohort (Predict Study), which focuses on ultrasound markers of (early) placentation (26). This study was conducted from January 2017 to March 2018 at the outpatient clinic of the Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Center, Rotterdam, the Netherlands. Women 18 years and older, before 10 weeks of gestation with a viable singleton pregnancy were eligible for participation. To validate our results in a larger cohort, we studied the associations between mode of conception and PV in the total Rotterdam Periconception Cohort. Other features of placental development were not measured in this study. The Predict study is an ongoing prospective cohort study, which has been conducted from November 2010 onwards after a pilot phase. Eligibility criteria were similar to those of the VIRTUAL Placenta study. Pregnancies after intrauterine insemination (IUI) or ovulation induction (OI) were included as natural conceptions, since fertilisation occurs *in vivo* and gonadotrophin levels are within physiological ranges. Pregnancies after oocyte donations, pregnancies resulting in a miscarriage and drop-outs were excluded.

Ethical approval

The studies were approved by the Medical Ethical and Institutional Review Board of the Erasmus University Medical Center, Rotterdam, The Netherlands (MEC-2004-227 and METC 2015-494). Prior to participation, written informed consent was obtained from women and their partners, as well as on behalf of their unborn child.

In-vitro fertilisation, cryopreservation and culture procedures

Procedures for ovarian stimulation, oocyte retrieval, IVF, intracytoplasmic sperm injection (ICSI) and assessment of embryo morphology were performed as described extensively in

two other publications (27, 28). Until November 2014 embryos were cultured in Vitrolife G5 series (Vitrolife, Goteborg, Sweden); thenceforth embryos were cultured in SAGE 1-Step™ (CooperSurgical, Trumbull, CT, United States of America). Evaluation and selection of embryos for transfer was performed on day 3 after oocyte retrieval, based on morphology. Supernumerary embryos of adequate quality were cultured until day 4, when selection for cryopreservation was performed based on the degree of compaction and presence of fragmentation (29). Firstly, selected embryos were incubated for 10 minutes in culture medium containing 1.0 di-methyl sulfoxide (DMSO) and loaded into straws (CBS High Security embryo straw, CryoBioSystem, Saint-Ouen-Sur-Iton, France). Secondly, cryopreservation was performed by slow-freezing the straws in a controlled rate freezer (Kryo 360, Planer, Sunbury-on-Thames, United Kingdom) to -40 °C at -0.3°C/min. Subsequently, the straws were cooled rapidly at -25 °C/min to -140 °C. Finally, after immersion in liquid nitrogen, the straws were stored in nitrogen vapour. Duration of storage varied between 1 month to 5 years.

If patients had a regular menstrual cycle, frozen-thawed ET was performed in a natural cycle. If patients had an irregular or absent menstrual cycle, frozen-thawed ET was performed in a hormonally-prepared endometrium. This was achieved by increasing dosages of intravaginal estrogens and optionally daily injections of a GnRH-agonist. Embryos were thawed four days after spontaneous ovulation, or after approximately 19 days of endometrial preparation. Until March 2016, this was accomplished by consecutive washes in decreasing concentrations of DMSO in buffered culture medium at room temperature. Thereafter, the Quinn's Advantage™ Thaw Kit (CooperSurgical) was used for thawing. One hour after thawing, embryos were checked for survival and cultured overnight in an atmosphere of 5% CO_2 and 7% O_2 in 1 ml of SAGE 1-Step culture medium at 37°C. The following day, morphology was evaluated and only embryos demonstrating developmental progression were transferred.

Study parameters

At enrolment, women completed a self-administered questionnaire regarding general characteristics and periconceptional lifestyle factors. All data were verified by a research nurse and anthropometric measurements were performed. Mean arterial pressure (MAP) was calculated using the following formula: *(systolic blood pressure + 2*diastolic blood pressure)/3*. In IVF/ICSI pregnancies, medical records were screened to assess conception mode (i.e. frozen-thawed ET or fresh ET) and fertilisation method (IVF or ICSI). Geographical origin was classified conform the definition of Statistics Netherlands, i.e. Western or non-Western. Educational level was categorised into low, intermediate and high. Use of cigarettes or alcohol was defined as any use during the periconception period, defined as 14 weeks prior to conception to 10 weeks after (30). Preconception daily use of folic acid supplements was defined as adequate, whereas no or post-conception initiation was defined as inadequate.

In pregnancies after IVF/ICSI, gestational age (GA) at ultrasound was determined based on the moment of transfer. For frozen-thawed ET, GA was calculated by adding 19 days to the date of transfer, since frozen-thawed embryos were transferred at day 5 of embryo development. For fresh ET, GA was calculated by adding 14 days to the date of oocyte retrieval. In pregnancies after natural conception, GA at ultrasound was based on the last menstrual period (LMP). If participants had a regular menstrual cycle of <25 or >32 days, GA was adjusted for the duration of the menstrual cycle. If LMP was unknown or if the GA based on LMP and GA based on crown-rump length (CRL) differed >7 days, GA was based on the CRL at ultrasound performed at 9 weeks of gestation.

Ultrasound data

All ultrasound scans were performed according to the international guidelines for safe use of (Doppler) ultrasound imaging in pregnancy (31). Ultrasound images were obtained by three trained researches who have their expertise in first-trimester imaging. All three also first conducted a learning curve and their reliability was tested, which was good for all three. Placental volume (PV, cm^3) was measured by using 3D ultrasound volumes of the whole gestational sac including the placenta, obtained at week 7, 9 and 11 of gestation, using a 6-12 MHz transvaginal probe compatible with the GE Voluson E8 and E10 Expert system in the total cohort. A minimum of two volumes were recorded in a perpendicular angle (90°) to increase the chances of obtaining a complete and high-quality volume. PV was measured offline by using Virtual Organ Computer-Aided AnaLyses (VOCAL) software. This technique has been previously described and is validated to measure PV in the first-trimester of pregnancy, with intra-class correlation coefficients (ICC) >0.97 (32).

uPVV was measured in 3D-PD ultrasound volumes in the subcohort, obtained by conform PV, with standardised settings (pulse repetition frequency 0.6 kHz, wall motion filter ‘low1’, quality ‘high’, gain -8.0) and expressed in cm^3. The uPVV was measured offline on a VR desktop system, which projects the ultrasound datasets as holograms that can be enlarged and rotated for more precision (33). By erasing the volume of the embryonic and myometrial vessels, only the vessels up to the myometrial-placental border remained, thus the uPVV. This technique has an ICC >0.94 (33).

The pulsatility index (PI) and resistance index (RI) of the uterine artery (UtA) was measured bilaterally in threefold at week 7, 9, 11 and 13 of gestation by using transvaginal pulsed wave Doppler ultrasound in the subcohort. The mean of these indices at each measurement moment was used for analysis. At week 22 and 32 of gestation, these indices were measured transabdominally by a single researcher with expertise in second- and third-trimester ultrasound imaging.

Statistical analyses

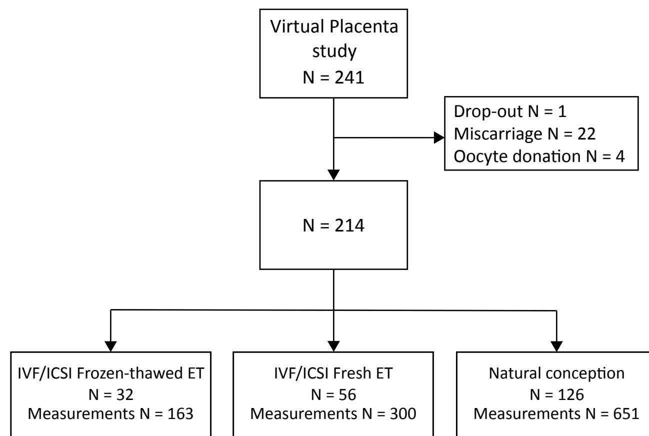
The study populations were stratified into three groups according to mode of conception: pregnancies after IVF/ICSI with 1) fresh ET or 2) frozen-thawed ET and 3) naturally conceived pregnancies. Baseline characteristics between these three groups were compared with either the Kruskall-Wallis test (continuous variables) or X^2 (categorical variables). The associations between conception mode and PV, uPVV, UtA-PI and UtA-RI were assessed by linear mixed models. Prior to analyses, PV and uPVV were cube-root transformed to obtain linearity. For the UtA indices, the linear mixed models were performed separately for first-, second- and third-trimester measurements. All linear mixed model analyses were performed using both pregnancies after fresh ET as well as naturally conceived pregnancies as reference. This approach enables comparison between all modes of conception. Two linear mixed models were constructed; the first was adjusted for GA only (model 1) and the second was additionally adjusted for confounders based on literature (model 2). Similar models were applied in our validation cohort. P-values <0.05 were considered statistically significant. All analyses were performed in SPSS 24.0 (IBM SPSS Statistics, Armonk, NY).

RESULTS

Baseline characteristics

The VIRTUAL Placenta study population comprised 32 pregnancies after frozen-thawed ET, 56 after fresh ET and 126 that were naturally conceived, corresponding to a total of 1114 measurements (**Figure 1**). Of the IVF/ICSI pregnancies, 49 (55.6%) were after the first cycle of ovarian stimulation. Six women in the frozen-thawed ET group did not have a fresh ET after fertilisation, four due to OHSS. In the frozen thawed-ET-group, eight received hormonal preparation and 22 did not. Of the 126 naturally conceived pregnancies, five were achieved by a natural IUI cycle, eight were by OI and two were from a combination of IUI and OI.

Figure 1. Flowchart of the Rotterdam Periconception subcohort: The VIRTUAL Placenta study.



PV, placental volume. ET, embryo transfer.

The women who were pregnant after natural conception were less often nulliparous than women pregnant after IVF/ICSI with either frozen-thawed ET or fresh ET (39.7%, 71.9%, and 81.8%, respectively, *p*<0.001) and had used folic acid supplements adequately less often (73.6%, 100% and 93.8%, respectively, *p*<0.001). Women pregnant after fresh ET were more often from a Western background compared to women pregnant after frozen-thawed ET and after natural conception (90.9%, 71.9% and 77.8%, respectively, *p*=0.048) (**Table 1**). Maternal age, BMI, MAP, level of education, use of alcohol and cigarettes and fertilisation method were comparable between the three groups.

Table 1. Baseline characteristics of the VIRTUAL Placenta subcohort, stratified for the mode of conception.

	IVF/ICSI Frozen-thawed ET N=32		IVF/ICSI Fresh ET N=56		Natural conception N=126			
	Median / N	**IQR /%**	**Median / N**	**IQR /%**	**Median / N**	**IQR /%**	**P-value**	**Missing**
Maternal characteristics								
Age, years	33.3	29.4-36.4	33.1	29.3-36.2	31.4	28.8-34.5	0.098	0
Body mass index, kg/m^2	24.2	21.9-28.4	23.9	21.8-27.3	25.3	22.5-29.7	0.118	0
MAP, mmHg	82.5	76.7-88.2	80	76.7-83.3	80	75.3-85.1	0.202	0
Nulliparous	23	71.9	45	81.8	50	39.7	**<0.001**[d,e]	1
Geographical origin, Western	23	71.9	50	90.9	98	77.8	**0.048**[c,e]	0
Education							0.698	6
Low	4	12.5	4	7.3	10	8.3		
Middle	8	28.1	22	40.0	38	31.4		
High	19	59.4	29	52.7	73	60.3		
Alcohol, yes[a]	13	40.6	10	17.9	34	27.0	0.067	0
Smoking, yes[b]	7	21.9	5	8.9	16	12.7	0.219	0
Folic acid, yes[b]	30	93.8	56	100	92	73.6	**<0.001**[d,e]	1
Fertilisation method, ICSI	15	46.9	38	67.9	NA		0.053	0

IQR, interquartile range. MAP, mean arterial pressure.

a. Any use during the 14 weeks prior to up to 10 weeks of gestation.
b. Daily use during the 14 weeks prior to up to 10 weeks of gestation.
c. Significantly different between pregnancies after frozen-thawed ET and after fresh ET.
d. Significantly different between pregnancies after frozen-thawed ET and naturally conceived pregnancies.
e. Significantly different between pregnancies after fresh ET and naturally conceived pregnancies.

The validation cohort of the Predict study comprised a total of 722 pregnancies; 96 after frozen-thawed ET, 184 after fresh ET and 442 were naturally conceived (**Supplemental figure 1**). In the frozen-thawed ET group, 18 received hormonal preparation and 78 did not. Maternal age, BMI, parity, periconceptional use of alcohol and folic acid and fertilisation method were significantly different between the three groups (**Supplemental table 1**).

Placental Volume (PV) and uterine Placental Vascular Volume (uPVV)

The first-trimester PV growth trajectories in pregnancies after frozen-thawed ET were comparable to growth trajectories in pregnancies after fresh ET (model 1: β -0.007 (95%CI -0.184, 0.170), *p*=0.936; model 2: β -0.026 (95%CI:-0.206, 0.155), *p*=0.778) and after natural conception (model 1: β 0.002 (95%CI -0.160, 0.163), *p*=0.983; model 2: β 0.006 (95%CI -0.162, 0.174), *p*=0.940) (**Table 2**). A β greater than 0 indicates increased growth, whereas a β

below 0 indicates reduced growth compared to the reference category. First-trimester PV in pregnancies after fresh ET was also comparable to PV in pregnancies after natural conception (**Table 2**). Similar results were observed in the validation cohort for both models (**Table 3**).

Table 2. First-trimester trajectories of placental development and uterine artery indices in VIRTUAL Placenta subcohort for pregnancies after frozen-thawed ET (n=32), fresh ET (n=56), and after natural conception (n=126).

		Model 1		Model 2	
		Beta (95%CI)	**P-value**	**Beta (95%CI)**	**P-value**
PV ($\sqrt[3]{cm^3}$)	Frozen-thawed ET	-0.007 (-0.184, 0.170)	0.936	-0.026 (-0.206, 0.155)	0.778
	Fresh ET	Reference		Reference	
	Frozen-thawed ET	0.002 (-0.160, 0.163)	0.983	0.006 (-0.162, 0.174)	0.940
	Fresh ET	0.003 (-0.127, 0.134)	0.962	0.013 (-0.132, 0.158)	0.857
	Natural conception	Reference		Reference	
uPVV ($\sqrt[3]{cm^3}$)	Frozen-thawed ET	0.121(-0.098, 0.340)	0.276	0.075 (-0.147, 0.297)	0.506
	Fresh ET	Reference		Reference	
	Frozen-thawed ET	0.071 (-0.121, 0.263)	0.465	0.112 (-0.085, 0.311)	0.262
	Fresh ET	-0.052 (-0.207, 0.103)	0.505	-0.009 (-0.180, 0.162)	0.916
	Natural conception	Reference		Reference	
UtA-PI	Frozen-thawed ET	0.028 (-0.159 0.215)	0.767	0.023 (-0.178, 0.223)	0.833
	Fresh ET	Reference		Reference	
	Frozen-thawed ET	-0.112 (-0.305, 0.080)	0.251	-0.130 (-0.330, 0.070)	0.200
	Fresh ET	-0.148 (-0.304, 0.009)	0.064	-0.125 (-0.298, 0.048)	0.155
	Natural conception	Reference		Reference	
UtA-RI	Frozen-thawed ET	-0.012 (-0.039, 0.015)	0.383	-0.012 (-0.041, 0.017)	0.408
	Fresh ET	Reference		Reference	
	Frozen-thawed ET	-0.022 (-0.050, 0.005)	0.112	-0.021 (-0.050, 0.007)	0.114
	Fresh ET	-0.011 (-0.034, 0.011)	0.322	-0.003 (-0.028, 0.021)	0.783
	Natural conception	Reference		Reference	

PV, placental volume. uPVV, utero-placental vascular volume. UtA-PI, uterine artery pulsatility index. UtA-RI, uterine artery resistance index. CI confidence interval.
Model 1: Adjusted for GA.
Model 2: Adjusted for GA, parity, periconceptional folic acid supplement use and smoking.

First-trimester uPVV in pregnancies after frozen-thawed ET were comparable to uPVV in pregnancies after fresh ET and naturally conceived pregnancies in both models (**Table 2**). First-trimester uPVV in pregnancies after fresh ET was also similar to uPVV in pregnancies after natural conception in both models (**Table 2**).

Table 3. First-trimester trajectories of placental volume in the total cohort for pregnancies after frozen-thawed ET (n=93), fresh ET (n=172), and after natural conception (n=435).

	Model 1		Model 2	
	Beta (95%CI) $\sqrt[3]{cm^3}$	**P-value**	**Beta (95%CI) $\sqrt[3]{cm^3}$**	**P-value**
Frozen-thawed ET	0.016 (-0.076, 0.108)	0.735	0.016 (-0.080, 0.111)	0.742
Fresh ET	Reference		Reference	
Frozen-thawed ET	0.030 (-0.057, 0.117)	0.493	0.021 (-0.071, 0.112)	0.657
Fresh ET	0.007 (-0.061, 0.075)	0.849	-0.018 (-0.092, 0.056)	0.632
Natural conception	Reference		Reference	

95%CI, 95% confidence interval.
Model 1: Adjusted for GA.
Model 2: Adjusted for GA, parity, periconceptional use of folic acid supplements and smoking.

Table 4. Second- and third-trimester trajectories of uterine indices in the VIRTUAL Placenta subcohort for pregnancies after frozen-thawed ET (n=32), fresh ET (n=56), and after natural conception (n=126).

		Model 1		Model 2	
		Beta (95%CI)	**P-value**	**Beta (95%CI)**	**P-value**
UtA-PI	Frozen-thawed ET	-0.122 (-0.238, -0.029)	**0.013**	-0.158 (-0.268, -0.048)	**0.005**
	Fresh ET	Reference		Reference	
	Frozen-thawed ET	-0.115 (-0.218, -0.012)	**0.029**	-0.104 (-0.211, 0.002)	0.055
	Fresh ET	0.019 (-0.063, 0.101)	0.647	0.048 (-0.042, 0.138)	0.291
	Natural conception	Reference		Reference	
UtA-RI	Frozen-thawed ET	-0.043 (-0.078, -0.008)	**0.018**	-0.052 (-0.089, -0.015)	**0.006**
	Fresh ET	Reference		Reference	
	Frozen-thawed ET	-0.033 (-0.066, -0.001)	**0.043**	-0.028 (-0.062, 0.005)	0.093
	Fresh ET	0.010 (-0.016, 0.035)	0.454	0.020 (-0.008, 0.048)	0.159
	Natural conception	Reference		Reference	

UtA-PI, uterine artery pulsatility index. UtA-RI, uterine artery resistance index. CI confidence interval.
Model 1: Adjusted for GA.
Model 2: Adjusted for GA, parity, periconceptional folic acid supplement use and smoking.

Uterine Artery Indices

First-trimester UtA-PI and UtA-RI trajectories were not significantly different between pregnancies after frozen-thawed ET, fresh ET and after natural conception in both models (**Table 2**).

In the second and third trimester, the decline of UtA-PI in pregnancies after frozen-thawed ET was stronger compared to the UtA-PI in pregnancies after fresh ET (Model 1: β -0.122 (95%CI -0.238, -0.029), *p*=0.013; Model 2: β -0.158 (95%CI -0.268, -0.048), *p*=0.005) indicating a lower distal vascular resistance (**Table 4**). This was also observed when compared to pregnancies after natural conception in the crude model (β -0.115 (95%CI -0.218, -0.012), *p*=0.029).

Second- and third- trimester UtA-RI decline in pregnancies after frozen-thawed ET was stronger than after fresh ET (Model 1: β -0.043 (95%CI -0.078, -0.008), *p*=0.018; Model 2: β -0.052 (95%CI -0.089, -0.015), *p*=0.006) (**Table 4**). Similar associations were observed when compared to naturally conceived pregnancies (model 1: β -0.033 (95%CI -0.066, -0.001), *p*=0.043). The second- and third-trimester UtA-PI and UtA-RI in pregnancies after fresh ET were similar to the indices in pregnancies after natural conception pregnancies, suggesting no difference in placental perfusion.

DISCUSSION

Summary of results

The current study is the first to investigate longitudinal differences in non-invasive parameters of placental development between pregnancies of different conception modes, i.e. after frozen-thawed ET, after fresh ET and after natural conception. In the first-trimester, placental developmental parameters (PV, uPVV, UtA-PI and UtA-RI) were comparable between pregnancies after frozen-thawed ET, after fresh ET and after natural conception.

In the second and third trimester, however, pregnancies after frozen-thawed ET showed a lower UtA-PI and UtA-RI compared to pregnancies after fresh ET, which suggests a lower distal vascular resistance. Interestingly, these indices were comparable between pregnancies after fresh ET and after natural conception. Validation analyses of the association between first-trimester PV and conception mode in a larger cohort demonstrated similar results.

Comparison with literature and Interpretation

Two previous studies have investigated differences in first-trimester PV between pregnancies after fresh ET and after frozen-thawed ET (23, 24). The study populations of these studies consisted of 532 (Rizzo et al.) and 252 (Choux. et al.) pregnancies. Contrary to our findings, both studies show that PV is smaller in pregnancies after frozen-thawed ET than in pregnancies after fresh ET. The method of cryopreservation may be of importance when investigating placental development. Research shows higher pregnancy rates for vitrification when compared to slow-freezing, but perinatal outcomes are comparable between the two methods (34, 35). As Choux et al. applied cryopreservation methods similar to ours, and Rizzo et al. did not report the method of cryopreservation, our results may not be generalizable to vitrification. There are also considerable differences between these studies and ours. The first difference is that the results of these studies were based on a single measurement from an abdominally acquired ultrasound, whereas we performed multiple vaginal ultrasound examinations in the first-trimester. Secondly, in both studies the ultrasound examination was performed at a later GA than in our study, i.e. between 11+0 and 13+6 weeks of gestation. To explain these differences further, we performed additional linear regression analyses of PV at 7, 9 and 11 weeks of gestation independently, showing no significant differences between pregnancies after fresh ET, after frozen-thawed ET and after natural conception (data not shown). Finally, the study by Rizzo et al., provided no information on endometrial preparation prior to frozen-thawed ET, whereas Choux et al. investigated PV in pregnancies after frozen-thawed ET in hormonally-induced cycles. Hence, these study populations are not comparable to ours, in which approximately 75% of all frozen-thawed ETs were performed in a natural cycle. A sensitivity analysis excluding frozen-thawed ETs in a hormonally-induced cycle also demonstrated no significant association between PV and conception mode (data not shown). However, another explanation might be that the current study was insufficiently powered to detect associations between PV and conception mode.

Nevertheless, there is emerging evidence that the endocrine uterine environment at the moment of ET plays a critical role in maternal vascular adaptation to pregnancy and thus might affect placental development (36-38). During hormonally-induced cycles, the hypothalamic-pituitary axis is suppressed by consecutive administration of estrogen and progesterone to prepare the endometrium for transfer. As a result, the corpus luteum is absent, whereas it is present in natural cycles as a major source of reproductive and vasoactive hormones, such

as prorenin. Conrad et al. suggested that the presence of a corpus luteum is essential for an optimal maternal hormonal environment during implantation and hemodynamic adaption to pregnancy, and its absence may impact the maternal physiology and pregnancy outcome (39). This might explain why we did not find any differences in first-trimester placental developmental parameters between naturally conceived pregnancies and pregnancies after frozen-thawed ET, as the corpus luteum is present in the majority of pregnancies after frozen-thawed natural cycle ET.

Since there are multiple corpora lutea after ovarian stimulation followed by fresh ET, one may also expect differences in first-trimester placental developmental parameters between pregnancies after fresh ET and naturally conceived pregnancies. However, we did not observe such differences. Most studies that have investigated first-trimester placental development after IVF do not distinguish between pregnancies after fresh ET and after frozen-thawed ET, which complicates comparison to previous results. Rifouna et al., investigated uPVV in relation to conception mode and showed no differences in uPVV between naturally conceived pregnancies and pregnancies after IVF (32). Their study population comprised 84 naturally conceived pregnancies, 59 pregnancies after fresh ET and 11 after frozen-thawed ET, but no distinction was made between pregnancies after fresh ET and after frozen-ET due to the small sample size. The lack of distinction between pregnancies after fresh ET and after frozen-thawed ET is also observed in studies regarding first-trimester uterine artery indices and conception mode. Only Cavoretto et al. demonstrated a lower first-trimester UtA-PI in pregnancies after frozen-thawed ET than in pregnancies after fresh ET, whereas Rizzo et al. found no differences (23, 40).

In the second and third trimester, we found a stronger decline in UtA-PI and UtA-RI in pregnancies after frozen-thawed ET than in pregnancies after fresh ET. This observation is in contrast with the reported increased risk for placenta-related pregnancy complications in pregnancies after frozen-thawed ET, which are associated with increased uterine artery indices (4, 5, 41). Although beyond the scope of this study, we found no associations with the incidence of placenta-related pregnancy complications in our cohort (data not shown). Moreover, we found decreased indices in pregnancies after frozen-thawed ET, which was also observed by Cavoretto et al. (40).

Recently, a major randomised controlled trial in 1508 women with polycystic ovarian syndrome (PCOS) showed that the risk of preeclampsia is higher after elective frozen-thawed cleavage ET than after fresh cleavage ET, which was not observed in 2157 ovulatory women (42, 43). This might be explained by the overt difference in study populations, as pregnant women with PCOS are at increased risk for preeclampsia (44). However, another considerable difference is the endometrial preparation preceding the elective frozen-thawed ET. For ovulatory women, the frozen-thawed ET was primarily performed in a natural cycle,

whereas for women with PCOS, this was in a hormonally-induced cycle. Together with our results, this supports the hypothesis that placental development after frozen-thawed natural cycle ET might be superior to placental development after frozen-thawed hormonal treatment cycle ET.

Strengths and limitations

The main strength of this study is the collection of longitudinal non-invasive measurements of early placental development using 3D ultrasound in combination with VR. Placental parameters were measured by experienced researchers and the applied innovative techniques have an excellent reproducibility (33) Furthermore, our results regarding the associations between conception mode and PV, a thoroughly studied non-invasive parameter of placental development, were validated in a larger cohort in the same setting and study design with over 700 participants. Finally, the evaluation of both naturally conceived pregnancies as well as pregnancies after fresh ET as a reference category enabled interpretation of our results in a wider perspective.

The main limitation of this study is the relatively small size of the VIRTUAL Placenta study to detect differences in placental development and placenta-related pregnancy outcomes between pregnancies of different modes of conception. Also, our frozen-thawed ET group consisted of both freeze-all cycles as well as cycles with an initial fresh ET. This may have implications, as it is not a homogeneous study population. Moreover, we performed cryopreservation on day 4 using a slow-freeze method and frozen-thawed ET was usually performed in a natural cycle. As this is not common practice for all clinics, this may have affected our results and their generalisability. Although we adjusted for multiple confounders to minimise the effect of differences between groups, e.g. previous unsuccessful ET, we cannot eliminate residual confounding, due to the observational character of this study. For example, the endometrium might have been a confounding factor in our analyses, but the small sample size and inconsistent data collection prevented adequate adjustment. Finally, as this study was conducted at a tertiary hospital, the naturally conceived pregnancies were at risk of developing pregnancy complications due to the presence of maternal comorbidities. This may have consequences for the extrapolation of our results to the general population.

Conclusion

In this study, we investigated differences in placental development between pregnancies resulting from fresh ET, frozen-thawed ET and natural conception, but did not find differences in first-trimester placental development. Furthermore, second- and third-trimester uterine artery indices in pregnancies after frozen-thawed ET were lower than indices in pregnancies after fresh ET, and this is associated with higher placental perfusion. This is in contrast with findings of other studies demonstrating a higher risk of placenta-related pregnancy complications after frozen-thawed ET (4, 5). These complications have serious implications

for short- and long-term maternal and offspring health (45). In conclusion, this study can be considered hypothesis generating. For example, the maternal hormonal environment at the moment of frozen-thawed ET may have an essential role in placental development. Larger longitudinal studies are necessary to study this hypothesis and should also include measurements of this maternal environment, e.g. endometrial thickness or quality. These studies are crucial, since approximately 1% of all newborns are conceived after frozen-thawed ET and the number of frozen-thawed ETs only continues to rise (46, 47).

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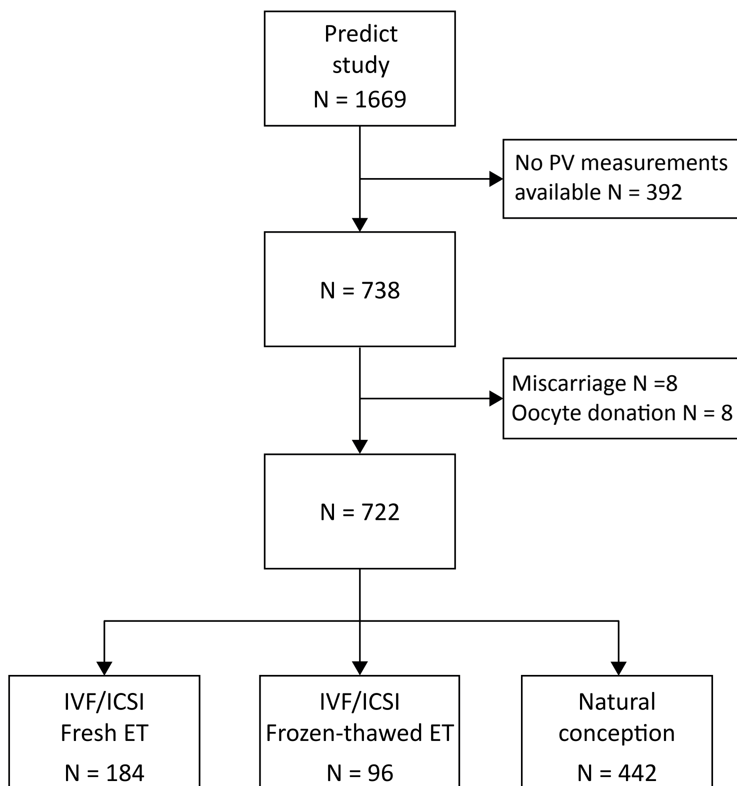
Supplemental material

Supplemental figure 1. Flowchart of the Rotterdam Periconception Cohort.



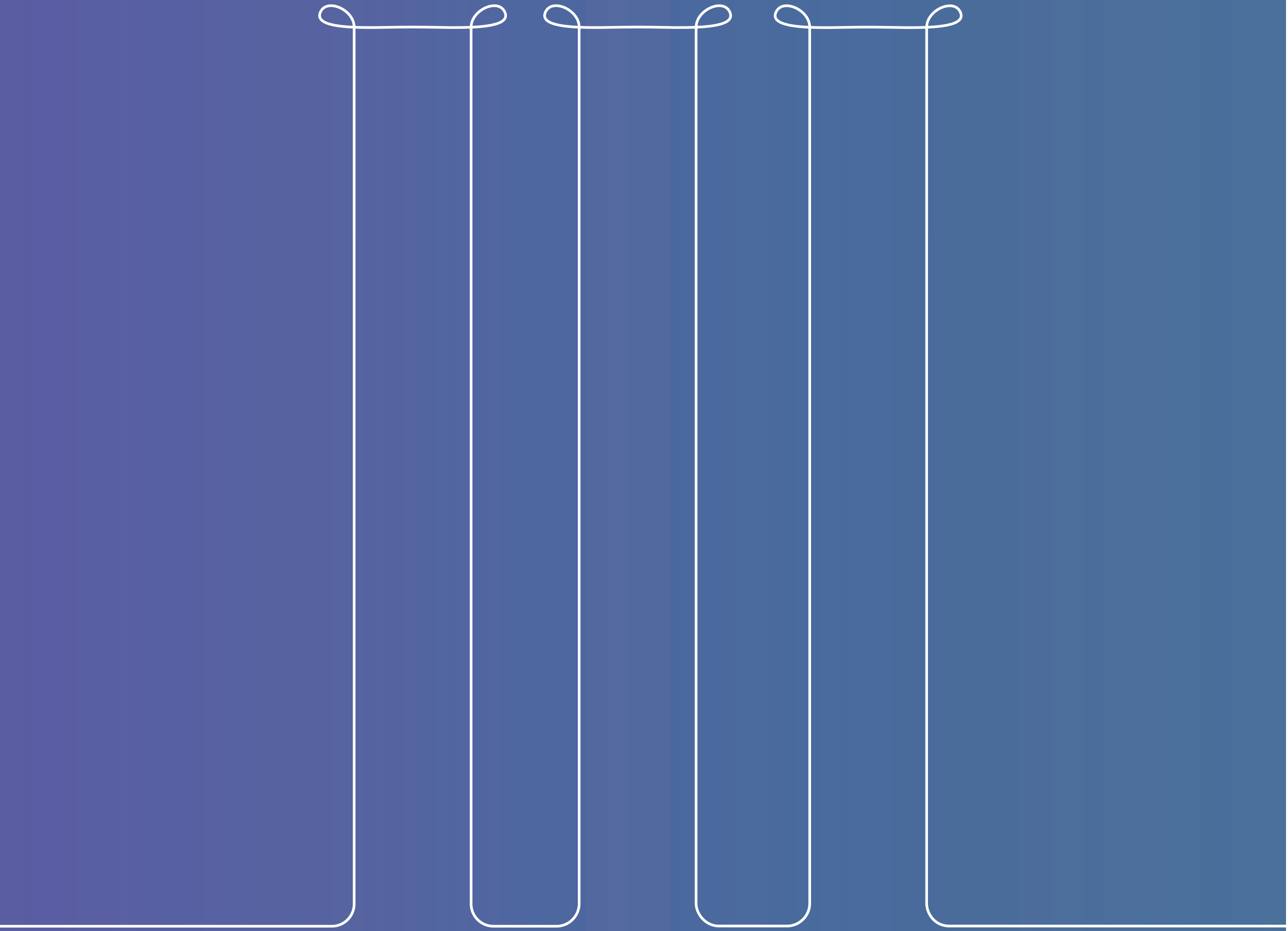
PV, placental volume. ET, embryo transfer.

Supplemental table I. Baseline characteristics of the total cohort population stratified for the mode of conception.

	IVF/ICSI Frozen-thawed ET N=96		IVF/ICSI Fresh ET N=184		Natural conception N=442			
	Median / N	**IQR /%**	**Median / N**	**IQR /%**	**Median / N**	**IQR /%**	**P-value**	**Missing**
Age, years	33.3	30.1-36.4	33.2	30.7-36.6	31.3	28.7-34.8	**<0.001**[d,e]	1
Body mass index, kg/m^2	24.1	21.8-27.2	23.9	21.7-27.6	24.9	22.2-29.0	**0.018**[d]	30
MAP, mmHg	80.0	75.0-86.3	80.0	76.0-86.0	80.0	76.7-86.7	0.530	30
Nullipara	61	63.5	138	75.4	192	43.6	**<0.001**[c,d,e]	3
Geographical origin, Western	74	77.1	161	87.5	376	85.1	0.066	0
Education							0.597	17
Low	4	4.3	16	8.9	37	8.5		
Middle	36	38.7	60	33.5	142	32.8		
High	103	57.5	53	57.0	254	58.2		
Alcohol, yes[a]	27	29.0	37	20.6	158	37.1	**<0.001**[e]	23
Smoking, yes[a]	12	12.9	28	15.4	72	16.9	0.629	22
Folic acid, yes[b]	89	95.7	176	98.9	314	73.2	**<0.001**[d,e]	22
Fertilisation method, ICSI	38	39.6	103	56.3	NA		**0.008**	1

IQR, interquartile range. MAP, mean arterial pressure.

a. Any use during the 14 weeks prior to up to 10 weeks of gestation.
b. Daily use during the 14 weeks prior to up to 10 weeks of gestation.
c. Significantly different between pregnancies after frozen-thawed ET and after fresh ET.
d. Significantly different between pregnancies after frozen-thawed ET and naturally conceived pregnancies.
e. Significantly different between pregnancies after fresh ET and naturally conceived pregnancies.



Discussion and appendices

General discussion

This thesis demonstrates that maternal BMI and differences in assisted reproductive technologies are associated with preimplantation and post-implantation embryo and placental development.

Maternal adiposity and embryonic growth and development

This thesis demonstrates a detrimental impact of a higher maternal BMI on periconceptional outcomes, thereby underlining the importance of a healthy weight prior to conception (**Figure 1**). An issue to consider in these associations is the classification of obesity by BMI, since it is not a uniform reflection of the amount of body fat for all populations. For example, an identical level of body fat, age and gender results in a higher BMI in Caucasians than in Chinese, meaning Caucasians are less adipose than other ethnicities for a similar BMI (1). Secondly, by only studying BMI, other critical components, e.g. the distribution of body fat, are neglected. Some researchers have even suggested that the waist to hip ratio (WHR), might be a stronger predictor for fertility than BMI (2, 3). Finally, nutritional quality should also be considered. Deficiencies in nutrients such as proteins, fibres, minerals and vitamins can further promote oxidative stress and impact periconceptional processes (4). This is supported by several studies showing that a strong adherence to Western or 'junk' diets are negatively associated with semen quality, fertility, fetal size and positively with the occurrence of congenital malformations, independent of BMI (5-8).

As numerous studies demonstrate that overweight and obesity are associated with lower odds of a healthy pregnancy, the majority of health care providers recommend preconceptional weight loss. However, the evidence underlying this recommendation remains inconclusive (9-11). Hence, we propose a more sustainable solution by shifting the focus from weight loss treatment to lifestyle improvements to gain a healthy weight, such as increasing vegetable and fruit intake. Since people are more motivated for behavioural changes during life-changing events such as pregnancy, the periconception period is an optimal moment to break the vicious cycle of transgenerational transmission of unhealthy lifestyle (12, 13). An example of such an intervention is www.smarterpregnancy.co.uk (Dutch: www.slimmerzwanger.nl), a web-based and cost-effective mHealth application for lifestyle improvement (14, 15). Nevertheless, without the support of solid public health strategies, such as nudging or food taxes to promote individual healthy lifestyle behaviour on a larger scale, such interventions are hard to implement (16, 17).

Targets for improving IVF/ICSI treatment outcomes: Transparency and collaboration

Studying associations between variables in IVF/ICSI treatment and reproductive outcomes can be challenging for several reasons. General differences in patient characteristics and lifestyle factors between women undergoing IVF/ICSI and those who conceive naturally in our cohort, e.g. age and folic acid use, can confound the associations with periconceptional

and perinatal outcomes. Next, several steps in the process from fertilisation to implantation are highly subjected to selection. Firstly, with ICSI, the spermatozoon is manually selected by humans instead of by the female reproductive tract (18). Secondly, when an IVF/ICSI cycle yields multiple good-quality embryos, only one or two are selected for transfer and the remaining are cryopreserved. Finally, transfer of a good-quality embryo does not guarantee implantation, which suggests a selective role for the endometrium as well. Another challenge is the aetiology underlying the subfertility, which can affect periconceptional and perinatal outcomes. For example, in women with severe endometriosis, fertilisation rate, embryo quality, and pregnancy rate are lower than in women with tubal infertility, possibly due to higher levels of local and chronic oxidative stress (19, 20). Additionally, it has been demonstrated that different phenotypes of PCOS have a heterogeneous effect on embryo development and pregnancy complications (21-24).

To overcome these challenges, it might be imperative to assemble a collaboration of IVF-clinics, so that it is easier to study homogenous (sub)groups and minimise the risk of bias. However, the field of infertility treatment is highly competitive. In this 20-billion-dollar industry, transparency and consensus are often lacking. For example, culture media are regulated as class III medical devices in Europe (25). To obtain a CE certificate, manufacturers have to submit an extensive technical file, including justification and risk of each component in the medium and (post-)clinical evaluation, to a Notified Body (26). Yet, it remains unclear how this system operates and how post-market surveillance is carried out. Additionally, for many stakeholders in this industry, the course of pregnancy and offspring health are often of secondary or minor importance to achieving a pregnancy. Although pregnancy rates might increase by modifications in culture media, ovarian stimulation protocols or transfer policies, it cannot be excluded that long-term offspring health is unaffected (27). In fact, this thesis indicates that there might be an (sex-specific) impact of such modifications (**Figure 1**). To meticulously investigate associations between different IVF practices and long-term offspring health, treatment data should be linked to national registries. As 1 out of 30 children born today is conceived through IVF/ICSI and as physicians have a responsibility towards both the prospective parents and the future child to provide the best care, there is an urgent need for transparency on culture media and other treatment variables, as well as for surveillance of long-term offspring health outcomes.

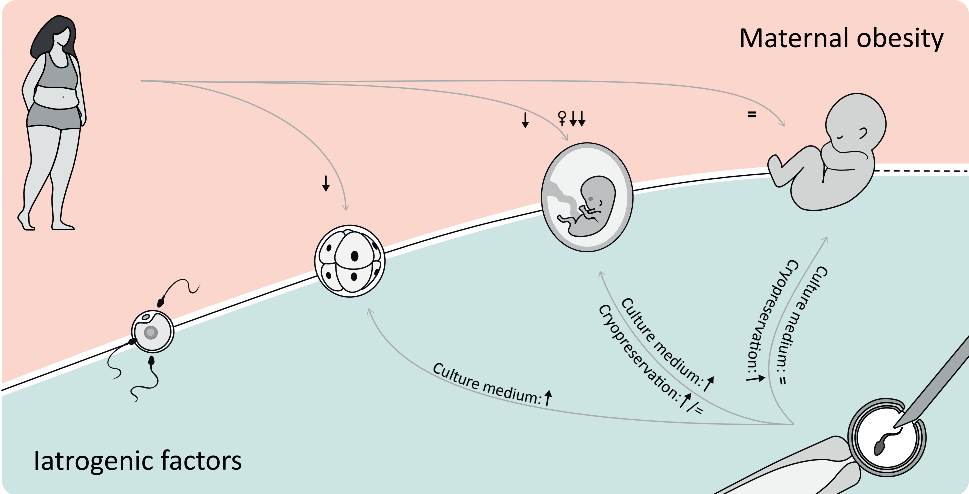
Future implications for research and clinical practice

Preimplantation embryo development

Visualising preimplantation embryo development can provide unique insights into the physiological processes involved in early embryo development. In naturally conceived pregnancies, this is virtually impossible, but during IVF/ICSI treatment it is vital for treatment success, since embryo selection is primarily based on morphology (28). However, the considerable variation in timing of embryo evaluation and applied scoring systems between

IVF-clinics remain major obstacles. These variations can impair objective classification of embryos, thereby potentially avoiding embryos with implantation potential, and hamper performing robust meta-analyses. Time-lapse imaging of embryo development allows undisturbed culture and a more objective assessment. As it monitors preimplantation embryo development more frequently, it has been proposed as a promising strategy for improving embryo selection, thereby reducing the time to pregnancy. This is beneficial for both patients and clinicians, as it reduces infertility-related stress and increases cost-effectivity. Yet, prediction of pregnancy solely based on embryo morphokinetics remains challenging. Although several prediction algorithms have been developed, the most accurate has an area under the curve of 0.69, which can be considered as a fair predictor (29). There are several external factors which can affect developmental kinetics, such as culture media and maternal BMI (**Figure 1**). It is relevant to study the impact of such exposures on kinetics, as their implementation in prediction models may improve the accuracy. Moreover, these models enable clinicians to inform couples of their personalised chances of implantation after transfer, thereby managing their expectations and potentially reducing grief after unsuccessful treatment.

Figure 1. Overview of the main findings in this thesis.



Legend: ↑ positive association; ↓ negative association; = no association

From blastocyst to heartbeat

It takes more than transferring a morphologically good embryo to achieve a pregnancy. Nonetheless, the processes between embryo transfer and ultrasound detection, i.e. implantation and early embryonic development, are complex and remain poorly understood. Since the post-implantation embryo can be properly examined at the earliest at six weeks of gestation, this period is considered a black box in periconceptional research.

Uncovering this box may provide unique insights into the role of the placenta in fetal growth, embryo-specific growth potentials and the relation between preimplantation and post-implantation development. In this thesis, such a relation is suggested by the comparable effects on preimplantation and post-implantation embryo development of external influences, such as culture medium or maternal BMI. Based on the resembling impact of elevated maternal BMI and Vitrolife culture medium on embryo development, it can even be hypothesised that increased glucose concentrations in the embryo environment reduce embryonic growth.

To properly investigate such hypotheses, information regarding individual preimplantation embryo development should be integrated with information regarding its post-implantation development. This can be achieved by combining (three-dimensional) VIRTUAL EmbryoScope data with post-implantation ultrasound markers from the Rotterdam Periconception Cohort. Although it is believed that numerous (epi-)genetic and molecular pathways play a crucial role during this critical period, the majority remains to be unraveled. Yet, upcoming techniques show promising results. For example, by *in vitro* culturing of human embryos beyond the blastocyst stage it is possible to investigate key events in early human development, such as lineage segregation and cell-fate specification (30). Another upcoming technique is single-cell sequencing of the (epi)genome, transcriptome and proteome, which is a powerful instrument for mapping the molecular landscape of embryonic and placental development (31). Although each technique has its own limitations, its application will contribute to understanding the (patho)physiological mechanisms in early human development.

Post-implantation embryonic and placental development

Accurate pregnancy dating is vital for providing optimal prenatal care. Traditionally, this is based on CRL measurements, since first-trimester growth was believed to be uniform for all embryos. (32, 33). However, first-trimester growth and development are affected by internal factors, such as maternal age and fetal sex, as well as by external factors, such as maternal nutrition or culture medium used during IVF/ICSI treatment (34, 35). By dating pregnancy based on first-trimester CRL measurements, gestational age can be incorrectly adjusted, thereby missing the potential opportunity for first-trimester identification of pregnancies at risk. A more eligible marker for gestational age might be the assessment by Carnegie stages, as it also takes morphological characteristics into account and is overall less impacted by external factors than CRL and EV. Currently, Carnegie measurements are not feasible for daily clinical practice, as they can only be performed by using VR. However, given the global advancements in VR, automatic assessment of the Carnegie stages may become a feasible method for pregnancy dating. Another, more practical, solution may be customizing first-trimester growth curves by including maternal and embryonic characteristics. Nevertheless, to increase the accuracy of pregnancy dating, further research is warranted.

Studies in this thesis also demonstrate a sex-specific impact of factors such as maternal BMI and culture media (**Figure 1**). Although the impact of fetal sex on first-trimester growth and development has only been demonstrated a few decades ago, differences in birth outcomes between male and female neonates were already reported in 1786 (36). Sex-specific differences occur as early as the second day after fertilisation and persist even decades after birth, affecting the pathophysiology of various non-communicable diseases (37). Proposed mechanisms are maternal-embryonic interactions, differences in expression of chromosomal content, sex-specific adaptations to developmental stressors and sex steroids influencing growth and development (38-41). Since many non-communicable diseases are related to adaption to prenatal stressors, including sexual dimorphism in transgenerational research will unravel sex-specific associations. By identifying these associations, we have the opportunity for prevention or early detection, thereby potentially reducing the burden of such diseases.

Finally, for optimal post-implantation growth and development, optimal placental development is crucial. More insight into the association between the maternal (hormonal) environment during implantation and subsequent placental development could further contribute to prevention and prediction of placenta-related pregnancy complications. Yet, future studies should not only focus on *in vivo* placental development but also on *in vivo* placental function, as function is more important than size.

Society and clinical care

Currently, the prevalence of overweight and obesity is historically high across all generations. This, in combination with the gradually increasing uptake of assisted reproductive technologies, has serious implications for embryonic and fetal development (42, 43). Clinicians should be aware of such associations, so they can properly inform and support their patients by offering effective lifestyle care. Currently, prenatal care starts from the end of the first-trimester onwards, when the opportunity to prevent adverse outcomes, i.e. the periconception period, has largely passed (13). The periconception period is a relatively undervalued period in reproduction, and therefore challenging to study. Hence, it is difficult to demonstrate its importance to the general population. To raise awareness in this group, education should ideally take place at a public level and prior to reproduction (44, 45). Promising interventions can be found in the school system or in contraceptive counselling (46, 47). A specific population are subfertile couples, as they are often more receptive to advice. Moreover, their gametes and embryos will be exposed to the additional stressors of IVF/ICSI treatment, which further stresses the importance of an adequate preconceptional diet and lifestyle (48-50). Preconceptional counselling on nutrition and lifestyle empowers subfertile couples and increases their chance of pregnancy (51). To conclude, improving the periconceptional *in vivo* environment, e.g. by preconceptional counselling and optimising the external, i.e. *in vitro,* embryo environment, will contribute to the health of future generations.

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Summary
Samenvatting

SUMMARY

The Developmental Origins of Health and Disease (DOHaD) paradigm postulates that the developing human fetus adapts to signals from the intra-uterine environment to increase the chances of extra-uterine survival. Initially, research on this paradigm focused on the second half of pregnancy and perinatal outcomes. However, due to increasing knowledge from molecular biology, epigenetics and translational research, the focus shifted towards the periconception period. Many adverse birth outcomes originate in this period, defined as 14 weeks prior to fertilisation until 10 weeks thereafter. Vital processes such as gametogenesis, fertilisation, implantation, embryogenesis and placentation take place in this timeframe. Disruption of the biological environment in which these processes take place can have a lifelong effect on offspring health. For example, an obese environment results in chronically elevated levels of oxidative stress and is associated with an increased risk of congenital malformations. Also, during *in vitro* fertilisation (IVF) with or without intracytoplasmic sperm injection (ICSI) treatment, the gametes and embryos are exposed to iatrogenic *in vitro* stressors. Therefore, the aim of this thesis is to increase our knowledge regarding the impact of environmental factors, in particular maternal obesity and features of IVF treatment, on both preimplantation as well as post-implantation embryo development and placenta development. The background is provided in the introduction of this thesis (**Chapter 1**).

In **Part I** of this thesis, we studied associations between maternal obesity and periconceptional outcomes, i.e. embryo growth and development.

In **Chapter 2**, we performed a systematic review of 75 studies and provided an overview of the evidence on the impact of periconceptional maternal BMI on oocyte quality, preimplantation embryo quality and post-implantation growth and development. In 19 out of 44 studies, high maternal BMI was shown to increase the risk of miscarriage. In 7 out of 25 studies in humans and in 1 out of 3 animal studies, a negative association between maternal BMI and the number of (mature) oocytes was demonstrated. Reduced pre-implantation embryo quality was associated with higher maternal BMI in 12 out of 35 human studies and in 8 out of 10 animal studies. These results suggest a detrimental impact of higher maternal BMI on periconceptional outcomes in humans. In animal studies, this association is more evident, due to standardised study conditions and minimised confounding by other factors. Therefore, clinicians should emphasise the importance of a healthy weight prior to pregnancy, as obesity is also associated with pregnancy complications.

A relatively new method to study preimplantation embryo development and quality is the use of time-lapse imaging. In **Chapter 3**, we investigated the impact of preconceptional maternal BMI on embryo developmental time-lapse parameters, predicted implantation potential, assessed with the KIDScore algorithm and clinical treatment outcomes. In 938 embryos of 268 couples undergoing IVF/ICSI treatment, we showed that a higher maternal

BMI is associated with faster early preimplantation embryo development, but not with the KIDScore or treatment outcomes. In embryos from couples with female or combined factor subfertility, higher maternal BMI is associated with a lower KIDScore, i.e. lower predicted implantation potential. As obesity is often the result of a complex interplay between nutrition, lifestyle and genetics, further research should focus on elucidating the underlying pathophysiological processes.

These observed differences in preimplantation embryo development could also have consequences for post-implantation development. Therefore, we investigated in more than 800 pregnancies whether there are associations between maternal BMI and post-implantation growth and morphological development (**Chapter 4**). Longitudinal three-dimensional ultrasound examinations were performed at 7, 9 and 11 weeks of gestation for offline measurements of crown-rump length (CRL), embryonic volume (EV) and embryonic developmental stages (Carnegie stages), using virtual reality techniques. We showed that periconceptional maternal underweight is associated with faster embryonic growth, especially in female embryos. In contrast, female embryos of women with obesity grow slower than those of women with normal weight. With these results, we further underline the importance of a healthy maternal weight, diet and lifestyle for optimal embryonic growth and development.

In **Part II** of this thesis, we investigated the impact of various IVF/ICSI treatment factors on embryo and placenta development.

In **Chapter 5**, we studied the impact of culture media on preimplantation morphokinetic parameters, predicted implantation potential, assessed with the KIDScore algorithm, and clinical treatment outcomes. This was assessed by comparing Vitrolife G-1 PLUS medium with SAGE 1-Step medium, using a patient registry with more than 400 treatment cycles. All cleavage divisions occurred earlier in SAGE-cultured embryos than in Vitrolife-cultured embryos. Since significantly more SAGE-cultured embryos had the highest KIDScore than Vitrolife-cultured embryos, culture medium also affects the predicted implantation probability. Clinical treatment outcomes were comparable between the two culture media. These findings add to the existing knowledge that IVF-clinics should validate morphokinetic-based selection tools prior to implementation or develop their own clinic-specific selection tool, since even optimal developmental kinetics are affected by culture medium.

It is known that culture media are associated with birthweight, but knowledge regarding the impact of embryonic and fetal growth and other perinatal outcomes is generally lacking. Therefore, **in Chapter 6**, we studied and compared these outcomes between pregnancies established after culturing embryos in Vitrolife G-1 PLUS medium and in SAGE 1-Step medium. Embryos cultured in SAGE 1-Step grow faster than those cultured in Vitrolife G-1 PLUS,

especially 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 male and female embryos separately. Our findings indicate a sex-specific susceptibility to stressors such as culture media. It remains to be studied however if these differences also translate to differences in long-term health outcomes.

We hypothesised that also cryopreservation may impact growth and development. Therefore, in **Chapter 7** we studied differences in embryonic and fetal growth trajectories and perinatal outcomes between IVF/ICSI pregnancies after frozen-thawed extended culture embryo transfer (ET) at day 5, after fresh ET at day 3 and naturally conceived pregnancies. After research in over 850 pregnancies, we demonstrated that embryonic growth in the first-trimester of pregnancy was not affected by cryopreservation of the embryo. It was notable that premature birth occurred less frequently in the freshly transferred embryo group compared to the frozen-thawed embryo group. This latter finding warrants further investigation of the underlying mechanisms and should be validated, as it may have implications for the transfer strategy of IVF-clinics.

Pregnancies after frozen-thawed ET are at increased risk of developing placenta-related pregnancy complications. In **Chapter 8**, we investigated whether or not cryopreservation prior to embryo transfer affects utero-placental (vascular) development, using three-dimensional (3D) ultrasound and virtual reality imaging techniques. In the first-trimester, placental size and growth were comparable between pregnancies resulting from frozen-thawed ET, fresh ET and natural conception. In the second and third trimester, uterine artery Doppler indices were lower in pregnancies after frozen-thawed ET compared to pregnancies after fresh ET and natural conception. These findings indicate that cryopreservation prior to transfer may not be as detrimental for placental perfusion as previous research has suggested. To reduce placental-related pregnancy complications after frozen-thawed ET, the optimal uterine environment prior to ET, including hormonal preparation, remains to be investigated.

Summarizing, in this thesis the evidence for the importance of a healthy maternal weight during the periconception period and optimal embryo environment during IVF/ICSI treatment is further substantiated. We showed that a high maternal BMI may be detrimental for several periconceptional outcomes, in particular preimplantation and post-implantation embryo growth and development. As maternal obesity also has a detrimental impact on perinatal outcomes and offspring health, there is an urgent need for both prevention as well as effective and sustainable treatment, such as digital and 'blended' lifestyle care (www. smarterpregnancy.co.uk and 'Healthy Pregnant').

Additionally, we demonstrated that culture medium alters both preimplantation as well as post-implantation embryo growth and development. Interestingly, embryonic and placental development in pregnancies after transfer of a cryopreserved embryo is comparable to that in pregnancies after fresh ET. These findings, in combination with studies reporting on the impact of culture media and cryopreservation on perinatal outcomes, ask for longitudinal follow-up of IVF/ICSI offspring, so that treatment safety and efficiency can be thoroughly evaluated.

To conclude, optimizing the environment in which periconceptional processes take place, by improving preconceptional diet and lifestyle and IVF/ICSI practices, are investments that will eventually advance the health of current and future generations during the life course.

SAMENVATTING

Het Developmental Origins of Health and Disease (DOHaD) paradigma gaat ervan uit dat de ontwikkelende foetus zich aanpast aan signalen uit de baarmoeder om de kansen op overleving na de geboorte te vergroten. Aanvankelijk richtte het onderzoek naar dit paradigma zich vooral op de periode rondom de geboorte en tweede helft van de zwangerschap. Echter, door toenemende kennis vanuit de moleculaire biologie en epigenetica is het aandachtsgebied verschoven naar de periconceptie periode. De meeste ernstige zwangerschapscomplicaties ontstaan in deze periode, gedefinieerd als 14 weken voorafgaand de bevruchting tot 10 weken erna. Essentiële processen als gametogenese, bevruchting, innesteling, embryogenese en placentatie vinden plaats in dit tijdsbestek. Verstoring van de biologische omgeving waarin deze processen plaatsvinden kan een levenslang effect hebben op de gezondheid van de volgende generatie. Bijvoorbeeld, een obese omgeving leidt tot chronisch oxidatieve stress en is geassocieerd met een verhoogd risico op o.a. aangeboren afwijkingen. Ook tijdens een *in vitro* fertilisatie (IVF) behandeling, met of zonder intracytoplasmische sperma injectie (ICSI), worden de gameten en embryo's blootgesteld aan iatrogene *in vitro* stressoren. Het doel van dit proefschrift is om meer kennis te verkrijgen over de invloed van omgevingsfactoren gedurende de embryonale ontwikkeling, in het bijzonder maternale obesitas en factoren van een IVF behandeling, op zowel pre- als post-implantatie embryonale ontwikkeling en op de ontwikkeling van de placenta. De achtergrond hiervan is beschreven in de introductie van dit proefschrift (**Hoofdstuk 1**).

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In **Deel I** van dit proefschrift bestudeerden we verbanden tussen maternale obesitas en periconceptionele uitkomsten, zoals embryonale groei en ontwikkeling.

In **Hoofdstuk 2** hebben we een systematisch literatuuronderzoek van 75 artikelen uitgevoerd en een overzicht gegeven van de invloed van periconceptionele maternale BMI op de kwaliteit van de eicel, het preimplantatie embryo en de post-implantatie embryonale groei en ontwikkeling. In 19 van de 44 studies werd aangetoond dat een hoge maternale BMI het risico verhoogt op het krijgen van een miskraam. In 7 van de 25 studies in mensen en in 1 van de 3 dierstudies werd een negatief verband aangetoond tussen de maternale BMI en het aantal (rijpe) eicellen. Een verminderde kwaliteit van het preimplantatie embryo is geassocieerd met een hogere maternale BMI in 12 van de 35 studies in mensen, en in 8 van de 10 dierstudies. Deze resultaten suggereren een nadelige invloed van een hogere maternale BMI op periconceptionele uitkomsten in de mens. In dierstudies is deze associatie duidelijker, door gestandaardiseerde condities van het onderzoek en minder verstoring door andere factoren. De conclusie is dan ook dat clinici meer aandacht zouden moeten hebben voor het belang van een gezond gewicht voorafgaand aan de zwangerschap, aangezien obesitas ook in verband wordt gebracht met zwangerschapscomplicaties.

Een relatief nieuwe methode om de ontwikkeling en kwaliteit van preimplantatie embryo's te bestuderen is het gebruik van time-lapse beeldvorming. In **Hoofdstuk 3** onderzochten we de invloed van de preconceptionele maternale BMI op de time-lapse parameters van de embryonale ontwikkeling, de implantatiekans, voorspeld met het KIDScore algoritme, en de klinische behandeluitkomsten. In 938 embryo's van 268 paren die een IVF/ICSI behandeling ondergingen, toonden we aan dat een hogere maternale BMI geassocieerd is met een snellere ontwikkeling van het preimplantatie embryo net na de bevruchting, maar niet met de KIDScore of behandeluitkomsten. Bij embryo's van subfertiele paren, waarbij een maternale of gecombineerde oorzaak voor de subfertiliteit gevonden werd, is een hogere maternale BMI geassocieerd met een lagere KIDScore, oftewel een lagere voorspelde implantatiekans. Aangezien obesitas vaak het resultaat is van een complexe wisselwerking tussen voeding, leefstijl en genen, zou verder onderzoek zich moeten toespitsen op het ophelderen van de onderliggende pathofysiologische processen.

Deze verschillen in preimplantatie embryonale ontwikkeling zouden ook gevolgen kunnen hebben voor de post-implantatie embryonale ontwikkeling. Daarom onderzochten we in meer dan 800 zwangerschappen of er associaties zijn tussen de maternale BMI en de embryonale groei en morfologische ontwikkeling na implantatie (**Hoofdstuk 4**). Longitudinale driedimensionale echoscopische onderzoeken werden uitgevoerd bij 7, 9 en 11 weken zwangerschap voor offline metingen van de kopstuitlengte (CRL), embryonaal volume (EV) en embryonale ontwikkelingsstadia (Carnegie stadia), waarbij we gebruik hebben gemaakt van virtual reality technieken. We toonden aan dat maternaal ondergewicht tijdens de periconceptie periode geassocieerd is met snellere embryonale groei, vooral bij vrouwelijke embryo's. Vrouwelijke embryo's van obese vrouwen groeien daarentegen langzamer dan vrouwelijke embryo's van vrouwen met een normaal gewicht. Met deze resultaten onderstrepen we nogmaals het belang van een gezonde voeding, leefstijl en gewicht van de moeder voor een optimale embryonale groei en ontwikkeling.

In **Deel II** van dit proefschrift onderzochten we de invloed van verschillende factoren van een IVF/ICSI behandeling op de embryonale en placentaire ontwikkeling.

In **Hoofdstuk 5** onderzochten we de invloed van het kweekmedium op preimplantatie morphokinetische parameters, de voorspelde kans op implantatie, berekend met het KIDScore algoritme, en op de klinische behandelingsresultaten. Dit werd onderzocht door het medium Vitrolife G-1 PLUS te vergelijken met het SAGE 1-Step medium, gebruikmakend van de patiëntenregistratie met meer dan 400 behandelingscycli. Alle celdelingen vonden eerder plaats bij in SAGE gekweekte embryo's dan bij embryo's gekweekt in Vitrolife. Aangezien aanzienlijk meer in SAGE gekweekte embryo's de hoogste KIDScore hadden dan in Vitrolife gekweekte embryo's, beïnvloedt het kweekmedium dus ook de voorspelde kans op implantatie. De klinische behandelingsresultaten waren vergelijkbaar tussen de

twee kweekmedia. Deze bevindingen dragen bij aan de bestaande kennis dat IVF-klinieken de op morphokinetiek gebaseerde selectiemodellen moeten valideren voorafgaand aan implementatie of juist kliniek specifieke selectiemodellen moeten ontwikkelen, omdat zelfs de optimale embryonale ontwikkeling wordt beïnvloed door het kweekmedium.

Bekend is dat kweekmedia geassocieerd zijn met het geboortegewicht, maar kennis over de invloed op de embryonale en foetale groei en andere perinatale uitkomsten ontbreekt grotendeels. Daarom hebben we in **Hoofdstuk 6** deze uitkomsten bestudeerd en vergeleken tussen zwangerschappen die tot stand kwamen na het kweken van embryo's in Vitrolife G-1 PLUS medium en in SAGE 1-Step medium. Embryo's die in SAGE 1-Step zijn gekweekt groeien sneller dan in Vitrolife gekweekte embryo's, met name mannelijke embryo's. Er werden geen significante verschillen vastgesteld in de foetale groei in het tweede trimester en de perinatale uitkomsten tussen de beide kweekmedia in de totale populatie en in mannelijke en vrouwelijke embryo's afzonderlijk. Onze bevindingen wijzen op een geslacht-specifieke gevoeligheid voor stressoren zoals het kweekmedium. Het moet echter nog worden onderzocht of deze verschillen zich ook vertalen naar verschillen in gezondheidsuitkomsten op lange termijn.

Ook het invriezen van embryo's zou de groei en ontwikkeling kunnen beïnvloeden. In **Hoofdstuk 7** onderzochten we daarom de embryonale en foetale groeipatronen en perinatale uitkomsten tussen IVF/ICSI zwangerschappen na terugplaatsing van een ingevroren embryo doorgekweekt tot dag 5, na verse embryo terugplaatsing (ET) op dag 3 en natuurlijk verwekte zwangerschappen. Na onderzoek van meer dan 850 zwangerschappen stelden we vast dat de groei van het embryo in het eerste trimester van de zwangerschap vergelijkbaar was tussen de vers teruggeplaatste embryo's en embryo's die waren ingevroren. Opvallend was dat vroeggeboorte minder vaak voorkwam in de groep met de teruggeplaatste verse embryo's dan in de groep met ingevroren embryo's. Deze laatste bevinding vraagt om verder onderzoek naar de onderliggende mechanismen en moet gevalideerd worden, aangezien het implicaties kan hebben voor de terugplaatsingsstrategie.

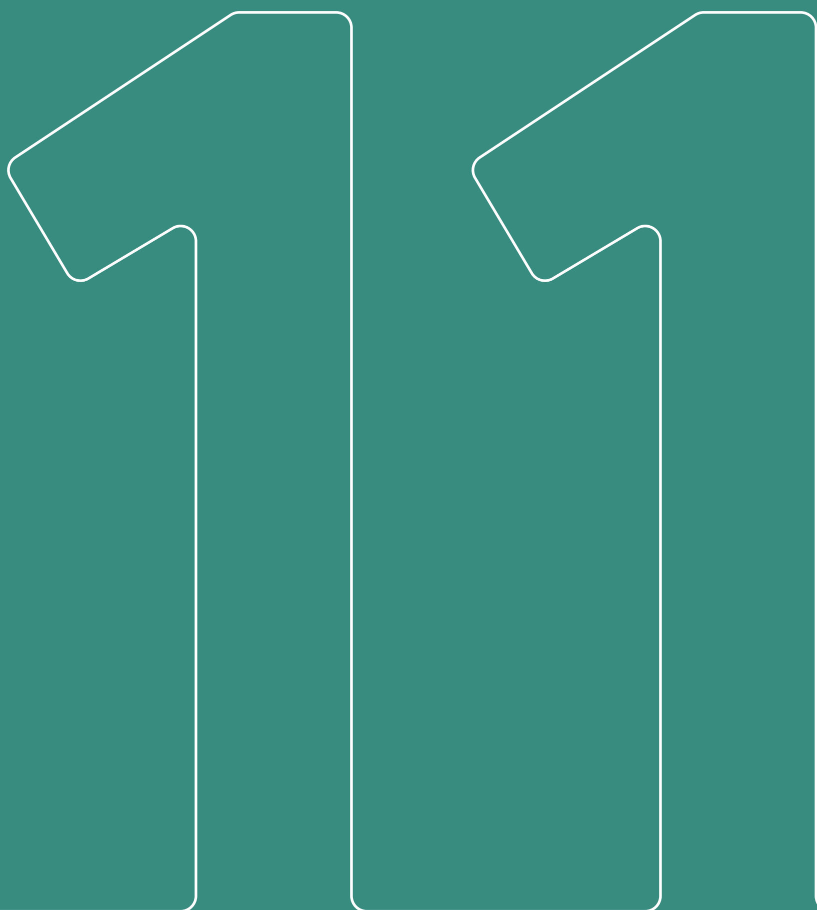
Zwangerschappen na terugplaatsing van een ingevroren embryo zouden mogelijk ook een verhoogd risico hebben op het ontwikkelen van placenta-gerelateerde zwangerschapscomplicaties. In **Hoofdstuk 8** bestudeerden we de invloed van het al dan niet invriezen van een embryo voorafgaand aan de terugplaatsing op de utero-placentaire (vasculaire) ontwikkeling met behulp van driedimensionale (3D) echografie en virtual reality beeldvormingstechnieken. In het eerste trimester was de grootte en groei van de placenta vergelijkbaar tussen zwangerschappen na terugplaatsing van een vers of ingevroren embryo en natuurlijke conceptie. In het tweede en derde trimester waren de Doppler-parameters van de arterie Uterina lager in zwangerschappen na ingevroren ET dan in zwangerschappen na verse ET en natuurlijke conceptie. Deze bevindingen wijzen erop dat het vooraf invriezen

van een embryo mogelijk niet zo schadelijk is voor de placentaire perfusie als eerder onderzoek suggereert. Om placenta-gerelateerde zwangerschapscomplicaties na ingevroren ET te verminderen, moet het optimale uterine milieu voorafgaand aan ET, met betrekking tot o.a. de hormonale voorbereiding, nader worden onderzocht.

Samenvattend, in dit proefschrift wordt het belang van een gezond maternaal gewicht tijdens de periconceptieperiode en een optimale embryonale omgeving tijdens de IVF/ICSI behandeling verder onderbouwd. We hebben aangetoond dat een hoge BMI van de moeder nadelig kan zijn voor de verschillende periconceptionele uitkomsten, waaronder de preimplantatie en post-implantatie embryonale groei en ontwikkeling. Aangezien maternale obesitas ook een nadelige invloed heeft op de perinatale uitkomsten en de gezondheid van het kind, is er dringend behoefte aan zowel preventie als een effectieve en duurzame behandeling tegen obesitas, zoals digitale en 'blended' leefstijlzorg (www.slimmerzwanger.nl en 'Gezond Zwanger').

Bovendien hebben we aangetoond dat het kweekmedium een effect heeft op zowel de preimplantatie als de post-implantatie embryonale groei en ontwikkeling. Interessant is dat de embryonale ontwikkeling in zwangerschappen na terugplaatsing van een ingevroren embryo vergelijkbaar is met die in zwangerschappen na terugplaatsing van een vers embryo. Deze bevindingen, in combinatie met studies naar de invloed van kweekmedia en cryopreservatie op perinatale uitkomsten, vragen om longitudinale follow-up van IVF/ICSI nakomelingen, zodat de veiligheid en efficiëntie van de behandeling uitvoerig geëvalueerd kan worden.

Concluderend kan worden gesteld dat het optimaliseren van omgeving waarin periconceptionele processen plaatsvinden, door verbetering van preconceptionele voeding en leefstijl en IVF/ICSI behandelingen, een investering is die uiteindelijk de gezondheid van de huidige en toekomstige generaties ten goede zal komen.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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AC	Abdominal circumference
AMH	Anti- Müllerian hormone
ART	Assisted reproductive techniques
BMI	Body mass index
CE	Conformité Européenne
CI	Confidence interval
CpG	Cytocyine (C) binded by phosphodiester to guanine (G)
CRL	Crown Rump Length
cm	Centimeter
CSF2	Colony stimulating factor 2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EFW	Estimated Fetal Weight
ESHRE	European Society of Human Reproduction and Embryology
ET	Embryo transfer
EV	Embryonic volume
FA	Folic acid
FL	Femur length
FSH	Follicle stimulating hormone
g	Gram
GA	Gestational age
GDM	Gestational diabetes mellitus
GnRH	Gonadotropin-releasing hormone
h	Hour
HC	Head circumference
hCG	Human chorionic gonadotropin
ICC	Intraclass correlation coefficient
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IQR	Interquartile range
IUI	Intrauterine insemination
IVF	*In vitro* fertilisation
kg	Kilogram
KID	Known implantation data
LED	Light Emitting Diode
LGA	Large for gestational age

LH	Luteinizing hormone
NT	Nuchal translucency
µg	Microgram
m	Meter
MAP	Mean arterial pressure
mg	Milligram
mHealth	Mobile health
MHz	Megahertz
min	Minute
µm	Micrometer
ml	Milliliter
mm	Millimeters
mRNA	Messenger ribonucleic acid
N	Number
n/a	Not applicable
NNT	Numbers needed tot threat
OHSS	Ovarian hyperstimulation syndrome
OI	Ovulation induction
OR	Odds ratio
PCOS	Polycystic ovary syndrome
PD	Power Doppler
PE	Preeclampsia
PGC	Primordial germ cells
PI	Pulsatility index
PIH	Pregnancy induced hypertension
PNa	Pronuclear appearance
PNf	Pronuclear fading
PTB	Preterm birth
PV	Placental volume
RI	Resistance index
RNA	Ribonucleic acid
SD	Standard deviation
SEM	Standard error of the mean
SGA	Small for gestational age
t	Timepoint
TESE	Testicular sperm extraction
uPVV	Utero-placental vascular volume
USA	United States of America
UtA	Uterine artery
VOCAL	Virtual Organ Computer-aided AnaLysis

VR	Virtual reality
WHO	World Health Organization
WHR	Waist-to-hip ratio

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Research integrity in science (ErasmusMC)	2017	0.3
e-BROK course (NFU BROK Academy)	2017	1.5
Biostatistical Principles (CC02) (NIHES)	2018	5.7
Motivational interviewing (Psychodidact)	2019	1.0
English Biomedical Writing and Communication (NIHES)	2020	3.0
'Stoppen met Roken' – Trimbos 2021	2021	0.3
'Rookvrije start' – Trimbos 2021	2021	0.3
Attended seminars, conferences and workshops		
10th DOHaD Congress, Rotterdam, The Netherlands	2017	1.0
64th Annual scientific meeting of the SRI, Orlando, USA	2017	1.0
Annual Sophia Research Day	2017-2020	1.0
Three monthly research meetings Rotterdam O&G	2017-2020	0.5
Two weekly research meeting of the department of O&G, subdivision of Fertility	2017-2020	0.5
Weekly research meeting of the department of O&G	2017-2020	0.5
54th Annual Gynaecongres, Amersfoort, The Netherlands	2018	0.5
11th DOHaD Congress, Melbourne, Australia	2019	1.0
66th Annual scientific meeting of the SRI, Paris, France	2019	1.0
67th Annual scientific meeting of the SRI, Vancouver, Canada	2020	1.0
Presentations at (inter)national conferences		
10th DOHaD Congress, Rotterdam, The Netherlands. Poster	2017	1.0
64th Annual scientific meeting of the SRI, Orlando, USA. Poster	2017	1.0
Annual Wladimiroff award meeting, department of O&G. Oral	2017-2019	3.0
Sophia Research Day 2017, Rotterdam, The Netherlands. Oral	2017	1.0
54th Annual Gynaecongres, Amersfoort, The Netherlands. Oral	2018	1.0

11th DOHaD Congress, Melbourne, Australia. Oral	2019	1.0
66th Annual scientific meeting of the SRI, Paris, France. Poster	2019	1.0
Sophia Research Day 2019, Rotterdam, The Netherlands. Oral	2019	1.0
67th Annual scientific meeting of the SRI, Vancouver, Canada. Poster	2020	1.0
37th Annual scientific meeting of the ESHRE, online. Oral	2021	1.0
68th Annual scientific meeting of the SRI, Boston, USA. Poster	2021	1.0

2. Teaching

Supervising students of the minor 'Mystery of Creation'	2017	0.5
Tutoring of 1st-year Medicine students	2017-2018	2.5
Supervising master thesis C.S Kramer, 4th year Diet & Nutrition student	2018	2.0
Coaching of Bachelor Medicine students	2019-2021	2.5
Supervising students of the minor 'Voeding en Leefstijl'	2019	0.5
Supervising master thesis M.J.J. van Trigt, 4th year Medicine student	2020	2.0

ABOUT THE AUTHOR

Linette van Duijn was born on the 16th of March 1993 in De Lier, The Netherlands. She completed her high school education at the Christelijk Lyceum Delft in 2010. During the last two years of high school, she participated in Junior Med School, an extra-curricular program at the ErasmusMC for high school students interested in medicine and research, where her enthusiasm for medicine and research started. After obtaining her VWO diploma, she started her medical training at the Erasmus University in Rotterdam.

During her Bachelor degree, Linette worked at the student team of Internal Medicine and was involved in teaching clinical practical skills. Her interest in Obstetrics and Gynaecology was sparked during the clerkships and resulted in a master research project on congenital cardiac malformations and periconceptional maternal vitamin D status at the Department of Obstetrics and Gynaecology, division Periconception Epidemiology, at the Erasmus MC.

After graduation in 2017 she continued research at this group and started her dissertation on periconceptional influences on early embryonic and placental development under the guidance of prof. dr. R.P.M. Steegers-Theunissen, prof. dr. J.S.E. Laven, dr. E.B. Baart en dr. M. Rousian. In 2021, Linette began working as a resident in Obstetrics and Gynaecology at Reinier de Graaf Hospital in Delft, followed by a residency in Internal Medicine. From 2023 onwards, she is working as a resident in Gastroenterology in the HagaZiekenhuis.

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