PERICONCEPTION PARENTAL LIFESTYLE, ASSISTED REPRODUCTIVE TECHNIQUES AND THE IMPACT ON (PREIMPLANTATION) EMBRYO AND PLACENTAL DEVELOPMENT

JEFFREY HOEK

Periconception parental lifestyle, assisted reproductive techniques and the impact on (preimplantation) embryo and placental development

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PART I

Introduction

CHAPTER 1

General introduction and aims

Modified from: J. Hoek, R.P.M. Steegers-Theunissen, K. Sinclair and S. Schoenmakers. Chapter 2: The science of preconception care. Preconception Health and Care: A Life Course Approach. 2020, Springer, pages 21-34. ISBN-10: 3030317528

General

The paradigm of the Developmental Origins of Health and Disease (DOHaD) states that maternal and paternal environmental factors, such as nutrition and lifestyle, influence the prenatal environment and development of the fetus, placenta and subsequent pregnancy outcomes with long-lasting consequences for health later in life (1). Most of the research to unravel this paradigm has been focused on birth outcomes and exposures during the second and third trimester of pregnancy. Research with a focus on the first trimester of pregnancy was initially hard to establish since visualization of the first trimester embryo was difficult. Furthermore obstetrical care mostly starts only at around 8–10 weeks of gestational age (GA) with a first ultrasound scan by a midwife or obstetrician. This also implies that the opportunity of prevention and treatment of harmful exposures to optimize periconception parental health conditions is often missed.

Hypothesis

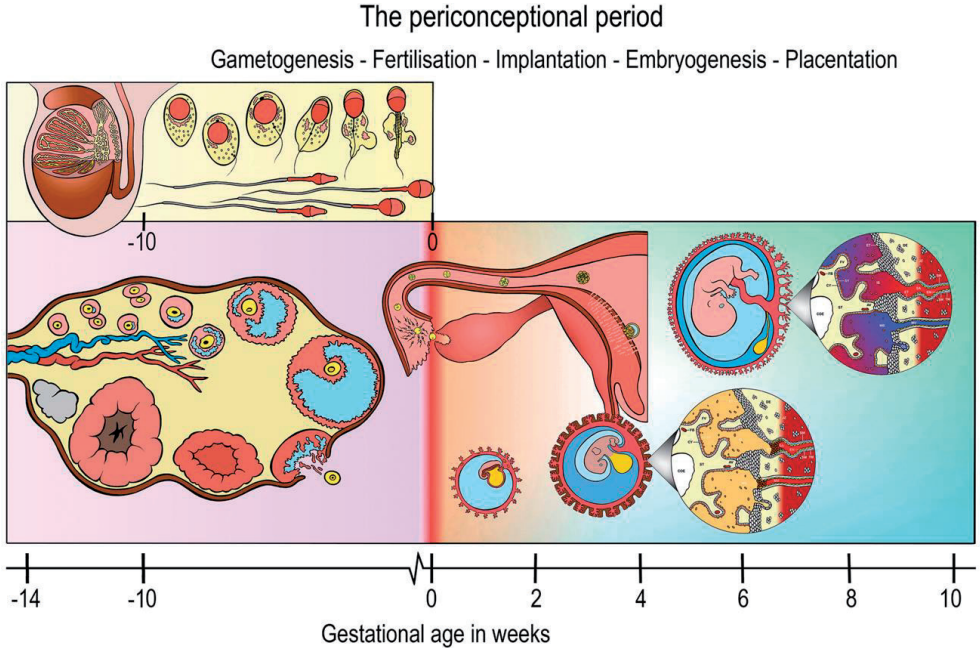
The largely ignored first trimester of pregnancy involves the period in which the most rapid cell multiplication and differentiation processes take place. This explains that the (extra) embryonic tissues are extremely vulnerable to environmental conditions and nutrition and lifestyle behaviors with short and long-lasting effects. We hypothesize that also the period before pregnancy is important since these stressors and behaviors affect oogenesis and spermatogenesis of which the effects are directly transferred to the conceptus and as such perturb the processes of embryonic and placental development (2-4).

Periconception period

General

The periconception period, defined as the period 14 weeks before until 10 weeks after conception, encompassed all essential developmental stages of the sperm, oocyte, (pre-implantation) embryo, implantation and placenta (5). These highly dynamic developmental stages are vulnerable to (ir)reversible and acute environmental influences (e.g. smoking, alcohol and nutrition) on molecular processes such as cell multiplication, epigenetic processes, DNA-replication and -repair as well as protein synthesis. Because of the demonstrated impact of the periconceptional findings, recent years also the DOHaD-related research has shifted its focus from the second half of pregnancy and birth to the periconception period (**Figure 1**).

Figure 1. Overview of the periconception period; 14 weeks before conception until 10 weeks thereafter.



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

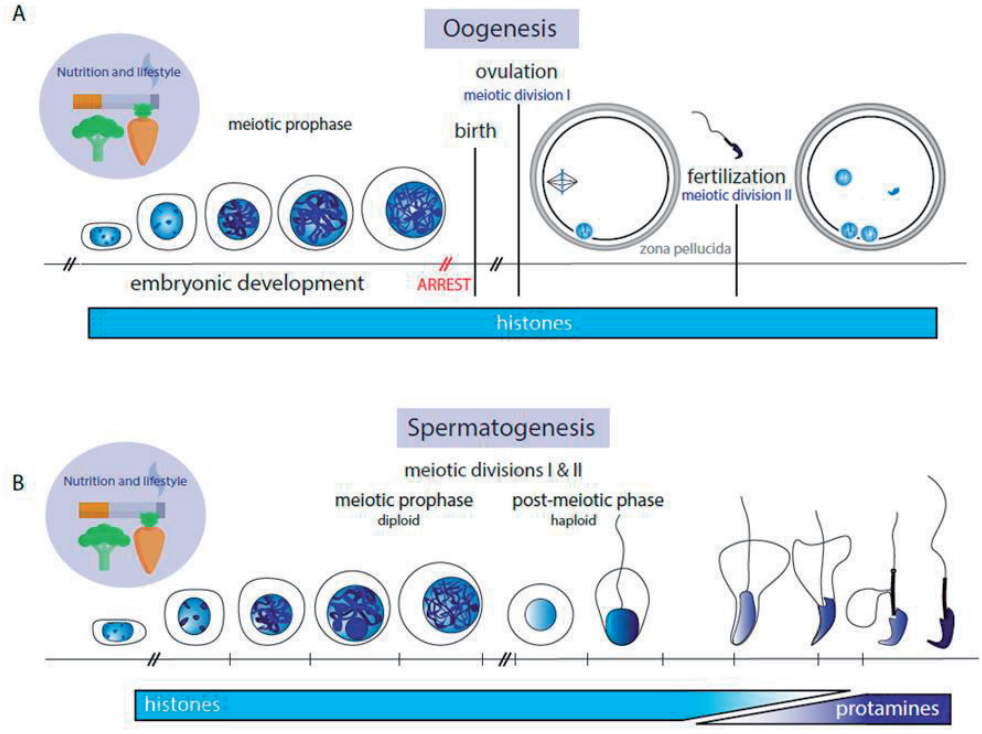
Processes in the preconception until preimplantation embryo phase

Oogenesis (6)

Early in the female embryonic period, primordial germ cells undergo multiple rounds of mitosis and differentiate into oogonia in the embryonic gonadal ridges. Already at the embryonic stage of development, all oogonia enter meiotic prophase, after which they are called primary oocytes. These primary oocytes progress through the early stages of the prophase of the first meiotic cycle and arrest at the so-called diplotene stage (**Figure 2a**). At this stage primordial follicles are formed in which arrested oocytes are enclosed until they are recruited for growth, often years later during postnatal reproductive life. During fetal life approximately 7 million primordial follicles are formed, of which most undergo apoptosis during the midpregnancy period. Only two million primordial follicles harboring these oocytes remain at birth and at menarche only 400.000 – 500.000 primordial follicles remain, of which ultimately 400-500 will reach the ovulatory stage. Since all primordial follicles are already formed and waiting to be recruited this emphasizes the need for a healthy environment and 1-C metabolism (**Chapter 2**) during the intra-uterine period and throughout the fertile lifespan. Only during the pre-ovulatory period (under the influence of follicle stimulating hormone (FSH) and luteinizing hormone (LH)) do these arrested

oocytes resume meiosis I, with the first meiotic oocyte division and the production of the first polar body occurring just before ovulation. This is immediately followed by the initiation of meiosis II until another period of arrest at metaphase II. Fertilization then results in completion of meiosis II with the production of a second polar body. The formation of two small polar bodies allows the fertilized oocyte to maintain maximal cytoplasmic volume. The cytoplasm contains multiple essential maternal factors, such as proteins and mRNA, required for the first cleavage divisions of the early embryo until the zygotic gene activation around the 8-cell stages takes over. Advanced maternal age is associated with decreased fecundity, where impaired quality of the cytoplasm (decrease in the endogenous metabolite pool) is believed to be a key factor besides chromosome segregation and alteration of gene expression.

Figure 2. Overview of spermatogenesis and oogenesis.



Legend:

A Oogenesis: The process of oogenesis starts a couple of weeks after fertilization after which all oocytes arrest in meiosis I already before birth. Meiosis I continues postnatally with the activation of the hypothalamic-pituitary-ovarian (HPO) axis and progresses until metaphase II. Directly after successful fertilization meiosis is completed.

B Spermatogenesis: The process of spermatogenesis starts with the activation of activation of the hypothalamic-pituitary-testes (gonad) (HPG) axis and the onset of puberty after which spermatogonial stem cells are recruited to enter meiotic cell divisions. The post-meiotic phase consists of mainly morphological changes of the spermatozoal head, including the histone to protamine change to ensure tight DNA compaction.

Spermatogenesis (7)

Just as during oogenesis the primordial germ cells (PGC) migrate to the genital ridges in the male embryo and keep proliferating until the gonadal regions are colonized. In contrast to oogenesis, meiosis in male germ cells is not initiated during the embryonic or fetal periods. Instead, spermatogenesis is initiated at the commencement of puberty by activation of the FSH and LH-stimulated testosterone axis and will continue until death. Importantly, in the context of paternal influences on embryonic health, the whole process of spermatogenesis takes place within a period of 2 to 3 months, indicating total renewal of all spermatozoa and its (epi)genetic content, every 3 months. In the human testes, outside the body, male PGCs develop into spermatids and eventually into spermatozoa (**Figure 2b**). The subsequent differentiation process, which takes place in the testis and epididymis, of spermiogenesis consists of major morphological and chemical alterations, such as the histone to protamine exchange, and is necessary to ensure that nuclear DNA is tightly compacted and fits in the spermatozoal head. Aberrations in the ratio of histones to protamines in the spermatozoal head are associated with lower sperm quality. Directly after fertilization this transitional protein process is reversed. Paternal protamines are replaced by histones of maternal origin in order to facilitate recondensation of the paternal DNA. This extensive recondensation is required for access to and the expression of important growth genes, essential for early embryonic development and growth. This also emphasizes the need for a healthy testicular environment during the periconception phase, allowing optimal production and formation of sperm. The production of millions of spermatocytes per day requires copious quantities of 1-C metabolism dependent moieties such as nucleotides, DNA and proteins (8). Paternal nutritional deficiencies of essential vitamins like folate, methionine and choline, but also lifestyle factors such as alcohol use and smoking, can have a direct measurable effect on spermatogenesis concerning total sperm counts, motility, percentage of DNA damage and epigenetic programming/markers (**Figure 2b**).

Fertilization and pre-implantation embryo.

Fertilization is the first important and initiation step in the process of embryogenesis (9, 10). Sperm binds to the ovulated oocyte at the zone pellucida (ZP), where the ZP protein 2 acts as an important receptor for the spermatozoa. After binding to the ZP the spermatozoal head releases acrosome content to allow fusing of the plasma membranes of the sperm and oocyte followed by entrance of the sperm nuclei into the oocyte cytoplasm. Directly following this membrane fusion, the membrane of the oocyte depolarizes to prevent polyspermy. Hereafter meiosis continued, the second polar body is formed and once haploid the maternal pronucleus is formed. Interestingly, while the oocyte finalizes meiosis II, the highly condensed and transcriptionally unreadable chromatin of the sperm undergoes major transformations. Nearly all protamines are replaced by maternally supplied histones to make the chromatin less condensed and ready for pairing with the maternal genetic material. The appearance of two pronuclei, seen usually after 18-20 hours, is the sign

of successful fertilization. After the pairing of the male and female genetic material the human zygote starts dividing. Around the 8-cell stage (embryo day 3), the embryo becomes compacted (morula stages), followed by the appearance of a cavity, the blastocyst stage. The blastocysts consist of an inner cell mass, which will develop into the embryo, and an outer cell layer, trophoblast, which will form the extra-embryonic compartments, such as the placenta. Around embryonic day 5, the developing blastocyst reaches the uterine cavity and, at embryonic day 7, after hatching the blastocyst invades the epithelium of the endometrium, initiating embryonic and placental development.

Processes in the post-implantation phase

Post-implantation embryonic developmental processes.

After invasion of the endometrium and successful implantation embryonic growth and development can be divided into two periods. The first period comprises day 7 till 20 after conception and consists of formation of the embryoblast, epiblast and hypoblast and formation of the amniotic cavity. After formation of the ectoderm, endoderm and mesoderm by gastrulation of the epiblast the embryo consists out of 3 germ layers. The second period lasts until embryonic day 60 and mainly consists of organogenesis and growth (cellular replication). With the advancements in ultrasound and 3D techniques, embryonic growth can nowadays be visualized and monitored more precisely from 6 weeks of gestation. Embryonic growth was believed to be uniform for everybody, but it is now apparent that many maternal and paternal factors can influence embryonic growth already during these early stages, with effects later in the life course. As example, being smaller in the first trimester of pregnancy is associated with an increased risk of preterm delivery, and being born small for gestational age is associated with postpartum cardiovascular risk factors at the age of six (11, 12).

Placental development.

After invasion of the endometrium and successful implantation, placental development originating from trophoblast cells is initiated (13, 14). The trophoblast cells are divided in cytotrophoblast and syncytiotrophoblast. By differentiation and fusion of cytotrophoblast cells, the syncytiotrophoblast is formed. Syncytiotrophoblasts on the embryonic side of the placenta develop quickly and start forming lacunas, while on the maternal side of the placenta they start signaling and reforming the maternal spiral arteries in the decidua basalis. Placental villi are formed by cytotrophoblast penetrating the syncytiotrophoblast and thereby forming columns of cytotrophoblast surrounded by syncytiotrophoblast. Subsequently mesoderm invades these columns and growth towards the decidua to form blood vessels. Subsequently extravillous trophoblast (EVT) invades into the endometrium to attach the placenta firmly to the uterine wall. On the maternal side of the placenta remodeling of the spiral arteries is important for normal functioning of the placenta. Differentiated EVTs, interstitial, endovascular and intramural EVTs, invade the

decidua and remove the endothelial cells from the spiral arteries. These cells are replaced by endovascular trophoblast and the spiral arteries are later again re-endothelialized. The formation and remodeling makes sure enough maternal blood passes through the placental villi with low pressure, which is necessary for optimal duration and exchange of gases and nutrients between mother and fetus in the intervillous space. Suboptimal implantation with impaired placental development and functioning is associated with a number of pregnancy complications with lifelong effects for both mother and child. The most important complications are pre-eclampsia, a disease characterized by high blood pressure and proteinuria in pregnancy, preterm birth and babies too small for gestational age (SGA).

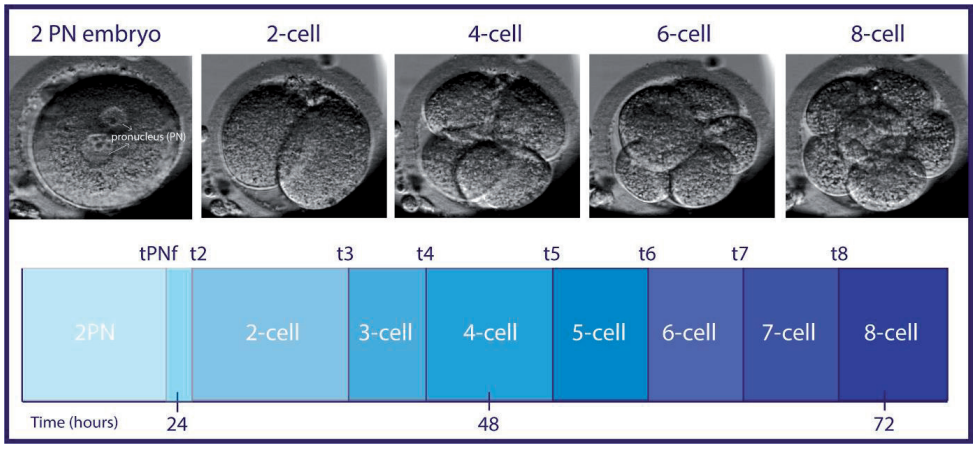
Visualization techniques in the periconception period

This thesis shows associations between periconception exposures and pre-implantation embryo development, post-implantation development, including fetal and placental growth and development. Adequate and novel visualization techniques are the foundations for collecting information and allowing to draw conclusions on pre-implantation embryonic, placental and fetal growth and development.

Pre-implantation embryo development

Pre-implantation embryo development can be studied using the EmbryoScope™, which incorporates a specialized built-in microscope designed for automated time-lapse embryo assessment by acquiring images (15-17). The EmbryoScope™ provides a controlled culture environment and captures comprehensive information on embryo development without the need for handling or disturbing the developing embryo. Embryo images are automatically recorded in seven focal planes every 10 minutes (**Figure 3**)

Figure 3. Embryonic development from 1 cell to 8 cell (embryonic day 1 till day 3) as seen from the EmbryoScope™.



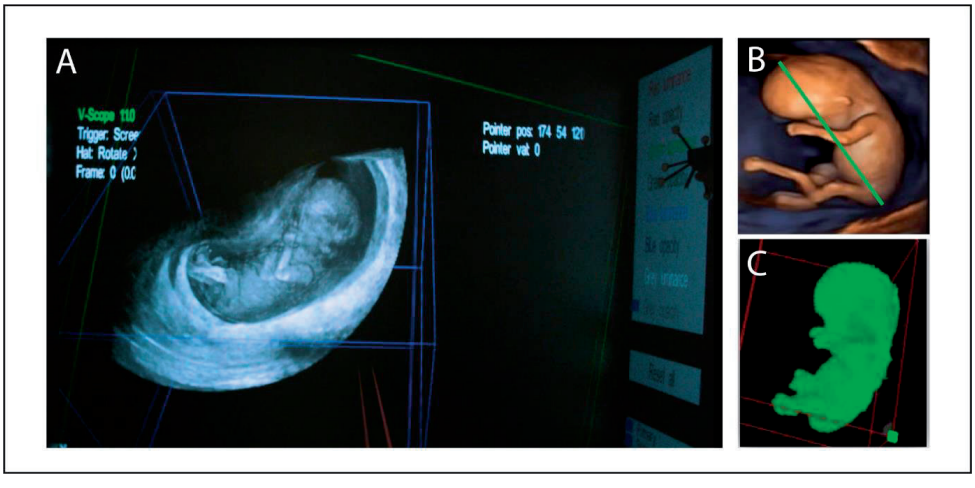
Post-implantation embryo development and placental development

Studying early post-implantation embryonic development is possible from 6 weeks of gestation onwards using two-dimensional (2D) transvaginal ultrasounds. Clinically first trimester ultrasounds are used as diagnostic tool to investigate pregnancy localization, the presence of an embryonic heart beat and embryonic growth and development. Adding three-dimensional (3D) graphics to the ultrasonography improves visualization and measurement possibilities during the first trimester of pregnancy. An important limitation of the 3D ultrasounds is the fact that these pictures usually are depicted on a 2D screen of paper, making no usage of the third dimension. To optimally make use of the depth information present in 3D-ultrasound data, images can be transferred to our Barco I-Space (a Cave Automatic Virtual Environment–like virtual reality system). Here an interactive virtual reality hologram, which allows depth perception and interaction with the images projected, is created (18-20).

3D transvaginal ultrasound techniques in combination with the Barco I-Space can be used to more precisely measure growth (Crown Rump Length (CRL)) and also gives the opportunity to measure volumes of both embryonic and extraembryonic structures (18, 20) (**Figure 4**).

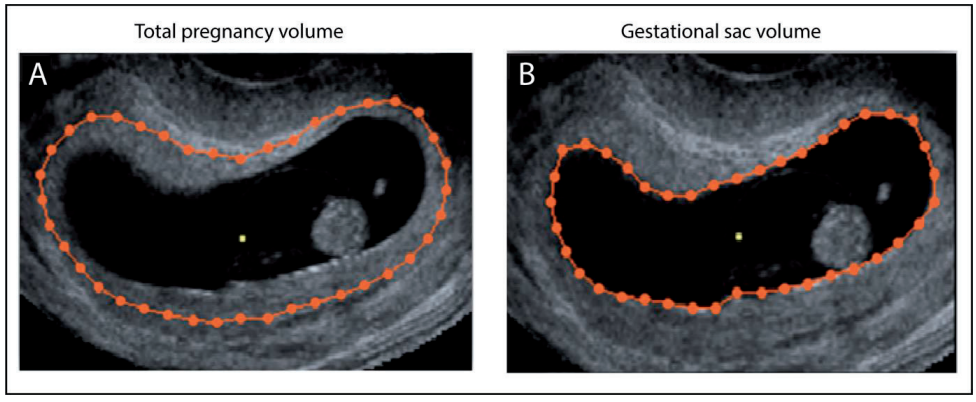
In early pregnancy, derivatives of placental development can be established using 2D and 3D ultrasound alone or in combination with power Doppler (PD) for visualization of vessels. Currently placental development can be monitored by either measuring the placental volume (PV; including vasculature and trophoblast) or utero-placental development (uPVV; including mainly maternal decidual vessels) (21). The Virtual Organ Computer-

Figure 4. Embryo measurements in the Barco I-Space with a. view of an embryo in the I-Space, b. measurement of the crown rump length and c. measurement of the embryonic volume.



aided AnaLysis (VOCAL) tool can be used offline to determine placental volume from 3D ultrasound volumes, by subtracting the gestational sac volume from the total pregnancy volume (**Figure 5**). In addition, 3D PD ultrasound can be used to measure the utero-placental vasculature. When combined with a Virtual Reality system, 3D PD recordings of the utero-placental vasculature can be depicted as a hologram, which enables measurement of the utero-placental vascular volume.

Figure 5. Placental volume measurement technique using 3D ultrasound volumes by subtracting a. total pregnancy volume and b. gestational sac volume.

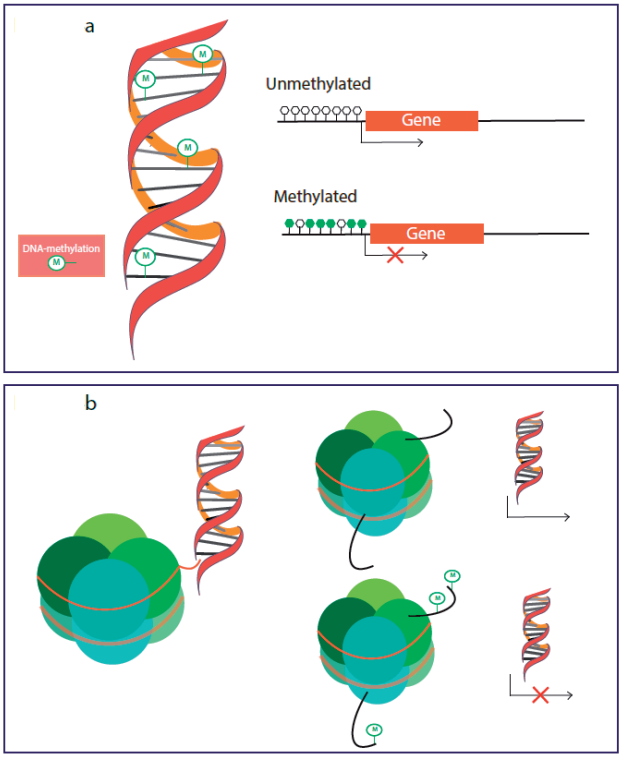


One carbon metabolism (5, 8, 22)

During the periconception period, gametes mature in the gonadal environment, influenced by genetic and environmental factors, i.e., diseases, medication, toxins and lifestyle, including nutrition. Optimal nutrition is essential for preconceptional parental health, as malnutrition (both undernutrition and overnutrition) is prevalent in many developing and developed countries, is known to disrupt important metabolic pathways, including one-carbon (1-C) metabolic pathways. One-carbon metabolism provides essential 1-C moieties for various preconception biological processes in order to successfully conceive and to accomplish optimal embryonic and fetal growth and development. 1-C metabolism is essential for synthesis of nucleotides, crucial as building blocks of DNA, and proteins and lipids required for most pathways, e.g. biosynthesis of building blocks of membranes, signaling, inflammation. These pathways also influence the methylation of DNA and associated (histone) proteins as well as RNA; such covalent modifications being referred to as epigenetics (**Figure 6**).

The most important dietary substrates/cofactors involved in 1-C metabolism include the B vitamins (particularly folate (B9 or B11), cobalamin (B12) and pyridoxine (B6)), together with methionine and choline. The main natural sources of folate are derived from fruits,

Figure 6. Overview of the epigenetic processes DNA methylation and histone modification associated with one-carbon metabolism.

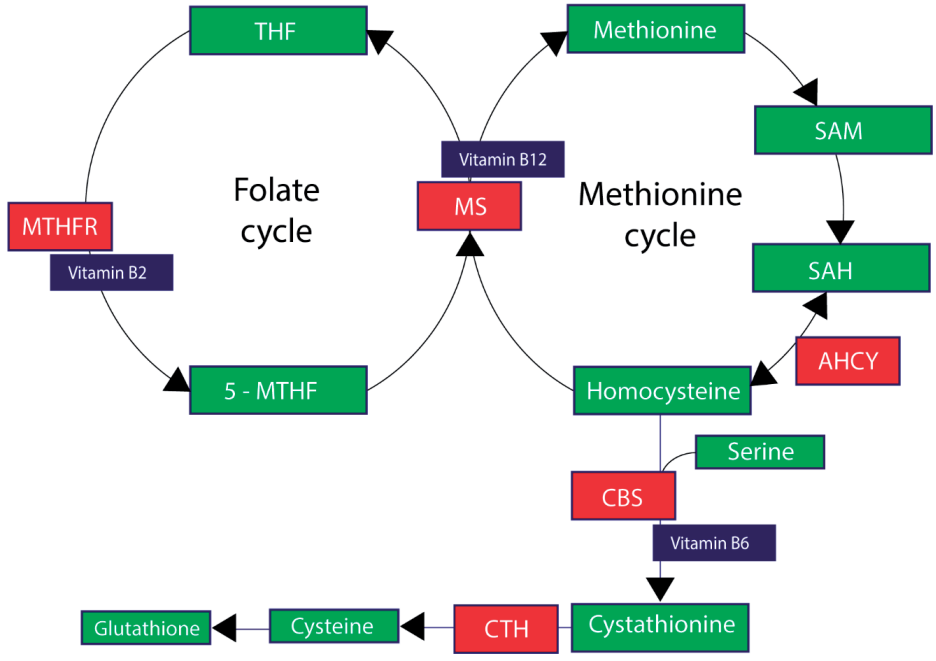


Legend: Green 'M' indicate methyl groups attached to either DNA (figure a) or histones (figure b).
A: The process of DNA methylation is characterized by the binding of methyl groups at CpG sites of DNA. DNA methylation at the promotor regions of genes causes inability to transcribe the gene.
B: Histone methylation is characterized by the binding of methyl groups on specific amino acids within peptide chains (so called histone tails) protruding from the nucleosome. Methylation of histone tails can cause either more or less condensed chromatin and is critical for the regulation of gene transcription, depending on the amount of methyl groups attached and which specific amino acids are methylated

vegetables and nuts, which are absorbed from the proximal jejunum as the biological active form of tetrahydrofolate (THF). Another source is synthetic folic acid (FA), derived from fortified foods in certain countries, such as Canada and the USA, and as various supplements. However, synthetic FA first needs to be converted by dihydrofolate reductase to the active form THF in the intestinal cells. The next essential step is the conversion of THF to 5-methyl-tetrahydrofolate (5-MTHF), by the enzyme methylenetetrahydrofolate reductase (MTHFR) with vitamin B2 (riboflavin) as cofactor. 5-MTHF provides a methyl group, transferred by vitamin B12 dependent methionine synthase, converting tHcy to methionine **(Figure 7)**. Methionine is transmethylated into S-adenosylmethionine (SAM), which is the

most important methyl donor in our body for numerous processes as mentioned above. After transfer of the methyl group from SAM, S-adenosylhomocysteine (SAH) is formed. The enzyme S-adenosyl-L-homocysteine hydrolase (AHCY) hydrolyzes SAH back to tHcy. The combined folate-methionine cycle is also involved in the production of gluthation, also known as the strongest scavenger of reactive oxygen species (ROS). In normal metabolic homeostasis, HCY is transsulphurated to cystathionine by the enzyme cystathionine ß-synthase (CBS) using vitamin B6 as cofactor. Cystathionine is converted by cystathionine gamma-lyase (CTH) to cysteine, which is a substrate that together with glutamate forms the antioxidant glutathione. Dependent on the availability of cofactors and enzyme activity, SAM regulates the activity of CBS **(Figure 7)**.

Figure 7. Summary of the 1-C metabolism.



Legend:
Green boxes: derivatives, red boxes: enzymes and blue boxes: cofactors. The first step is the conversion of THF to 5-methyl-tetrahydrofolate (5-MTHF), by the enzyme methylenetetrahydrofolate reductase (MTHFR) with vitamin B2 (riboflavin) as cofactor (folate cycle (left)). 5-MTHF together with total homocysteine (tHcy) is then converted to methionine by methionine synthase (MS) with vitamin B12 acting as cofactor (methionine cycle (right)).
Abbreviations: THF; Tetrahydrofolate, 5-MTHF; 5-Methylenetetrahydrofolate, MTHFR; Methylene-tetrahydrofolate reductase; MS; Methionine synthase, SAM; S-adenosylmethionine, SAH; S-adenosylhomocysteine, AHCY; S-adenosyl-L-homocysteine hydrolase, CBS; cystathionine ß-synthase and CTH; cystathionine gamma-lyase.

Since a reduced bioavailability of substrates and cofactors inhibit the remethylation and transsulfuration of tHcy, mild to moderate hyperhomocysteinemia can be used as a sensitive marker for impaired function of 1-C metabolism. FA supplements and a folate-rich diet can overcome relative folate deficiencies and single nucleotide polymorphisms (SNPs) in essential genes, such as MTHFR, MS and methionine synthase reductase (MTRR), can affect enzymatic activities resulting in altered folate levels. The supply of 1-C moieties depending on the availability of substrates, cofactors and genetics, affect the (patho)physiology of reproduction such as gamete quality. For example specific SNPs in these genes are associated with either a decreased or increased fertility (**Table 1**). Moreover, epigenetic modifications to DNA and associated proteins can be induced during spermatogenesis and oogenesis, as well as during early embryogenesis and placentation due to functioning of 1-C metabolism. This nicely explains that 1-C metabolism is essential in the periconception period for early development as well as future health of the offspring.

Table 1. Overview of different SNP genotypes of enzymes involved in the folate cycle as part of 1-C metabolism.

Genotype	Impact on fertility
MTHFR	
MTHFR C677T	Decreased fertility
MTHFR A1298C	Decreased fertility
MTHFR G1793A	Increased fertility
MS	
MS A2756G	Decreased fertility
MTRR	
MTRR A66G	Decreased fertility

Legend: Abbreviations: MTHFR; Methylenetetrahydrofolate reductase, MS; Methionine synthase and MTRR; 5-methyltetrahydrofolate-homocysteine methyltransferase reductase.

Assisted reproductive techniques

This thesis does not only describe naturally conceived pregnancies, but also pregnancies after assisted reproductive techniques (ART). If couples fail to achieve a naturally conceived pregnancies, ART are offered to couples suffering from subfertility, including in vitro fertilization (IVF), with or without intracytoplasmic sperm injection (ICSI). Since the birth of the first IVF baby in 1978, worldwide more than ten million children are conceived via assisted reproductive techniques ART. Subfertility can be subdivided into female factor, male factor and a combination of these factors. Although in quite a few cases no direct explanation for the subfertility will be found and these couples suffer from unexplained subfertility. Examples of female factor subfertility are endometriosis, anovulation and tubal

factor subfertility. Male factor subfertility is related to a low amount or low quality of sperm characterized as oligoasteno(terato)zoospermia or an azoospermia with no available sperm in the ejaculate. Examples of underlying causes are genetic disorders, obstructions in the vas deferens or post infection.

IVF and ICSI procedures all start with ovarian stimulation using exogenous FSH to stimulate the growth of multiple oocytes for later harvesting. To prevent a premature LH peak, which initiates the irreversible process of ovulation, gonadotropin-releasing hormone (GnRH) agonists or antagonists are started during the stimulation phase. Final oocyte maturation is triggered either by administering human chorionic gonadotropin (hCG) or by triggering an endogenous LH peak trough an GnRH agonist administration around 30 hours before oocyte harvesting. Subsequently oocytes are harvested via transvaginal fine needle aspiration guided by ultrasound. Sperm used for ART can have different origins dependent on the underlying cause or diagnosis in case of a male subfertility factor. In cases with good or reasonable amount of sperm, physiologically freshly, ejaculated sperm can be used for IVF or ICSI. In cases of azoospermia, sperm can only be collected by surgical techniques dependent on the underlying cause of the azoospermia. Patients with non-obstructive azoospermia (NOA), where testicular insufficiency is thought to be the cause, sperm can be retrieved using testicular sperm extraction (TESE). In patients with an obstructive azoospermia, as is the case after male sterilization or congenital absence of the vas deferens, sperm can be retrieved from the epididymis by a technique called Microsurgical Epididymal Sperm Aspiration (MESA).

Summarizing IVF, the sperm and oocytes are artificially brought together in a petri dish in the laboratory after which spontaneous fertilization is pursued. In ICSI, one spermatozoa is artificially and directly injected into the cytoplasm of the oocyte. Subsequent fertilization and early pre-implantation embryo development occurs as described above. After successful fertilization and acceptable pre-implantation development the embryo is transferred back to the uterus of the mother-to-be. In the Netherlands the standard is to transfer only one embryo per cycle, depending on the age of women. The remaining good quality pre-implantation embryos can be cryopreserved, which, after thawing, can be used at a later time. Artificial interventions, such as IVF and ICSI, in the periconception period also influence the intra-uterine development of the fetus and thereby health later in life. Some studies show that children conceived after ART are at increased risk of cardiovascular risk factors with increased blood pressure, vascular dysfunction and obesity as compared to naturally conceived children (23).

Lifestyle interventions

Unhealthy nutrition and lifestyle, characterized by a high caloric intake, low vitamin intake and minimal physical exercise contribute to obesity as phenotype through derangements of various metabolic and endocrine pathways. All factors related to these unhealthy behaviors result in higher risks of non-communicable diseases, such as cardiovascular and metabolic diseases (24). In the periconception period inadequate nutrition and lifestyle behaviors have a strong negative impact on fertility and the development of the embryo and fetus (3, 25). Previous years multiple studies repeatedly showed that couples contemplating pregnancy are often not aware of their inadequate nutrition and lifestyle (26-28). Furthermore, inadequate nutritional behaviors of the mother during pregnancy can also have detrimental consequences for the health of the offspring later in life, where earlier age of puberty-onset, a decline in ovarian follicle reserve and increased incidence of childhood asthma, cardiovascular disease and obesity have been reported (29, 30). Paternal obesity and especially smoking cigarettes and drinking alcohol are negatively associated with sperm quality and epigenetic profiles of the sperm, possibly affecting embryo quality (31-33).

The periconception outpatient clinic 'Healthy Pregnancy' designed to improve nutrition and lifestyle, showing promising results, i.e. 30% reduction of inadequate nutrition and lifestyle and a 65% increased chance of ongoing pregnancy after IVF treatment, supports the great importance of advocating a healthy lifestyle already before pregnancy (34). Periconception counseling at an outpatient clinic is usually only possible on a small scale and it does not reach all couples. Furthermore, men and women need to be willing to visit the hospital and caregiver. To implement periconception care on a larger scale regarding healthy nutrition and lifestyle new mobile technology approaches have been implemented, such as www.smarterpregnancy.co.uk (www.slimmerzwanger.nl). Smarter pregnancy provides individual and tailored information where it identifies the most important risk factors and subsequently provides information and motivational coaching by text and e-mail messages. Recent year the smarter pregnancy program has proven to be (cost)effective in reducing the amount of unhealthy nutrition and lifestyle factors in both the general population as in subfertile couples (28, 35, 36). The smarter pregnancy program can also be used to effectively asses nutrition and lifestyle factors which can be used by caregivers to quickly screen and counsel couples into a healthier lifestyle. Lastly, the smarter pregnancy program can also be used to asses nutrition and lifestyle factors and as intervention tool for research purposes.

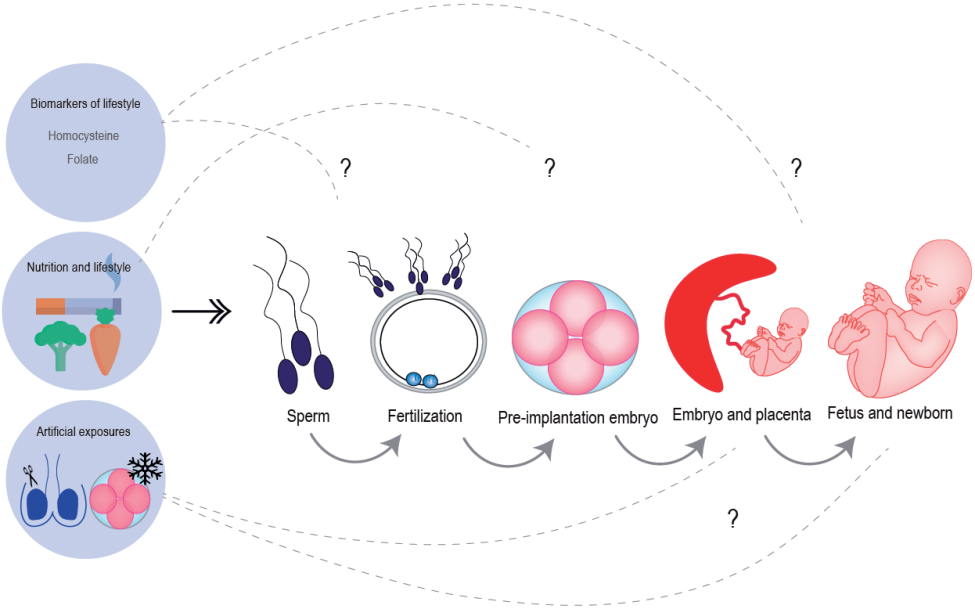
Aims of this thesis

The main aim of this thesis is to unravel the impact of periconception parental lifestyle and in vitro fertilization treatment on semen quality, pre- and postimplantation embryo and placental development (**Figure 8**).

The specific objectives of this thesis are to investigate the

I. impact of the periconception paternal folate status on sperm quality, fertilization, and embryo and placental development (Part 1)
II. paternal and maternal lifestyle in association with pre-implantation embryo development (Part 2).
III. post-implantation embryo development (Part 3), and placenta development (Part 4).

Figure 8. Hypotheses and aims of this thesis.



Setting and used studies

Setting

All studies described in this thesis are conducted in the research group Periconception Epidemiology at the department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center, Rotterdam, the Netherlands.

Rotterdam Periconception Cohort

The Rotterdam Periconception Cohort (Predict study) is an ongoing prospective cohort study including both men and women, embedded in the patient-care of the Erasmus MC, University Medical Center in Rotterdam (37). Participants were recruited from November 2010 onwards. Inclusion criteria for both women and their partners were as follows: at least 18 years of age, a singleton pregnancy and the ability to speak and read the Dutch language. Women and their partners were included before the 10th week of gestation, with pregnancies conceived either naturally or after In Vitro Fertilization (IVF) with or without Intracytoplasmic Sperm Injection (ICSI). The follow up of women is until 1 year after delivery. The study population consists of couples visiting our tertiary university-based hospital. Extensive questionnaires regarding medical history, general health and food and lifestyle habits are given to women and men at study enrollment. In the first trimester of pregnancy women undergo three-dimensional (3D) transvaginal ultrasounds at 7, 9 and 11 weeks of gestation. To precisely measure the embryo (crown rump length (CRL) and embryonic volume (EV)) and placenta (placental volume (PV)), images were transferred to the Barco I-Space (see above).

Virtual Reality embryoscope study

From 2017 the virtual Reality embryoscope study is a subcohort embedded in the Predict study. Women and their partner, who are undergoing in-vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) are included preconceptionally. Extensive questionnaires regarding medical history, general health and food and lifestyle habits are given to women and men at study enrollment. After ovarian stimulation and oocyte pick-up the resulting embryos are cultured in the EmbryoScope™ (see above).

Virtual Reality placenta study 1.0

Since 2015 the Virtual Reality placenta study 1.0 is also a subcohort of the Predict study. This study focuses on the investigation of placental health using imaging techniques from the first 7 weeks of pregnancy onwards for which the same in- and exclusion criteria are used. Three dimensional power Doppler ultrasound techniques are used to visualize the Utero-Placental Vascular Volume at 7, 9 and 11 weeks of gestation (see above).

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CHAPTER 2

Paternal folate status and sperm quality, pregnancy outcomes and epigenetics; a systematic review and meta-analysis

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BACKGROUND: The effectiveness of maternal folate in reducing the risk of congenital malformations during pregnancy is well established. However, the role of the paternal folate status is scarcely investigated. The aim of this study is to investigate the evidence of associations between the paternal folate status and sperm quality, sperm epigenome and pregnancy outcomes.

METHODS: Databases were searched up to December 2017 resulting in 3682 articles, of which 23 were retrieved for full-text assessment.

RESULTS: 4 out of 13 human and 2 out of 4 animal studies showed positive associations between folate concentrations and sperm parameters. An additional meta-analysis of 4 randomized controlled trials in subfertile men showed that the sperm concentration increases (3.54 95%CI (-1.40 to 8.48)) after 3-6 months of 5mg folic acid use per day compared to controls. Moreover, 2 out of 2 animal and 1 out of 3 human studies showed significant alterations in the overall methylation of the sperm epigenome. 1 animal and 1 human study showed associations between low folate intake and an increased risk of congenital malformations.

CONCLUSIONS: This systematic review and meta-analysis shows evidence of associations between the paternal folate status and sperm quality, fertility, congenital malformations and placental weight.

Abstract

Introduction

The last three decades of research have shown overwhelming evidence that the B vitamin folate is essential for reproduction, pregnancy, health and disease. In preconception care, maternal folic acid (FA) supplement use is well known for its role in the prevention of congenital malformations, in particular neural tube defects and congenital heart defects (1). Due to the proven protective role of FA in human reproduction, the World Health Organization advises all women to use 0.4mg FA from the moment of contemplating pregnancy up to 12 weeks of gestation. Van Uitert et al. showed in a systematic review that red blood cell (RBC) folate concentrations and FA supplement use is positively associated with an increased birthweight and inversely associated with the risk of low birthweight and small for gestation age infants (2). These effects can be explained by impaired cell multiplication, DNA synthesis and programming due to (ir)reversible changes of the epigenome, such as DNA methylation, histone modifications and chromatin remodeling**,** induced during gametogenesis and the first weeks after conception. The periconception epigenome of both men and women, together with transcription factors, RNA and one-carbon (1-C) moieties play key roles in molecular biological processes, such as programming of gene expression, involved in embryonic, fetal and placental growth and development. (**Figure 1a**) (3).

Folate, but also methionine and choline, are important substrates of the 1-C metabolism, which provides essential 1-C moieties for processes such as lipid, nucleotide, protein and DNA synthesis, but also for methylation of DNA and histones (4). The main natural sources of folate are fruits, vegetables and nuts, which are absorbed from the jejunum as the biological active form of tetrahydrofolate (THF). Another source is synthetic FA derived from fortified foods and supplements, that first need to be converted in the intestinal cells by dihydrofolate reductase (enzyme commission number (ECN): 1.5.1.3) to the active form, THF. The next essential step is the conversion of THF into 5-methyl-tetrahydrofolate (5-MTHF), by the enzyme methylenetetrahydrofolate reductase (MTHFR, ECN: 1.5.1.20). 5-MTHF together with homocysteine is converted into methionine by methionine synthase (MS) (ECN: 2.1.1.13) using vitamin B12 as cofactor. The folate dependent 1-C metabolism is necessary for the production of essential 1-C moieties (**Figure 1b**). Single nucleotide polymorphisms (SNPs) in essential genes of the folate dependent one-carbon metabolism, such as MTHFR (ECN: 1.5.1.20), can affect enzymatic activities and the availability of 1-C moieties. Altogether, differences in the intake of folate, FA and individual SNPs, in tissues and target organs and the combination of all these factors greatly influence the availability of 1-C moieties.

Since the embryo and fetus develops within the maternal environment, it is not surprising that previous research has mainly focused on the maternal folate status in relation to

Figure 1. Overview of the a. spermatogenesis, embryogenesis, the corresponding histone-protamine exchange and the methylation level of non-imprinted genes and b. folate related one-carbon metabolism.

Figure 1a

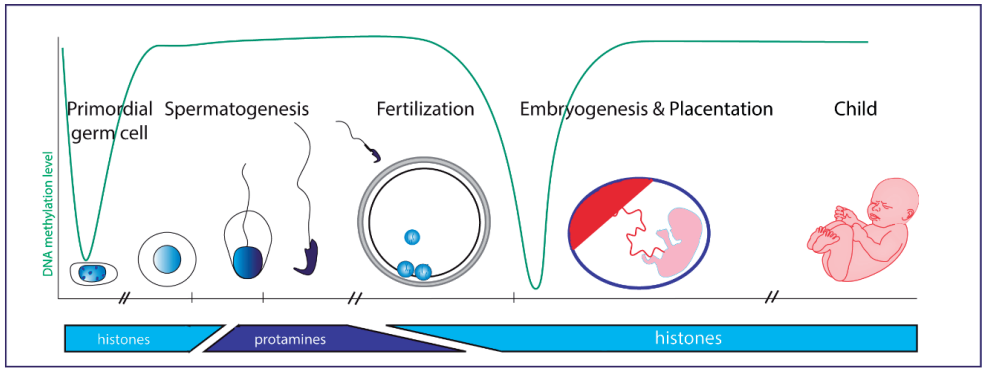
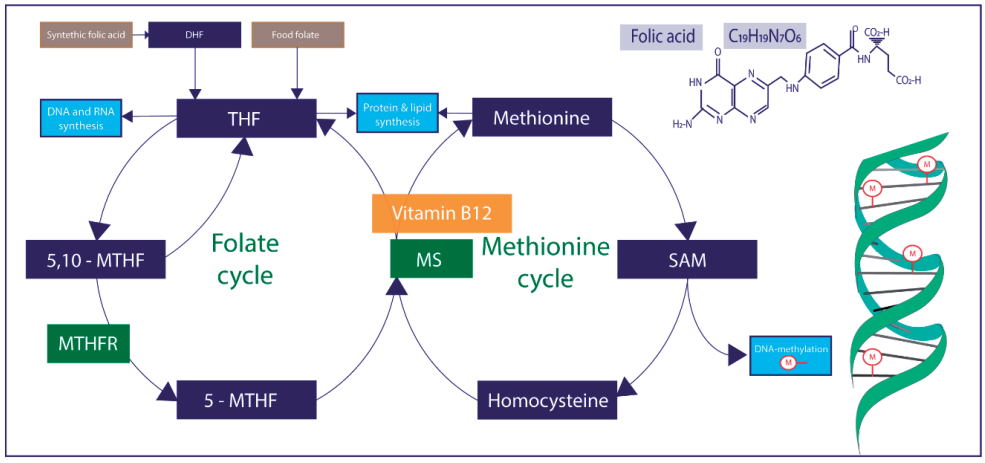


Figure 1b



Legend:
Dark blue box: proteins, green box: enzymes, yellow box: vitamin/cofactor, Light blue box: processes.

periconception and pregnancy outcomes. Although the father-to-be also contributes half of the genetic material to the offspring and the placenta, the periconception paternal folate status has hardly been investigated. This is surprising whereas it is known that paternal folate concentrations can affect sperm quality including its DNA integrity and epigenome (5-7). Therefore, we hypothesize that the paternal folate status could not only affect DNA methylation and sperm quality, but also fertility, and after successful conception, miscarriage risk, embryonic growth, fetal and placentation development and pregnancy outcome.

Spermatogonial stem cells are present from birth but the process of spermatogenesis only takes place in approximately 2-3 months. During spermatogenesis, millions of spermatozoa are produced per day, indicating that the production of proteins and DNA are needed on a large scale. In the human testes the male germ cells develop into spermatids and eventually into spermatozoa (sperm), during spermiogenesis. The differentiation process of spermiogenesis consists of major morphological and chemical alterations and is necessary to ensure that the nuclear DNA will be tightly compacted in the spermatozoal head. The histone to protamine exchange, in which most histones are replaced by protamines, allows a more condensed chromatin structure allowing the tight formation of DNA (**Figure 1a**) (8). Interestingly, retained histones with epigenetic information from the father can be transferred to the conceptus. Since spermatogenesis takes place in a relatively short time period, we hypothesize that paternal nutrition and lifestyle can have a relatively direct impact on reproductive success and pregnancy outcomes with short and long-term health effects for the offspring. Herein, we aim to give an overview of the evidence on associations between the periconception paternal folate status and sperm quality, pregnancy outcomes and epigenetics (**Figure 1a**).

Methods

Search strategy

Searches were performed in the databases of Embase, Medline, PubMed, Web of Science, the Cochrane databases and Google Scholar. The protocol for this systematic review was designed and registered a priori at the PROSPERO registry (PROSPERO 2017: CRD42017080482). The search strategy terms used the following MeSH-terms including but not limited to FA, folate, sperm, fertility, miscarriage, placenta and pregnancy outcome (**supplemental table 2**). These were combined using the Boolean operator 'or'.

Systematic review eligibility criteria

The paternal folate status was defined as folate concentrations measured in blood or seminal plasma. Determinants of folate status included in the search are intake of FA, folate intake and 1-C metabolism.

The main outcomes are divided in preconceptional and postconceptional outcomes. The preconceptional outcomes consisted of sperm parameters (sperm concentration, sperm count), sperm DNA damage and sperm DNA-methylation. Fertility, time-to-pregnancy, miscarriage, fetal growth (small for gestational age, intra-uterine growth restriction, and birthweight), placentation and (preterm) birth were considered as postconceptional outcomes. Databases were searched up and till December 2017. The results of all the

outcome searches were combined with 'or'. The results of the paternal folate status and outcome searches were then combined with 'and'.

Animal and human studies comprising experimental studies, observational cohort, case control studies and randomised controlled trials (RCTs) were eligible for inclusion in the review.

We have excluded letters, conference abstracts, editorials and case reports and restricted the search to English language papers.

Articles describing male participants with or without sperm dysfunction were included, as were papers investigating administration of high or low doses of FA compared to a control dose. Studies measuring folate concentrations in blood or seminal plasma as exposure variable were also included. We excluded maternal only as well as combined paternal and maternal FA interventions.

Study selection, full text review and data extraction

JH and SS reviewed the titles and abstracts independently from each other and selected papers for the full-text review. Next, full text reviewing and data extraction were also independently performed by JH and SS. Data were put into a template, specifically for this review. Differences were resolved by discussion between these authors. Any disagreements concerning the eligibility of particular studies were resolved through discussion with a third reviewer (RST). Data extracted included the country of origin, year of publication, study design, study population (including human or animal), sample size, exposures of interest, outcome data, exclusion criteria, statistical analysis, potential confounders, results, and conclusion.

Quality of study and risk of bias

To assess the quality of the human studies included in our review we used the ErasmusAGE quality score for systematic reviews a tool composed of five items based on previously published scoring systems. Each of the five items can be allocated either zero, one or two points giving a total score between zero and ten, with a score of ten representing a study of the highest quality. The five items include study design (0=cross-sectional study, 1=longitudinal study, 2=intervention study), study size (0=<50, 1=50-150, 2=>150 participants), method of measuring exposure (0=not reported, 1=moderate quality exposure, 2=good quality exposure), method of measuring outcome (0=no appropriate outcome reported, 1=moderate outcome quality, 2=adequate outcome quality) and analysis with adjustments (0=no adjustments, 1=controlled for key confounders, 2=additional adjustments for confounders) (**Supplemental table 1**) (9).

Meta-analysis

We conducted an additional meta-analysis of only human data to investigate the effects of 5mg FA per day supplement use for 3 to 6 months in subfertile males on sperm concentration, sperm motility and normal sperm morphology. For the other outcomes considered in this systematic review, unfortunately, not enough information was available for meta-analysis.

We extracted and pooled the difference-in-difference of three outcomes: sperm concentration, sperm motility and normal sperm morphology. The difference-in-difference is the difference between the effects of the treatment in the intervention and the control group, where the effect of the treatment is measured as the difference between the outcome after and before the intervention. When no information was available of the effect on the outcomes, it was computed based on the published baseline and follow-up measurements. When standard deviations were not given, they were calculated based on standard error and sample size or approximated using the interquartile range (using the assumption of normality). None of the studies published the standard error of the difference between the pre- and post-intervention outcomes. To compute these we based our estimates of the correlation between the two time points on the data of Wong et al. The pooling of effects was done using a random-effects model estimated by restricted maximum likelihood and the heterogeneity was assessed using the I^2-value. Pooled effects with a p-value of 5% were considered significant. We did not apply any multiplicity adjustment.

Results and discussion

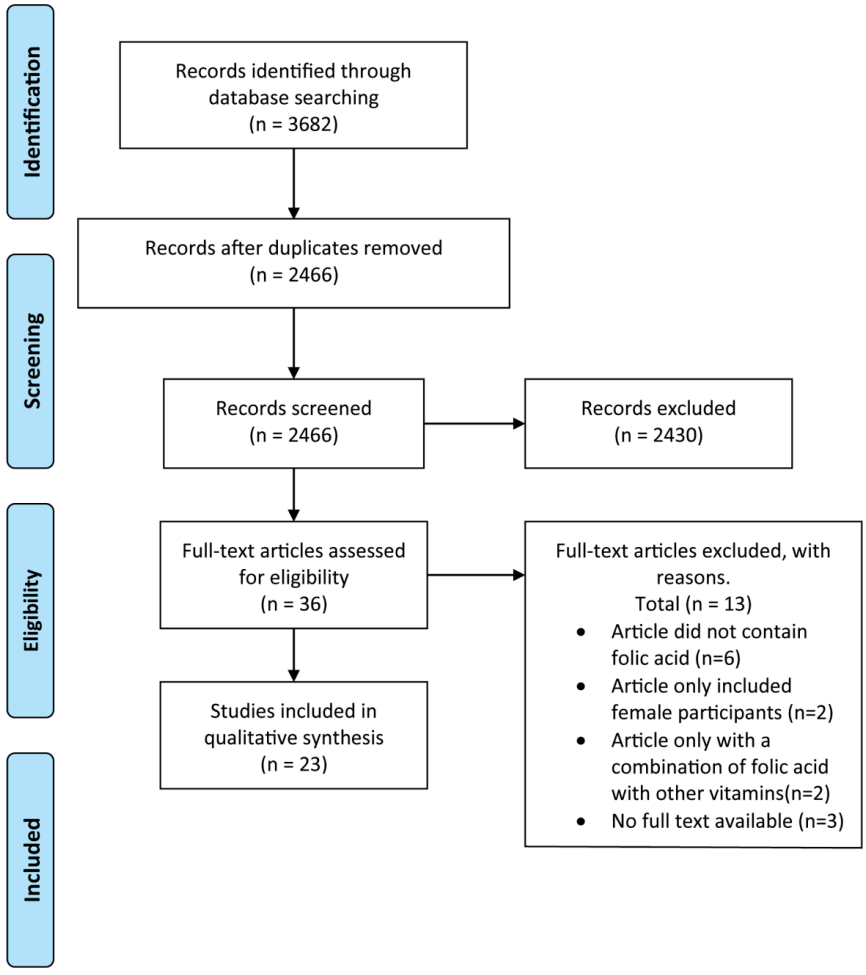
Study selection

The flowchart summarizes the process of literature search and selection of studies **(Figure 2)**. The initial search identified 3,682 records of which 1,216 were duplicates. Of the remaining 2,466 records, a total of 2,430 publications were excluded because they did not fulfil the selection criteria. The full text of 36 papers were read, 13 papers were excluded, resulting in 23 remaining articles for analysis. The general characteristics of all included studies and all specific concentrations of FA supplemented/deficient animal diets are shown in **Table 1**. Of these 23 articles, 6 are animal studies, 1 article combined a human and animal study and 16 represent human studies, including 6 randomised controlled trials (RCTs), 4 case-control studies, 3 cross sectional studies, 2 intervention studies, 1 pre-post analysis and 1 prospective cohort study.

Preconceptional

A total of 4 studies investigated the associations between folate status and sperm parameters in animals (10-13). Furthermore, 13 articles reported on the association

Figure 2. Flowchart of in- and excluded studies



between folate status and sperm parameters in human (5-7, 12, 14-22). A total of 5 studies investigated the association between folate status and sperm epigenetics, of which 2 animal studies (10, 13) and 3 human studies (5, 12, 23). 6 studies investigated the associations between folate status and sperm DNA damage and apoptosis, including 2 animal studies (11, 13) and 4 human studies **(Table 2)** (7, 19, 21, 24).

Sperm parameters

Animal studies

One study in mice comparing a 20-fold FA fortified diet (40mg/kg) with a 7-fold FA deficient diet (0.3mg/kg), starting during pregnancy through maternal exposure and continued postnatally with a control diet (2mg/kg), found that both diets resulted in decreased sperm

Table 1. Main characteristics of 23 included studies

Author	Year	Country	Study population	Study design	Sample size	Exposure(s)	Outcome(s)	Quality score
Aarabi *et al.*	2015	Canada	Healthy normozoospermic men presenting with idiopathic infertility stratified for 3 types of MTHFR gene polymorphisms	Prospective intervention study	30	6 months high dose (5mg) FA	Sperm quality according to WHO, sperm DNA damage and sperm epigenetics	4
Boonyarangkul et al.	2015	Thailand	Men with abnormal sperm analysis	RCT	68	FA supplementation 5mg/ day for 3 months	Sperm quality according to WHO and sperm DNA-damage	9
Boxmeer *et al.*	2007	The Nether-lands	Fertile and subfertile men	Cross-sectional	111	Folate concentrations in serum, RBC and seminal plasma	Sperm quality according to WHO	7
Boxmeer *et al.*	2009	The Nether-lands	Fertile and subfertile men	Cross-sectional	279	Folate concentrations in serum, RBC and seminal plasma	Sperm quality according to WHO and sperm DNA-damage	8
Chan *et al.*	2017	Canada	Men exposed to FA food fortification for years	pre-post study	27	Food fortification	Sperm epigenetics	4
Crha *et al.*	2010	Czech Republic	Men with azoospermia and normozoospermia controls	Cross-sectional	134	Folate concentrations in serum and seminal plasma	Sperm quality according to WHO and testicular volume	5
Da Silva *et al.*	2013	Brazil	Subfertile men 20-55 years	RCT	70	FA supplementation 5mg/ day for 3 months	Sperm quality according to WHO	8
Ebisch *et al.*	2006	The Nether-lands	Fertile and subfertile men	RCT	87	4 groups: FA 5mg, zinc 66mg zinc, zinc and FA and placebo for 26 weeks	Sperm quality according to WHO	4
Ebisch *et al.*	2005	The Nether-lands	Fertile and subfertile men	RCT	164	4 groups: FA 5mg, zinc 66mg zinc, zinc and FA and placebo for 26 weeks	Sperm quality according to WHO and seminal annexin A5 (apoptosis marker)	9
Kim *et al.*	2011	Korea	Male rats on either a folate supplemented or folate deficient diet	Animal study	14	Folate deficient (0mg) or folate rich (8mg/kg diet) diet for 4 weeks	Fetal growth, fetal liver and placenta folate content. Folate receptor alfa expression	N/A
Kim *et al.*	2013	Korea	Male rats on either a folate supplemented or folate deficient diet	Animal study	12	Folate deficient (0mg) or folate rich (8mg/kg diet) diet for 4 weeks	Fetal growth, total folate content in fetal liver and brain and IGF2 expression in fetal brain	N/A
Lambrot *et al.*	2013	Canada	Male mice (C57BL/6) on either a folate supplemented or folate deficient diet already in utero exposed to the same feeding regime	Animal study	128	Folate deficient (0,3mg/kg) or folate rich (2mg/kg) diet	Sperm morphology, sperm epigenome, pregnancy rate, miscarriage, fetal growth and congenital malformations	N/A
Landau et al.	1978	Israel	Men with normo- and oligospermia	Prospective intervention study	40	FA 10 mg for 30 days	Sperm concentration and motility, DNA content of the spermatozoa	3

Continue

Continued

Author	Year	Country	Study population	Study design	Sample size	Exposure(s)	Outcome(s)	Quality score
Ly *et al.*	2017	Canada	Male mice (BALB/c) already in utero exposed to 4 feeding regimens and during life fed the same regimen	Animal study	60	4 feeding regimens (control; 2mg/kg FA, 0,3mg/kg) FA, 20mg/kg FA and 40mg/kg FA) from mothers and during life fed the same regimen.	Sperm count and DNA methylation, Fetal placenta and brain DNA methylation and miscarriage	N/A
Mejos *et al.*	2013	Korea	Male and female rats who got folate supplemented or deficient diet.	Animal study	40	Folate deficient (0mg) or folate supplement (8mg/kg) diet for 4 weeks	Postnatal hepatic folate content and DNA methylation and hepatic FR alfa, IGF-2 and IGF-1R expression	N/A
Murphy *et al.*	2011	Sweden	Infertile men who are 20-45 year old, having regular sexual intercourse>1 year without a pregnancy. Fertile men: who are 20-45 year old and conceived at least 1 pregnancy who now stopped birth control.	Case-control study	337	Folate concentrations in serum and seminal plasma	Sperm quality according to WHO and SNP genotyping in genes related to folate metabolism.	8
Pauwels *et al.*	2017	Belgium	Caucasian men	Prospective cohort study	51	Paternal methyl group intake	Paternal and offspring global DNA methylation and offspring IGF2 methylation and birthweight	8
Raigani et al.	2014	Iran	Subfertile oligoasthenoteratozoospermic men	RCT	83	4 groups: FA 5mg, zinc 220mg zinc, zinc and FA and placebo for 16 weeks	Sperm quality according to WHO and sperm DNA damage	9
Ratan *et al.*	2008	India	Neonates with neural tube defects as cases. Controls: neonates with other congenital malformations and neonates with no abnormalities	Case-control study	90	Serum and RBC folate concentrations	congenital malformations	3
Swayne *et al.*	2012	Canada	Male mice (BALB/c) were given control, folate deficient or folate supplemented diet already started in utero and switched to control diet during weaning.	Animal study	96	Control diet with FA 2mg/kg. FA deficient diet contained 0 mg/kg FA and FA supplemented contained 6 mg/kg FA	Cauda epididymal sperm counts and sperm DNA damage	N/A
Wallock *et al.*	2001	USA	Healthy male smokers and non-smoker with a low intake of vegetables and fruit, aged 20-50 year.	Case-control study	48	Serum and seminal folate concentrations	Sperm count and density	6
Wong *et al.*	2002	The Netherlands	Fertile men: no history of fertility problems and a current pregnant partner. Subfertile men: failure to conceive after 1 year of unprotected intercourse and a sperm count between 5 and 20 million per mL.	RCT	194	4 groups: FA 5mg, zinc 66mg zinc, zinc and FA and placebo for 26 weeks	Sperm quality according to WHO	9
Yuan *et al.*	2017	China	Human: Male subfertile patients aged 18-55 years with azoospermia and normospermia. Animals: Female mice (C57BL/6) were given a folate deficient diet or control diet already started in utero and male offspring were fed the same regimen	Case-control study & Animal study	269	Human: seminal folate concentrations Animal: FA deficient (0,3mg/kg) diet and control diet	Human: Sperm quality according to WHO, DNA methylation and protein expression. Animal: Sperm counts, testis histology and proteins.	8

Table 2. Description and summary of data from 19 studies that investigated associations between folate and sperm quality and sperm epigenetics.

Author	Year	Study type	Synthetic/ Natural folate	Sperm parameters	Sperm epigenetics	Sperm DNA damage	Sperm apoptosis
Lambrot et al.	2013	Animal study	Synthetic	=	+/-	-	=
Swayne et al.	2012	Animal study	Synthetic	=		-	
Ly et al.	2017	Animal study	Synthetic	+/-	+/-		
Yuan et al.	2017	Animal study & Case-control study	Synthetic Natural	+ +	=		
Murphy et al.	2011	Case-control study	Natural	+			
Wallock et al.	2001	Case-control study	Natural	+			
Boxmeer et al.	2007	Cross sectional study	Natural	=			
Boxmeer et al.	2009	Cross sectional study	Natural	-		-	
Crha et al.	2010	Cross sectional study	Natural	=			
Ebisch et al.	2006	Randomized controlled trial. *Data used is cross sectional*	Synthetic	=			
Ebisch et al.	2005	Randomized controlled trial.	Synthetic				=
Boonyarangkul et al.	2015	Randomized controlled trial	Synthetic	+		-	
Da Silva et al.	2013	Randomized controlled trial	Synthetic	=			
Raigani et al.	2014	Randomized controlled trial	Synthetic	=		=	
Wong et al.	2002	Randomized controlled trial	Synthetic	=			
Chan et al.	2017	Retrospective intervention study	Synthetic		=		
Landau et al.	1978	Prospective intervention study	Synthetic	=			
Aarabi et al.	2015	Prospective intervention study	Synthetic	=	+/-	=	

Legend: Abbreviations: +; positive association, -; negative association, =; no association.

counts (10). One study showed that a folate deficient diet (0.3mg/kg) resulted in decreased sperm counts compared to a control (2mg/kg) diet ($9.3 \pm 1.2x10^6$ vs $13.0 \pm 1.1x10^6$) (12). Furthermore *Swayne et al.* found no significant differences regarding sperm count when comparing a 6mg/kg FA supplemented diet, starting during early developmental in utero until just after weaning, compared to a 2mg/kg control diet ($14.0 \pm 1.5x10^6$ vs $13.0 \pm 1.1x10^6$) (11).

Another study showed no significant difference in sperm count when mice received a folate deficient diet (0.3mg/kg already started in utero through maternal exposure) (13).

In conclusion, animal studies show that both a FA supplemented and depleted diet can result in decreased sperm counts.

Human studies

A total of 5 studies in human were designed as randomised controlled trials investigating the effect of FA supplement use on sperm parameters (6, 15, 16, 19, 21). Of these 5 RCT's, we could only use cross-sectional data from one study for this systematic review (16). 3 RCT's

reported no significant differences regarding sperm volume, motility and morphology in the FA supplement user group (all 5mg FA per day) as compared to the control group (6, 15, 19). On the other hand, 1 of the RCTs showed a significant increase in sperm motility from 11.4% to 20.4% after 3 months of 5mg/day FA supplement use (21). Only *Raigani et al.* showed that FA supplement use also caused a significant increase in serum FA from 4ng/mL at baseline to 32.4ng/mL after the intervention. Two non-randomised intervention studies did not notice any effect on the same sperm parameters after a 30-day trial of 10mg FA supplementation and after 6 months of 5mg FA supplementation (5, 17).

The remaining 7 human studies were either case-control studies or cross-sectional study designs (7, 12, 14, 16, 18, 20, 22). Four of these studies showed significant associations between FA supplement use and sperm parameters (7, 12, 18, 20). *Wallock et al.* showed that in healthy males folate concentrations measured in seminal plasma (17.5nmol/L) correlated significantly with blood plasma folate (10.3nmol/L; r=0.76*, P<0.001)* and that seminal plasma folate significantly correlated with sperm density (r=0.37*, P<0.05)* and sperm total count (r=0.31, *P<0.05).(20)* In line with this paper, *Boxmeer et al*. showed positive associations between seminal plasma folate (25.3nmol/L) and blood plasma folate (15.7nmol/L) in both fertile and subfertile men (r=0.47*, P<0.001)*. Significant associations were found between blood folate concentrations and sperm parameters, although seminal plasma folate concentration was inversely correlated with ejaculate volume (r=-0.20*, P<0.01*) (7). One case-control study showed that serum and red blood cell (RBC) folate concentrations were significantly lower in subfertile compared with fertile males (serum: 12.9±5.9nmol/L and 14.7±6.0nmol/L (*P=0.006* respectively and RBC: serum: 649.1±203.6nmol/L and 714.5±223.4nmol/L (*P=0.044)* respectively (18). In the logistic regression model, serum folate was a significant predictor of subfertility, especially among non-users of vitamins (Odds ratio 0.36 (95% confidence interval 0.16 to 0.78). In addition, men with azoospermia showed significantly lower seminal plasma folate concentrations than men with normozoospermia (respectively 24.0nmol/L (interquartile range (IQR) 19.84-30.69) versus 26.2nmol/L (IQR 21.7-34.8))(12) and seminal plasma folate concentrations were significantly correlated with sperm density (r=0.19*, P<0.01)*, but not with other sperm parameters.

The other 3 out of these 7 studies did not find any associations between paternal folate status and sperm parameters (14, 16, 22). *Chra et al*. showed no significant differences regarding both blood and seminal plasma folate on sperm parameters, although in men with obstructive azoospermia, higher seminal plasma folate concentrations were found compared to non-obstructive azoospermia (31.5nmol/L vs 20.7nmol/L respectively) (22). When comparing blood and seminal plasma folate concentrations between fertile and subfertile males, two studies found no significant associations with sperm parameters (7, 16).

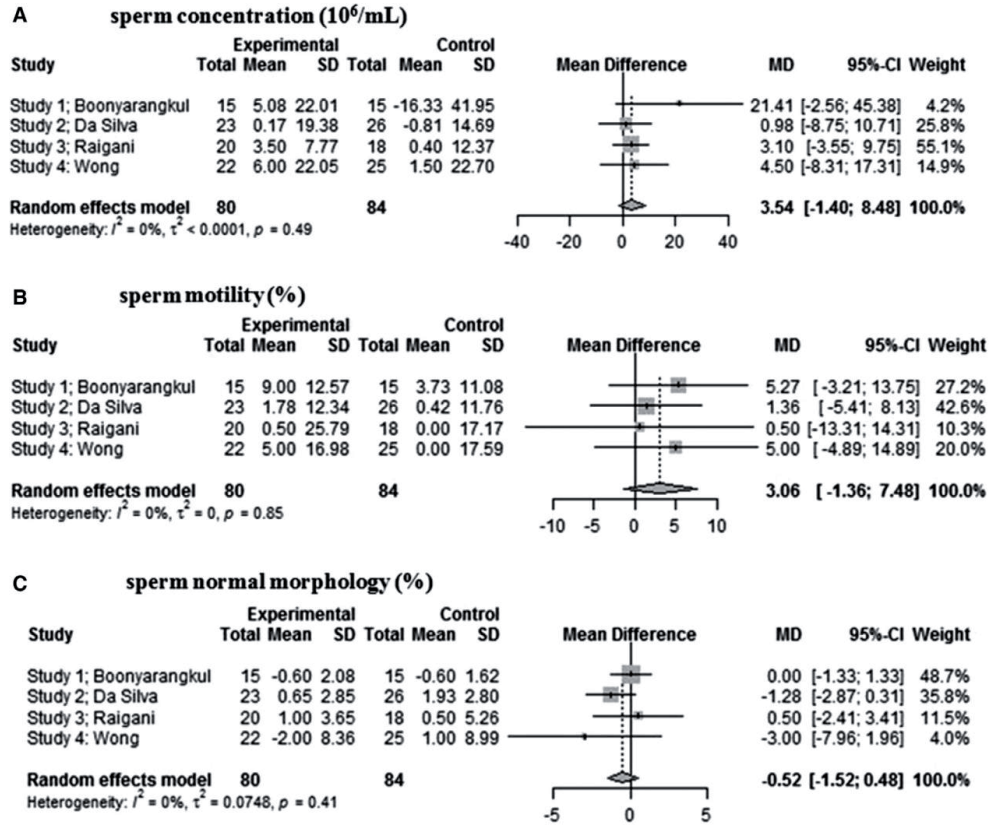
The quality of abovementioned studies according to the ErasmusAGE quality score ranged between 3 and 9, with the majority (54%) having a score above 7. Although, the RCTs were adequately designed according to the CONSORT statements (25), the number of included participants was low. Significantly positive associations were reported in the large case-control studies of Yuan et al. (n=269) and Murphy et al. (n=337), whereas the smaller studies failed to show significance, which might be due to underpowerment. The study of *Aarabi et al*. was initiated to investigate effects on methylation status of the sperm, but without correcting for confounders. Of the remaining 7 human studies, only two adjusted their statistical model for confounders to allow adequate interpretation of the results; the studies of *Boxmeer et al*. and *Murphy et al*. corrected for at least paternal age and smoking. It is important to take confounders into consideration since previous studies have shown that a diversity of conditions and factors, such as smoking, alcohol use, age and BMI also influence sperm parameters, which is in line with the induction of excessive oxidative stress (26-29). Although, less research is performed on paternal influences on pregnancy outcomes, we assume that the same confounding factors should be considered.

Only 7 out of 13 studies reported blood folate concentrations in the study population, ranging from 9 nmol/L to 73 nmol/L, while two reported concentrations before and after intervention. Unfortunately, the effects of normal values of folate concentrations regarding sperm quality are not mentioned. One might hypothesize that only men with low folate concentrations benefit from FA supplementation. This is supported by *Murphy et al*., who showed that an increase of folate from 13 nmol/L to 25 nmol/L was associated with a significant increase in sperm parameters. However, the study of *Raigani et al.* found an increase from 9 nmol/L to 73 nmol/L without a significant effect on sperm parameters.

Meta-analysis of folic acid supplement use and sperm parameters.

Four studies were eligible for a meta-analysis to assess the combined effect of FA supplement use on sperm parameters in subfertile males (6, 15, 19, 21). Data of sperm concentration, motility and normal morphology were respectively analyzed in a random-effects model to estimate the effect of daily 5mg FA treatment on each sperm parameter (**Figure 3)**. The results show that the sperm concentration was higher in patients after FA supplement use compared to control (3.54 95%CI (-1.40 to 8.48)), however these results were not significantly different (*p=0.16)*. Sperm motility also did not significantly differ after FA supplement use compared to controls (3.06 95%CI (-1.36 to 7.48)) (*p=0.17)*. A non-significant decrease after FA supplement use (-0.52 95%CI (-1.52 to 0.48)) was shown regarding sperm normal morphology (*p=0.31)*. There was no evidence of significant heterogeneity in the study populations regarding concentration, motility and normal morphology (I^2 all 0%).

Figure 3. Forest plot of the effect of 5 mg folic acid supplement use in subfertile men: a. sperm concentration, b. sperm motility and c. sperm morphology.



In conclusion, some human studies show associations between paternal folate status and sperm parameters. A meta-analysis of 4 RCTs showed no significant differences regarding sperm parameters after 5mg per day FA supplementation.

Discussion

Decreased folate concentrations alter the 1-C metabolism resulting in a reduced availability of 1-C groups and building blocks for DNA synthesis and repair, which are essential for successful spermatogenesis and genomic stability. Supporting this hypothesis, all non-randomized controlled trials studies showed significant associations between folate concentrations and sperm parameters (7, 12, 18, 20). The suggestion that adequate folate concentrations could serve as protection against DNA damage is supported by a RCT showing a decrease in sperm DNA damage after 3 months of 5mg/day FA supplement use (21). To compensate for a possible folate deficiency, FA supplement use will provide essential building blocks that could improve sperm quality parameters. Although, three out of the four RCTs did not find any significant improvements in sperm parameters after FA

supplement use (6, 15, 19), Boonyarankul et al., showed a significant increase in percentage of sperm motility (11.40% to 20.40%) after 3 months of 5mg per day FA supplement use. Three out of the four RCTs did not report whether folate concentrations in either blood or RBCs increased after the FA intervention, while Raigani et al. showed a significant increase in serum folate concentrations. Taking measurement of folate concentrations along, either in blood or RBCs, are especially interesting since there is heterogeneity in the data between before mentioned studies, which might be explained by either subjects not daily taking FA, differences in folate absorption or in the conversion of dihydrofolate to tetrahydrofolate by intestinal dihydrofolate reductase.

In the additional meta-analysis, we did notice a trend indicating that 3-6 months of daily FA treatment of 5mg per day improves sperm volume and the percentage of sperm motility. The non-significance of the meta-analysis can be explained by the relatively small numbers of patients in each trial (N=160 in total), since in studies in women around 6500 participants were needed to show significant effect on neural tube defects for example (1).

The relationship between low folate concentrations and sperm quality seems explainable, however, the possible detrimental effect of too high folate concentrations is less clear. It is hypothesized that excessive FA supplement use gives an increase in dihydrofolate, with a negative feedback signal on the MTHFR enzyme, thereby downregulating the biosynthesis of 1-C groups. Without knowing the beneficial and detrimental effects of exposure to different concentrations of FA, we should be cautious with the global administration of high doses of FA due to possible teratogenicity (30).

Sperm DNA damage and methylation

Animal

Sperm DNA damage was investigated in two independent studies in mice, showing that a folate deficient diet results in increased DNA damage (11, 13). *Lambrot et al.* showed an increase in the expression of a histone variant (γH2AX) involved in repair of DNA double strand breaks, while the total number of DNA double strand breaks in spermatocytes remained comparable between the groups indicating that DNA damage was correctly repaired (13).

Another way to measure DNA damage is via the DNA fragmentation index (DFI), where a higher DFI indicates more DNA damage. *Swayne et al*. showed that mice weaned to a folate deficient diet (see **Table 1** for exact folic acid concentrations) had an increased percentage DFI, compared to a control diet (5.0%±0.9 vs 2.6%±0.1, *P=0.04*) (11). Furthermore, two other animal studies showed that a low intake of dietary folate resulted in increased as well as decreased sperm DNA-methylation (10, 13). *Lambrot et al*. showed that DNA methylation concentrations in general were both increased and decreased for various genes in the

folate deficient group as compared to the control, while histone methylation was primarily downregulated. No differences regarding sperm apoptosis or in methylation status of imprinted genes were reported (13). *Ly et al*. showed that both a high FA supplemented and depleted diet resulted in increased variance in methylation across imprinted genes, required for normal fetal development (10).

In conclusion, in animal models a FA depleted diet results in more sperm DNA damage and both increased and decreased sperm DNA methylation.

Human

Sperm DNA damage was reported by 4 studies (5, 7, 19, 21). Two RCTs reported a decrease in sperm DNA damage after FA treatment (19, 21). *Boonyarangkul et al*. showed a significant decrease in DNA tail length based on a Comet assay, indicating less DNA damage, from 14.59µm to 4.04µm ($p<0.05$) after 3 months of 5mg per day FA treatment (21). *Raigani et al.* showed a non-significant decrease in DFI after a 16-week 5mg per day FA intervention compared to placebo (from 31.7±14.8% to 24.3±12% vs from 34.5±19.7% to 29.5±10% respectively) (19). Same results were shown in the *Aarabi* study. One cross sectional study showed that only in the fertile male group, the seminal plasma folate concentrations were negatively associated with DFI (r=-0.36, $P<0.05$) (7).

Annexin A5 (AnxA5) is commonly used to detect apoptotic cells by its ability to bind to phosphatidylserine, a marker of apoptosis, which presents on the exterior part of the plasma membrane. Hence, a high concentration of AnxA5 indicates high concentrations of apoptosis. Seminal AnxA5 was determined in one RCT to assess the effect of FA supplement use on seminal apoptosis. After a 26 week period of 5mg per day FA intervention there was a slight decrease in AnxA5 in both fertile (from 5.6µg/mL to 5.4µg/mL) and subfertile (from 5.4µg/mL to 5.2µg/mL) males, however it failed to reach significance (24).

A total of 3 studies investigated the effect of folate status on sperm DNA methylation (5, 12, 23). The recent study of *Chan et al*. showed that multiple years of FA food fortification in Canada had no significant influence on sperm overall DNA methylation (23). However, 6 months of 5mg per day FA supplement use caused genome wide hypomethylation and hypermethylation covering intergenic regions, introns and exons of the sperm DNA (5). This effect is aggravated in individuals who are homozygous for the *MTHFR 677C>T* polymorphism. *Aarabi et al.* found no effect of FA supplement use on the differentially methylated regions of several imprinted genes (5). A case-control study showed no differences in methylation pattern of the promotor regions of some spermatogenesis key-genes (*Esr1, Cav1* and *Elavl1*) in males with low versus high seminal plasma and blood folate concentrations (12).

The articles reporting on DNA damage were of good quality (75% received a quality score of 8 or higher), whereas all studies investigating methylation of sperm were of low quality (66% received a quality score of 4 or lower). Concerning potential confounders in these studies, two studies are designed as RCTs in which correction for confounders is not needed. Boxmeer et al. correctly adjusted for several confounding factors such as age, BMI, smoking and alcohol use, while the Aarabi et al. study did not apply any correction for confounders.

In conclusion, in humans multiple studies show that FA supplementation results in a reduction in sperm DNA damage with some studies showing that folate status is associated with the sperm epigenome.

Discussion

Chronic exposure to high dose synthetic FA and low folate concentrations seems to induce excessive oxidative stress and as such cause increased cellular apoptosis and seminal DNA damage. Techniques used to measure sperm DNA damage included the sperm chromatin structure assay (SCSA; 2 studies), the comet assay (1 study) and acridine orange staining (AO-test; 1 study). While the SCSA and comet assay are both sensitive and reliable, the AO-test appears to have a relative lack of reproducibility (31). A recent guideline regarding DNA damage does not recommend using a specific technique, but mentions that SCSA is one of the most used techniques. The low number of studies (4 in total) and the usage of different tests, do not allow comparison between studies.

Folate is as substrate involved in the synthesis of lipids, proteins, DNA and RNA, the scavenging of reactive oxidative radicals, DNA repair and epigenetic. These mechanisms are involved in cell multiplication and cell differentiation, apoptosis, signaling and programming and as such in spermatogenesis and embryogenesis. Elevated levels of reactive oxygen species (ROS), caused by various chronic diseases, obesity, genetic variations, medication use, ageing and an unhealthy diet and lifestyle, will lead to oxidative stress, which is an important cause of DNA damage. A crucial function of the 1-C cycle is scavenging of these reactive oxygen species (ROS) by the anti-oxidant glutathione, which is synthesized from folate together with homocysteine. Importantly, an unhealthy diet is associated with a decreased intake of folate. Only when concentrations of methionine and especially folate are sufficient, glutathione is formed. Low intake of FA and folate are associated with an increase in oxidative stress thereby altering DNA-integrity and subsequent molecular processes involved in spermatogenesis and embryogenesis. The studies in this review show that FA supplement use can result in decreased sperm DNA damage.

Postconceptional

7 articles reported on associations between paternal folate status and the post-conceptional outcomes, such as fertility, embryonic growth, miscarriage, fetal development, congenital

malformations, placentation and pregnancy outcomes, of which 5 animal studies (10, 13, 32-34) and 2 human studies **(Table 3)** (35, 36).

Table 3. Description and summary of data from 9 studies that investigated associations between folate and postconceptional outcomes.

Author	Year	Study type	Synthetic/ Natural folate	Fertility	Miscarriage	Birth-weight	Fetal liver	Fetal brain	Placenta	Congenital malformations
Kim et al.	2011	Animal study	Synthetic			=	+		+	
Kim et al.	2013	Animal study	Synthetic			+	+	+		
Lambrot et al.	2013	Animal study	Synthetic	+	-	=			=	-
Ly et al.	2017	Animal study	Synthetic		+	=		+	=	=
Mejos et al.	2013	Animal study	Synthetic			=	+			
Pauwels et al.	2017	Prospective cohort study	Natural			=				=
Ratan et al.	2008	Case-control study	Natural							-

Abbreviations: +; positive association, -; negative association, =; no association.

Fertility

Animal

Only one animal study showed that a folate deficient diet in mice resulted in decreased pregnancy rates compared to mice fed control diet (52.4% and 85.0% respectively) (13).

Human

There are no human studies reporting on fertility in relation to paternal folate status.

Discussion

The overall results of the selected articles in this review show that paternal folate status is often positively associated with sperm parameters. The sperm parameters concentration and percentage of mobile sperm are associated with fertility and ongoing pregnancy rates (37, 38). Therefore, the reasoning is that in future fathers optimization of folate status has the potential to beneficially influence male fertility and pregnancy chances of a couple. Unfortunately, until now no human studies have shown any effect of the paternal folate status on pregnancy-chance. However, strong adherence of a couple to a diet very rich in natural folate, like the Mediterranean diet, increases the chance of an ongoing pregnancy after an IVF/ICSI treatment (39, 40).

Embryonic growth and development

Animal

Two animal studies in mice investigated the association between the paternal FA supplement use and embryonic growth and miscarriages (10, 13). Lambrot et al. showed that the offspring of male mice, which had received a folate deficient diet from early

embryonic development onward (0.3mg FA/kg), did not differ regarding embryonic weight and crown rump length (CRL) compared to male mice on a control diet (2mg/kg). However, they found that a paternal folate deficient diet resulted in a twofold increase of post-implantation embryonic loss in mice (13). Another animal study showed that male mice fed a highly FA fortified diet (40mg/kg) have an increased risk of post-implantation embryonic loss and their offspring show growth restriction compared to control diet (2mg/kg) (10). In conclusion, in animals both very high and very low FA intake is associated with an increased miscarriage rate.

Human

There are no human studies reporting on the association between paternal folate status and embryonic growth and development or miscarriage.

Discussion

Shortly after conception a global loss of methylation at the level of DNA and histones takes place (**Figure 1a**) (41). However both paternally and maternally imprinted genes, such as IGF-2, are unaffected by this demethylation wave (42). Since imprinting of these genes occurs during the process of male and female gametogenesis, studying the effects of periconception lifestyle factors on embryonic health and health later in life, makes these genes of special interest. Imprinted genes have a parent-of-origin effect by preferential expression of either maternal or paternal inherited allele and emphasize the influence of the father during the periconception period. This epigenetic mechanism can possibly explain our results, where sperm DNA methylation in genes for normal growth and development of embryonic growth and development are altered.

Fetal liver & brain

Animal

The insulin-like-growth factor 2 (IGF-2) gene is paternally expressed and encodes for a protein that plays a major role in regulating embryonic growth and development (43). Three animal studies investigated the effect of a paternal folate deficiency on fetal liver outcomes (32-34). All studies showed that the fetal liver folate content was decreased after a paternal folate deficient diet compared to control diet. Mejos et al. showed that a folate deficient diet significantly decreased global hepatic DNA- methylation concentrations with 37,9%, although no significant differences in hepatic IGF-2 expression when compared to a folate sufficient diet were detected (34).

Two other animal studies investigated the association between paternal folate status and brain development (10, 33). One study showed that the total folate content of the fetal brain was comparable in rats on a folate deficient and control diet, whereas the IGF-2 protein expression in the fetal whole brain was decreased in former group (33). Interestingly, they

also found a significant decrease in whole brain DNA-methylation, as measured by the quantity of 5-methylcytosine (5-MC). The percentage of 5-MC decreases from 4.5% to 2.6% when comparing a folate sufficient diet with a folate deficient diet. Another animal study that investigated global brain methylation failed to see an effect of a paternal high or low folate diet (10). They did, however, find a significant increase in variance of DNA methylation on a locus of the Paternally Expressed Gene 1 (PEG1) in the group supplemented with high FA.

In conclusion, in animals all studies show an effect of paternal folate diets on fetal liver contents while some indicate effects on diverse fetal brain measurements.

Human
There are no human studies reporting on the fetal development of brain and liver.

Congenital anomalies
Animal
Two animal studies investigated the association between paternal folate status and congenital anomalies, of which 1 found an association between a FA deficient diet compared to control mice (13). Lambrot et al. showed that in fathers on a folate deficient diet the percentage of litters with congenital malformations is significantly increased when compared to a control diet (27% and 3%). The abnormalities consisted of craniofacial abnormalities, limb defects, muscle and skeletal malformations (13). Another study found no significant differences between male mice on a control diet compared to those on high FA or low FA diet (10).

In conclusion, in animals some studies show a negative association between FA intake and congenital malformations.

Human
One human study investigated the association between paternal folate status and congenital malformations (36). They found that the fathers of children born with neural tube defects had significantly lower folate concentrations compared to fathers of children born with other or without congenital malformations. Although, they reported an odds ratio for neural tube defects of 5.2 (95%CI: 1.3-20.8) of offspring of fathers with low folate concentrations, the effect diminished when adjusting for potential confounders, which were unfortunately not mentioned.

In conclusion, in humans the only study showed a negative association between folate intake and congenital malformations.

Discussion

Low intake of folate and low folate concentration are associated with increased sperm DNA damage and alterations in sperm epigenetics, which in case of successful fertilization could interfere with embryonic development at later stages. The human study underlines that paternal folate status can effect embryonic growth and development, most likely by altering the sperm epigenome and thereby inducing adverse pregnancy outcomes (36). However, results need to be interpreted with caution since residual confounding cannot be excluded due to the lack of mentioning of adjusted confounders (ErasmusAGE quality score of 3).

The number of women needed to use FA supplements periconceptionally to prevent one child with a neural tube defect is 847 (NNT=847) (44). For men, this number is most likely much higher, since FA use by women directly affect the intrauterine environment and could potentially compensate for or correct occurring paternal effects of folate deficiencies. Males most likely pass on folate effects via sperm DNA methylation changes and concentration in seminal fluid. Seminal fluid contains folate and it is known that previous contact of the seminal fluid with the endometrium will lead to better embryo implantation and developments, possibly via an adaptive immune response (45). More diverse and intensive human research is necessary before we can translate the results of the mouse models to humans.

Placentation

Animal

3 animal studies describe the effect of paternal folate on general aspects of placentation, such as weight, size and folate content (10, 13, 32). Two studies did not find any significant differences when comparing placenta weight and size between a paternal FA deficient and control diet (see **Table 1** for exact folic acid concentrations) (10, 13). Another study found a lower placental weight and a lower total placental folate content in the folate deficient diet group compared to control (32). Surprisingly, Lambrot et al. reported two fused placenta's, which is considered to be abnormal, out of the group op 35 pregnancies.

Regarding the methylation status of the placenta, one study found no significant differences in global placental methylation concentrations when comparing both low and high FA paternal content diets compared to control (10). However, in the group with very high FA fortified diets (folate concentration 20 times higher as compared to the control) compared to control diet, inter-individual alterations in methylation across the paternally expressed genes Small Nuclear Ribonucleoprotein Polypeptide N and Paternally Expressed Gene 3 were found.

Placental transporter proteins are necessary and essential for the transport of micronutrients over the placental barrier. Of these proteins, the placental folate receptor alpha enzyme is

crucial for the transport of folate over the placenta. Interestingly, Kim et al. showed that a paternal folate deficient diet resulted in a significant upregulation of this enzyme expression compared to wildtype rats (2.3 times higher expression)

In conclusion, in animals some studies show a negative association between paternal FA intake and placenta weight and development and an association with alterations in placenta epigenetics.

Human

There are no human studies reporting on associations between paternal folate status and placental development.

Discussion

Paternally imprinted genes, which are predominantly expressed in the placenta, are in general excluded from the postconceptional de- and remethylation wave (**figure 1A**). External influences such as nutrition, lifestyle and folate status throughout the preconception stage can heavily influence the definitive programming of (non) imprinted genes, with potential negative effects on embryonic but also placental development postconceptionally**.** The function of the placenta is essential for embryonic and fetal growth and development all throughout pregnancy and sub-optimal human placental development is associated with adverse maternal, fetal and perinatal outcomes, such as preeclampsia, intrauterine growth restriction and (iatrogenic) preterm birth (46). Micronutrients like iron, vitamin D, vitamin A, folate and vitamin B12 are necessary for normal placental development and deficiencies of these micronutrients in women are associated with impaired placental development (47). In women, FA supplement use is also associated with placental development, since placental weight at birth between women using FA supplements versus women not using FA supplements is different (643 grams vs 626 grams, respectively) (48). The causal effect of these paternal factors remains to be elucidated, but epigenetic programming of paternal origin is a plausible mechanism.

Pregnancy outcome

Animal

Five animal studies investigated the association between paternal folate status and birthweight (10, 13, 32-34). Four studies did not find an association between a folate deficient diet and birthweight (10, 13, 32, 34), whereas a very high FA fortified diet also did not alter birthweight compared to controls (10). One animal study, however, showed that a folate deficient diet compared to control diet resulted in lower birthweight (2.1gram – 2.3gram ($p<0.001$)) and smaller crown rump length (CRL) (3.3cm – 3.4cm ($p<0.05$)) (33). Interestingly, one study found an increase in postnatal deaths when comparing both very high and low FA fortified diets compared to control mice (10).

In conclusion, in animals a minority of studies showed an association between paternal folate diet and pregnancy outcomes.

Human
There is 1 human study (ErasmusAGE quality score of 8) reporting on birthweight, which found no significant association between paternal folate intake, as measured by food questionnaires, and birthweight of the offspring (35).

Strengths and limitations

The present work is the first to systematically review the currently available evidence on the impact of the paternal folate status on male fertility factors from sperm quality to pregnancy outcomes. Due to the lack of human studies on paternal effects of FA supplement use, we included animal studies to gain more insight into the (patho)physiologic mechanisms and the (epi)genetic effects of FA supplement use, resulting in a translational systematic review. The review also includes an additionally performed meta-analysis on the associations between paternal FA supplement use ranging from 3 to 6 months and sperm parameters concentration, motility and normal morphology. Despite our extensive literature search, the amount of evidence and quality of the studies was relatively low. Regarding the included animal studies; in a number of studies the FA intervention already started *in utero*, during the key time of parental erasure and reprogramming of the germ cell epigenome and continuing postnatally for varying amounts of time. The extended exposure might have lifelong effects on the male germ cell, perturbing prenatal and postnatal germ cell development and epigenetics. Nevertheless, this review provides some evidence that the periconception paternal folate status or diet, can influence sperm parameters, fertility, embryonic growth and pregnancy outcomes possibly explained via an impaired embryonic and/or placental DNA synthesis and repair, epigenetic programming or cell multiplication.

Unfortunately, optimal ranges of folate concentrations in males are lacking in both human and animals, making comparisons between studies difficult. Several study results indicate that either too low or too high concentrations are not beneficial. Before any general recommendations for paternal FA supplement use can be issued, further investigation is necessary to better understand the contribution of the paternal folate status on fertility and pregnancy outcomes, including placentation.

Main Conclusion

This translational systematic review shows that the paternal folate status in humans and animals might be associated with sperm quality and subsequent pregnancy outcomes, like fetal development, placentation and congenital malformations. As in women, not only low but also high folate concentrations are associated with negative outcomes in men, such as poorer sperm quality and an increased risk of congenital malformations. In general, low paternal folate status is associated with poorer outcomes, while deficiencies can easily be supplemented with FA tablets and fortified diets. However, in recent years the concerns of high folate concentrations are increasing,(30) especially with the worldwide increase of the use of multivitamin supplements and FA fortified foods. Therefore, we have to be increasingly aware of also the risk of harmful effects of too high (supplementary) folate concentrations in developed countries. Furthermore, the results of this systematic review make it clear that human data on paternal folate status and fertility and pregnancy outcome is very scarce. More research is necessary into the periconception roles of paternal micronutrients. We need to understand the effects of paternal folate status on sperm epigenome and periconception outcomes, so we can optimally counsel future parents during the periconception period.

Supplemental Table 1. ErasmusAGE quality score form for systematic reviews.

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 (Carter et al, 2010 and the Quality Assessment Tool for Quantitative Studies*)*.
The quality score is composed of 5 items, and each item is allocated 0, 1 or 2 points. This allows a total score between 0 and 10 points, 10 representing the highest quality.

The version presented below is a general version and needs to be adapted for each review separately, e.g. concerning what study size is large or small 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 before 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 (<50 patients)
   1 intermediate population for analysis (50-150 patients)
   2 large population for analysis (>150 patients)

3. **Exposure**
   Observational studies
   0 if the study used no appropriate exposure measurement method or if not reported
   1 if the study used moderate quality exposure measurement methods
   2 if the study used adequate exposure measurement methods

   Intervention studies
   0 if the intervention was not described or not blinded
   1 if the intervention was adequately single blinded.
   2 if the intervention was adequately double-blinded.

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 (BMI, smoking, alcohol, age)
   1 if findings are controlled for key confounders (BMI, smoking, alcohol, age)
   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)

Supplemental Table 2. Key search terms

Keywords		
EXPOSURE	**OUTCOME**	
Folic acid	**Preconceptional**	**Postconceptional**
Folate	Sperm concentration	Fertility
MTHFR	Sperm quality	Male infertility
Vitamins	Sperm	Male fertility
Diet	Sperm dysfunction	Time to pregnancy
Antioxidants	Sperm count	Implantation
One carbon metabolism	VCM	Pregnancy
Homocysteine	Seminal plasma	Fetal growth
Metabolome	Ejaculate volume	Placenta
	Asthenozoospermia	Placentation
	Oligozoospermia	Pregnancy outcome
	Oligoasthenozoospermia	Life birth ratio
	Teratozoospermia	Miscarriage
	Epigenetics	Congenital malformations
	DNA methylation	Fetal growth retardation
	DNA damage	Fetal growth restriction
	Histone modifications	Small for Gestational Age
		Intrauterine growth restriction
		Premature birth
		Preterm birth
		Intrauterine death

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PART II

Pre-implantation embryo development

CHAPTER 3

Preconceptional maternal vegetable intake and paternal smoking are associated with pre-implantation embryo quality

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BACKGROUND: Inadequate nutrition and lifestyle behaviours, particularly during the periconception period, are associated with a negative impact on embryonic and subsequent fetal development. We investigated the associations between parental nutritional and lifestyle factors and preimplantation embryo development.

METHODS: A total of 113 women and 41 partners, with a corresponding 490 embryos, who underwent intracytoplasmic sperm injection (ICSI) treatment subscribed to the mHealth coaching platform 'Smarter Pregnancy'. At baseline, nutrition and lifestyle behaviours (intake of fruits, vegetables, folic acid, and smoking and alcohol use) were identified and risk scores were calculated. A lower risk score represents healthier behaviour. As outcome measure, a time lapse morphokinetic selection algorithm (KIDScore) was used to rank pre-implantation embryo quality on a scale from 1 (poor) to 5 (good) after being cultured in the EmbryoscopeTM time-lapse incubator until embryonic Day 3. To study the association between the nutritional and lifestyle risk scores and the KIDScore in men and women, we used a proportional odds model.

RESULTS: In women, the dietary risk score (DRS), a combination of the risk score of fruits, vegetables and folic acid, was negatively associated with the KIDScore (OR 0.86 (95 % CI: 0.76 to 0.98), p=0.02). This could mainly be attributed to an inadequate vegetable intake (OR 0.76 (95 % CI: 0.59 to 0.96), p=0.02). In men, smoking was negatively associated with the KIDScore (OR 0.53 (95 % CI: 0.33 to 0.85), p<0.01).

CONCLUSIONS: We conclude that inadequate periconception maternal vegetable intake and paternal smoking significantly reduce the implantation potential of embryos after ICSI treatment. Identifying modifiable lifestyle risk factors can contribute to directed, personalized and individual recommendations that can potentially increase the chance of a healthy pregnancy.

Abstract

Introduction

Subfertility is still an increasing problem in the Western world, which can be attributed to postponing pregnancy, but also to a decline in sperm count, increasing age of women at the time of conception, obesity, smoking and other poor lifestyles (1-3). Despite novel developments in assisted reproductive technology (ART), high rates of implantation failure and early pregnancy loss are still seen after the transfer of selected, morphologically high quality, pre-implantation embryos (4).

Pre-implantation embryo development can be studied using the EmbryoScope™, which incorporates a specialized built-in microscope designed for automated time-lapse embryo assessment by acquiring images (5). The EmbryoScope™ provides a controlled culture environment and captures comprehensive information on embryo development without the need for handling or disturbing the developing embryo. The use of embryo morphokinetics by timing of embryo developmental events, available through continuous time-lapse monitoring, has added another dimension to current traditional morphology classification scores used to predict embryo implantation potential and viability (6, 7). Animal studies have shown that single embryo developmental kinetics at the cleavage stage are reflective of culture conditions, but also of embryo metabolism, genetic integrity and blastocyst formation and quality (8-11). To assist in embryo selection as part of IVF treatment, the KIDScore algorithm was developed as a generally applicable morphokinetic algorithm suitable to rank Day 3 embryos, originating from different culture conditions and fertilization methods. Embryos are ranked in five groups predicting their ability to develop into a blastocyst with an area under the curve (AUC) of 0.75 and implantation potential with an AUC of 0.65 (indicative of intermediate prediction) (12). Interestingly, a recent study showed that the KIDScore was superior regarding predicting implantation and ongoing pregnancy rates when compared to only scoring embryo morphology (13).

Most reproductive challenges, such as fertility problems, miscarriages, congenital malformations and fetal growth restriction, largely originate in the periconception period, which ranges from at least 14 weeks before conception until 10 weeks after conception (14-16). Inadequate nutrition and lifestyle behaviours particularly during the periconception period are associated with a negative impact on the development of the embryo and subsequent fetal development (17). Nutrition and lifestyle behaviours are specifically of clinical interest since these factors are modifiable. Couples contemplating pregnancy are often not aware of their inadequate nutrition and lifestyle behaviours and the detrimental effects on reproduction (18). To investigate the effect of adherence to general dietary recommendations in couples undergoing IVF/ICSI treatment, our group studied the association with the chance of ongoing pregnancy. Improvement of adherence to the nutritional recommendations of the Dutch Nutrition Centre (covering the intake

of six main food groups namely fruits, vegetables, meat, fish, whole wheat products and fats) resulted in a 65% increase of ongoing pregnancy after IVF/ICSI treatment (19). Furthermore, inadequate nutritional behaviours of the mother during pregnancy can also have detrimental consequences for the health of the offspring later in life, where earlier age of puberty-onset and a decline in ovarian follicle reserve have been reported (20). Furthermore paternal obesity and nutritional factors are also linked to sperm quality and epigenetic profiles, possibly also affecting embryo quality and pregnancy outcomes (21, 22). We hypothesize that paternal nutrition and lifestyle factors affect multiple pathways involved in the (patho)physiology of sperm quality, such as inflammation, vascular pathways and epigenetics, that can also influence the development of pre-implantation embryos.

Nutrition and lifestyle behaviours can be easily assessed using the online mHealth program Smarter Pregnancy, which effectively improves the intake of fruits, vegetables, folic acid supplements, stop smoking and use of alcoholic drinks (23). The effect of nutrition and lifestyle behaviours on fertility and pregnancy outcomes are widely studied, however, their influence on pre-implantation embryo development is limited. Investigating pre-implantation embryo parameters *in vitro*, provides a unique insight into the direct impact of maternal and paternal factors through oocyte and sperm, respectively, independent of the *in vivo* utero maternal environment. Therefore, the aim of this study is to investigate the associations between the five Smarter Pregnancy lifestyle behaviours (vegetables, fruit, folic acid, smoking and alcohol use) of both men and women and the quality of development of pre-implantation embryos cultured in the EmbryoScope™ as marker of implantation potential and assessed by the KIDScore algorithm.

Materials and methods

Study design, population and patient inclusion

In a prospective cohort study, couples that underwent ICSI treatment were included when embryos were cultured in the EmbryoScope™ time-lapse incubator and baseline data on the five nutrition and lifestyle behaviours of the mHealth program Smarter Pregnancy were available. Couples were included between October 2014 until December 2017 at the Erasmus MC, University Medical Center, Rotterdam, the Netherlands (23).

Patients had to be at least 18 years of age and had to have good understanding of Dutch speaking and writing. After the introduction of the EmbryoScope™ time-lapse incubator in our clinic, it was mostly used for cycles from patients undergoing ICSI treatment, as in this situation oocytes can be submitted to time lapse culture directly after injection and resulting embryos can benefit optimally from undisturbed culture. Only in the more recent years we also cultured some embryos from IVF treatment cycles in the EmbryoScope™. In

this case, fertilized oocytes are submitted to time lapse culture the day after insemination, after pronuclear inspection. In these cases, the time point of fertilization is less accurate than after ICSI and pronuclear appearance cannot be assessed. We therefore excluded these cycles for the current analysis. Furthermore, we excluded patients with no available data of the Smarter Pregnancy program.

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 Institutional Review Board of the Erasmus, University Medical Centre, Rotterdam, the Netherlands. Written informed consent was obtained from all female and male participants at enrolment.

In vitro fertilization procedures

Ovarian stimulation, oocyte retrieval, the ICSI procedures and assessment of embryo morphology were performed as described previously (24). Inseminated oocytes were cultured in the EmbryoScope™ in Sage 1-step culture medium (Origio/Cooper Surgical™, Denmark) at 36.8°C, 7% oxygen and 5% carbon dioxide. Embryo evaluation and selection for transfer was carried out on day 3 after oocyte retrieval, where selection was based on developmental stage and morphology. Embryos were ranked according to number of blastomeres, fragmentation, size equality, and signs of early compaction. Top ranked embryos consisted of 8 equally sized blastomeres with no to little fragmentation. 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.

Time-lapse imaging and analysis of morphokinetic parameters

Embryo images were automatically recorded in seven focal planes (15 µm intervals, 1280 x 1024 pixels, 3 pixels per µm, monochrome CCD camera, single red LED 635 nm duration <0.1 sec per image, total light exposure time <50 sec per day per embryo) every 10 minutes until embryo Day 3. Manual annotations were performed by specifically trained members of our team according to the definitions and guidelines by Ciray and colleagues (25). Time of pronuclei appearance (tPNa) was defined as the appearance of both pronuclei, whereas the time of pronuclei fading (tPNf) was the first frame where both pronuclei were faded. The time points t2, t3, t4, t5, t6, t7 and t8 render the exact timing of reaching the 2, 3, 4, 5, 6, 7- and 8-cell stage of an individual embryo (**Figure 1**). Team members performed a proficiency test to check consistency of annotations within and between observers. Extremely close agreement (ICC>0.95) was observed for the pronuclear stage and the first cleavage divisions until the 5-cell stage, moderate agreement was observed for identifying the 6 to 8cell stage (ICC 0.23-0.40).

Application of the KIDScore algoritm

To assess embryo quality and the implantation potential of a pre-implantation embryo, the KIDScore was used (12). The KIDScore ranges from 1 till 5 and is based on six annotations: the number of pronuclei equals 2 at the 1-cell stage, time from insemination to pronuclei fading (tPNf). Time to the 2, 3 or 5-cell stage (t2, t3, t5) and the number of cells 66 hours after insemination (**Figure 1**). The KIDScore is a deselection algorithm, where a decision tree with specific cut-off values determines which score is allocated to an individual embryo. Embryos classified as score 1 show a developmental pattern indicative of a low developmental and implantation potential (an observed average chance of implantation of 5 %), whereas score 5 embryos follow a pattern indicative of a high potential (36 %) (12).

mHealth program Smarter Pregnancy

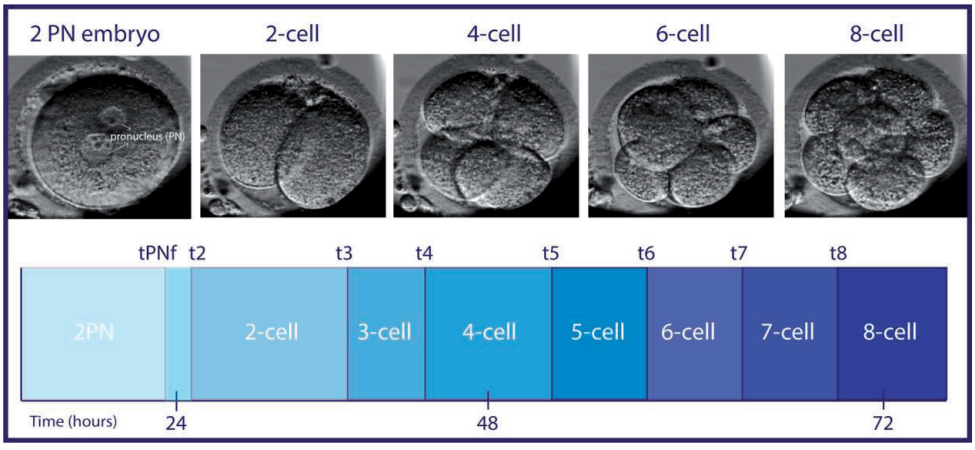
The mHealth program Smarter Pregnancy (https://www.slimmerzwanger.nl; https://www.smarterpregnancy.co.uk) is a (cost) effective tool to improve nutrition and lifestyle (26). Couples who wanted to participate in the program were subscribed to the mHealth program at the moment of fertility intake in the outpatient clinic of the Erasmus MC University Medical Center, Rotterdam. The Smarter Pregnancy program offers online coaching for a period of six months, focusing on four of the most prevalent inadequate behaviours, i.e., vegetables, fruits, alcohol and folic acid supplement intake, and smoking as the behaviour with the strongest detrimental effects on fertility and pregnancy outcome (17, 27-29). The guidelines of the Netherlands Nutrition Centre were used to set the adequate daily intakes of at least 200 grams of vegetables, two pieces of fruit, 400 micrograms by a folic acid supplements, no smoking and no use of alcohol. A baseline questionnaire was used to determine the presence of these five nutrition and lifestyle behaviours, which each were translated in a risk score. A high risk score represents unhealthy nutrition or lifestyle. Intake of fruits, vegetables, folic acid supplements and alcohol use were depicted on a scale from 0-3 and smoking was depicted on a scale from 0-6. Vegetable and fruit intake were both subdivided into a risk score of 0, 1.5 or 3, in which 0 represents an adequate daily intake (**Table 1**). The total risk score (TRS) was defined as the sum of all risks per behaviour. The dietary risk score (DRS) is the combined total of fruits, vegetables and folic acid supplement intake risk scores, while the lifestyle risk score (LRS) is the combined total of alcohol and smoking risk scores.

Study parameters

Exposure variables as described above were extracted from the Smarter Pregnancy database. Electronic patient files were used to extract data on age and standardized anthropometric measurements carried out at intake, including maternal height with 0.1 cm accuracy and weight with 0.1 kg accuracy (anthropometric rod and weighing scale; SECA, Hamburg, Germany), as well as information about diagnosis of subfertility and oocyte retrieval date.

As outcome variable, we used the KIDScore as described above. In our hospital the KIDScore is not used as a decision tool to select embryos for either transfer or cryopreservation. This decision is made by the embryologist based on a single morphological assessment at 66-68 hours post fertilization. Only embryos with normal fertilization, as evidenced by the appearance of two pronuclei, that were subsequently transferred or cryopreserved were respectively annotated for research purposes.

Figure 1 Embryo development from 1 cell to 8 cell (embryonic day 1 till day 3) as seen from the EmbryoScope™ with the corresponding time points used for the KIDScore.



Statistical analysis

Baseline characteristics of the female and male population in the current study are depicted as median or number with the corresponding interquartile range (IQR) or percentage. All analyses were performed using SPSS package 21.0 (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). To study the association between the nutritional and lifestyle risk scores and the KIDScore in men and women, we used a proportional odds model (30). This is a model for ordinal outcomes like the KID-score, using the ordinal package in R (Rune Haubo B Christensen). Challenging in pre-implantation analysis is the fact that couples usually have multiple embryos per cycle and normal regression analysis does not account for this clustering. Random subjects effects are used in the proportional odds model to account for this clustering. To adjust for potential confounders, two different models were constructed for the analysis. In the first model, no adjustments were made (crude model). The second model was adjusted for the covariate maternal age in the study population of women and for maternal age and for the risk score of the corresponding couples' female risk score in the population of males. Subgroup analyses were performed in women with overweight/obesity (BMI ≥25 kg/m^2) and normal weight (BMI <25 kg/m^2). The effect estimates of the models were transformed into odds ratios using the exponential

function on the effect estimate. This odds ratio represents the chance of an individual embryo proceeding to a 1 point higher KIDScore given the associated maternal risk score. In the proportional odds model this odds ratio is assumed to be constant across all levels of the KIDScore.

To study the associations between the fraction of discarded embryos and the nutrition and lifestyle risk scores, we used a generalized linear mixed model (GLMM) approach. Similar to the models discussed above, in the first model no adjustments were made (crude model) where the second model was additionally adjusted for the covariate maternal age. The effect estimates of the models were again transformed into odds ratios using the exponential function on the effect estimate. This odds ratio represents the chance of ICSI treatment resulting in a discarded embryo based on the individual risk score. Since nearly all women used folic acid supplements and nearly none were smokers analyzing these two behaviours was not possible.

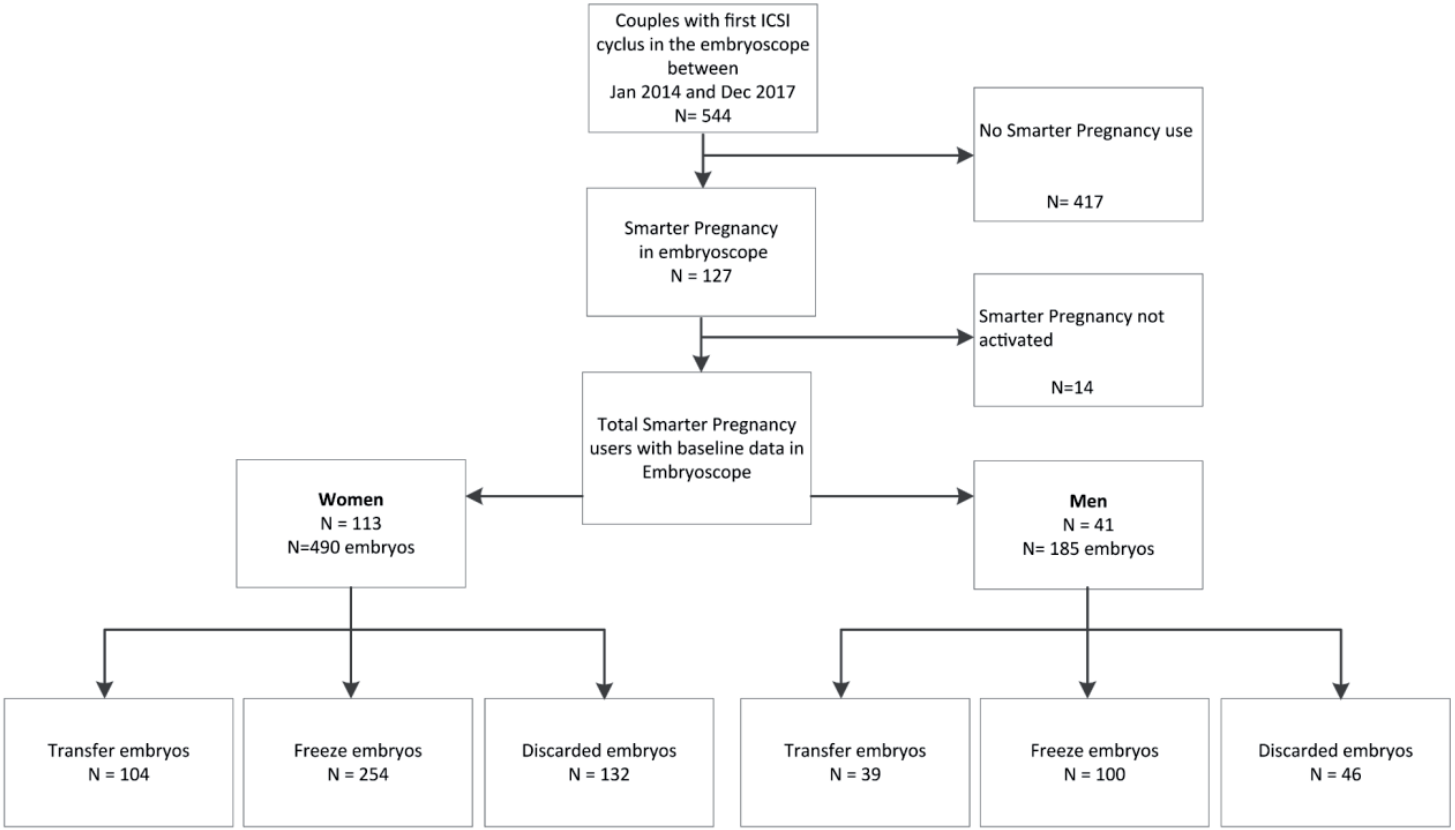
Results

Between January 2014 and December 2017, embryos of 544 couples who underwent their first IVF/ICSI cycle were cultured in the EmbryoScope™. Of these couples, 417 (76.5 %) were not subscribed to Smarter Pregnancy. In the end 113 couples were included, of which 41 of the men also participated. In all included couples, fertilization was performed by using intracytoplasmic sperm injection (ICSI), which resulted in a total of 490 embryos that were cultured in the EmbryoScope™. Of these 490 embryos, 104 embryos were transferred, 254 were frozen and 132 were of poor quality and thus discarded **(Figure 2)**. From the 41 couples of which the male partner also participated, a total of 185 embryos were cultured in the EmbryoScope™. Of these 185 embryos (which is a subset of the total amount of 490 embryos), 39 embryos were transferred, 100 were frozen and 46 were of poor quality and thus discarded **(Figure 2)**.

Women

The median age and BMI of the women was respectively 32.4 (IQR: 29.1-35.1) years and 23.2 (IQR: 21.7-26.0) kg/m^2, of which 66 (59 %) women had a normal BMI and 46 (41 %) were overweight/obese (BMI >25 kg/m2). Most women were of Western origin (84 %) and were highly educated (51 %). The main reason for ICSI treatment was male factor subfertility (81 %), of which in 55% of the cases sperm was retrieved surgically. Other indications included unexplained subfertility (2 %), female factor subfertility (2%) and combined male-female factor subfertility (15 %). The average time between completing the screening of the Smarter Pregnancy program and oocyte retrieval was 49 days (IQR: 35-126 days). Most women had inadequate intake of vegetables (n=79 (72 %)) and of fruits (n=63 (58 %)). Only

Figure 2. Flowchart of in- and exclusions of the study population



two women did not take folic acid supplements (2 %). The vast majority of all women did not smoke (n=107 (98 %)) and did not consume alcohol (n=67 (61 %)) **(Table 1)**.

The results from the proportional odds model, indicative of the odds for an individual embryo getting a 1 point higher KIDScore given the individual risk scores, show that the vegetable risk score for the total population of women was negatively associated with the KIDScore with an effect estimate of -0.28 and an odds ratio of 0.76 (95 % CI: 0.59 to 0.96) **(Table 2a)**. After adjustment for maternal age, the effect remained statistically significant with an effect estimate of -0.28 and an odds ratio of 0.76 (95 % CI: 0.59 to 0.96). No significant associations were observed for fruit intake and alcohol consumption. Furthermore, the DRS was also significantly associated with the KIDScore with an effect estimate of

-0.15 and a corresponding odds ratio of 0.86 (95 % CI: 0.76 to 0.98). Subgroup analysis showed that the effect of vegetable intake alone was only pronounced in overweight/obese women, with an effect estimate of -0.55 and an odds ratio of 0.58 (95 % CI: 0.37 to 0.91). This effect even increased after adjustment for maternal age with an effect estimate of -0.63 and an odds ratio of 0.53 (95 % CI: 0.32 to 0.87) **(Table 2b).** The associations between vegetables intake, fruit intake and DRS and KIDScore are visually depicted in **Supplemental figure 1.** In women with a normal BMI, no significant associations were observed between the KIDScore and vegetables and fruit intake and alcohol consumption (**Supplemental table 1)**. When analysing the risk of developing embryos with poor quality given the individual risk scores, the effect estimate for the TRS was 0.10 with a corresponding odds ratio of 1.11 (95 % CI: 0.92 to 1.33) after adjustment for maternal age **(Supplemental table 2**), indicating that the TRS was not associated with the chance of a discarded embryo.

Males

The median age and BMI of the male partner was respectively 34.0 (IQR: 29.5-41.5) years and 24.0 (IQR: 22.6-27.5). In males 24 (59 %) had a normal BMI (>18.5 to <25 kg/m^2) and 17 (41 %) were overweight. All men were of Western origin and most were highly educated (59 %). The main indication for ICSI treatment was male factor subfertility (78 %), of which in 53 % of the cases sperm was retrieved surgically. The average time between completing the Smarter Pregnancy screening and oocyte retrieval was 45 days (IQR: 34.5-97). The vast majority of men had an inadequate intake of vegetables (n=27 (66 %)) and fruits (n=23 (56 %)). A total of 38 (93 %) were non-smokers, while refraining from alcohol was reported by only 13 men (32 %) **(Table 1)**.

The results from the proportional odds model show that the risk score for smoking was negatively associated with the KIDScore with an effect estimate of -0.63 and an odds ratio of 0.54 (95 % CI: 0.34 to 0.85) for the crude model and an effect estimate of -0.63 and an odds ratio of 0.53 (95 % CI: 0.33 to 0.85) in the adjusted model, respectively **(Table 2c).**

Table 1: Baseline characteristics of women and men in the total study population.

	Women (N=113)	Missing	Men (N=41)	Missing
Age, years	32.4 (29.1-35.1)	0	34.0 (29.5-41.5)	0
Etnicity				
Western	95 (84 %)	0	41 (100 %)	
Non-Western	18 (16 %)		0 (0 %)	
Education				
Low	8 (7 %)	0	3 (7 %)	
Middle	47 (42 %)		14 (34 %)	
High	58 (51 %)		24 (59 %)	
BMI (measured), kg/m^2	23.2 (21.7-26.0)	4	24.0 (22.6-27.5)	0
BMI category				
<25	66 (59 %)	1	24 (59 %)	0
≥25	46 (41 %)		17 (41 %)	
Diagnosis category:		0		0
Only male factor	92 (81 %)		32 (78 %)	
Of which surgically retrieved sperm	*51*		*17*	
Only female factor	2 (2 %)		1 (2 %)	
Anovulation	*2*		*1*	
Tubal pathology	*0*		*0*	
Both male and female	17 (15 %)		7 (18 %)	
Of which surgically retrieved sperm	*6*		*3*	
Anovulation	*16*		*6*	
Tubal pathology	*1*		*1*	
Unexplained	2 (2 %)		1 (2 %)	
Time between activation Smarter Pregnancy and oocyte retrieval, days	49 (35-126)	0	45 (34.5-97)	0
Vegetable risk score				
0 (≥200 gram/day)	31 (28 %)	3	14 (34 %)	0
1.5 (150-199 gram/day)	22 (20 %)		8 (20 %)	
3 (<150 gram/day)	57 (52 %)		19 (46 %)	
Fruit risk score				
0 (≥2 pieces/day)	46 (42 %)	4	18 (44 %)	0
1.5 (1,5-1.9 pieces/day)	13 (12 %)		8 (20 %)	
3 (<1.5 pieces/day)	50 (46 %)		15 (36 %)	
Folic acid risk score				
0 (0.4 mg/day)	111 (98 %)	0	n/a	
3 (no usage)	2 (2 %)			
Smoking risk score				
0 (no smoking)	107 (98 %)	4	38 (93 %)	0
1 (1-5 cigarettes/day)	0 (0 %)		0 (0 %)	
3 (6-14 cigarettes/day)	2 (2 %)		2 (5 %)	
6 (≥ 15 cigarettes/day)	0 (0 %)		1 (2 %)	
Alcohol risk score				
0 (no alcohol)	67 (61 %)	4	13 (32 %)	0
1.5 (1-2 units/day)	39 (36 %)		24 (59 %)	
3 (≥ 2 units/day)	3 (3 %)		4 (9 %)	

Data are presented as medians (IQR) or number of subjects (%). Abbreviations: IQR, interquartile range; BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

Table 2. Effect estimates and odds ratios from the proportional odds model for the nutrition and lifestyle risk scores on the KIDScore for **a.** total study population of women, **b.** overweight women only and **c.** total study population of males

	Effect estimate	**Odds ratio (95 % CI)**	**P-value**	**Effect estimate**	**Odds ratio (95 % CI)**	**P-value**
Table 2a	**Crude**			**Adjusted***		
Total Risk Score	-0.13	0.88 (0.78 to 1.00)	**0.049**	-0.13	0.88 (0.78 to 1.00)	**0.047**
Dietary Risk Score	-0.15	0.86 (0.76 to 0.98)	**0.025**	-0.15	0.86 (0.76 to 0.98)	**0.024**
Lifestyle Risk Score	0.15	1.16 (0.78 to 1.71)	0.47	0.07	1.08 (0.76 to 1.53)	0.42
Vegetable intake	-0.28	0.76 (0.59 to 0.96)	**0.03**	-0.28	0.76 (0.59 to 0.96)	**0.02**
Fruit intake	-0.14	0.87 (0.68 to 1.11)	0.25	-0.16	0.86 (0.70 to 1.05)	0.24
FA supplement use	n/a			n/a		
Alcohol use	0.15	1.16 (0.78 to 1.71)	0.62	0.07	1.08 (0.76 to 1.53)	0.68
Smoking	n/a			n/a		
Table 2b	**Crude**			**Adjusted***		
Total Risk Score	-0.29	0.75 (0.57 to 0.98)	**0.04**	-0.30	0.74 (0.56 to 0.98)	0.04
Dietary Risk Score	-0.31	0.73 (0.56 to 0.97)	**0.029**	-0.31	0.74 (0.55 to 0.98)	0.033
Lifestyle Risk Score	0.06	1.06 (0.52 to 2.19)	0.87	0.02	1.01 (0.47 to 2.17)	0.97
Vegetable intake	-0.55	0.58 (0.37 to 0.91)	**0.02**	-0.63	0.53 (0.32 to 0.87)	**0.01**
Fruit intake	-0.18	0.84 (0.56 to 1.24)	0.37	-0.18	0.84 (0.56 to 1.26)	0.39
FA supplement use	n/a			n/a		
Alcohol use	0.06	1.06 (0.52 to 2.19)	0.87	0.01	1.01 (0.47 to 2.17)	0.97
Smoking	n/a			n/a		
Table 2c	**Crude**			**Adjusted#**		
Total Risk Score	-0.13	0.88 (0.73 to 1.06)	0.18	-0.11	0.90 (0.74 to 1.09)	0.28
Dietary Risk Score	-0.08	0.92 (0.74 to 1.15)	0.45	-0.03	0.97 (0.76 to 1.24)	0.83
Lifestyle Risk Score	-0.23	0.80 (0.57 to 1.11)	0.18	-0.27	0.76 (0.55 to 1.06)	0.11
Vegetable intake	-0.21	0.81 (0.58 to 1.13)	0.22	-0.13	0.88 (0.57 to 1.37)	0.57
Fruit intake	0.02	1.02 (0.74 to 1.42)	0.90	0.06	1.06 (0.76 to 1.48)	0.75
Alcohol use	0.14	1.15 (0.72 to 1.83)	0.57	0.08	1.08 (0.67 to 1.73)	0.75
Smoking	-0.63	0.54 (0.34 to 0.85)	**<0.01**	-0.63	0.53 (0.33 to 0.85)	**<0.01**

Crude model: no adjustments made. Adjusted model *: Model 1 + adjusted for maternal age. Adjusted model #: Model 1 + adjusted for maternal age and the corresponding risk score from the women.

Although paternal vegetables intake and the DRS and TRS show negative associations with the KIDScore, the effect diminished when adjusting for maternal age combined with the risk scores of women and failed to reach significance.

Discussion

In this study, we showed that inadequate maternal vegetable and fruit intake, as well as paternal smoking during the periconception period, are associated with the quality and developmental morphokinetics of pre-implantation embryos as outcome of implantation potential. Inadequate periconception maternal vegetable intake was negatively associated with the quality of resulting ICSI embryos. Moreover, the effect size was more than doubled in women with a BMI > 25 kg/m^2. In men, we observed that smoking was negatively associated with embryo quality as measured by the KIDScore. Importantly, our results highlight that a majority of subfertile couples undergoing ICSI treatment are not adherent to a healthy diet and lifestyle in the months preceding oocyte retrieval, semen collection and subsequent fertilization. Despite the fact that many couples score low on a healthy diet and lifestyle, we did not observe associations between these factors and the proportion of underdevelopment of embryos, which were discarded.

Our results are in line with the study of Braga *et al.,* which showed that periconception intake of fruits and vegetables significantly improved pre-implantation embryo quality after ICSI treatment, whereas fruit intake was also positively associated with blastocyst formation (31). No effects of fruit and vegetable intake were seen regarding clinical ongoing pregnancy rates, which might be attributed to the fact that this study is underpowered for this outcome. However, Braga *et al*. did not use the KIDScore as outcome parameter, but only performed conventional, static, embryo morphology assessment on day 3. They showed an effect of maternal smoking and alcohol use, although the proportion of women that smoked in their study was not mentioned. The absence of a clear effect of alcohol consumption on pre-implantation embryo quality, could be explained by the fact that in our cohort only a few women reported to use alcohol. Moreover, as few women reported smoking and nearly all women used folic acid in our study population, statistical analysis on smoking and folic acid use was not meaningful.

The correlation between a maternal healthy diet and periconception outcomes have been well established (3). Vujkovic *et al*. showed that adherence to a healthy "Mediterranean" dietary pattern, which consists of high intake of vegetable oils, vegetables, fish, and legumes and low intake of snacks, is associated with an increased chance (odds ratio: 1.4) of pregnancy in couples undergoing an IVF/ICSI treatment (32). Inversely, an unhealthy diet characterized by low levels of folate, zinc and antioxidants are associated with a decreased chance of pregnancy (33). Importantly, a Western diet with high intakes of pizza, potatoes and low intake of fruit was associated with a nearly two-fold increase in developing a congenital cleft lip (34). In line with these findings, Oostingh *et al.* recently reported in a review that inadequate maternal nutrition is associated with lower fecundity and that an optimal maternal vitamin status is associated with decreased risk of first-trimester

miscarriage (3). The positive associations in our study between maternal vegetables, fruits, paternal smoking and pre-implantation embryo quality indicate that both maternal and paternal factors already influence initiation of embryo potential and development capacity directly postconceptionally. These effects could be explained by the fact that fruits and vegetables are rich in exogenous antioxidants, such as vitamin C and vitamin E, and also in elements with antioxidant properties such as folate and zinc. Antioxidants can provide protection of DNA against oxidative stress caused by reactive oxygen species (ROS), which are produced as a byproduct in the process of aerobic metabolism necessary for normal physiological function of DNA replication. However, excessive oxidative stress can result in DNA-damage (single and double strand breaks and chromosomal rearrangements) and in sperm also leads to decreased mitochondrial function necessary for seminal propulsion with resulting impaired motility (35). Interestingly, our results are only present in the overweight/obese group as compared to the normal weight group. Overweight and obesity can be considered a chronic inflammatory state, with fat cells releasing inflammatory factors and thereby inducing a pro-inflammatory state and oxidative stress (36). Possibly obesity and inadequate fruit and vegetable intake work synergistically and the combination of both induces too much oxidative stress during oogenesis, which results in embryos with less optimal developmental potential.

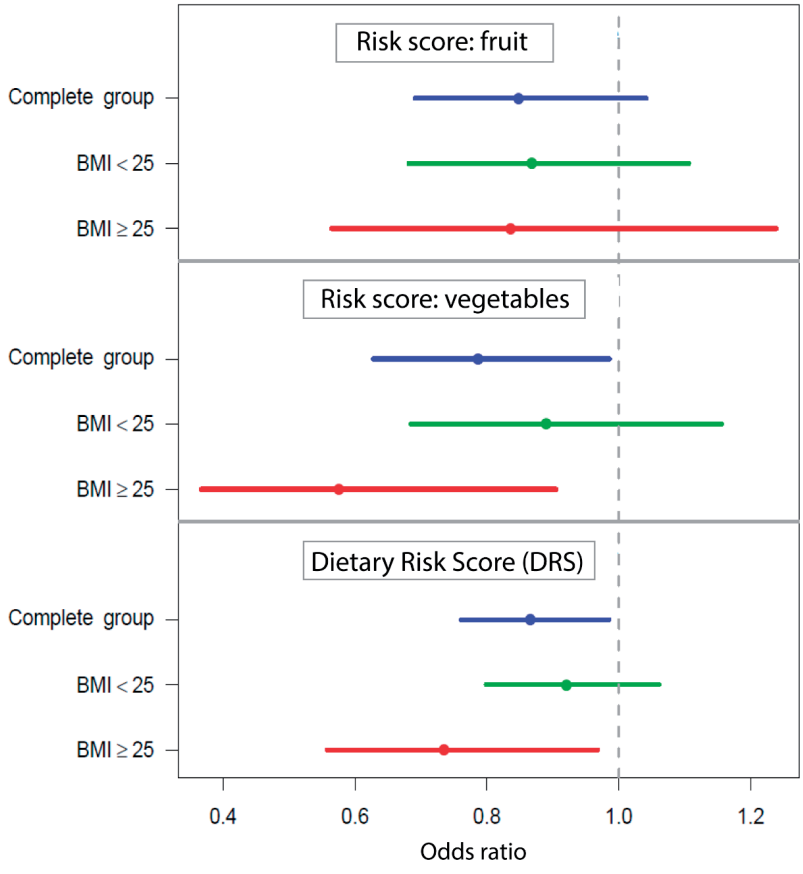
Paternal smoking and high BMI can have a detrimental effect on all semen parameters like volume, density, concentration and morphology (37, 38), which are linked to reproductive success. It is known that smoking can increase DNA damage and aneuploidies present in sperm and is associated with, or even can be the cause, of congenital malformations (39). Recent literature shows that DNA damage and sperm epigenetic information are also transferred to the embryo (40, 41). Our results point to an effect of paternal smoking on sperm that is directly carried over to the pre-implantation embryo as it is associated with less optimal early development and hence lower embryo quality based on the scored morphokinetic parameters. Although we find associations regarding paternal vegetable intake and the embryo KIDScore with similar effect estimates and odds ratios as for the women, these associations disappear when corrected for maternal total risk scores. Since similar odds ratios were seen for men and women, this could possibly be attributed to a power problem. We decided to correct for the maternal risk scores, since we assume that the corresponding effect estimates are due to high correlation between couples regarding their eating habits and lifestyle factors. We are therefore unable to determine who of both does contribute most to the detrimental effects on early developments, the woman or the man. However, since the number of participating women in the current study is far larger than men, we decided to adjust for maternal risk scores in the male analysis. In larger cohorts, we want to argue for the interpretation of the paternal results without corrections for their female partners.

A strength of this study is the use of the validated mHealth Smarter pregnancy program using the dietary risk score. Another strength is the use of the KIDScore to evaluate embryo quality, which is generally used and easily applicable. A KIDScore of 5 is associated with high implantation rates, where a low KIDScore of 1 is associated with low implantation rates. Despite its general and easy applicability, the predictive capability of the KIDScore on implantation has an AUC of 0.65, which could be classified as a fair predictor. In addition, we have to consider for inference of our data that developmental kinetics at the cleavage stages are also reflective of genetic integrity and blastocyst quality. Our study was conducted on embryos cultured until day 3 in a time period that culturing until day 3 was routine clinical practice in most hospitals and also in our hospital. From 2019 onwards we are culturing the embryos until the blastocyst stage (day 5). Therefore, future research should also investigate the associations between periconception parental nutrition and lifestyle factors and the outcome after culturing until day 5, i.e., blastocyst quality, pregnancy rates and outcomes. In our study we did not investigate the association between nutritional and lifestyle scores on clinical pregnancy rate (detectable embryonic heartbeat), which should be done in larger cohorts. Another strength is that using pre-implantation embryo development *in vitro* allows studying early development outside of the maternal body, allowing contribution and identification of paternal as well as maternal factors from both parental gametes. The uterine environment makes identification of paternal factors impossible, since factors of the uterine environment could bypass and dampen paternal effects, while following early development and implantation *in utero* is still technically impossible. Importantly, we show that paternal lifestyle significantly impacts on embryo developmental kinetics during the cleavage stages. Since the study population consists of subfertile couples visiting a tertiary university based hospital, although it does not mean all couples are in need of tertiary referral or care, the results cannot be automatically extrapolated to the general fertile population and this might have consequences for the external validity of our study. All couples underwent ICSI treatment, so little information is available on IVF and female factor subfertility. However, studying the effects of pre-implantation embryo development can only be performed in fertility centres which have availability of time-lapse imaging. Bias to our results cannot be excluded, because only 36 % of the male partners were willing to participate. This study revealed the magnitude of effect sizes which will help us to better calculate the sample size for future larger studies or randomized controlled trials

We realized that specific subfertility-related diseases, such as endometriosis and polycystic ovary syndrome (PCOS), are also associated with oocyte and embryo quality. Endometriosis negatively affects the oocyte quality, which could be caused by increased oxidative stress (42). PCOS is associated with decreased embryo quality, defined as slower development during the cleavage stage (43). Although our study population consisted of 113 women, it was statistically not possible to correct or stratify for these possible confounders. Future studies in larger populations should take these confounders into consideration.

This study showed that both periconception maternal and paternal lifestyle and nutritional factors already have an impact on pre-implantation embryo quality based on morphokinetic evaluation after time-lapse culture. As more than 80 % of the reproductive population has one or more inadequate nutrition and lifestyle behaviours, studying and modifying the associations by evidence based interventions is becoming increasingly important. Moreover, the live birth rate per started IVF/ICSI cycle has remained stable at around 30 % per cycle for the last decade. With the increase of subfertility in the Western world, it is essential to determine how to stop the increasing subfertility numbers, improve ART chances and prevent and overcome subfertility causes. Therefore, we conclude that identifying modifiable risk factors, as a first step in the behavioral change pathway to stimulate awareness, can have already a clinical impact by increasing the chance of a healthy pregnancy and having a live born baby.

Supplemental figure 1. Odds ratios and confidence intervals of the associations between the risk scores for vegetable intake, fruit intake and the dietary risk score and the KIDScore in the female population depicted for the total group of women (n=113), and women with BMI ≥25 kg/m^2 (n=46) and BMI<25 kg/m^2 (n=66).



Supplemental table 1. Effect estimates and odds ratios from the proportional odds model for the nutrition and lifestyle risk scores and the impact on the KIDScore for normal weight women only (n=66).

	Crude			**Adjusted**		
	Effect estimate	Odds ratio (95 % CI)	P-value	Effect estimate	Odds ratio (95 % CI)	P-value
Total Risk Score	-0.06	0.94 (0.82 to 1.08)	0.39	-0.06	0.94 (0.82 to 1.08)	0.39
Dietary Risk Score	-0.08	0.92 (0.80 to 1.06)	0.25	-0.09	0.92 (0.80 to 1.06)	0.24
Lifestyle Risk Score	0.15	1.16 (0.78 to 1.71)	0.47	0.16	1.18 (0.79 to 1.74)	0.42
Vegetable intake	-0.12	0.89 (0.69 to 1.15)	0.38	-0.12	0.89 (0.68 to 1.17)	0.39
Fruit intake	-0.14	0.87 (0.68 to 1.11)	0.25	-0.14	0.87 (0.68 to 1.10)	0.24
Folic acid supplement use	n/a			n/a		
Alcohol use	0.15	1.16 (0.78 to 1.71)	0.47	0.16	1.18 (0.79 to 1.74)	0.42
Smoking	n/a			n/a		

Model 1: crude model
Model 2: Model 1 + adjusted for maternal age and the corresponding risk score from the women

Supplemental table 2. Effect estimates and odds ratios from the generalized linear mixed model for the association between the nutrition and lifestyle risk scores on the proportion of discarded embryos.

	Crude			**Adjusted**		
	Effect estimate	Odds ratio (95 % CI)	P-value	Effect estimate	Odds ratio (95 % CI)	P-value
Total Risk Score	-0.02	0.98 (0.88 to 1.08)	0.68	0.10	1.11 (0.92 to 1.33)	0.53
Dietary Risk Score	0.01	1.01 (0.91 to 1.13)	0.83	0.07	1.07 (0.89 to 1.29)	0.93
Lifestyle Risk Score	-0.28	0.75 (0.56 to 1.02)	0.06	0.27	1.31 (0.77 to 2.20)	0.10
Vegetable intake	-0.01	1.00 (0.82 to 1.21)	0.96	-0.08	0.92 (0.64 to 1.33)	0.69
Fruit intake	0.06	1.07 (0.89 to 1.28)	0.49	0.26	1.29 (0.96 to 1.75)	0.60
Folic acid supplement use	n/a			n/a		
Alcohol use	-0.28	0.76 (0.56 to 1.02)	0.06	0.27	1.31 (0.77 to 2.20)	0.10
Smoking	n/a			n/a		

Model 1: crude model
Model 2: Model 1 + adjusted for maternal age

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CHAPTER 4

A higher preconception paternal body weight influences fertilization rate and preimplantation embryo development: The Rotterdam Periconception Cohort

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BACKGROUND: Paternal obesity is negatively associated sperm parameters and altered sperm epigenetics, however the effect on pre-implantation embryo morphokinetic development is less studied. The aim of this study is to investigate the association between preconceptional paternal body mass index (BMI) and sperm quality, embryo morphokinetics using a time-lapse incubator and in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatment outcomes.

METHODS: A total of 211 men were included from the prospective virtual embryoscope cohort study. Eighty-six were of normal weight (BMI <25.0), 94 were overweight (BMI 25-29.9), and 41 were obese (BMI≥30). Couples underwent IVF or ICSI treatment with ejaculated sperm, resulting in 757 embryos submitted to time-lapse culture. Main outcome parameters included sperm quality parameters, including the total motile sperm count (TMSC), and IVF/ICSI treatment outcomes as measured by fertilization rate, embryo developmental morphokinetics, embryo quality as assessed by a time lapse prediction algorithm and live birth rate after fresh transfer.

RESULTS: TMSC in normal weight men (25.8x106) was higher compared to overweight and obese men (12.1x106 and 8.0x106 respectively), however not significantly (p=0.09). Increased BMI was associated with significantly faster development of the preimplantation embryo, especially during the first cleavage divisions (t2: -0.11 (p=0.05) and t3: -0.19 (p=0.01)). Embryo quality using the KIDScore was not altered. In linear regression analysis, after adjustment for maternal and paternal confounders, paternal BMI was a significant inverse predictor for fertilization rate (effect estimate: -0,01 (p-value: 0,002), but not for live birth rate.

CONCLUSIONS: These data demonstrate that a higher paternal BMI is associated with lower fertilization rates and faster preimplantation embryo development. Our findings underline the importance and effect of the paternal BMI during the preconception period.

Introduction

Overweight and obesity are worldwide problems affecting the health of millions of people throughout the life course (1). In general, obesity is strongly linked to the development of type 2 diabetes, cardiovascular disease and neurological diseases later in life (2). The pathophysiology of obesity is complex and results from the interplay between inadequate dietary intake, limited exercise, genetic predisposition and sometimes medication use (3). The prevalence of obesity is also high in the reproductive population, with estimates up to 50%. The influence of obesity on reproductive health is widely studied in women, showing lower oocyte and embryo quality, a longer time to pregnancy, and increased risks of congenital malformations, miscarriages (4-6), preeclampsia, preterm birth and fetal death (7). However, the negative effects of obesity in men in the reproductive period are largely neglected and understudied. With the global burden of male obesity (8), we hypothesize that periconception paternal obesity also affects reproductive outcome.

A recent systematic review and meta-analysis showed a significant decrease of 35% in live birth rate in obese compared to normal weight fathers-to-be after assisted reproduction (9). Other studies revealed that obesity impairs sperm concentration and motility, and sperm DNA quality (9, 10). Obesity is characterized by a systemic and chronic inflammatory state, with adipocytes continuously releasing inflammatory factors and thereby inducing a pro-inflammatory state and excessive oxidative stress (11) that increases sperm DNA damage (12), with an impact on DNA integrity. The increased exposure to reactive oxidative species due to excessive oxidative stress is also associated with changes in DNA methylation patterns and chromatin constitution during spermatogenesis, with a potential impact on a paternal epigenetic contribution on subsequent embryogenesis (13-15).

The influence of paternal obesity on preimplantation embryo development has scarcely been studied in human. Two studies didn't find any significant difference between obese and non-obese men regarding embryonic parameters. However, one study reported a decreased blastulation rate with increased BMI (16). In mice, obesity negatively influences preimplantation embryo quality and blastocyst formation (17). In vitro preimplantation embryo development is of interest since it provides a unique insight into the direct impact of paternal factors through sperm, undisturbed by the effects of the maternal in vivo uterine environment. Since obesity itself is associated with sperm quality parameters and pregnancy chance, we hypothesize that preconception paternal weight is also associated with preimplantation embryo development and fertility treatment outcomes.

Preimplantation embryo development can be studied using time-lapse embryo culture, which uses incubators with a built-in microscope designed for automated time-lapse embryo assessment by acquiring images. This provides a controlled culture environment

and captures comprehensive information on embryo development without the need for handling or disturbing the developing embryo. The use of timing of embryo developmental events, also referred to as embryo morphokinetic parameters has added another dimension to current traditional morphology classification scores. This has led to the development of algorithms predicting embryo implantation potential and viability, such as the KIDScore (18).

Because of the epidemic burden of obesity which also involves men of reproductive age, the main aim of this study is to investigate associations between preconception paternal obesity and developmental morphokinetics of preimplantation embryos cultured in the EmbryoScope™ and specific IVF/ICSI treatment outcomes.

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 Institutional Review Board of the Erasmus, University Medical Centre, Rotterdam, the Netherlands. Written informed consent was obtained from all participants at enrolment.

Study design, population and patient inclusion

Couples were enrolled in the prospective Virtual Embryoscope study, which is embedded in the Rotterdam Periconception Cohort (Predict study) (19). The Predict study is an ongoing prospective tertiary hospital-based cohort embedded in the outpatient clinic of the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center Rotterdam, the Netherlands. Patients were eligible for inclusion in the Virtual Embryoscope study if they were subfertile, based on either male of female factor subfertility, with an indication for IVF treatment, with or without ICSI. Furthermore, participants needed to be at least 18 years of age and had to read and understand good Dutch. Criteria for exclusion consisted of oocyte donation and not able to understand the Dutch language. Couples were included between May 2017 until December 2019 at the Erasmus MC, University Medical Centre, Rotterdam, the Netherlands.

In vitro fertilization procedures

Ovarian stimulation, oocyte retrieval, the IVF/ICSI procedures and assessment of embryo morphology were performed as described previously (20, 21). After ICSI treatment, injected oocytes were directly placed in the EmbryoScopeTM in Sage 1-step culture medium (Origio/Cooper Surgical™, Denmark) at 36.8oC, 7% oxygen and 5% carbon dioxide. Embryos after IVF treatment were cultured in the EmbryoScopeTM after the appearance of both pronuclei.

Embryo images were automatically recorded in seven focal planes (15 µm intervals, 1280 x 1024 pixels, 3 pixels per µm, monochrome CCD camera, single red LED 635 nm duration <0.1 sec per image, total light exposure time <50 sec per day per embryo) every 10 minutes until embryo Day 3.

Embryo evaluation and selection for transfer was carried out on day 3 after oocyte retrieval, based on developmental stage and morphology as assessed on the last time-lapse image acquired 66-68 hours after fertilization. Embryo selection for transfer was not aided by time-lapse information. Embryos were ranked according to number of blastomeres, fragmentation, size equality, and signs of early compaction. Top ranked embryos consisted of 8 equally sized blastomeres with little (<10%) or no fragmentation.

Study parameters

Standardized 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). Participants completed a self-administered questionnaire covering details on age, ethnicity, educational level and preconceptional use of alcohol, cigarettes and folic acid supplements. All data were verified at study entry and anthropometrics were measured by a researcher.

Before processing for IVF or ICSI, quality of the semen sample was routinely assessed. Total motile sperm count (TMSC), which is obtained by multiplying the volume of the ejaculate in milliliters by the sperm concentration and the proportion of A (fast forward progressive) and B (slow progressive) motile sperms divided by 100%. All semen parameters were assessed according to the WHO guidelines.

Time-lapse parameters were annotated manually according to the definitions and guidelines of the ESHRE consensus for dynamic monitoring of human preimplantation development (22). All freshly transferred and cryopreserved embryos were individually annotated for the following morphokinetic parameters: tPNf, t2, t3, t4, t5, t6, t7 and t8. tPNf was defined as the first frame in which both pronuclei had faded. The timing of reaching the 2-, 3-, 4-, 5-, 6-, 7-, and 8-cell stage were defined as t2, t3, t4, t5, t6, t7 and t8, respectively. These parameters were used by the Vitrolife® embryo-viewer software to calculate intervals between cleavages as well as to assign each embryo a Known Implantation Data (KID) Score (18). 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 (18). Internal validation of inter-observer reproducibility of annotations between team members demonstrated extremely close agreement for the timings of the pronuclear stage until the 5-cell cleavage

divisions (intraclass correlation coefficient (ICC) >0.95). A moderate agreement was found for cleavage divisions between the 6- and 8-cell stage (ICC 0.23-0.40).

Fertility treatment outcomes were retrieved from medical records. Fertilization rate was calculated by dividing the number of fertilized oocytes by the total number of metaphase II oocytes retrieved. Embryo usage rate was calculated by dividing the total number of usable embryos per patient, i.e. all embryos transferred or cryopreserved, by the number of fertilized oocytes. Additionally, positive pregnancy test, visible fetal heartbeat around 12 weeks of gestation and livebirth data were collected after fresh transfer.

Statistical analysis

Baseline characteristics of men are depicted as median or number with the corresponding interquartile range (IQR) or percentage. All analyses were performed using SPSS package 25.0 (IBM SPSS Statistics, Armonk, NY). R (R: A language and Environment for Statistical Computing, version 3.1.3, 2015 for Windows, R Core Team, Vienna, Austria) was used to perform analysis on the KIDScore using the proportional odds model.

Baseline data were tested for the assumption of normality. If continuous data did not fulfil the assumption of normality, a Kruskal-Wallis test was performed and estimates are reported as medians and interquartile range (IQR). Categorical baseline data were analyzed using a Chi-square test/Fishers exact test.

All analyses were performed with BMI as continuous variable. Analyses on the developmental time points of reaching the different cell stages were performed on transferred and cryopreserved embryos. Linear mixed models were applied to study the association between paternal BMI and preimplantation developmental timings. To study associations between paternal BMI and the KIDScore, we used a proportional odds model, which is a model for ordinal outcomes like the KIDScore, using the ordinal package in R (Rune Haubo B Christensen). Random subject effects are used in the proportional odds model to account for the clustering of multiple embryos of one couple.

The dichotomous treatment outcomes; positive pregnancy test, fetal heartbeat and live birth, were analyzed using logistic regression and the continuous treatment outcomes like fertilization rate and embryo usage rate were analyzed using standard linear regression.

For linear mixed models, proportional odds models, logistic regression and linear regression we used different models to account for confounding factors. In the crude model we did not adjust for potential confounders and in model 1 we additionally adjust for potential paternal confounders (age, ethnicity, smoking, alcohol use and education) and maternal

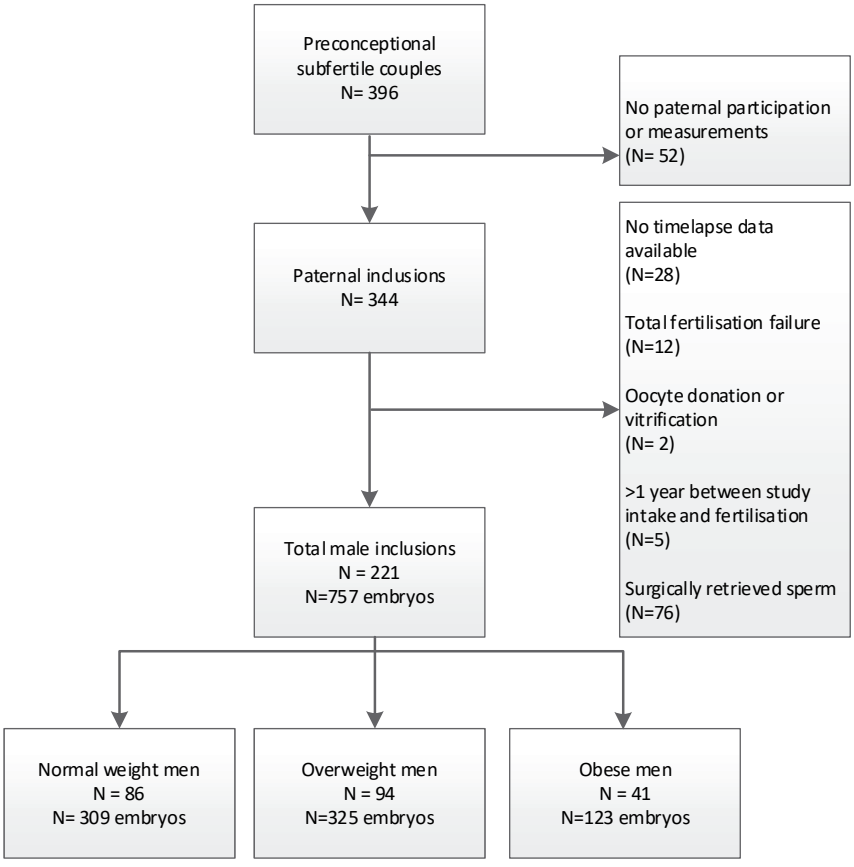
confounders (BMI, age, smoking, alcohol use and education) and fertilization type. P-values <0.05 were considered statistically significant.

Results

Baseline

After inclusion of a total of 396 preconceptional subfertile couples, patients were excluded because of no paternal participation (n=52), no available time-lapse data (n=28), total fertilization failure (n=12), oocyte donation/vitrification (n=2), more than 1 year between study intake and fertilization (n=5) and use of surgically retrieved sperm (n=76). In total 221 (n=757 embryos) men were included of which 86 (n=309 embryos) were of normal weight, 94 (n=325 embryos) were overweight and 41 (n=123 embryos) were obese (**Figure 1)**.

Figure 1: Flowchart of the in- and exclusions of the study population.



At baseline we found no significant differences between normal weight, overweight and obese men regarding the indication of the subfertility treatment (male factor in 33.7%, 36.2% and 26.8% respectively (resp.) (p=0.17)), or the ovarian stimulation protocol used by the female partner (GnRH-antagonist co-treatment in 76.1%, 81.9%, 65.9% resp. (p=0.47)) (**Table 1**). Differences were observed between normal weight, overweight and obese men regarding age (34.5, 35.4 and 36.6 years resp. (p=0.01)), geographic origin (88.4%, 77.7% and 73.2% Dutch origin resp. (p=0.01)) and alcohol use (62.8%, 71.3% and 48.8% resp. (p=0.01)) (**Supplemental table 1**). Significantly more men were highly educated in the normal weight group (54.7%) compared to the obese group (22.0%) (p=0.01).

In normal weight men, TMSC was 25.8x106 IQR (2.7-110.3), whereas this was lower in overweight men (12.1x106 IQR (0.2-85.8)) and obese men (8.0x106 IQR (0.6-55.4)), however not significantly (p=0.09) (**Table 1**). In linear regression analysis, paternal BMI was negatively predictive for TMSC (beta: -2.48 (p=0.11)), however not significantly (**Table 2**).

Embryo morphokinetic parameters

The crude results of the linear mixed models for paternal BMI as continuous variable showed that the effect estimates of all morphokinetic time points were negative, indicating that for every increase in BMI point embryos develop faster from t2 (-0.13 hours (95% confidence interval (CI) (-0.24 to -0.03)), till t7 (-0.18 hours (95% CI (-0.37 to 0.01)) and t8 (-0.15 hours (95% CI (-0.34 to 0.05)) (**Table 3**). After correcting for maternal and paternal confounders (model 1) these negative associations remain, but are only significantly in the first cleavage divisions t2 (-0.11 hours (95% CI (-0.21 to 0.001)) and t3 (-0.19 hours (95% CI (-0.33 to -0.04)).

Embryo quality and treatment outcomes

Embryo morphokinetic quality was assessed using the KIDScore. In the proportional odds model, paternal BMI was not associated with the KIDScore (beta: -0.01 (p=0.64)), which remained also after adjustment for confounders (beta: -0.01 (p=0.62)) (**Table 2**).

In normal weight men of the total study group the fertilization rate was 0.88, whereas the rate decreased to 0.81 and 0.76 in the overweight and obese group, this decrease was also present in subgroups of IVF or ICSI embryos (**Supplemental table 2**). In linear regression analysis paternal BMI was negatively associated with fertilization rate (beta -0.01 (p=0.001)), meaning that with every increase in paternal BMI point, the fertilization rate decreased with 1%. After adjustment for both maternal and paternal confounders, the negative association remained significant (beta -0.01 (p=0.002)) (**Table 2**). The embryo usage rate was not associated with paternal BMI after adjustment for confounders (beta -0.001 (p=0.84). In logistic regression (model 1) paternal BMI was not associated with a positive pregnancy test (OR=1.03 (p=0.49)), fetal heartbeat (OR=1.03 (p=0.51)) and live birth (OR=1.01 (p=0.82)).

Table 1: Subfertility parameters of men and women in the study population of the Virtual Embryoscope study.

	Normal weight men N=86	**Overweight men N=94**	**Obese men N=41**	p-value
Indications for subfertility treatment: n (%)				
Male factor	29 (33.7%)	34 (36.2%)	11 (26.8%)	0.17
Combined male-female	10 (11.6%)	18 (19.6%)	14 (34.1%)	
Female factor	32 (37.2%)	33 (35.1%)	10 (24.4%)	
Unexplained	15 (17.4%)	9 (9.6%)	6 (14.6%)	
Male factors: n (%)	**N=39 (29 male and 10 combined female-male)**	**N=52 (34 male and 18 combined female-male)**	**N=25 (11 male and 14 combined female-male)**	0.35
OA(T)	38	49	25	
An- and retrograde ejaculation	1	3	0	
Female factors: n (%)	**N=42 (32 female and 10 combined female-male)**	**N=51 (33 female and 18 combined female-male)**	**N=24 (10 female and 14 combined female-male)**	0.51
Tuba factor	7	10	6	
Ovulation disorder	18	23	8	
Endometriosis	11	13	9	
Others	6	5	1	
Oocytes aspired	9 [5-14]	8 [5.5-13]	8 [5-11.5]	0.59
Ovarian stimulation: n (%)				0.47
GnRH-Agonist	22 (25.6%)	17 (18.1%)	14 (34.1%)	
GnRH-Antagonist	64 (76.1%)	77 (81.9%)	27 (65.9%)	
Fertilization type n (%)				0.35
IVF	46 (53.5%)	42 (44.7%)	14 (34.1%)	
ICSI	40 (46.5%)	52 (55.3%)	27 (65.9%)	
TMSC (x 10^6), IQR	25.8 [2.7 – 110.3]	12.1 [0.2 – 85.8]	8.0 [0.6 – 55.4]	0.09

Total motile sperm count (TMSC) as a sperm quality parameter

Table 2. Periconception paternal BMI and associations with sperm quality, IVF/ICSI treatment outcomes and pregnancy outcomes.

	Crude			**Model 1**		
Table 3a	Beta (se)		p-value	Beta (se)		p-value
TMSC[a] *linear regression*	-2.61 (1.50)		0.08	-2.48 (1.53)		0.11
KIDScore *ordinal model*	-0.01 (0.02)		0.64	-0.01 (0.02)		0.62
Fertilization rate *linear regression*	**-0.01 (0.003)**		**0.001**	**-0.01 (0.004)**		**0.002**
Embryo usage rate *linear regression*	-0.001 (0.004)		0.99	-0.001 (0.004)		0.84
Table 3b	Beta	OR	p-value	Beta	OR	p-value
Positive pregnancy test *logistic regression*	0.02	1.02	0.57	0.03	1.03	0.49
Fetal heartbeat *logistic regression*	0.01	1.01	0.80	0.03	1.03	0.51
Live birth *logistic regression*	-0.01	0.99	0.84	0.01	1.01	0.82

Model 1: Model 1 + paternal adjustments (age, ethnicity, smoking, alcohol use and education) and maternal adjustments (BMI, age, ethnicity, smoking, alcohol use, education). [a] Only adjustments for paternal confounders in model 2.

Discussion

In this study, we show that a higher paternal BMI is associated with a reduced fertilization rate and faster development of the preimplantation embryo, with stronger effects on the first cleavage divisions as compared to the 6- to 8-cell stage. Paternal BMI was not associated with overall morphokinetic embryonic quality of the day 3 embryo, measured by the KIDScore, the embryo usage rate, positive pregnancy test and live birth rate.

Our results regarding embryo morphokinetics are only supported by previous studies in obese women showing a delay in the first cleavage divisions of embryos (23, 24). In addition, Leary et al. showed that embryos from women with overweight and obesity develop faster into a blastocyst. In this study, alterations in glucose and pyruvate metabolism of the embryo were suggested as an underlying cause, as embryo metabolism is determined by the oocyte and the oocyte may directly inherit such impairments from the maternal environment (25). Before fertilization is carried out, sperm are selected by density gradient centrifugation and washing, removing the seminal fluid. The influence on embryo development of any nutrients present in seminal fluid is therefore limited.

Table 3. Results of the linear mixed model with periconception paternal BMI as continuous variable and morphokinetic parameters of the preimplantation embryo.

Morphokinetic parameters	Crude		Model 1	
	Beta [95% CI] hours	**p-value**	**Beta [95% CI] hours**	**p-value**
tPNa	-0.01 [-0.12 to 0.09]	0.81	-0.01 [-0.08 to 0.07]	0.86
tPNf	**-0.10 [-0.19 to -0.01]**	**0.048**	-0.07 [-0.17 to 0.03]	0.15
tPNf – tPNa	-0.06 [-0.17 to 0.06]	0.32	-0.04 [-0.15 to 0.07]	0.46
t2	**-0.13 [-0.24 to -0.03]**	**0.02**	-0.11 [-0.21 to 0.001]	0.05
t3	**-0.20 [-0.34 to -0.06]**	**0.01**	**-0.19 [-0.33 to -0.04]**	**0.01**
t4	-0.13 [-0.28 to 0.01]	0.07	-0.10 [-0.25 to 0.05]	0.17
t5	-0.14 [-0.32 to 0.05]	0.15	-0.12 [-0.32 to 0.08]	0.23
t6	-0.14 [-0.33 to 0.04]	0.12	-0.14 [-0.34 to 0.05]	0.14
t7	-0.18 [-0.37 to 0.01]	0.07	-0.15 [-0.36 to 0.06]	0.15
t8	-0.15 [-0.34 to 0.05]	0.14	-0.11 [-0.33 to 0.10]	0.29

Model 1: crude model + paternal adjustments (age, ethnicity, smoking, alcohol use and education) and maternal adjustments (BMI, age, ethnicity, smoking, alcohol use, education and conception method)

We show negative associations between paternal body weight and fertilization rate. Reports in the literature are conflicting with studies in less than 300 participants reporting no association (26, 27), and studies with over 600 participants showing negative associations (28). While we show no association between paternal BMI and live birth rate, a recent meta-analysis showed that the paternal obesity is linked to a decreased live birth rate (OR 0.88) (29). The seven included studies combined included more than 13.000 IVF/ICSI cycles. Importantly, each individual study (ranging from 170 cycles to 25,000 cycles) found non-significant results, indicating a power problem, as could be the case in our study with 221 IVF/ICSI cycles.

Obesity can negatively influence sperm quality, sperm DNA damage and sperm epigenetic programming, which all impact on fertilization success. Different underlying mechanisms, such (epi)genetic, endocrinological and environmental effects, are described in the literature linking obesity to sperm quality (28). Obesity is strongly linked to decreased

sperm count via hyperinsulinemia, increased scrotal temperature and increased oxidative stress (30). The increased oxidative stress is caused by reactive oxygen species (ROS), which are produced as a byproduct in the process of aerobic metabolism necessary for normal physiological function of DNA replication. Obesity is characterized by chronic exposure of tissues to excessive oxidative stress, which can result in DNA-damage (single and double strand breaks and chromosomal rearrangements). Furthermore it is also associated with an increased sperm DNA fragmentation and also leads to decreased mitochondrial function in sperm necessary for seminal propulsion with resulting impaired motility (31).

Our results regarding early embryo morphokinetics are most likely explained by alterations in epigenetics, such as DNA methylation and/or chromatin constitution, since they are thought to affect cleavage divisions up to the 4-cell stage (32). Obesity related epigenetic changes in sperm are reported in current literature. Significantly lower (MEG3 and NDN), but also higher (H19) methylation percentages in imprinted genes were observed in sperm of obese men compared to normal weight men, possibly altering embryo development (33). The epigenetic alterations seem to occur only at the level of DNA methylation since one study showed similar histone positioning between sperm of obese and normal weight men (34). Interestingly, the methylation status of sperm is dynamic and under environmental pressure. Sperm DNA methylation profiles in 6 men undergoing bariatric Roux-en-Y gastric bypass surgery was determined before surgery, 1 week post-surgery and 1 year post-surgery. Already 1 week after surgery more than 1500 unique genes had altered DNA methylation profiles, which remained until at least 1 year after surgery (34).

It remains unknown which factors cause obesity related sperm DNA-methylation differences, however some hypothesis are proposed in current literature. Sperm DNA methylation in male mice born from mothers with either diabetes or obesity showed significant alterations in imprinted genes H19 and Peg3. Several studies have demonstrated that the sperm epigenome is responsive to dietary factors and that negative and positive influences are transferred to future generations (35-37). Obesity in general is strongly associated with elevated estrogen levels, both in women and men. Results from animal studies suggest that increased exposure to estrogen, by increased activity of aromatase present in fat tissue, may lead to abnormal methylation patterns in sperm cells providing a possible mechanism how body fat mass can impact DNA methylation (30, 38).

Recently, a novel potential epigenetic mechanism was identified (39, 40). Sperm cells carry different types of ribonucleic acid (RNA) and also the epididymal epithelium produces exosome vesicles, which are able to transfer RNA molecules to the passing sperm cells (41). In mice, such RNAs have been shown to be critical for fertilization and pre- and post-implantation embryo development (42, 43). A study in human sperm cells identified the

level of expression of a large number of these human sperm RNAs to be responsive to BMI (40).

From these data, we hypothesize that obesity-related molecular mechanisms, hormonal imbalances, diet, and other obesity-related factors can be involved in the causation of (epigenetic) changes in sperm quality of obese men. The exact underlying pathophysiologic mechanism in our study remains to be elucidated and future research should focus on investigating the role of underlying pathophysiological mechanisms.

We do not show any significant effect of paternal BMI on pregnancy rate, fetal heartbeat at 12 weeks and live birth rate. This can be explained by the IVF-ICSI procedure itself, either the ovarian stimulation, culture medium or the fact that with ICSI the embryologist selects the sperm cell, which are additional factors influencing pregnancy and live birth rate and could overrule the epigenetic effects of paternal obesity on sperm.

A strength of this study is the use of a standardized method to determine BMI by the same two researchers over the complete study period, instead of relying on self-reported data. We adhered to the WHO definitions of BMI categories to make comparing between studies more convenient. A statistical strength is the fact that we controlled paternal effects for important maternal clinical variables such as BMI and age, allowing us to present paternal effects independent of the maternal effects. In this light, preimplantation embryo development in vitro allows studying early developmental factors outside of the maternal body, allowing contribution and identification of paternal as well as maternal factors from both parental gametes. The uterine environment makes identification of paternal factors impossible, since factors of the uterine environment could diminish paternal effects, while following early development and implantation in utero is still technically impossible. It remains however important to also take the influence of determinants of maternal health into consideration. Female germ cells enter and undergo the first part of meiosis during fetal development, and resulting oocytes are then exposed to the intrinsic maternal environment, determined by multiple biologic pathways and exposures for many years before meiotic resumption and ovulation. For example, maternal obesity is associated with excessive chronic oxidative stress which can also lead to decreased oocyte quality as evidenced by lower rates of normally fertilized oocytes and decreased ongoing pregnancy rates.

A limitation is that our study was conducted in a time period that culture until day 3 was routine clinical practice in most hospitals as well as in ours. Therefore, future research should also investigate the associations between periconception parental nutrition and lifestyle factors and the outcome after culturing until day 5, i.e., blastocyst quality. Since the study is an observational cohort, we adjusted for potential maternal and paternal confounders, however residual confounding cannot be fully excluded. Other potential

important confounders such as stress and medication use are not corrected for in our study. A limitation of our study remains a relatively low number of patients in the different subgroups, potentially missing the power to investigate birth outcomes. Also other differences in the groups are present, such as the cause of infertility. Since our study population is too small to correct or stratify for these factors, future larger studies should incorporate these factors in sample size calculation and analyses. Furthermore it remains unknown what the impact of early alterations in human embryo development implies. Future research should investigate if incorporating paternal BMI in prediction models is of additional value. Although male obesity is linked to adverse reproductive outcome, it is unknown which specific conditions, such as intrinsic excessive chronic oxidative stress exposure affects spermatozoa, or is a mediator in the causation. Our study did not investigate if different levels of reactive oxygen species in semen of men with different weight classes that could lead to oxidative stress in spermatozoa. To further validate our findings we recommend to add the measurement of oxidative stress in seminal plasma in future studies on the same topic.

Conclusion

In this study, we show that paternal body weight has a strong negative association with fertilization rate and a positive association on the development of embryos especially in the first cleavage divisions. We found no associations between paternal body weight and pregnancy chance and live birth rate. Explanations for our findings might be the induced alterations in sperm quality, DNA damage and epigenetic programming caused by chronic exposure to excessive oxidative stress and altered glucose and estrogen levels. Our results demonstrate a paternal impact on pre-implantation embryo development with potential consequences for the post-implantation embryo. Therefore, more research has to be done to investigate if there is a direct impact of paternal obesity on the reproductive outcomes through mechanisms such as excessive intrinsic oxidative stress. In general, it remains important to advise overweight and obese men to achieve a healthy nutritional state and lose weight prior to treatment to optimize the outcome of a time intensive and expensive fertility treatment.

Supplemental table 1: Baseline characteristics of the study population of men participating in the Virtual Embryoscope cohort.

	Normal weight men	Overweight men	Obese men	p-value
	N=86	N=94	N=41	
Age (years)	34.5 [31.8– 38.3]	35.4 [32.1– 40.4]	36.6 [30.9– 40.8]	**0.01**
Geographic origin				**0.01**
Dutch	76 (88.4%)	73 (77.7%)	30 (73.2%)	
Western	3 (3.5%)	2 (2.1%)	1 (2.4%)	
Non-Western	7 (8.1%)	19 (20.2%)	10 (24.4%)	
Educational level				**0.01**
Low	12 (14.0%)	17 (18.3%)	9 (22.0%)	
Intermediate	27 (31.4%)	43 (46.2%)	23 (56.1%)	
High	47 (54.7%)	33 (35.5%)	9 (22.0%)	
BMI, measured (kg/m^2)	23.2 [22.0-24.0]	27.2 [26.1–28.1]	32.2 [30.9-35.3]	**0.001**
Waist-hip ratio	0.86 [0.82-0.89]	0.89 [0.84-0.93]	0.94 [0.90-0.97]	**0.001**
Folic acid supplement use				0.20
–0,4-0,5 mg/day	6 (7.0%)	8 (8.5%)	1 (2.4%)	
–5mg/day	2 (2.3%)	2 (2.1%)	0 (0%)	
Vitamin use, yes	17 (22.7%)	27 (32.5%)	9 (23.7%)	0.06
Alcohol consumption, yes	54 (62.8%)	67 (71.3%)	20 (48.8%)	**0.01**
Smoking, yes	15 (17.4%)	20 (21.3%)	8 (19.5%)	0.87

Data are presented as median [interquartile range (IQR)] or n (%).

Supplemental table 2. Fertilization and embryo usage rates in the total, IVF and ICSI study population of men stratified for categories of normal, overweight and obesity.

Treatment outcomes:	Normal weight men			Overweight men			Obese men		
	Total	IVF	ICSI	Total	IVF	ICSI	Total	IVF	ICSI
Fertilisation rate	0.88 (0.74-1.00)	0.89 (0.85-1.00)	0.78 (0.67-1.00)	0.81 (0.67-1.00)	0.87 (0.80-1.00)	0.72 (0.58-0.86)	0.76 (0.51-1.00)	0.77 (0.46-1.00)	0.72 (0.54-1.00)
Embryo usage rate	0.67 (0.44 – 0.86)	0.64 (0.42 – 0.84)	0.67 (0.50 – 0.90)	0.71 (0.50–1.00)	0.73 (0.56–1.00)	0.66 (0.50 –0.96)	0.67 (0.50 -1.00)	0.74 (0.50–1.00)	0.65 (0.40–1.00)

Data are presented as median [interquartile range (IQR)]

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PART III

Post-implantation embryo development

CHAPTER 5

Does the father matter? The association between the periconception paternal folate status and embryonic growth

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BACKGROUND: The periconception folate metabolism is critical for synthesis of DNA, proteins and epigenetic programming. Maternal folate status influences embryonic growth and DNA methylation of the neonate. Moreover, paternal folic acid status can influence the seminal epigenome. Therefore, we hypothesize that paternal folate status also affects embryonic growth due to changes of the seminal DNA methylation pattern. Here we investigate the association between periconception paternal folate status and first-trimester embryonic growth.

METHODS: A total of 507 singleton pregnancies, 205 spontaneously conceived and 302 after IVF/ICSI treatment, were selected from the prospective Rotterdam Periconception Cohort in which couples participated. Women underwent longitudinal transvaginal three-dimensional ultrasound examinations at 7, 9 and 11 weeks of gestation. Crown-rump-length (CRL) and embryonic volume (EV) measurements were performed offline using a virtual reality system (Barco I-Space). At the first visit, long-term paternal folate status was assessed by measuring red blood cell (RBC) folate levels. We analyzed associations between RBC folate divided into quartiles and CRL and EV measurements using linear mixed models and adjusted for paternal and maternal covariates.

RESULTS: Using the 3rd quartile of paternal red blood cell (RBC) folate levels as reference, we found significantly negative associations between RBC folate and longitudinal CRL measurements in the 2nd (beta: -0.14 √mm (95% CI: -0.28 to -0.006)) and 4th quartile (beta: -0.19 √mm (95%CI: -0.33 to -0.04)) in spontaneously conceived pregnancies. Comparable results were found for longitudinal EV measurements in the 4th quartile (beta: -0.12 $\sqrt[3]{}$cm3 (95% CI: -0.20 to -0.05)). No significant associations were observed between RBC folate levels and embryonic growth trajectories in IVF/ICSI pregnancies.

CONCLUSIONS: These data demonstrate for the first time that low and high periconception paternal RBC folate levels are associated with reduced embryonic growth trajectories in spontaneously conceived pregnancies. These data underlines also the importance of the paternal folate status during the periconception period.

Abstract

Introduction

One of the micronutrients of most interest is the B-vitamin folate serving as an essential substrate of 1-carbon metabolism involved in protein and lipid synthesis, DNA synthesis, repair and epigenetic programming (1, 2). The intake of folate is derived from foods and synthetic folic acid (FA) through supplements and as such provides 1-carbon moieties (2). During the last three decades, maternal FA supplement use, but also strong adherence to a folate-rich diet was shown to be beneficial for the prevention of congenital malformations, such as neural tube defects and congenital heart defects (3, 4). This evidence has contributed to the World Health Organization (WHO) recommendation of the use of low dose FA supplement (0.4 milligrams) from the preconception period up until the 10th week after conception (5). In many countries this has also resulted in mandatory FA food fortification, which already revealed substantial reductions in the birth prevalence rate of neural tube defects (NTD) (6).

Maternal FA supplement use and dietary folate intake also positively affect fetal growth (7). Pastor-Valero et al. showed that mothers with a dietary folate intake in the highest quintiles have a significantly lower risk of a neonate born small for gestational age (SGA). This is in line with a review by Van Uitert et al. showing that the maternal folate status is positively associated with birthweight and negatively with SGA (8). Moreover, our group previously demonstrated that both low and high periconception maternal folate levels determined by red blood cell (RBC) folate are associated with reduced growth trajectories of the embryo and embryonic cerebellum (9, 10).

Previous studies in man have largely been focused on the influence of the paternal folate status on semen parameters. Mainly cross-sectional studies show that serum and seminal folate levels as well as FA supplement use have a positive effect on semen quality (11, 12). Two randomized trials confirmed this data by also demonstrating that FA supplement use improved semen quality (13, 14). Boonyarangkul et al. showed that after a three month treatment with 5 mg FA per day the percentage of motile semen increased from 11.4% to 20.4% (13). With regard to pregnancy chance and outcome it is important to be aware that also men contribute half of their genetic material to the embryo. Therefore, their folate status is also critically involved in biological processes, such as DNA synthesis and epigenetic (re)programming of the seminal and subsequent embryonic genome (15). Of interest is that a study in humans revealed that a six-month treatment with 5 mg of FA supplements significantly changed the overall seminal DNA methylation pattern (16). A study in mice also showed that a paternal low dietary folate intake resulted in changes of the seminal epigenome, but also in an increase of malformations like craniofacial, muscle and skeletal anomalies (17). These findings contribute to our hypothesis that the periconception paternal folate status affects the seminal and embryonic epigenome with

consequences for embryonic growth trajectories. Associations between paternal folate levels and pregnancy outcomes are scarcely investigated. Pauwels et al. found no significant associations between paternal food folate intake, birthweight and DNA methylation in offspring (18). However, Ratan et al. found lower folate levels in fathers of children born with NTD when compared to a control group (19).

An technological advantage of the last two decades is that embryonic growth and development can be measured by use of state-of-the-art three-dimensional ultrasound (3D-US) techniques in combination with virtual reality technology. This approach provides an opportunity to study in very early pregnancy embryonic crown rump length (CRL) and embryonic volume (EV) more precisely using depth perception and three dimensional interaction (20).

As a first step to explore the impact of paternal folate status on pregnancy outcome, we investigate the association between the periconception paternal folate status and embryonic growth trajectories in spontaneously conceived pregnancies and those conceived after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Pregnancies conceived after IVF/ ICSI are associated with increased risks of preterm delivery and low birthweight (21). Moreover, from previous research it is known that embryonic growth patterns differ between spontaneously conceived pregnancies and IVF/ICSI (22). Therefore, and because of the relatively large group of IVF/ICSI pregnancies in the current study we stratified the analysis in spontaneous and IVF/ICSI pregnancies.

Methods

Study population

The data used for this study were collected as part of the Rotterdam Periconception Cohort (Predict study). This is an ongoing prospective tertiary hospital-based cohort embedded in the outpatient clinic of the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center Rotterdam, the Netherlands. The rationale and design of this cohort study have previously been published (23). Briefly, women with an ongoing intrauterine singleton pregnancy and less than 10+0 weeks GA were eligible for participation and recruited from November 2010 onwards. Inclusion criteria are that the woman and her partner have to be at least 18 years of age and familiar with the Dutch language in speaking and reading. All male partners of the study participants were explicitly asked to participate along with their partner.

For the current analysis, we only selected 1) women with a strict regular menstrual cycle between 25 and 31 days who conceived spontaneously, and 2) women who conceived through IVF/ICSI treatment.

Pregnancies conceived in women with a strict regular menstrual cycle are dated based on their first day of last menstrual period. In women with an irregular cycle, dating of a pregnancy using this method is not possible since the exact conception date is unknown. Therefore, in these women pregnancy dating is based on crown-rump length (CRL) by ultrasound measurements. Since, in our study CRL is one of the main outcome parameters, inclusion of pregnancies dated based on CRL would introduce a bias. In all other pregnancies, GA was either based on the reported last menstrual period (for spontaneously conceived pregnancies) or on the conception date (embryonic transfer date for pregnancies conceived after IVF or ICSI). Pregnancies conceived through intrauterine insemination (IUI) and hormonal therapy were considered spontaneous pregnancies. We excluded pregnancies conceived using donor semen, oocyte donation, with congenital malformations and intrauterine fetal deaths.

Study parameters

Both parents completed a self-administered questionnaire covering details on age, ethnicity, educational level and periconception FA supplement use, nutrition and lifestyle behaviour. Women also completed additional items on their obstetric history. All data were verified at study entry by a researcher or research nurse. Questions regarding paternal FA supplement use included if FA supplements were used, during which time-span (more or less than 10 weeks before conception), in which dosage (0.4mg, 0.5mg or 5 mg) and whether it was used as part of a vitamin supplement. Anthropometrics (i.e. weight and height) were measured by a research nurse.

Blood samples were taken at study entry between 7^{+0} and 9^{+6} weeks of gestation. Folate status was measured in RBC. The venous blood was collected in Vacutainer ethylenediaminetetraacetic acid (EDTA) tubes. Directly after blood sampling, the hemolysate was prepared by diluting 0.1mL full blood in 0.9mL fresh 1.0% ascorbic acid. The hematocrit of the remaining EDTA full blood was determined. After the hemolysate was centrifuged at 1000g for five minutes at 18 degrees Celsius, the serum folate levels were measured using electrochemiluminescence immunoassay (Modular E170, Roche GmbH, Mannheim, Germany). The following formula was applied to calculate the RBC folate concentration: (nM hemolysate folate * 10/hematocrit) – (nM serum folate * (1-haematocrit)/hematocrit)) = nM RBC folate.

Ultrasound data

From November 2010 till December 2012 women received weekly transvaginal 3D ultrasound scans from enrollment up to the 13th week of pregnancy. Since our pilot study showed that three ultrasound scans are sufficient to model embryonic growth curves accurately, from December 2012 onwards, the number of ultrasounds was reduced to three, precisely in the 7th, 9th and 11th week of gestation (24).

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). We have developed a protocol with standardized ultrasound settings for the image acquisition of the embryo and instructions for the patient to minimize movements. All trained sonographers followed this protocol very strictly to optimize image quality.

To optimally make use of the depth information present in 3D-ultrasound data, images were transferred to our Barco I-Space (a Cave Automatic Virtual Environment–like virtual reality system) (25). Here we create an interactive virtual reality hologram, which allows depth perception and interaction with the images projected. Per embryo and time point, CRL was measured three times, and the mean of these measurements was used for analysis. EV measurements were performed once at each time point using a semi-automatic method based on gray levels (25). All measurements were performed by trained research staff. The accuracy and reliability of this technique have been investigated at the start of this cohort study and were excellent with intraclass correlation coefficients of 0.99 for interobserver and intraobserver agreement (25).

Statistical analysis

The folate levels in RBC of the entire male study population were divided into four quartiles (Q), which led to the following categories: 524-874 nmol/L, 875-1018 nmol/L, 1019-1195 nmol/L and 1196-4343 nmol/L for Q1, Q2, Q3 and Q4, respectively. Main paternal characteristics in the four quartiles were compared using the Kruskal Wallis test for continuous variables and the chi-square test for categorical variables. Since a previous study by our group showed that maternal RBC folate status in Q3 was associated with the largest embryonic growth trajectories, we also selected the third quartile as reference category in the current study (9).

Correlation between the paternal and maternal folate status was assessed using the Pearson correlation coefficient. To take into account the correlation between measurements of the same pregnancy, we used a linear mixed model to assess the associations between paternal RBC folate levels and embryonic growth trajectories, assessed by longitudinal CRL and EV measurements between 7-12 weeks GA. For analysis, we used a square root transformation for the CRL measurements and a third root transformation for the EV measurements,

which led to linearity with GA. For the graphs of these embryonic growth trajectories we retransformed the modelled CRLs and EVs to the original values.

In the first model, we adjusted for GA at the time of the ultrasound. In the second model, we additionally adjusted for the paternal covariates age, smoking (yes/no), alcohol (yes/no) and geographic origin (Western/Non-Western). In the third model, we also adjusted for the maternal covariates age, BMI, smoking (yes/no), alcohol (yes/no), parity (nulliparous/multiparous), RBC folate levels, educational level (low/middle/high), geographic origin (Western/Non-Western) and fetal gender (male/female).

P-values ≤0.05 were considered statistically significant. All analyses were performed using SPSS package 21.0 (IBM SPSS Statistics, Armonk, NY) and R (R: A language and Environment for Statistical Computing, version 3.5, for Windows, R Core Team, Vienna, Austria).

Ethical approval

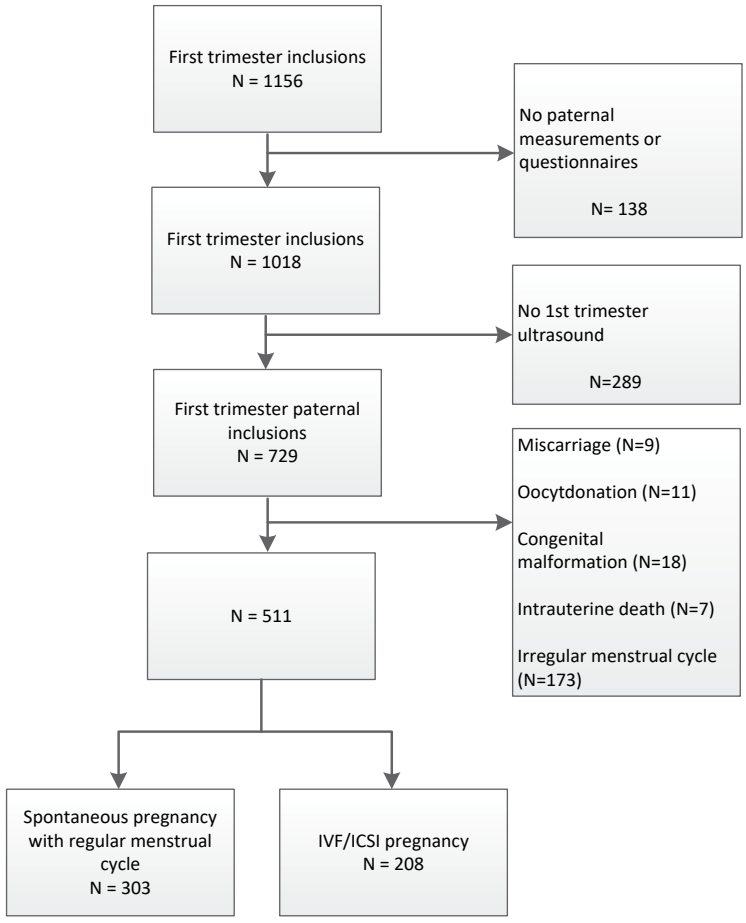
This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving patients were approved by the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Centre, Rotterdam, the Netherlands. Written informed consent was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

Results

From a total of 1,156 included pregnant women in the cohort, 1,018 (88.1%) participated with their male partner. Due to missing ultrasound data and women who did not undergo a first trimester ultrasound we excluded 289 patients. Furthermore, 218 pregnancies were excluded because of miscarriage (n=9), oocyte-donation (n=11), congenital malformations (n=18), intrauterine fetal death (n=7) and irregular menstrual cycle (n=173) **(Figure 1)**. From the remaining 511 included pregnancies, a total of 303 were spontaneous pregnancies and 208 were IVF/ICSI pregnancies. Of the spontaneously conceived pregnancies, 13 occurred after intra-uterine insemination and 12 after ovulation induction.

The median age of the men was 34.2 years and the majority (n=448; 87.8%) was of Western origin and highly educated (n=248; 48.7%). A total of 374 (75.6%) men consumed alcohol and 151 (30.6%) men smoked during the periconception period. A total of 42 (8.1%) men used FA supplements and 28 (5.5%) of them used FA as part of a vitamin supplement; 29 (80.6%) men used the supplements for more than 10 weeks **(Supplemental table 1)**.

Figure 1: Flowchart of in- and exclusions of the study population.



legend: Abbreviations: IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection

The median age of the women was 32.4 years and the majority (n=450; 88.8%) was of Western origin and highly educated (n=295; 58.1%). Alcohol consumption and smoking were reported by 167 (33.1%) and 75 (14.8%) women, respectively. The vast majority (n=502; 98.3%) of all women used FA supplements, either 0.4 mg (49.1%), 5 mg (2.2%) or as a part of multivitamin use (47.0%) **(Supplemental table 1)**. The correlation between maternal and paternal folate levels was 0.191 (p <0.01).

Paternal age, geographic origin, alcohol consumption and FA supplement use were significantly different among the four quartiles of RBC folate levels **(Table 1)**. Posthoc analysis showed significant differences between Q1 and Q4 regarding geographic origin,

Table 1 Baseline characteristics of the male study population stratified for red blood cell (RBC) folate level quartiles (Q).

	Q1 (524-874 nmol/L) N=108	Q2 (875-1018 nmol/L) N=108	Q3 (1019-1195 nmol/L) N=113	Q4 (1196-4343 nmol/L) N=99	*p-value*
RBC folate levels (nmol/L)	772	939	1103	1338	<0.001
Age, years	33.7 (31.3-37.3)	33.8 (30.7-37.2)	34.1 (31.4 – 39.1)	35.9 (32.6-40.0)	0.026[a]
Missing	0	0	0	0	
Parity, nulliparous	46 (43.8)	46 (44.7)	54 (49.1)	55 (59.1)	0.13
Missing	4	5	6	3	
Geographic origin					
Western	88 (82.2)	98 (90.7)	99 (87.6)	96 (97.0)	0.007[a]
Non-Western,	19 (17.8)	10 (9.3)	14 (12.4)	3 (3.0)	
Missing	1	0	0	0	
Educational level					
High	44 (42.5)	52 (48.1)	57 (50.4)	51 (51.5)	0.35
Intermediate	40 (37.7)	41 (38.0)	44 (38.9)	34 (34.3)	
Low	22 (19.8)	14 (13.0)	10 (8.8)	12 (12.1)	
Missing	2	1	2	2	
BMI (measured), kg/m^2	26.0 (23.1-28.5)	25.8 (23.8-28.1)	25.4 (23.7-27.4)	26.9 (24.4-28.4)	0.12
Missing	11	4	4	9	
Mode of conception					
Spontaneous	59 (54.6)	61 (56.5)	62 (54.9)	50 (50.5)	0.39
IVF/ICSI	34 (31.5)	35 (32.4)	41 (36.3)	43 (43.4)	
Missing	15	12	10	6	
Alcohol use	71 (69.6)	72 (71.3)	91 (82.0)	84 (85.7)	0.013[a]
Missing	6	7	1	2	
Smoking	42 (40.8)	33 (32.7)	29 (26.6)	27 (27.6)	0.11
Missing	5	7	1	4	
Folic acid supplement use					
Yes, with vitamin	2 (1.9)	1 (0.9)	9 (8.0)	14 (14.1)	0.004[a,b]
Yes, 0.4/0.5mg folic acid	1 (0.9)	2 (1.9)	4 (3.6)	4 (4.0)	0.82
Yes, 5.0 mg folic acid	0	0	0	0	
More than 10 weeks	2 (100)	2 (66.7)	9 (81.8)	13 (76.5)	
Less than 10 weeks	0	1 (33.3)	2 (18.2)	4 (23.5)	
Missing	0	1	1	0	

Data are presented as medians (IQR) or number of subjects (%). Significant differences in the post-hoc analysis over the various groups: a Q1 vs Q4, b Q2 vs Q4

alcohol consumption and folic acid supplement use (p=0.001, p=0.006 and p=0.003 respectively), and between Q2 and Q4 regarding folic acid supplement use (p=0.002).

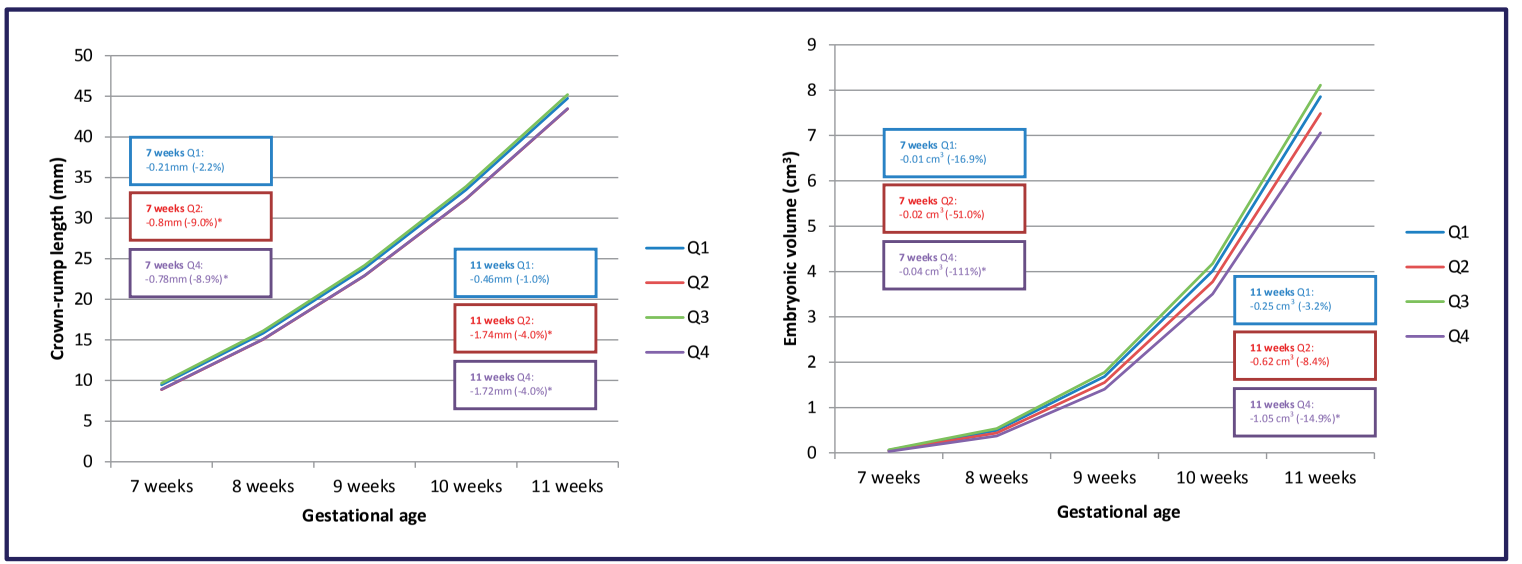
Regression analysis (Model 1) in the entire study population (i.e. IVF/ICSI pregnancies and spontaneous pregnancies) showed a significantly negative association between paternal RBC folate status and the CRL trajectories in Q2 (beta: -0.10 (95%CI: -0.18 to -0.02)) compared

Table 2 The group of spontaneously conceived pregnancies; betas from the models for the different paternal red blood cell (RBC) folate level quartiles with respect to crown-rump length (CRL) and embryonic volume (EV).

	Model 1				**Model 2**				**Model 3**			
	CRL		EV		CRL		EV		CRL		EV	
Variables	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P
RBC Folate Q1	-0.03 (-0.15 to 0.08)	0.57	-0.02 (-0.08 to 0.04)	0.49	-0.02 (-0.14 to 0.10)	0.77	-0.01 (-0.07 to 0.05)	0.70	-0.01 (-0.15 to 0.12)	0.84	-0.009 (-0.08 to 0.06)	0.79
RBC Folate Q2	-0.13 (-0.25 to -0.01)	0.03	-0.05 (-0.11 to 0.01)	0.08	-0.10 (-0.23 to 0.02)	0.09	-0.04 (-0.10 to 0.02)	0.20	-0.14 (-0.28 to-0.006)	0.04	-0.06 (-0.12 to0.01)	0.11
RBC Folate Q3	Ref		Ref		Ref		Ref		Ref		Ref	
RBC Folate Q4	-0.13 (-0.25 to -0.01)	0.04	-0.09 (-0.15 to -0.03)	0.004	-0.16 (-0.29 to -0.03)	0.01	-0.10 (-0.17 to -0.04)	0.001	-0.19 (-0.33 to -0.04)	0.012	-0.12 (-0.20 to -0.05)	0.001

Model 1: Adjusted for gestational age
Model 2: Model 1 + paternal covariates (age, smoking, alcohol, geographic origin)
Model 3: Model 2 + maternal covariates (age, body mass index, smoking, alcohol, nulliparity, red blood cell folate levels, education, geographic origin, fetal gender)
Abbreviations: IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; CI, confidence interval.

Figure 2: Embryonic growth trajectories in the spontaneously conceived pregnancy group for crown-rump length (CRL) and embryonic volume (EV). Third quartile (Q3, green line) versus Q1, Q2 and Q4 (blue, red and purple line, respectively) are depicted, including the absolute and relative differences at 7 and 11 weeks of gestational age.



Legend: * Significantly different compared to Q3 ($p<0.05$)

to Q3 **(Supplemental table 2)**. For the EV trajectories a significantly negative association was found in Q4 (beta: -0.05 (95%CI: -0.09 to -0.005)) compared to Q3. After adjusting for paternal and maternal covariates these negative associations remained for the CRL trajectories when comparing Q2 with Q3 (beta: -0.11 (95%CI: -0.21 to -0.02)) and for EV trajectories when comparing Q4 with Q3 (beta: -0.06 (95%CI: -0.10 to -0.007)).

In the fully adjusted (Model 3) there was a significantly negative association in the spontaneously conceived pregnancies for CRL trajectories in Q2 (beta: -0.14 (95%CI: -0.28 to -0.006)) and Q4 (beta: -0.19 (95%CI: -0.33 to -0.04)) and for EV trajectories in Q4 (beta: -0.12 (95%CI: -0.20 to -0.05)) compared to Q3 **(Table 2)**. We did not observe any significant associations between paternal RBC folate status and CRL and EV trajectories in IVF/ICSI pregnancies **(Supplemental table 3)**.

Retransformation of the effect estimates in the spontaneous pregnancy group to the original scale showed that CRL and EV trajectories are largest in Q3 **(Figure 2).** For example, at 11 weeks gestation the CRL in Q3 was on average 0.46 mm (increase of 1.0%), 1.74 mm (increase of 4.0%) and 1.72 mm (increase of 4.0%) larger compared to Q1, Q2 and Q4, respectively. EV in Q3 was on average 0.25cm^3 (increase of 3.2%), 0.62cm^3 (increase of 8.4%) and 1.05cm^3 (increase of 14.9%) larger compared to Q1, Q2 and Q4 respectively at 11 weeks gestation.

Discussion

We show for the first time that both a low and high long-term paternal folate status, assessed by RBC folate levels, is associated with reduced embryonic growth trajectories based on longitudinal CRL and EV measurements between 7 and 11 weeks of GA. The paternal Q3 RBC folate level is associated with the largest embryonic growth trajectories compared with the Q2 and Q4 RBC folate. These associations were only present in spontaneously conceived pregnancies but not in IVF/ICSI pregnancies.

The main strengths of our study are the collection of multiple longitudinal three dimensional ultrasound scans very early in pregnancy, the precise embryonic CRL and EV measurements, paternal RBC folate levels that represent long-term folate status covering the preconception period, and detailed information regarding baseline characteristics. Several statistical models were developed in order to adjust for many confounders, in particular maternal RBC folate status to study the independent association with paternal folate status. Furthermore, our patients were included from a single hospital, standardized protocols were used for the outcome measurements and all RBC folate levels were measured in the same laboratory. Our study also has some limitations. Because participants were recruited in a tertiary referral

hospital, the possibility of a selected study population has to be taken into account. This tertiary population mainly consists of high risks pregnancies, which implies a high internal but low external validity by design. Extrapolating our findings to a general population therefore needs further investigation in another study. In our analysis we adjusted for numerous maternal and paternal covariates, minimizing the effects of residual confounding. However, residual confounding can never be fully excluded because of the observational character of this study.

Despite these methodological limitations, our results are very much in line with previously published data showing that maternal RBC folate levels in Q3 are associated with larger embryonic growth trajectories and with increased embryonic cerebellar growth trajectories (9, 10). In these women, folate levels in the Q3 ranged from 1513 – 1812 nmol/L, while the Q3 in our male population was lower and ranged from 1019- 1195 nmol/L. This difference might be explained by the fact that the vast majority of women used FA supplements in early pregnancy. Paternal and maternal folate levels were however weakly correlated in our study.

Surprisingly, we did not show a linear dose-response relationship, in which the first quartile resulted in reduced embryonic growth trajectories compared to the second quartile, and so on. We did however observe that all quartiles revealed reduced embryonic growth trajectories when compared to the third quartile. The clinical consequences of paternal RBC folate of each quartile for pregnancy outcomes like SGA, preterm birth, fetal congenital malformations and birth weight are not yet known. We do, however, know from previous studies that embryonic growth during the first trimester is correlated with estimated fetal weight in the second trimester of pregnancy (Pearson's correlation coefficient: 0.57) and with birthweight (Pearson's correlation coefficient: 0.15 (p=0.04)) (24, 26). Our study shows that embryonic volume at 11 weeks of gestation is reduced with 1% for Q1, 4% for Q2 and 4% for Q4 when compared with Q3 (**Figure 2**). CRL at 11 weeks of gestation is reduced with even 14.9% in Q4 when compared with Q3. These differences may be extrapolated to birthweights, an important determinant of health later in life. However, this necessitates more investigation to establish an optimum level of the periconception maternal and paternal folate status for embryonic growth. These optimum levels could be very helpful to determine the best folate levels and thereby also optimal embryonic growth.

The normal value of RBC folate ranges from 340-1020 nmol/L, which covers the first, second and partly the third quartile of our study population (27). The folate levels in our study population are comparable with studies that investigated the association between folate levels and semen parameters (28, 29). Compared with Q3, we did not see a significant effect of folate levels on embryonic growth parameters in the first quartile. This may be

explained by the fact that none of the men in our study had folate levels under the normal value (range 524 to 4343 nmol/L).

Although the percentage of men using FA supplements increases over the FA level quartiles, i.e. from 2.8% in the first quartile to 18.1% in the fourth quartile, the majority of men in our study did not use FA supplements. Thus, differences in folate levels in these men need to be attributed to different determinants of folate status, such as intake of dietary folate and lifestyle factors interacting with folate status. It is important to realize that, in contrast to countries such as Canada and the United States, folic acid food fortification is not as common in the Netherlands and the total number of food products with fortification is still relatively low.

Another explanation for differences in folate levels can be genetic variations for example in the methylenetetrahydrofolate reductase MTHFR-enzyme gene. The MTHFR enzyme is critical for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and thus essential in FA metabolism. Single nucleotide polymorphisms (SNPs) in the MTHFR-gene effect the enzymatic activity and the resulting folate levels, which can range from 648 nM to 811 nM (30). However, we did not investigate SNPs of the MTHFR-gene in our study population.

The findings of this study substantiate our hypothesis that the folate status during spermatogenesis influence the paternal epigenome of the embryo with consequences for embryonic growth trajectories. This is in line with a study showing a global loss of seminal methylation after 6 months of high dose FA supplement use of 5 mg per day (16). In addition, Wong et al. reported an increase in abnormal morphology of spermatozoa, implying that a high FA dose also can have detrimental effects on spermatogenesis (31). A recent study in mice showed that both very high (20 times the normal dosage; corresponding to 10 mg/day in humans) as well as very low dosage (0.7 times the normal dosage; corresponding to 0.3mg/day in humans) negatively affect sperm count and global methylation patterns. Strikingly is that this study also showed an increase in postnatal mortality in both groups compared to the control group (32). Other animal studies suggest that different paternal folate levels result in transgenerational decreased fetal liver and placental folate content with corresponding alterations in DNA methylation (33-35).

So far it remains largely unclear which embryonic genes are influenced by the paternal periconception folate status, though candidate genes are those involved in general growth and differentiation. Of special interest are genes that have a differential parental of origin expression of the alleles (imprinting). These genes are subject to epigenetic changes and make them a target for environmental interactions with the genome. The insulin-like growth factor 2 (IGF2) gene is maternally imprinted and paternally expressed and

is especially interesting since it is involved in growth and development. Periconception maternal FA supplement use is associated with differences in genomic methylation of the IGF2-differentially methylation region (IGF2 DMR) in the offspring (36).

Our data show that the differences in embryonic growth trajectories are clearly present in the group with spontaneously conceived pregnancies, but not in IVF/ICSI pregnancies. The most likely explanation is that the procedure of IVF/ICSI itself, including hormonal therapy, overrules the influence of the paternal folate status on the epigenetic (re)programming of the embryo and receptivity status of the endometrium. This is supported by the impact shown of the culture medium on the epigenetic programming of the embryo and in particularly of the imprinted genes (37).

With the worldwide increase of the use of fortified food, FA supplements and multivitamins, the risk of harmful effects of very high synthetic folate levels is becoming an issue of concern (38). Exposure to high dose synthetic FA can induce excessive oxidative stress in tissues and cells resulting in derangements of DNA synthesis and loss of methylation of amongst others the seminal epigenome. Because an increasing number of men used FA supplements over the four quartiles, the future measurement of synthetic FA levels can be helpful to further understand the benefits and risks of synthetic folic acid supplement use on embryonic growth trajectories, pregnancy outcomes like SGA and preterm birth, but also of health and disease risks in later life.

Conclusions

This study strongly suggests that in spontaneously conceived pregnancies both low and high levels of paternal periconception RBC folate are associated with reduced embryonic growth trajectories. This data underlines also the importance of a strong adherence to a folate rich diet and supports the need of involving couples in preconception care. Further research is needed in the general population to further validate our findings.

Supplemental table 1: Baseline characteristics of men and women in the entire study population.

	Men (N=511)	Missing	Women (N=511)	Missing
Age, years	34.2 (31.5-38.3)	14	32.4 (29.6-35.7)	5
Parity, nulliparous	-	-	231 (47.1)	21
Geographic origin				
Western	448 (87.8)	1	450 (88.8)	4
non-Western	62 (12.2)		57 (11.2)	
Educational level				
High	248 (48.7)	2	295 (58.1)	3
Intermediate	187 (36.7)		177 (34.8)	
Low	69 (13.6)		35 (6.9)	
BMI (measured), kg/m^2	25.9 (23.9-28.3)	59	24.2 (21.8-27.9)	32
Mode of conception				
Spontaneous	-	-	303 (59.3)	-
IVF/ICSI			208 (40.7)	
Alcohol use	374 (75.6)	16	167 (33.1)	6
Smoking	151 (30.6)	18	75 (14.8)	5
Folic acid supplement use				
Yes, as part of multivitamin preparation	28 (5.5)	2	240 (47.0)	2
Yes, 0.4/0.5mg folic acid	14 (2.6)	5	251 (49.1)	
Yes, 5.0 mg folic acid	0		11 (2.2)	
More than 10 weeks	29 (80.6)			
Less than 10 weeks	7 (19.4)			

Data are presented as medians (IQR) or number of subjects (%). Abbreviations: IQR, interquartile range; BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection;

Supplemental table 2: Total group of spontaneously conceived pregnancies and IVF/ICSI pregnancies; betas from the models for the different paternal folate level quartiles (Q) with respect to crown-rump length (CRL) and embryonic volume (EV).

	Model 1				**Model 2**				**Model 3**			
	CRL		EV		CRL		EV		CRL		EV	
Variables	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P
RBC Folate Q1	-0.03 (-0.11 to 0.05)	0.41	-0.01 (-0.05 to 0.03)	0.56	-0.02 (-0.11 to 0.06)	0.60	-0.009 (-0.05 to 0.03)	0.69	-0.02 (-0.11 to 0.08)	0.70	-0.003 (-0.05 to 0.05)	0.89
RBC Folate Q2	-0.10 (-0.18 to -0.02)	0.01	-0.04 (-0.08 to 0.0)	0.05	-0.09 (-0.17 to -0.006)	0.04	-0.03 (-0.08 to 0.01)	0.13	-0.11 (-0.21 to -0.02)	0.02	-0.04 (-0.08 to 0.01)	0.14
RBC Folate Q3	Ref		Ref		Ref		Ref		Ref		Ref	
RBC Folate Q4	-0.06 (-0.14 to 0.02)	0.15	-0.05 (-0.09 to -0.005)	0.03	-0.07 (-0.16 to 0.01)	0.08	-0.05 (-0.09 to -0.003)	0.03	-0.09 (-0.19 to 0.003)	0.06	-0.06 (-0.10 to -0.007)	0.03

Model 1: Adjusted for gestational age
Model 2: Model 1 + paternal covariates (age, smoking, alcohol, geographic origin)
Model 3: Model 2 + maternal covariates (age, body mass index, smoking, alcohol, nulliparity, red blood cell folate levels, education, geographic origin, fetal gender). Abbreviations: IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; CI, confidence interval.

Supplemental table 3: The group with pregnancies conceived after IVF/ICSI treatment; betas from the models for the different paternal folate level quartiles with respect to crown-rump length (CRL) and embryonic volume (EV).

	Model 1				**Model 2**				**Model 3**			
	CRL		EV		CRL		EV		CRL		EV	
Variables	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P	Beta (95%CI) √mm	P	Beta (95%CI) $\sqrt[3]{cm^3}$	P
RBC Folate Q1	-0.03 (-0.12 to 0.05)	0.47	0.004 (-0.04 to 0.05)	0.86	-0.03 (-0.12 to 0.07)	0.56	0.003 (-0.05 to 0.05)	0.91	-0.03 (-0.14 to 0.08)	0.62	-0.002 (-0.06 to 0.06)	0.95
RBC Folate Q2	-0.05 (-0.14 to 0.03)	0.19	-0.02 (-0.07 to 0.02)	0.35	-0.05 (-0.14 to 0.04)	0.24	-0.01 (-0.06 to 0.03)	0.56	-0.08 (-0.18 to 0.03)	0.16	-0.02 (-0.08 to 0.04)	0.56
RBC Folate Q3	Ref		Ref		Ref		Ref		Ref		Ref	
RBC Folate Q4	0.02 (-0.06 to 0.10)	0.57	0.009 (-0.03 to 0.05)	0.69	0.03 (-0.05 to 0.12)	0.44	0.02 (-0.02 to 0.07)	0.30	0.03 (-0.07 to 0.13)	0.55	0.03 (-0.03 to 0.08)	0.32

Model 1: Adjusted for gestational age
Model 2: Model 1 + paternal covariates (age, smoking, alcohol, geographic origin)
Model 3: Model 2 + maternal covariates (age, body mass index, smoking, alcohol, nulliparity, red blood cell folate levels, education, geographic origin, fetal gender). Abbreviations: IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; CI, confidence interval.

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CHAPTER 6

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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BACKGROUND: Worldwide, an increasing number of fertility clinics are applying a 'freeze-all strategy' as a standard approach to IVF/ICSI treatment. The rationale for this is that pregnancy rates per transfer for frozen and fresh ET are similar, whereas maternal morbidity is significantly decreased after frozen-thawed ET by a reduction of the risk of ovarian hyperstimulation syndrome (OHSS). While there is a global increase in the use of frozen-thawed ET, concerns are being raised concerning its safety as increased odds for large for gestational age offspring are observed. The aim of this study is to investigate differences in embryonic and fetal growth trajectories and birth outcome between pregnancies conceived after in vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) treatment with either fresh embryo transfer (ET) or frozen-thawed ET, and naturally conceived pregnancies?

METHODS: 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 and 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% and 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.

CONCLUSIONS: 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.

Abstract

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 synchronization 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 synchronization 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 fertilization (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.

Materials and method

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. Written informed consent was obtained from all women and their male partner at enrolment.

Study population

The data for this study was 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 and known last menstrual period. Pregnancies conceived in women with a strict regular menstrual cycle are dated based on their first day of last menstrual period. In women with an irregular cycle, determining the gestational age based upon their menstrual cycle is not reliable since the expected time of ovulation is more uncertain. Therefore, in these women 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 fertilization 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-StepTM (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, conform 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% of 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 vapour. 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 the manufactures' 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% CO2 and 7% O2. 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

Standardized 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 periconception lifestyle behaviours. Extracted

data included maternal age, parity, geographic origin, educational level, periconception use of cigarettes, alcohol and folic acid (FA) supplements.

GA of pregnancies after frozen-thawed ET was calculated based on the time of fertilization 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 categorized 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 categorized 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. FA supplement use was defined as daily periconception use of FA supplements (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.

Ultrasound data

From November 2010 till 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-square 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, periconception 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-square test.

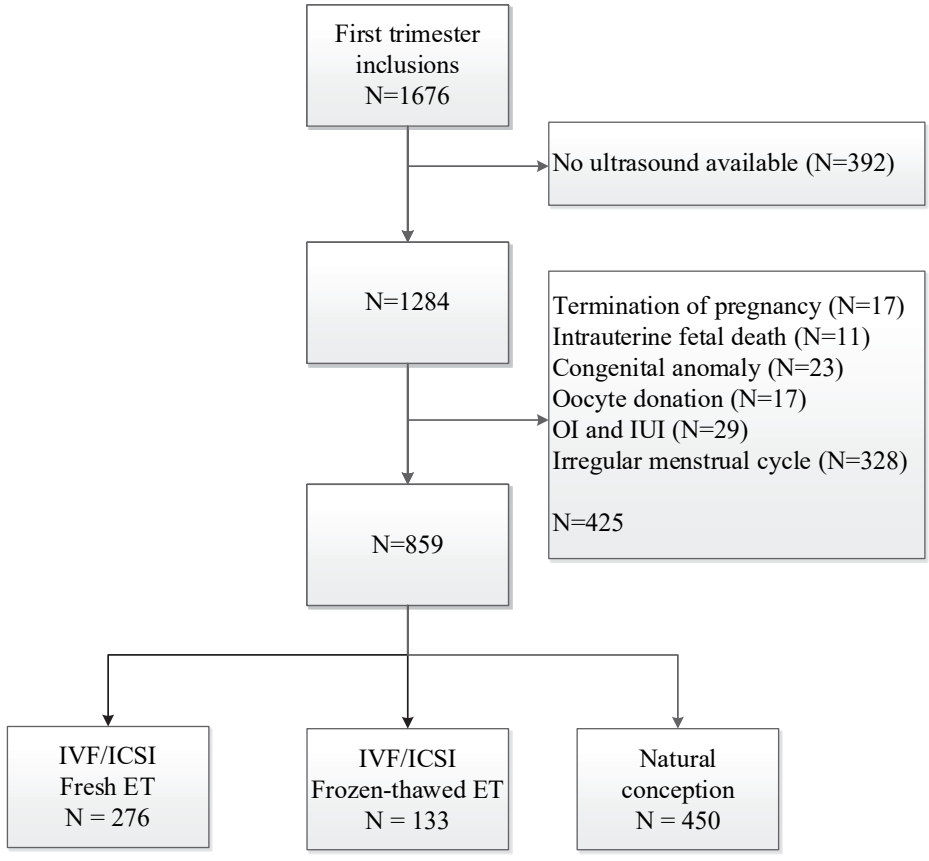
P-values <0.05 were considered statistically significant. All analyses were performed using SPSS package 25.0 (IBM SPSS Statistics, Armonk, NY) and R (R: A language and Environment for Statistical Computing, version 3.1.3, 2015 for Windows, R Core Team, Vienna, Austria).

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 malformations (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 within the groups of pregnancies complicated by congenital malformations and pregnancies without congenital malformations between fresh ET, frozen ET and naturally conceived pregnancies (p=0.211) (data not shown).

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

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



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), parity (52.3% vs. 45.1% nulliparous), geographic origin (83.1% vs. 75.2% Western) and periconception use of folic acid supplements (83.2% vs. 75.3% adequate use) 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 (3.7% vs. 13.4%, p=0.003), however the incidence of a vanishing twin syndrome was comparable (0.3% vs. 0.8%, p=0.601).

Other baseline characteristics were not significantly different between the two groups.

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<0.01)) (**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<0.01))

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

	IVF/ICSI Frozen-thawed ET n = 133		IVF/ICSI Fresh ET n = 276			
	Median	IQR	Median	IQR	*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, n/%	81	60.9	200	72.5	**0.018**	0
Geographic origin: Western, n /%	103	77.4	233	84.4	0.084	0
Education, n/%					0.650	14
Low	7	5.4	20	7.7		
Middle	53	41.4	100	37.6		
High	69	53.5	146	54.9		
Periconception lifestyle: n/%						
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	3.7	37	13.4	**0.003**	0
Vanishing twin syndrome	1	0.3	1	0.8	0.601	0

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.

and alcohol consumption (28.6%, 19.2% and 37.9%, respectively (p<0.01)). Women who conceived naturally also used folic acid significantly less adequate than women pregnant after fresh ET or after frozen ET (71.1%, 96.2% and 96.7%, respectively (p=<0.01)).

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 aforementioned confounders (model 2), 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: β 0.046 (95%CI: 0.018, 0.075)) (**Table 2**).

Second trimester:

The z-scores of midpregnancy EFW were comparable in pregnancies after frozen-thawed ET and after fresh ET (median z-score: 74.5 and 66.9 (p=0.068)). When compared to naturally conceived pregnancies, it was demonstrated that fetuses after frozen-thawed ET have a higher midpregnancy EFW than naturally conceived fetuses (median z-score: 74.5 and 68.1(post-hoc: 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 (48.0 and 38.0 (p=0.048)), whereas the gestational age at birth was lower (275 and 276 days (p=0.044)) (**Table 3**). There were no significant differences regarding the incidence of PIH, PE and GDM between the two groups. However, when iatrogenic causes of PTB (induction of 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)).

When compared to naturally conceived neonates, it was found that neonates after fresh ET have a lower relative birthweight (birthweight percentile (38.0 and 48.0 (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 incidence of PIH, PE and GDM between the three groups. However, the prevalence of non-iatrogenic PTB was lower in pregnancies after fresh ET compared to naturally conceived pregnancies (4.7% and 9.2% (post-hoc: p=0.033)).

Table 2. Beta's from the models for the different modes of conception with respect to estimates of growth trajectories of serial embryonic 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) $\sqrt[3]{cm^3}$	*P*-value	Beta (95%CI) √mm	*P*-value	Beta (95%CI) $\sqrt[3]{cm^3}$	*P*-value
Naturally conceived	reference		reference		reference		reference	
Fresh ET, day 3	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, day 5	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**
Fresh ET, day 3	reference		reference		reference		reference	
Frozen-thawed ET, day 5	-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

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)

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					
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	%		N	%		
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 causes	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.
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 (%).

Discussion

We observed that pregnancies after fresh ET compared with pregnancies after frozen-thawed ET exhibit similar first trimester growth trajectories and midpregnancy 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 confirmed by 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 in 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 on fresh embryos transferred 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 report no enhanced risk of PTB after cleavage-stage ET compared to blastocyst-stage ET (35, 36), whereas one large 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 is not yet investigated and 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 synchronization 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 use innovative methods and standardized protocols to precisely measure the outcome variables. We corrected for multiple confounders to minimize residual confounding, yet due to the observational character of the study this can never be completely excluded. The analyses were adjusted to minimize 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 ET are 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).

Conclusions

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 optimize 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.

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
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		
Periconception 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

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PART IV

Post-implantation placenta development

CHAPTER 7

The impact of the origin of surgical sperm retrieval on placental and embryonic development: The Rotterdam Periconception cohort.

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BACKGROUND: In patients with azoospermia, pregnancy can be achieved after surgical techniques using sperm retrieved from the testis or epididymis, which can impact on DNA integrity and epigenetics. DNA of the fetus and placenta is equally derived from both parents, however genes important for placental development are expressed from the paternal alleles. Therefore, the origin of sperm may affect fetal and placental development. The objective of this study is to investigate whether first-trimester trajectories of embryonic and placental development of pregnancies conceived after intracytoplasmic sperm injection(ICSI) with testicular sperm extraction(TESE) or microsurgical epididymal sperm aspiration(MESA), are different from pregnancies after ICSI with ejaculated sperm or natural conceptions.

METHODS: A total of 147 singleton ICSI pregnancies, including pregnancies conceived after TESE (n=23), MESA (n=25) and ejaculated sperm (n=99), and 380 naturally conceived and 140 after IVF treatment without ICSI were selected from the prospective Rotterdam periconception cohort. Crown-rump length(CRL), embryonic volume(EV), Carnegie stages and placental volume(PV) at 7, 9 and 11 weeks of gestation were measured using 3D ultrasound and virtual reality technology.

RESULTS: Linear mixed model analysis showed no differences in trajectories of CRL, EV and Carnegie stages between pregnancies conceived after ICSI with testicular, epididymal and ejaculated sperm. A significantly positive association was demonstrated for PV between pregnancies conceived after TESE-ICSI (adjusted beta: 0.28(95%CI: 0.05-0.50)) versus ICSI with ejaculated sperm. Retransformation to original values showed that the PV of pregnancies after TESE-ICSI is 14.6% (95%CI: 1.4%-25.5%) larger at 11 weeks of gestation compared to ICSI pregnancies conceived with ejaculated sperm.

CONCLUSIONS: Here we demonstrate that the first trimester growth trajectory of the placenta is increased in pregnancies conceived after TESE-ICSI compared to those conceived after ICSI with ejaculated sperm. Findings are discussed in the light of known differences in sperm DNA integrity, epigenetics, and placental gene expression.

Abstract

Introduction

Male factor subfertility is an increasing problem due to ageing, obesity and poor lifestyle, which can often be treated with lifestyle interventions (1, 2). In severe male factor subfertility, however, intracytoplasmic sperm injection (ICSI) is often successfully used to achieve pregnancy. Sperm used for assisted reproductive treatment (ART) can have different origins dependent on the underlying cause or diagnosis of the male factor. In cases of non-obstructive azoospermia (NOA) sperm can be retrieved surgically by testicular sperm extraction (TESE). TESE can also be performed in the case of post-vasectomy, iatrogenic, congenital or post-infectious obstructive azoospermia (OA), or when vasovasostomy or microsurgical epididymal sperm aspiration (MESA) failed (3). In both cases the sperm is not (TESE) or only partially (MESA) transported through the epididymis. It is becoming increasingly clear that this transport through the epididymis is a key factor in sperm maturation and functioning (4). The epididymis secretes epididymal specific proteins, hormones, small non-coding RNAs and these factors combined can influence sperm epigenetics, gene expression and modify the sperm surface (5). Although testicular sperm has not entered the anatomical part of the epididymis, it has the ability to activate the oocyte after ICSI. However, testicular sperm morphological quality is usually low and several studies indicate that the incidence of chromosomal abnormalities is increased in testicular sperm (6, 7). These abnormalities are associated with a decreased chance of implantation and lower ongoing pregnancy rates, possibly due to an increased aneuploidy rate in embryos (7, 8). However, a recent study in more than 340.000 IVF/ICSI cycles showed no clinical differences between pregnancies conceived with testicular, epididymal or ejaculated sperm regarding pregnancy rate and full-term delivery (9). To overcome issues of timing and multiple surgical procedures for men, sperm can also be cryopreserved and thawed for later use. Several studies show similar treatment outcomes between fresh and frozen sperm (10, 11).

The period of embryonic and placental growth in the first trimester of pregnancy is characterized by rapid cell multiplication and therefore vulnerable for alterations in both maternal- and paternal-originated DNA integrity and epigenetic (re)programming. Interestingly, paternally imprinted genes are predominantly expressed in the placenta (12).

Embryonic and placental growth can be reliably measured using three-dimensional ultrasound (3D-US) techniques in combination with virtual reality technology (13). This combination allows in-depth perception and assessment of growth by measuring Crown Rump Length (CRL) and embryonic volume (EV). Furthermore, it is possible to make an accurate assessment of the Carnegie stage based on internal and external morphological characteristics, as a marker of embryonic development (14).

Since there are a number of differences regarding chromosomal constitution and epigenetics between sperm retrieved after TESE and MESA as compared to ejaculated sperm combined with a large preferential paternal expression profile of the placental genome, we hypothesize that embryonic and placental growth and development might differ between these groups. The aim of this study is to investigate whether placental and embryonic growth trajectories of ICSI pregnancies conceived with TESE and MESA are different from pregnancies after ICSI with ejaculated sperm, IVF or naturally conceived.

Materials 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 (MEC-2004-227). Written informed consent was obtained from all female and male participants at enrolment.

Study population

Participants selected for this study were enrolled in the Rotterdam Periconception Cohort (Predict study) (15). This is an ongoing open prospective tertiary hospital-based cohort embedded in the outpatient clinic of the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center Rotterdam, the Netherlands. The design of the cohort study has previously been published (15). Women and their partners were eligible for inclusion if they were at least 18 years of age and had an ongoing intrauterine singleton pregnancy and were less than 10+0 weeks of gestational age (GA). Participants were recruited for inclusion from November 2010 onwards.

For the current analysis, we included pregnancies of women who conceived after ICSI in combination with testicular, epididymal, cryopreserved or ejaculated sperm. For a general reference group, we included pregnancies of women who conceived through IVF with ejaculated sperm and women who conceived naturally. GA was either based on the exact conception date for ICSI and the reported last menstrual period for naturally conceived pregnancies. We excluded pregnancies conceived after using donor semen or oocyte donation, and pregnancies complicated by congenital malformations and intrauterine fetal demise. Furthermore, we excluded pregnancies of women with an irregular menstrual cycle (menstrual cycle of less than 25 days or more than 31 days) or from which no first day of the last menstrual period was known, since in these pregnancies GA is based on CRL, which is our main outcome parameter.

Sperm retrieval

Men with non-obstructive azoospermia (NOA) all underwent TESE. In general, an obstructive component was excluded on medical history, physical examination, reproductive hormones (luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone) and scrotal ultrasound. Karyotyping and Y-chromosomal microdeletions were determined in all of these patients. All men with a diagnosed obstructive azoospermia (OA) initially underwent MESA. When motile sperm were microscopically detected, sperm cryopreservation was performed. When no motile sperm was found, TESE was performed. The latter group was excluded for analysis to create a homogenous TESE-ICSI group with only NOA patients.

TESE was performed under local anesthesia with a standard bilateral open surgical biopsy technique. A transverse two centimeter scrotal incision was made, the tunica albuginea was incised for one centimeter (cm) and a small fragment (approximately 1.5 cm(3) of testicular tissue was dissected using sterile glass slides. The collected tissue was subsequently minced and the resulting spermatogenic cell suspension was washed with HEPES-HTF, followed by centrifugation at 900 g (Thermo Scientific Centrifuge) for 10 minutes. The pellet was resuspended in one ml HEPES-HTF and subsequently diluted 1:1 with cryoprotectant (Test Yolk Buffer, Irvine Scientific, United States of America). When in 1 microliter suspension in 250 fields of view (FOV) at least one normal spermatozoa was found, cryopreservation was performed in straws (Cryo Bio System™, Irvine Scientific, United States of America) by placing them in liquid nitrogen vapor.

MESA was performed under local anaesthesia with a scrotal incision and microsurgically opening of one or more tubuli in the caput epididymii. Spermatozoa were aspirated with a micropipette attached to a tuberculin syringe filled with HEPES-buffered medium. The retrieved sperm was immediately diluted in HEPES-buffered medium and cryoprotectant was added before cryopreservation.

For logistic reasons, our clinic always freezes TESE and MESA sperm. In men requesting semen cryopreservation because of upcoming spermato-toxic treatments like chemotherapy for malignant diseases, fresh ejaculated sperm was diluted 1:1 with cryoprotectant (Test Yolk Buffer) and cryopreservation was performed in straws (Cryo Bio System™) by placing them in liquid nitrogen vapor. Semen cryopreservation was always performed prior to spermato-toxic treatment.

Ovarian stimulation and embryo culture

Ovarian stimulation was performed by either a GnRH-agonist or –antagonist followed by recombinant follicle stimulating hormone (FSH). Human chorionic gonadotrophin or a GnRH agonist were used as a trigger for final maturation of the oocyte and ovulation. Testicular, epididymal and cryopreserved sperm were thawed and the sperm cells with

best morphological and motile characteristics were selected for oocyte injection. After ovum pick-up, the collected oocytes were injected with either frozen-thawed testicular, epididymal or cryopreserved sperm or fresh ejaculated sperm. For the IVF treatment, fresh ejaculated sperm was washed and added to the dishes with oocytes.

All inseminated oocytes were cultured in G-1 PLUS cleavage stage medium ((Vitrolife, Goteborg, Sweden) between January 2010 to November 2014 and SAGE 1-StepTM medium (Origo/Cooper Surgical) from December 2014 onwards. All inseminated oocytes were cultured at 36.8 degrees Celsius with 7% oxygen and 5% carbon dioxide. The transfer of fresh embryos was performed on day 3 and the supernumerary embryos were cultured until day 4 and then cryopreserved. If a cryopreserved embryo was transferred, embryos were thawed and cultured overnight and transferred the following day corresponding to day 5 of embryo development.

Study parameters

Baseline characteristics and obstetric history were retrieved through self-administered questionnaires covering details on age, ethnicity and educational level. All data were verified at study entry by a researcher. Anthropometrics were measured by a researcher at study entry. Smoking and alcohol were defined as any consumption during the periconception period. Details regarding subfertility diagnoses, method of sperm retrieval and whether IVF or ICSI was used, were retrieved from the electronic patient files.

Ultrasound data

From November 2010 onwards women underwent three transvaginal ultrasounds, in the 7^{th}, 9^{th} and 11^{th} week of gestation. 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).

To optimally make use of the depth information present in 3D-ultrasound data, images were transferred to our Barco I-Space (a Cave Automatic Virtual Environment–like virtual reality system) (13). In the Barco I-space, an interactive virtual reality hologram, which allows depth perception, was created. All measurements were performed by trained research staff.

Outcome variables

Outcome variables were generated multiple times in the first trimester of pregnancy, which gives the opportunity to study growth trajectories over time. CRL was measured three separate times per time point and embryo, and the mean of these measurements was used for analysis. EV measurements were performed once at each time point per embryo using a semi-automatic method based on gray levels (16). Carnegie stages were determined once per time-point to assess external morphological features of the embryo such as the

development of the limbs and the curvature of the embryo, according to the protocol which was published previously (14). Placental volume (PV) in first trimester, also known as trophoblast volume, was measured once per time-point per pregnancy using Virtual Organ Computer-aided AnaLysis (VOCAL) (TM; GE Medical Systems, Zipf, Austria) (17). In short, twelve sections of the placenta were obtained using a rotational step of 15º. Trophoblast and myometrium can be distinguished by their difference in echogenicity, thereby calculating the total pregnancy volume. The volume of the placenta can be calculated by subtracting the gestational sac volume from the total pregnancy volume.

Statistical analysis

To take the correlation between measurements of the same pregnancy into account, we used a linear mixed model. In the first trimester of pregnancy, we used this linear mixed model to assess the associations between the different origins of sperm and CRL, EV, Carnegie stage and PV.

Furthermore, we used naturally conceived pregnancies and pregnancies after IVF with ejaculated sperm as a reference group for the different origins of sperm for ICSI.

For analysis, we used a square root transformation for the CRL measurements and a third root transformation for the EV and PV measurements, which led to linearity with GA. Carnegie stages were not transformed and used as continuous variable. For the graphs showing the EV and PV trajectories, the modelled EV and PV were retransformed to the original values.

In the first model, we adjusted for GA only. In the second model, we additionally adjusted for the paternal covariates age, smoking and alcohol use and maternal parity selected based on the characteristics of the study groups and literature.

P-values <0.05 were considered statistically significant. All analyses were performed using SPSS package 24.0 (IBM SPSS Statistics, Armonk, NY).

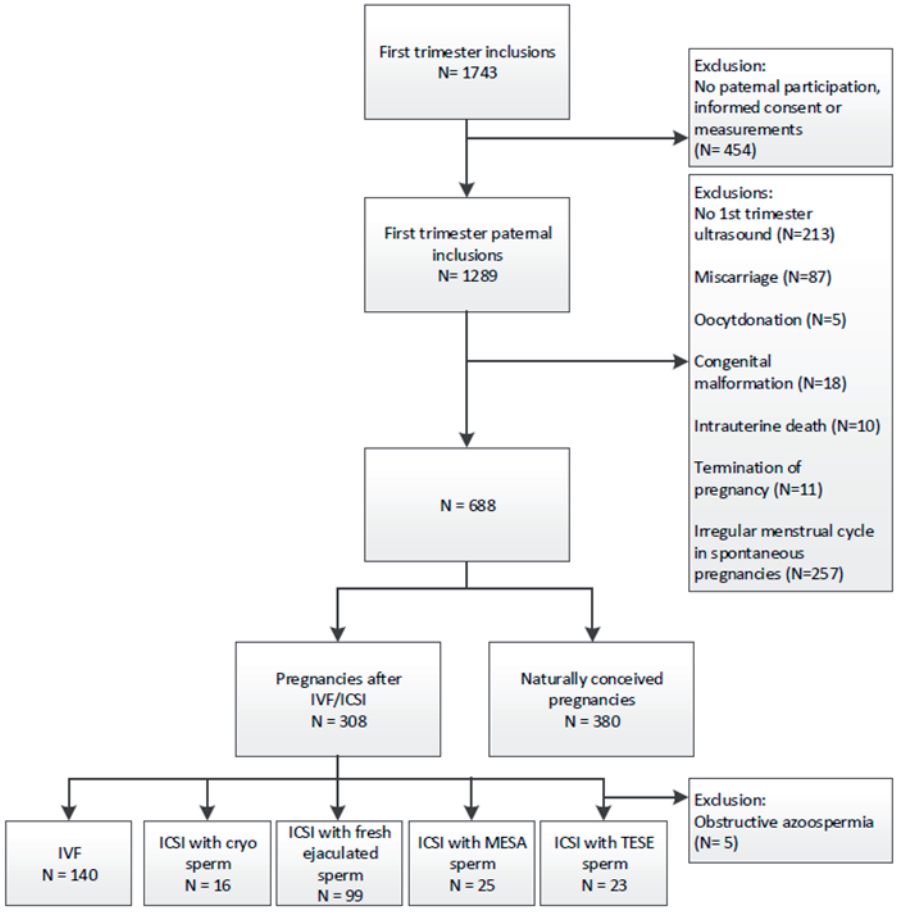
Results

Baseline

From a total of 1,743 pregnant women included in the cohort, 1,289 participated together with their male partner. Due to missing ultrasound data and women who did not undergo a first trimester ultrasound, 213 pregnancies were excluded. Furthermore, we excluded pregnancies because of miscarriage (n=87), oocyte-donation (n=5), congenital malformations (n=18), intrauterine fetal death (n=10) and termination of pregnancy

(n=11) and cases with an irregular menstrual cycle prior to natural conception (n=257). The remaining 688 included pregnancies comprised of 380 naturally conceived pregnancies, 140 pregnancies after IVF, and 168 pregnancies after ICSI. Of the included ICSI pregnancies, 28 were conceived after ICSI with TESE sperm. Five patients were excluded because of an obstructive azoospermia. The included 23 patients all underwent TESE because of a non-obstructive azoospermia. In the ICSI pregnancy group 25 resulted after ICSI with MESA sperm, 16 with cryopreserved sperm and 99 with freshly ejaculated sperm **(Figure 1)**. There are no significant differences regarding the distribution of the origin of the sperm between pregnancies complicated by congenital malformations and pregnancies without congenital malformations (p=0.09) and between pregnancies complicated by intra uterine fetal demise and pregnancies without intra uterine fetal demise (p=0.73) (data not shown).

Figure 1: Flowchart of the in- and exclusions of the study population.



Legend: Abbreviations: IVF; in vitro fertilization, ICSI; intracytoplasmic sperm injection, TESE; testicular sperm extraction, MESA; microsurgical epididymal sperm aspiration.

At baseline there were no significant differences regarding paternal age, BMI, geographical background, education, alcohol use and smoking between the different ICSI groups. Paternal age was significantly lower in the naturally conceived pregnancy group compared to the IVF group (33.6 vs 36.2 year respectively (*p<0.01*) (**Table 1**). Regarding maternal factors at baseline, we found no statistical differences for age, BMI, geographical origin, education and alcohol use. There was a significant difference regarding parity, where the percentage nulliparous in the naturally conceived group was lower compared to all other groups (*p<0.001*).

Of the included pregnancies resulting after TESE-procedures, the indication for ICSI treatment was because of either male (82.6%) or combined male-female factor subfertility (17.4%), with 100% being a non-obstructive azoospermia (**Supplemental table 1**).

No differences were seen regarding the embryonic growth parameters CRL and EV when comparing the TESE and MESA ICSI groups with freshly ejaculated sperm-ICSI (**Table 2 and Figure 2b**). Also, when comparing TESE-ICSI with naturally conceived pregnancies, EV trajectories were not different (Model 2: TESE-ICSI beta: 0.06 (95% confidence interval (CI): -0.01 to 0.13)) (**Table 3a**). A significantly positive association was found for Model 1 regarding embryonic development estimated in Carnegie stages when comparing MESA-ICSI with ICSI with freshly ejaculated sperm (beta: 0.38 (95%CI: 0.02 to 0.74)), however this effect attenuated after correction for confounders (beta: 0.17 (95%CI: -0.18 to 0.52)) (**Table 2**).

Model 1 showed a significantly positive association (beta: 0.23 (95% confidence interval (CI): 0.02 to 0.44)) regarding PV in the TESE-ICSI group when compared to ICSI with freshly ejaculated sperm (**Table 2**). After adjustment for paternal covariates age, smoking, alcohol and maternal parity (Model 2) the significantly positive association remains (beta: 0.28 (95%CI: 0.05 to 0.50)). Retransformation of the betas to the original values showed that PVs of pregnancies after TESE-ICSI are 14.6% (95%CI: 1.4% to 25.5%) larger at 11 weeks of gestation as compared to pregnancies after fresh ejaculated-ICSI (**Figure 2a**).

To investigate the effect of cryopreservation, we pooled the groups with TESE and MESA treatment and cryopreserved sperm, and compared them with the group of ICSI with freshly ejaculated sperm. Again, we found no statistically significant differences regarding trajectories of CRL, EV, PV and Carnegie stage (**Table 3b**).

Table 1. Baseline characteristics of the study population of (sub)fertile couples

Conception mode	**ICSI with TESE sperm** N=23	**ICSI with MESA sperm** N=25	**ICSI with cryopreserved sperm** N=16	**ICSI with freshly ejaculated sperm** N=99	***p-value*** *Differences over the ICSI groups*	**IVF** N=140	**naturally conceived** N=380	***p-value*** *Differences between all the groups*
Men								
Age, years median (IQR)	32.8 (29.9-36.5)	36.3 (32.9-41.8)	35.2 (30.6-38.1)	35.2 (32.1-38.6)	0.08	36.2 (32.6-39.9)	33.6 (30.5-37.3)	**<0.01**[a]
BMI (measured), kg/m^2 median (IQR)	26.2 (22.4-29.1)	27.3 (23.9-29.9)	26.6 (22.8-31.2)	26.5 (24.4-28.4)	0.59	26.0 (24.0-28.6)	25.6 (23.7-28.0)	0.23
Geographical origin					0.31			0.24
Western, n (%)	18 (78.3%)	19 (76.0%)	15 (93.8%)	86 (86.9%)		108 (77.1%)	317 (83.4%)	
non-Western, n (%)	5 (21.7%)	6 (24.0%)	1 (6.3%)	13 (13.1%)		32 (22.9%)	63 (16.6%)	
Education					0.99			0.77
High, n (%)	10 (45.5%)	9 (37.5%)	8 (50.0%)	40 (43.5%)		65 (50.0%)	179 (49.6%)	
Intermediate, n(%)	9 (40.9%)	11 (45.8%)	6 (37.5%)	40 (43.5%)		53 (40.8%)	126 (34.9%)	
Low, n(%)	3 (13.6%)	4 (16.7%)	2 (12.5%)	12 (13.0%)		12 (9.2%)	56 (15.5%)	
Alcohol use, n (%)	17 (81.0%)	17 (70.8%)	11 (73.3%)	60 (69.0%)	0.75	94 (75.2%)	272 (76.6%)	0.73
Smoking, n (%)	7 (33.3%)	4 (18.2%)	5 (33.3%)	20 (23.0%)	0.56	34 (27.2%)	120 (33.8%)	0.26
Women								
Age, years median (IQR)	30.0 (28.1-33.2)	31.1 (28.8-35.7)	30.8 (24.5-35.2)	32.5 (29.5-35.0)	0.09	34.6 (31.5-38.1)	31.7 (29.1.-34.9)	**<0.01**[g]
BMI (measured), kg/m^2 median (IQR)	24.4 (21.7-30.6)	22.5 (21.2-28.2)	25.6 (22.8-28.8)	24.9 (21.5-28.0)	0.43	24.2 (21.7-26.6)	24.2 (22.2-28.2)	0.39
Parity, nulliparous, n (%)	18 (81.8%)	17 (68.0%)	12 (75.0%)	70 (70.7%)	0.70	94 (67.6%)	150 (39.5%)	**<0.001**[f]
Geographical origin					0.08			0.11
Western, n (%)	16 (69.6%)	22 (88.0%)	15 (93.8%)	88 (88.9%)		113 (80.7%)	333 (87.6%)	
non-Western, n (%)	7 (30.4%)	3 (12.0%)	1 (6.3%)	11 (11.1%)		27 (19.3%)	47 (12.4%)	
Education					0.12			0.06
High, n (%)	7 (33.3%)	13 (54.2%)	5 (31.1%)	55 (57.9%)		84 (61.3%)	240 (63.3%)	
Intermediate, n(%)	12 (57.1%)	9 (37.5%)	11 (68.8%)	33 (34.7%)		45 (32.8%)	117 (30.9%)	
Low, n(%)	2 (9.5%)	2 (8.3%)	0 (0.0%)	7 (7.4%)		8 (5.8%)	22 (5.8%)	
Alcohol use, n (%)	6 (30.0%)	6 (24.0%)	5 (33.3%)	19 (19.8%)	0.57	30 (22.1%)	149 (39.6%)	0.06
Smoking, n (%)	7 (35.0%)	2 (8.0%)	5 (33.3%)	8 (8.3%)	**0.002**[b,c,d,e]	14 (10.2%)	60 (16.0%)	0.07

Posthoc test significant differences between: [a] naturally conceived and IVF, [b]TESE-ICSI and fresh ejaculated sperm, [c]TESE-ICSI and MESA-ICSI, [d]cryo sperm-ICSI and MESA-ICSI, [e]cryo sperm-ICSI and fresh ejaculated sperm, [f] naturally conceived and all other growth, [g]IVF and all other growth.

Abbreviations: IQR; interquartile range, IVF; in vitro fertilization, ICSI; intracytoplasmic sperm injection, TESE; testicular sperm extraction, MESA; microsurgical epididymal sperm aspiration, BMI; body mass index.

Table 2: Betas of embryonic development (CRL, EV, Carnegie stages) and placental growth trajectories (PV) of different origins of sperm retrieval compared to ICSI with ejaculated sperm

	Model 1								Model 2							
	CRL		EV		Carnegie		PV		CRL		EV		Carnegie		PV	
	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	**Beta (95% CI)**	P-value	**Beta (95% CI)**	P-value
ICSI with ejaculated sperm	Reference		Reference		Reference		Reference		Reference		Reference		Reference		Reference	
ICSI with TESE sperm	0.02 (-0.08 to 0.11)	0.70	0.02 (-0.03 to 0.06)	0.54	0.14 (-0.25 to 0.52)	0.49	**0.23 (0.02 to 0.44)**	**0.03**	0,01 (-0.08 to 0.11)	0.80	0.02 (-0.03 to 0.07)	0.40	0.20 (-0.16 to 0.57)	0.28	**0.28 (0.05 to 0.50)**	**0.02**
ICSI with MESA sperm	0.01 (-0.08 to 0.09)	0.83	0.02 (-0.03 to 0.06)	0.51	**0.38 (0.02 to 0.74)**	**0.04**	-0.01 (-0.21 to 0.20)	0.93	-0.02 (-0.11 to 0.08)	0.73	-0.003 (-0.05 to 0.05)	0.91	0.17 (-0.18 to 0.52)	0.34	0.03 (-0.20 to 0.27)	0.77
ICSI with cryopreserved sperm	-0.001 (-0.11 to 0.11)	0.98	-0.002 (-0.06 to 0.06)	0.95	0.21 (-0.25 to 0.67)	0.37	0.01 (-0.27 to 0.29)	0.95	-0.02 (-0.14 to 0.09)	0.71	-0.01 (-0.07 to 0.05)	0.72	0.19 (-0.24 to 0.63)	0.38	0.06 (-0.23 to 0.35)	0.67

Model 1: Adjusted for gestational age. Model 2: Model 1 + adjusted for paternal age, smoking and alcohol and maternal parity
Abbreviations: CRL; crown rump length, EV; embryonic volume, PV; placental volume, CI; confidence interval, ICSI; intracytoplasmic sperm injection, TESE; testicular sperm extraction, MESA; microsurgical epididymal sperm aspiration

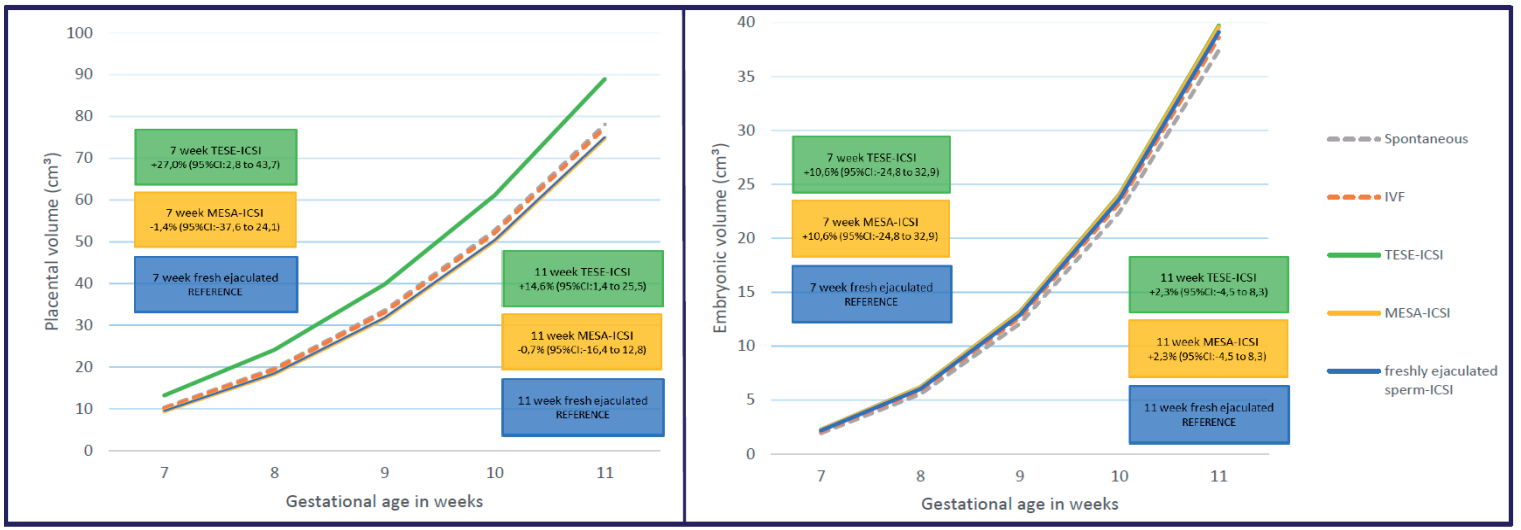
Table 3. Betas of embryonic development (CRL, EV, Carnegie stages) and placental growth trajectories (PV) of different origins of sperm retrieval compared to 3A. naturally conceived pregnancies and 3B. all cryopreserved sperm compared to freshly ejaculated sperm.

Table 3A	Model 1								Model 2							
	CRL		EV		Carnegie		PV		CRL		EV		Carnegie		PV	
	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	**Beta (95% CI)**	P-value	**Beta (95% CI)**	P-value
Naturally	Reference		Reference		Reference		Reference		Reference		Reference		Reference		Reference	
IVF	**0.06 (0.003 to 0.12)**	**0.04**	**0.04 (0.01 to 0.06)**	**0.02**	**0.21 (0.01 to 0.41)**	**0.04**	-0.01 (-0.11 to 0.08)	0.77	0.03 (-0.03 to 0.10)	0.32	0.03 (-0.01 to 0.06)	0.11	0.15 (-0.06 to 0.36)	0.16	0.03 (-0.07 to 0.14)	0.52
ICSI with ejaculated sperm	**0.08 (0.01 to 0.14)**	**0.02**	**0.05 (0.02 to 0.08)**	**0.003**	**0.27 (0.05 to 0.50)**	**0.02**	-0.06 (-0.17 to 0.06)	0.34	0.06 (-0.01 to 0.13)	0.10	**0.04 (0.01 to 0.08)**	**0.02**	0.19 (-0.05 to 0.42)	0.12	-0.07 (-0.19 to 0.06)	0.28
ICSI with TESE sperm	0.09 (-0.03 to 0.22)	0.13	**0.07 (0.01 to 0.13)**	**0.04**	0.41 (-0.01 to 0.84)	0.06	0.19 (-0.01 to 0.39)	0.06	0.09 (-0.04 to 0.22)	0.17	0.06 (-0.01 to 0.13)	0.11	0.27 (-0.16 to 0.71)	0.22	0.21 (-0.002 to 0.42)	0.05
ICSI with MESA sperm	0.08 (-0.04 to 0.20)	0.17	**0.06 (0.004 to 0.12)**	**0.04**	**0.65 (0.25 to 1.04)**	**0.001**	-0.06 (-0.26 to 0.13)	0.53	0.01 (-0.11 to 0.14)	0.83	0.03 (-0.03 to 0.10)	0.34	0.26 (-0.16 to 0.68)	0.22	-0.04 (-0.27 to 0.18)	0.71
ICSI with cryo sperm	0.08 (-0.07 to 0.23)	0.29	0.05 (-0.02 to 0.12)	0.18	0.49 (-0.03 to 1.00)	0.07	-0.02 (-0.29 to 0.24)	0.86	0.08 (-0.08 to 0.23)	0.33	0.05 (-0.03 to 0.13)	0.20	0.46 (-0.07 to 0.98)	0.09	-0.01 (-0.27 to 0.26)	0.97
Table 3B	Model 1								Model 2							
	CRL		EV		Carnegie		PV		CRL		EV		Carnegie		PV	
	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	**Beta (95% CI)**	P-value	**Beta (95% CI)**	P-value
ICSI with ejaculated sperm	Reference		Reference		Reference		Reference		Reference		Reference		Reference		Reference	
ICSI with cryo-preserved sperm (TESE, MESA and cryo)	0.01 (-0.05 to 0.07)	0.75	0.01 (-0.02 to 0.04)	0.50	0.26 (-0.01 to 0.52)	0.06	0.09 (-0.06 to 0.24)	0.26	-0.01 (-0.07 to 0.06)	0.84	0.004 (-0.03 to 0.04)	0.82	0.19 (-0.06 to 0.43)	0.14	0.14 (-0.02 to 0.31)	0.09

Model 1: Adjusted for gestational age. Model 2: Model 1 + adjusted for paternal age, smoking and alcohol and maternal parity

Abbreviations: CRL; crown rump length, EV; embryonic volume, PV; placental volume, CI; confidence interval, IVF; in vitro fertilization, ICSI; intracytoplasmic sperm injection, TESE; testicular sperm extraction, MESA; microsurgical epididymal sperm aspiration.

Figure 2: Growth trajectories for the pregnancies conceived after ICSI (TESE, MESA, ejaculated), naturally conceived pregnancies and IVF pregnancies regarding A. placental volume and B. embryonic volume.



Legend: Abbreviations: CI; confidence interval, IVF; in vitro fertilization, ICSI; intracytoplasmic sperm injection, TESE; testicular sperm extraction, MESA; microsurgical epididymal sperm aspiration.

Discussion

In this study, we show that pregnancies conceived after TESE-ICSI compared with ejaculated sperm-ICSI are associated with trajectories of increased PV in the first trimester of pregnancy, with an estimate of 14.6% increase at 11 weeks GA. Furthermore, we found no differences between TESE-ICSI and ICSI with ejaculated sperm regarding embryonic growth and development as measured by CRL, EV and Carnegie stages.

The placenta is initiated with the formation of trophectoderm, which is part of the developing blastocyst. After fusion of the trophoblast cells, two placental lineages are formed, the syncytiotrophoblasts and the cytotrophoblasts. After invasion of the syncytiotrophoblasts into decidualised endometrium, the syncytiotrophoblast cells come in contact with the maternal blood supply. The role of paternally expressed (and thus maternally imprinted) genes on early placental growth and development is only partially established (12). Imprinted genes have a parent-of-origin effect by preferential expression of either the maternal or paternal inherited allele, emphasizing the essential influence of paternally expressed genes for early placental and embryonic development. Shortly after conception, a global loss of DNA-methylation is initiated, followed by a remethylation starting from the blastocyst stage (18). However, since imprinted genes are largely unaffected by the demethylation wave, the epigenetic profile of the sperm cells at imprinted genes obtained during spermatogenesis is directly passed on to the resulting zygote and maintained in the (extra)embryonic tissues. Several paternally expressed genes, such as the *Paternally Expressed Gene* (*PEG*) *3* and *PEG 1*, all important in early growth and development, have shown to already be expressed in trophoblast cells (19, 20). Animal studies showed that a knockout model of *PEG 3* resulted in a significant reduction of placental size, whereas a knock out model of *PEG 1*, which is normally expressed in the invasive syncytiotrophoblasts, showed severe embryonic and placental growth restriction (21). Interestingly, previous research showed reduced DNA-methylation in testicular sperm of azoospermic men of the imprinted gene *H19* compared to sperm of fertile men (22). The first trimester of pregnancy represents a period of rapid cell division and development, where differences in epigenetic regulation of genes involved in growth can have a large impact. So far, no studies are known investigating differences in epigenetic marks, such as DNA-methylation, in neonates conceived after using TESE sperm. Considering our observation on the early developmental differences in placental volume in the first trimester, this would be of interest.

All testicular and epididydimal extracted sperm is cryopreserved after collection and thawed at the moment of oocyte pickup. Cryopreservation of sperm has been found to significantly alter sperm DNA methylation (23, 24). However, despite these reported epigenetic variations, we found no differences regarding embryonic and placental growth and development after pooling of all groups using cryopreserved sperm (TESE, MESA

and cryopreserved sperm) compared to ICSI with freshly ejaculated sperm. We cannot distinguish between the influence of the origin of the sperm or cryopreservation since in our clinic cryopreservation of the sperm is always used after TESE and MESA.

The finding of larger PV trajectories in early pregnancies conceived after TESE-ICSI can also be explained by differences in DNA integrity due to DNA damage. Although, the epididymis is thought to play an important role in sperm maturation, a recent meta-analysis showed that sperm DNA damage, measured by sperm DNA fragmentation, can be significantly higher in freshly ejaculated sperm compared to testicular sperm (25). The lower sperm DNA fragmentation can explain the larger placental development, in terms of better development with improved sperm DNA integrity. PV is associated with birth outcomes: pregnancies ending in miscarriage had smaller placental volumes during the first trimester as compared with those that result in a livebirth, indicating the potential beneficial role of using testicular sperm, since we found placental volume to be increased (17, 26). A confounding factor can be maternal smoking, since significantly more women smoked in the TESE-ICSI group. However since smoking is associated with smaller placental growth in the first trimester of pregnancy, PV is hypothesized to be even larger after using testicular sperm for ICSI in women who do not smoke.

Previous research investigating the association between surgically retrieved sperm and pregnancy outcomes comprised of far more participants than our study and mentioned no significant differences between testicular, epididymal and ejaculated sperm regarding the birth outcomes birthweight and preterm birth (27-29). The aim of our study was to gain more insight into the early (patho)physiology of the role of the origin of sperm and embryonic and placental development, not to study pregnancy outcomes. Our study was not powered to detect differences regarding pregnancy outcomes. The posthoc sample size calculation using an α-level of 0.05 and power of 80%, revealed that at least 80 participants in the total study group are needed to accurately show significance regarding placental volume, which were present in our study. This study revealed the magnitude of effect sizes which will aid to determine an optimal sample size for future larger studies or randomized controlled trials.

No effects were established regarding embryonic growth, as measured by CRL and EV, between the different sperm origins. Since paternally expressed genes are predominantly expressed in the placenta, we expected to show periconception paternal effects on PV trajectories. Our group previously showed that IVF/ICSI pregnancies exhibit larger embryonic growth compared to naturally conceived pregnancies indicating that the procedure itself can induce differences regarding embryonic development (30). Another possible explanation for a lack of detectable effect of TESE sperm on CRL and EV, could be

due to the strong impact of the IVF/ICSI procedure itself on epigenetic reprogramming of the embryo and endometrial receptivity (31, 32).

Our study has several strengths and limitations. A strength is the availability of multiple serial 3-D ultrasounds in the first trimester of the same pregnancy and the possibility to precisely assess several morphogenic features of embryonic and placental growth and development. Our group previously showed that early PV can reliably be measured with very high intra-class correlation coefficients (ICC>0.95) (33). In the present study, we confirm other studies that showed no significant differences regarding PV between naturally conceived pregnancies (n=84) and IVF/ICSI pregnancies (n=70) (39.8cm^3 and 40.2cm^3 respectively) (34, 35). Moreover, unique in our study is the comparison of differences in embryonic and placental growth trajectories in subgroups of ICSI with TESE, MESA and ejaculated sperm versus IVF (with ejaculated sperm) with naturally conceived conceived pregnancies as reference groups. Furthermore, we were able to differentiate between the effects of the cryopreservation procedure and the direct effect of TESE sperm itself by showing results of all cryopreserved sperm compared to ICSI with freshly ejaculated sperm. A limitation of our study remains a relatively low number of patients in the different subgroups. Since this study is an observational cohort, we adjusted for potential confounders, however residual confounding cannot be fully excluded. We used self-administered questionnaires, which are verified face to face at the first consultation by a trained research nurse, to obtain information regarding baseline variables. Social desirable answers can be expected regarding smoking and alcohol use. However, because of an expected random effect on both patient groups suffering from subfertility, we assume that confounding of the effect estimates is limited. In epidemiology, observational studies are performed as a first step for hypothesis testing and showing associations. Therefore, the results should be interpreted with causation and causality should be shown in a randomized controlled design. Our study population was recruited from a tertiary hospital. The indication for cryopreservation of sperm for example include hematological or testicular cancer, which limits extrapolating of our results to a general population. Despite homogenization of the groups per different surgically retrieval procedure (only cases with NOA in the TESE group, and OA in the MESA group), other differences between groups are present, such as the cause of infertility. Since our study population is too small to correct or stratify for these factors, future larger studies should incorporate these factors in sample size calculation and analyses. Furthermore we did not investigate birth outcomes, which was not an outcome in this study and therefore not powered accordingly.

Conclusion

We show that placental growth trajectories in the first trimester of pregnancy are increased in pregnancies conceived after TESE-ICSI as compared to ejaculated sperm. No significant differences are shown regarding embryonic development measured as CRL, EV and

Carnegie stages. These findings might be partially explained by differences in DNA damage, chromosomal constitution and epigenetics of testicular sperm as compared to ejaculated sperm. After this explorative study, future research should validate these findings in larger cohorts, including investigating possible associations with birth outcomes. Exploring the underlying pathofysiological mechanisms by measuring sperm DNA damage, placental and also the neonatal epigenome will provide insights that help optimize preconceptional health and counseling. This will help to further improve pregnancy chances and birth outcomes in subfertile couples with male factor subfertility.

Supplemental table 1: Diagnosis category per ART treatment

	ICSI TESE Sperm (n=23)	**ICSI MESA Sperm (n=25)**	**ICSI ejaculated sperm (n=99)**	**IVF (n=140)**
Female factor subfertility	0 (0.0%)	0 (0.0%)	21 (21.2%)	119 (85.0%)
Combined female-male subfertility	4 (17.4%)	3 (12.0%)	17 (17.2%)	10 (7.1%)
Male factor subfertility	19 (82.6%)	22 (88.0%)	56 (56.6%)	10 (7.1%)
Missing	0 (0%)	0 (0%)	3 (3.0%)	1 (0.7%)
Female factor diagnosis of both female only and combined male-female	N=4	N=3	N=38	N=129
Tuba	1 (25%)	0 (0%)	4 (10.5%)	21 (16.3%)
Endometriosis	0 (0%)	0 (0%)	5 (13.2%)	22 (17.1%)
Unexplained	0 (0%)	0 (0%)	14 (36.8%)	64 (49.6%)
Anovulation	3 (75%)	3 (100.0%)	15 (39.5%)	22 (17.1%)
Male factor diagnosis of both male only and combined male-female	N=23	N=25	N=73	N=20
oligozoospermia	0 (0.0%)	0 (0.0%)	73 (100%)	20 (100%)
Obstructive azoospermia	0 (14.3%)	25 (100 %)	0 (0.0%)	0 (0.0%)
Non-obstructive azoospermia	23 (100%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Of which:				
Normal karyotype, no Y chromosome microdeletions	22 (95.7%)	19 (76.0%)	99 (100%)	140 (100%)
Karyotype 47, XXY	1 (4.3%)	0 (0%)	0 (0%)	0 (0%)
CFTR gene mutation	0 (0%)	6 (24.0%)	0 (0%)	0 (0%)

Data are presented as number of subjects (% of total). Abbreviations: IQR; interquartile range, IVF; in vitro fertilization, ICSI; intracytoplasmic sperm injection, TESE; testicular sperm extraction, MESA; microsurgical epididymal sperm aspiration, CFTR; cystic fibrosis transmembrane conductance regulator.

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CHAPTER 8

Periconceptional maternal and paternal homocysteine levels and early utero-placental (vascular) growth trajectories: The Rotterdam periconception cohort

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BACKGROUND: Maternal plasma total homocysteine (tHcy) is a central component of 1-C metabolism. tHcy serves as biomarker of excessive oxidative stress and as such is involved in the developmental origin of several pregnancy complications, including those related to impairments of the placenta. The first trimester is a sensitive period for the development of the placenta, where also paternal influences and fertility treatment play a role. The aim of this study is to investigate associations between periconception parental tHcy and utero-placental growth trajectories in the first trimester of pregnancy after natural conception and In Vitro Fertilization (IVF) with or without Intracytoplasmic Sperm Injection (ICSI).

METHODS: Pregnant women and their partners were enrolled before 10 weeks of gestation in the Virtual Placenta study, embedded as a sub-cohort of the Rotterdam Periconception cohort (Predict study). In the Virtual Placenta study, we included a total of 190 women with a singleton pregnancy, of which 109 conceived naturally and 81 after IVF/ICSI treatment. We measured serial utero-placental vascular volumes (uPVV) and placental volumes (PV) at 7, 9 and 11 weeks of pregnancy. Validation of first trimester trajectories of PV was performed in 662 pregnancies from the Predict study. Associations between tHcy and uPVV and PV were identified using linear mixed models, correcting for gestational age and maternal covariates age, BMI, alcohol use, ethnicity, education and parity.

RESULTS: In the Virtual Placenta study, no significant associations revealed between parental tHcy and first-trimester trajectories of uPVV and PV in the total study population and subgroup of naturally conceived pregnancies. In the IVF/ICSI subgroup we observed significantly negative associations between maternal tHcy and uPVV trajectories in the 3rd and 4th quartile (beta: -0.38 (95%CI -0.74 to -0.02) and beta: -0.42 (95% CI -0.78 to -0.05), respectively) using the 1st quartile as reference. In addition, the uPVV/PV ratio in this subgroup was also significantly smaller. The validation analysis in the total Predict study group showed a significantly negative association between maternal tHcy and PV trajectories for the 3rd and 4th quartile (beta -0.12 (95% CI -0.22 to -0.01) and beta -0.12 (95% CI -0.23 to -0.02) respectively), using the same 1st quartile as reference. No significant associations were shown between paternal tHcy and first trimester trajectories of uPVV and PV in both cohorts.

CONCLUSIONS: The inverse associations between periconceptionally elevated maternal tHcy and trajectories of PV substantiate the recommendation of preconceptional folic acid supplement use for the prevention of neural tube defects. Folic acid lowers mild to moderate tHcy and as such might also reduce the risk of placenta-related (vascular) pregnancy complications. The stronger effects of elevated tHcy on the utero-placental vasculature in IVF/ICSI pregnancies suggests a higher sensitivity of these tissues due to the fertility treatment or a vascular underlying cause of the subfertility, which needs further investigation.

Abstract

Introduction

The placenta plays a crucial role in pregnancy, being essential to embryonic growth and development by facilitating gas-exchange of oxygen and carbon dioxide and the supply of important nutrients, such as glucose, amino acids and calcium (1). Following conception, placentation starts with endometrial invasion by the embryonic trophoblast cells, followed by the formation and remodeling of maternal utero-placental vessels (2). Impaired early placentation is associated with placenta-related vascular pregnancy complications, such as miscarriage, fetal growth restriction, and (early-onset) preeclampsia with high morbidity and mortality in both mother and child (3, 4).

Homocysteine, part of the one-carbon (1-C) metabolism, is known to play a role in early placentation involving cellular metabolism and proliferation, as well as in the regulation of gene expression through epigenetic mechanisms (5). Plasma total homocysteine (tHcy) is a sensitive biomarker of the 1-C metabolism. tHcy, together with folate and vitamin B12, is converted into methionine, which is needed for the synthesis of proteins and (phospho)lipids. Methionine is converted to S-adenosylmethionine (SAM), which is a 1-C donor for methyl groups, important for methylation of substrates and epigenetic processes (5). Subsequently, low levels of B-vitamins or folate, are associated with an increase of tHcy. An important function of tHcy is the scavenging of reactive oxygen species (ROS) by the production of glutathione, and as such elevated tHcy can also serve as a biomarker of excessive oxidative stress. Excessive oxidative stress is strongly linked to endothelial dysfunction and thrombosis ultimately causing cardiovascular diseases (6, 7). Preconceptional maternal hyperhomocysteinemia is associated with placenta-related vascular pregnancy complications, yet the impact on early placental development has not yet been examined. (8, 9).

In men, plasma concentrations of tHcy affect sperm parameters and high tHcy is associated with increased damage to the sperm DNA, which is relevant since paternally imprinted genes are predominantly expressed in the placenta (10, 11). Also, alterations in 1-C metabolism in men have been shown to affect embryonic development and growth (12).

Since plasma levels of tHcy are strongly associated with placenta-related vascular pregnancy complications in women and sperm parameters in men, we hypothesize that parental periconceptional elevated plasma tHcy concentrations are negatively associated with early pregnancy placental vascular development. Therefore, the aim of this study is to investigate the associations between both periconception maternal and paternal plasma tHcy and early placental (vascular) development to identify factors which optimize placental vascular development.

Materials and methods

Ethical approval

This study was approved by the Medical Ethical and Institutional Review Board (MEC 2015-494) of the Erasmus MC, University Medical Centre, Rotterdam, the Netherlands. Both women and men provided written informed consent at enrolment.

Study design

Participants were enrolled in the Virtual Placenta Study (registration number Dutch Trial Register: NTR6854), which focuses on placental imaging from the first trimester of pregnancy onwards (13). Participants of the study were recruited from January 2017 until March 2018 and participants of the Rotterdam Periconception Cohort (Predict study) (validation cohort) were recruited from November 2010 onwards. Since 2016 the virtual Placenta study, embedded in the Rotterdam periconception cohort (Predict study) is designed for in-depth investigation of placental development using ultrasound imaging, performing measurements of the utero-placental vascular volume (uPVV). These detailed vascular volumes were not part of and collected in the original Predict study. The Predict Study is an ongoing prospective cohort study, performed at the Department of Obstetrics and Gynecology at the Erasmus MC, University Medical Center Rotterdam, The Netherlands (13). Inclusion criteria for both women and their partners were as follows: at least 18 years of age, a singleton pregnancy and the ability to speak and read the Dutch language. Women and their partners were included before the 10th week of gestation, with pregnancies conceived either naturally or after In Vitro Fertilization (IVF) with or without Intracytoplasmic Sperm Injection (ICSI) treatment.

Study parameters

After enrollment participants filled out a general questionnaire, addressing topics like geographical background, education, smoking, alcohol use and the use of vitamins. At inclusion, length, weight and blood pressure of the couples were measured and a non-fasted blood sample was taken. Blood samples were collected in Vacutainer ethylenediaminetetraacetic acid (EDTA) tubes. Centrifugation of the EDTA-plasma took place within 10 minutes to prevent an artificial increase of tHcy (14).

After centrifugation, determination of tHcy was performed with a Waters Quattro Premiere liquid chromatography-tandem mass spectrometry system (Waters, Milford, Massachusetts, United States). The interassay coefficient of variation was <5,5%.

For naturally conceived pregnancies, gestational age (GA) was determined by the first day of the last menstrual period (LMP). For participants who had a regular menstrual cycle of less than 25 days or more than 31 days, GA was adjusted for duration of the menstrual

cycle. When GA determined by LMP and GA determined by measurement of the crown rump length (CRL) indicated a difference of more than 7 days, or when LMP was missing, GA was estimated by the CRL at the 9 weeks ultrasound scan.

When pregnancies were conceived after IVF/ICSI, GA was calculated by adding 14 days to the date of oocyte pick-up for IVF with or without ICSI. If a cryopreserved embryo was used, GA was calculated by adding 19 days to the transfer day, since thawed cryopreserved embryos were transferred at day 5 of embryo development.

Ultrasound and outcome variables

Placental volume (PV, cm^3) was measured in both the Virtual Placenta and Predict study using 3D ultrasound volumes of the whole pregnancy obtained at 7, 9 and 11 weeks of gestation, using a 6-12 MHz transvaginal probe compatible with the GE Voluson E8 Expert System. The utero-placental vasculature was visualized using power Doppler ultrasound with standardized settings (power Doppler gain '-8.0', pulse repetition frequency '0.6 kHz', wall motion filter 'low 1', quality 'high'). Furthermore constant default instrument settings were used throughout the examinations with the following settings: frequency, low; dynamic, set 5; balance, 180; smooth, 4/5; ensemble, 12; line density, 8; power Doppler map, 5; artifact suppression, on; power Doppler line filter, 1; quality, high.

We measured PV offline, using Virtual Organ Computer-aided AnaLysis (VOCAL), which was proven to be a valid technique to measure PV in the first trimester of pregnancy (intraclass correlation coefficients (ICC) >0.97) (15). To determine PV, 12 sections of the trophoblast were made with a rotational step of 15°. Subsequently the total pregnancy volume and the gestational sac in each section were measured manually and PV was calculated by subtracting the gestational sac from the total pregnancy volume (15).

Utero-Placental Vascular Volume (uPVV, cm^3) was exclusively measured in the Virtual Placenta Study. In short, to measure uPVV, 3D PD ultrasound volumes of the pregnancy were obtained at 7, 9 and 11 weeks of gestation using a 6-12 MHz transvaginal probe compatible with the GE Voluson E8 Expert System. Offline using a semi-automated technique, assessment of the placental vascular volume in VR enabled more accurate measurement by projecting the volume as real-life image, 'a hologram', which could be rotated and enlarged for more precision (16). This procedure also proved to be a valid technique (intraclass correlation coefficients ICC between 0.94 and 0.97). At first, all embryonic structures were removed from the hologram and secondly the myometrium was removed, leaving only the vessels up to the utero-placental border, and thus the uPVV. After measurement of both uPVV and PV, we calculated the ratio of those parameters, which was used to determine the amount of vessels per cm^3 of placental volume.

Statistical analysis

Pregnancies were excluded from analysis after oocyte donation, miscarriage or congenital abnormality. Participants without available tHcy levels or placental measurements were also excluded.

First, we analyzed maternal and paternal baseline characteristics of the total study population, in natural conceptions and in IVF/ICSI pregnancies. We compared the distribution of baseline variables between IVF/ICSI and naturally conceived pregnancies using the medians and interquartile ranges for continuous variables and percentages for categorical variables. We tested for differences using the Mann-Whitney U test or the Chi-squared test, respectively. To assess the association between longitudinal measurements of PV, uPVV and uPVV/PV with tHcy of both women and men, we used linear mixed models. For analysis of the trajectories of PV, uPVV and uPVV/PV ratio, we used a third root transformation of these variables to establish linearity between these parameters and GA.

To further investigate the association of tHcy with PV, uPVV and uPVV/PV ratio, we divided the tHcy of the Virtual Placenta study into quartiles (Q) and used Q1 as reference category. Maternal quartiles were as following: Q1: 3,80-5,39 µmol/L; Q2: 5,40-6,29 µmol/L; Q3: 6,30-7,29 µmol/L; and Q4: 7,30-53,70 µmol/L.

For the linear mixed models analyzing the association of maternal tHcy on PV, uPVV, uPVV/PV ratio, we first adjusted only for GA (Model 1). Subsequently, in model 2 we adjusted for the maternal covariates age, parity, BMI, alcohol use, ethnicity, education, which were selected based on literature and baseline differences. When analyzing the association of paternal tHcy on PV, uPVV, uPVV/PV ratio, we additionally adjusted for the paternal covariates age, alcohol use and education. Furthermore, we adjusted for paternal tHcy when analyzing the effect of the maternal tHcy and vice versa and additionally adjusted for GA at blood withdrawal. P-values <0.05 were considered statistically significant. All analyses were performed using SPSS package 24.0 (IBM SPSS Statistics, Armonk, NY).

Validation cohort

To validate PV trajectories, we analyzed the associations between parental periconception tHcy and PV trajectories in the Predict study. Subsequently, the associations between maternal and paternal tHcy on PV using linear mixed models with the same models and confounders as the Virtual Placenta study were evaluated. The association of the different maternal tHcy quartiles was analyzed using the cut-off points from the Virtual Placenta study since these were nearly similar (**Supplemental table 1**).

Results

Virtual Placenta study

In the Virtual Placenta study, we included 241 participants (**Figure 1**). Prior to analysis, we excluded participants because of oocyte donation (n=3), miscarriage (n=22), congenital malformation (n=9) and drop-out (n=1). Furthermore, we excluded women without available blood samples (n=8) or placental measurements (n=8), leaving a total of 190 women for analysis, of which 81 pregnancies were conceived after IVF/ICSI and 109 pregnancies were naturally conceived.

We included a total of 146 men, of which 65 pregnancies were conceived after IVF/ICSI and 81 pregnancies were naturally conceived. (**Figure 1**)

At baseline, only a significant difference in the number of nulliparous women between the IVF/ICSI group and the natural pregnancy group (77.8% vs 44% respectively (p<0.001)) was noted (**Table 1**). The median tHcy was 6.3 µmol/L (IQR 5.4–7.3 µmol/L) for the total group,

Figure 1: Flowchart of the study population of the Virtual Placenta Study

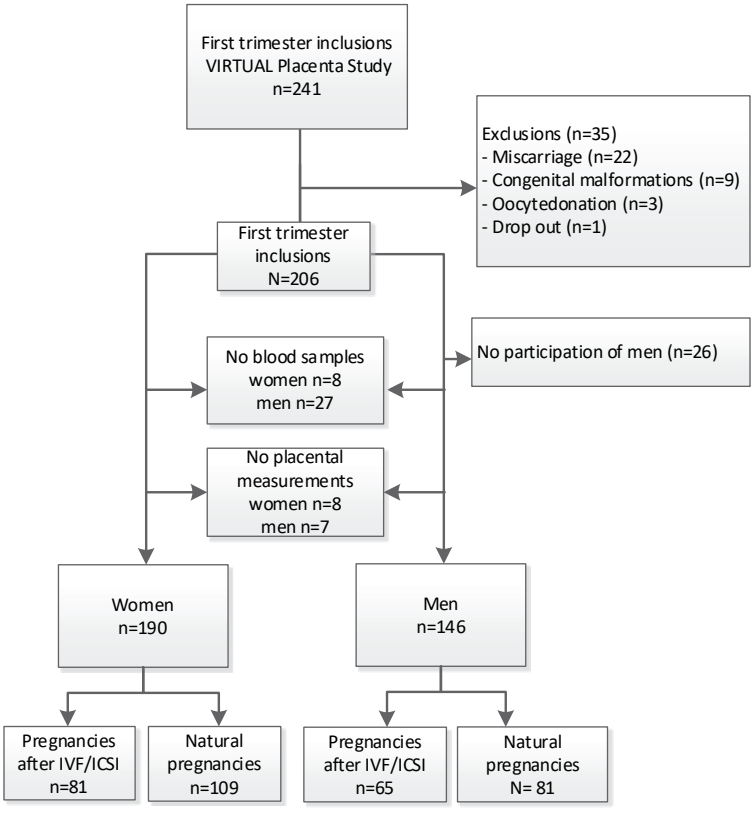


Table 1: Virtual Placenta Study population: Baseline characteristics of the female (n=190) and male participants (n=146) stratified for mode of conception.

	Total study population	IVF/ICSI pregnancies	Naturally conceived pregnancies	P-value
Women	n=190	n=81	n=109	
Age (years)	31.9 [29.0-35.3]	32.9 [29.3-36.3]	31.5 [28.9-34.5]	0.084
Nulliparous	111 (58.4)	63 (77.8)	48 (44)	0.001*
Geographic origin				0.487
Dutch	147 (78.6)	67 (82.7)	80 (75.5)	
Western	6 (3.2)	2 (2.5)	4 (3.8)	
Non-Western	34 (18.2)	12 (14.8)	22 (20.8)	
Educational level				0.729
Low	13 (7.0)	6 (7.4)	7 (6.6)	
Intermediate	64 (34.2)	30 (37.0)	34 (32.1)	
High	110 (58.8)	45 (55.6)	65 (61.3)	
At the first trimester study entry: BMI, measured (kg/m^2)	24.9 [22.2-28.0]	24.1 [22.0-27.7]	25.2 [22.5-29.2]	0.139
Folic acid supplement use	188 (98.9)	81 (100.0)	107 (98.2)	0.220
Started preconceptionally	158 (83.2)	79 (97.5)	79 (72.5)	0.001*
Use of other vitamins, yes	115 (60.5)	48 (59.3)	67 (61.5)	0.277
Alcohol consumption, yes	51 (26.8)	21 (25.9)	30 (27.5)	0.806
Smoking	26 (13.7)	11 (13.6)	15 (13.8)	0.971
tHcy, plasma, uMol/L	6.3 [5.4-7.3]	6.1 [5.5-7.0]	6.4 [5.4-7.3]	0.240
Men	n=146	n=65	n=81	
Age (years)	33.8 [30.9-38.5]	35.3 [31.6-38.6]	33.2 [30.3-38.4]	0.135
Geographic origin				0.584
Dutch	109 (80.1)	49 (81.7)	60 (78.9)	
Western	3 (2.2)	2 (3.3)	1 (1.3)	
Non-Western	24 (17.6)	9 (15.0)	15 (19.7)	
Educational level				0.878
Low	16 (11.8)	7 (11.7)	9 (11.8)	
Intermediate	58 (42.6)	27 (45.0)	31 (40.8)	
High	62 (45.6)	26 (43.3)	36 (47.4)	
At the first trimester study entry: BMI, measured (kg/m^2)	26.1 [23.7-29.2]	26.6 [23.8-29.3]	25.8 [23.6-29.0]	0.446
Folic acid supplement use	6 (4.4)	3 (5.0)	3 (4.0)	0.779
Use of vitamins	29 (21.5)	14 (23.3)	15 (20.0)	0.639
Alcohol consumption	95 (70.4)	43 (71.7)	52 (69.3)	0.768
Smoking	43 (31.9)	19 (31.7)	24 (32.0)	0.967
tHcy, plasma, uMol/L	12.2 [10.0-15.2]	12.6 [10.5-15.4]	11.9 [9.7-15.2]	0.058

Data are presented as median [interquartile range (IQR)] or n (%). *statistically significantly different between the three groups.

6.1 µmol/L (IQR 5.5 – 7.0 µmol/L) for the IVF/ICSI group and 6.4 µmol/L (IQR 5.4 – 7.3 µmol/L) for the naturally conceived pregnancy group (p=0.24).

Regression analysis in the total study population of women and in the naturally conceived pregnancy group showed no statistically significant association between tHcy and PV, uPVV or uPVV/PV ratio. In IVF/ICSI pregnancies, there was a significantly negative association between tHcy and uPVV and uPVV/PV ratio (beta -0.132 (95% CI: -0.235 to -0.029) and beta -0.032 (95% CI -0.064 to -0.001) respectively) after adjustment for the confounders specified above (model 2) (**Table 2**). The regression analyses of Model 1 for the IVF/ICSI group showed negative associations regarding uPVV trajectories when comparing Q3 and Q4 with Q1 as reference (Q3 beta: -0.21 (95%CI -0.52 to 0.11), Q4 beta: -0.29 (95% CI -0.61 to 0.03)) (**Table 3**). After adjusting for confounders these negative associations are significantly different for Q3 and Q4 with Q1 as reference (Q3 beta: -0.38 (95%CI -0.74 to -0.02), Q4 beta: -0.42 (95% CI -0.78 to -0.05)). The confounders with the strongest effect were maternal educational level and alcohol use. We found no significant associations between tHcy and uPVV when stratified for fetal sex (**Supplemental table 2**). Retransformation of the effect estimates from the IVF/ICSI group to the original scale showed that maternal tHcy in Q4 the uPVV was reduced with 16.69 cm^3 (86.7%) at 11 weeks of gestation compared to women with tHcy in Q1 (**Figure 2**). We found no significant differences in tHcy concentrations between women with or without pre-eclampsia (6.4 vs 6.3 µmol/L respectively (p=0.71)), with or without pregnancy-induced hypertension (7.2 vs 6.3 µmol/L respectively (p=0.32)) and with or without preterm birth (6.6 vs 6.3 µmol/L respectively (p=0.16)).

Men

At baseline there were no significant differences in the study population (**Table 1**). Median tHcy was 12.2 µmol/L (IQR 10.0 – 15.2 µmol/L) for the total study population. Regression analysis (Model 2) in the total study population showed no significant associations between paternal tHcy and PV and uPVV (beta: -0.006 (95% CI: -0.018 to 0.007) and beta: -0.011 (95%CI: -0.026 to 0.004)) respectively (**Table 2**).

Predict study

In total 662 women were included, of which 252 conceived after IVF/ICSI and 410 after natural conception. At baseline there were statistically significant differences between naturally conceived pregnancies and pregnancies after IVF/ICSI regarding maternal age, parity, tHcy, BMI and alcohol use, paternal age and vitamin use (**Supplemental table 2**). When using maternal Q1 of the Virtual Placenta study as reference, we found a significantly negative association between maternal tHcy in the total study population (Model 2) and PV for Q3 and Q4 (beta -0.116 (95% CI -0.220 to -0.012) and beta -0.122 (95% CI -0.226 to -0.019) respectively) (**Table 4**). Regression analysis (model 1) in the IVF/ICSI group showed a significant negative association, when using maternal Q1 as reference, for tHcy in Q3 and

Table 2: Virtual Placenta Study: Effect estimates for associations between periconception parental tHcy and trajectories of utero-placental (vascular) volumes and stratified for mode of conception.

	Model 1						Model 2					
	uPVV ($\sqrt[3]{cm^3}$)		**PV ($\sqrt[3]{cm^3}$)**		**uPVV/PV ($\sqrt[3]{\ }$)**		**uPVV ($\sqrt[3]{cm^3}$)**		**PV ($\sqrt[3]{cm^3}$)**		**uPVV/PV ($\sqrt[3]{\ }$)**	
	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 study population												
Women	0.001	0.87	-0.012	0.12	0.002	0.31	-0.003	0.91	-0.028	0.25	0.003	0.74
n=190	(-0.016 to 0.019)		(-0.027 to 0.003)		(-0.002 to 0.007)		(-0.060 to 0.054)		(-0.075 to 0.020)		(-0.013 to 0.018)	
Men	-0.008	0.26	-0.006	0.28	-0.001	0.52	-0.011	0.16	-0.006	0.38	-0.003	0.20
n=146	(-0.022 to 0.006)		(-0.017 to 0.005)		(-0.005 to 0.003)		(-0.026 to 0.004)		(-0.018 to 0.007)		(-0.007 to 0.001)	
IVF/ICSI pregnancy												
Women	-0.078	0.08	-0.049	0.17	-0.011	0.40	**-0.132**	**0.01**	-0.002	0.96	**-0.032**	**0.046**
n=81	(-0.166 to 0.009)		(-0.12 to 0.022)		(-0.036 to 0.015)		**(-0.235 to -0.029)**		(-0.090 to 0.086)		**(-0.064 to -0.001)**	
Men	-0.017	0.19	-0.004	0.65	-0.005	0.19	-0.020	0.10	-0.005	0.59	-0.005	0.15
n=65	(-0.041 to 0.008)		(-0.022 to 0.014)		(-0.012 to 0.002)		(-0.044 to 0.004)		(-0.025 to 0.014)		(-0.012 to 0.002)	
Naturally conceived pregnancy												
Women	0.005	0.56	-0.010	0.21	0.003	0.20	0.020	0.57	-0.031	0.37	0.013	0.19
n=109	(-0.012 to 0.023)		(-0.026 to 0.006)		(-0.002 to 0.008)		(-0.052 to 0.092)		(-0.098 to 0.037)		(-0.007 to 0.032)	
Men	-0.004	0.67	-0.007	0.38	0.001	0.90	-0.006	0.54	0.001	0.98	-0.003	0.28
n=81	(-0.021 to 0.014)		(-0.022 to 0.009)		(-0.004 to 0.005)		(-0.027 to 0.014)		(-0.020 to 0.021)		(-0.008 to 0.003)	

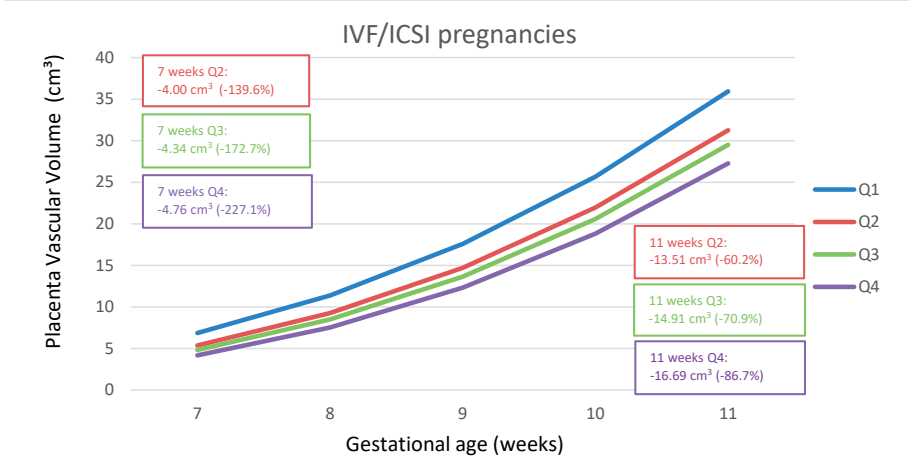
Model 1: adjusted for gestational age.
Model 2 women: Model 1 + maternal covariates age, parity, BMI, alcohol use, ethnicity, education, time of blood withdrawal and paternal covariate tHcy.
Model 2 men: Model 1 + maternal covariates age, age, parity, BMI, alcohol use, ethnicity, education, homocysteine and paternal covariates age, education and alcohol use.
Abbreviations: uPVV: utero-Placental Vascular Volume, PV: Placental Volume, CI: Confidence Interval

Table 3: Virtual Placenta Study: Effect estimates for associations between tHcy divided in quartiles (Q) and trajectories of placental (vascular) volumes for the women in the IVF/ICSI population.

	Model 1						Model 2					
IVF/ICSI pregnancy	**uPVV ($\sqrt[3]{cm^3}$)**		**PV ($\sqrt[3]{cm^3}$)**		**uPVV/PV ($\sqrt[3]{\ }$)**		**uPVV ($\sqrt[3]{cm^3}$)**		**PV ($\sqrt[3]{cm^3}$)**		**uPVV/PV ($\sqrt[3]{\ }$)**	
homocysteine	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
Q1 3,80-5,39 µmol/l n=21	Reference		Reference		Reference		Reference		Reference		Reference	
Q2 5,40-6,29 µmol/l n=20	-0.15 (-0.45 to 0.15)	0.31	**-0.24** **(-0.47 to -0.01)**	**0.046**	-0.01 (-0.09 to 0.08)	0.92	-0.24 (-0.58 to 0.11)	0.18	-0.01 (-0.30 to 0.27)	0.92	-0.05 (-0.15 to 0.05)	0.34
Q3 6,30-7,29 µmol/l n=20	-0.21 (-0.52 to 0.11)	0.19	-0.038 (-0.28 to 0.21)	0.76	-0.05 (-0.14 to 0.03)	0.23	**-0.38** **(-0.74 to -0.02)**	**0.04**	0.04 (-0.27 to 0.27)	0.81	**-0.11** **(-0.22 ton -0.01)**	**0.03**
Q4 7,30-53,70 µmol/l n=20	-0.289 (-0.61 to 0.03)	0.08	-0.20 (-0.45 to 0.05)	0.12	-0.04 (-0.13 to 0.05)	0.35	**-0.42** **(-0.78 to -0.05)**	**0.03**	0.03 (-0.29 to 0.35)	0.85	-0.10 (-0.21 to 0.01)	0.06

Model 1: adjusted for gestational age.
Model 2: Model 1 + maternal covariates age, parity, BMI, alcohol use, ethnicity, education, time of blood withdrawal and paternal covariate tHcy.
Abbreviations: uPVV: utero-Placental Vascular Volume, PV: Placental Volume, CI: Confidence Interval, Q: Quartile.

Figure 2: Trajectories of utero-placental vascular volumes (uPVV) in IVF/ICSI pregnancies. tHcy in the first quartile (Q1, blue line, reference) versus Q2, Q3 and Q4 (red, green and purple lines, respectively) are depicted, including the absolute and percentage differences at 7 and 11 weeks of gestational age.



Legend: tHcy in quartiles: Q1: 3.80-5.39 µmol/L; Q2: 5.40-6.29 µmol/L; Q3: 6.30-7.29 µmol/L; and Q4: 7.30-53.70 µmol/L.

Q4 (beta -0.14 (95% CI -0.26 to -0.01) and beta -0.18 (95% CI -0.32 to -0.04) respectively). After correction for confounders this effect attenuated for Q3 and Q4 as compared to Q1 (beta -0.09 (95% CI -0.24 to 0.06) and beta -0.09 (95% CI -0.26 to 0.07) respectively), also showing larger effects in the IVF/ICSI group as compared to the total and natural conception population. Retransformation of the effect estimates for PV of the IVF/ICSI group to the original scale showed that PV of maternal tHcy in Q4 was reduced by 10.5 cm3 (13.0%) at 11 weeks of gestation compared to the reference of women with a tHcy in Q1.

Discussion

Principal findings:

This study shows associations between periconception maternal plasma tHcy and uPVV and PV in the first trimester of pregnancy, specifically showing that tHcy in the lowest quartile is associated with the largest uPVV and PV. No associations were found between periconception paternal plasma tHcy and placental (vascular) development. These results support the benefit of periconception optimalisation of maternal tHcy-levels, which will clinically result in better placentation, with potential reduced maternal and fetal risks of placental-related vascular pregnancy complications at a later stage of gestation.

Table 4: Validation in the Predict Study: effect estimates for associations between periconception maternal tHcy in quartiles (Q) and placental volume in the total study population and subgroups of IVF/ICSI and naturally conceived pregnancies.

	Model 1		Model 2	
Maternal tHcy	**PV ($\sqrt[3]{cm^3}$) Beta (95% CI)**	**P-value**	**PV ($\sqrt[3]{cm^3}$) Beta (95% CI)**	**P-value**
Total study population				
Q1 n=166	Ref		Ref	
Q2 n=165	-0.06 (-0.15 tot 0.02)	0.157	-0.08 (-0.18 to 0.02)	0.131
Q3 n=165	**-0.14 (-0.23 tot -0.06)**	**0.001**	**-0.12 (-0.22 to -0.01)**	**0.030**
Q4 n=166	**-0.13 (-0.21 tot -0.04)**	**0.004**	**-0.12 (-0.23 to -0.02)**	**0.020**
IVF/ICSI pregnancy				
Q1 n=63	Ref		Ref	
Q2 n=63	-0.07 (-0.20 tot 0.05)	0.252	0.01 (-0.14 to 0.15)	0.953
Q3 n=63	**-0.14 (-0.26 tot -0.01)**	**0.039**	-0.09 (-0.24 to 0.06)	0.249
Q4 n=63	**-0.18 (-0.32 tot 0.04)**	**0.010**	-0.09 (-0.26 to 0.07)	0.246
Naturally conceived pregnancy				
Q1: n=103	Ref		Ref	
Q2 n=102	-0.06 (-0.18 tot 0.06)	0.300	**-0.15 (-0.29 to -0.01)**	**0.045**
Q3 n=102	**-0.14 (-0.26 tot -0.03)**	**0.013**	-0.13 (-0.27 to 0.01)	0.071
Q4 n=103	-0.10 (-0.21 tot 0.01)	0.076	-0.12 (-0.26 to 0.02)	0.093

tHcy quartile references: Q1: 3.80-5.39 µmol/L; Q2: 5.40-6.29 µmol/L; Q3: 6.30-7.29 µmol/L; and Q4: 7.30-53.70 µmol/L.
Model 1: adjusted for gestational age.
Model 2: Model 1 + maternal covariates age, parity, BMI, alcohol use, ethnicity, education, time of blood withdrawal and paternal covariate tHcy.
Abbreviations: PV: Placental Volume, CI: Confidence Interval

Previous research showed that elevated levels of plasma tHcy significantly increases the odds of any placenta-related vascular pregnancy complications (8, 17, 18). Plasma levels of tHcy influence placental weight at birth, with plasma levels of tHcy of >8.3 µmol/L being associated with a lower placental weight of 30 grams (19). These finding are in agreement with our study, where maternal plasma tHcy in Q4 (plasma tHcy >7.3 µmol/L) was strongly

associated with reduced PV and uPVV, compared to maternal tHcy in Q1 (plasma tHcy <5,40 µmol/L). We show a clear concentration-dependent effect, since maternal tHcy in Q2 (plasma tHcy between 5.40 and 6.29 µmol/L) did not show an effect on PV and uPVV when compared to maternal tHcy concentrations in Q1.

These associations could be explained by the dynamics of the 1-C metabolism. An unhealthy diet and lifestyle usually result in a low intake of vitamins necessary for normal functioning of the 1-C metabolism, altering epigenetics and synthesis of nucleotides and lipids. Vascular endothelial growth factor (VEGF) is one of the most important growth factors stimulating neovascularization, which is essential for placental development. Differences in epigenetic profiling of VEGF, and its related gene expression, can therefore possibly have an effect on the amount and volume of vessels in the developing placenta. Hyperhomocysteinemia is also associated with decreased concentrations of glutathione causing excessive oxidative stress, which is associated with apoptosis and dysfunction of the endothelium (20). In cardiovascular medicine, elevated tHcy is associated with endothelial dysfunction, thrombosis and atherosclerosis ultimately leading to coronary artery disease (21). Decreased vascularization due to reduced expression levels of VEGF combined with apoptosis, can interfere with placental development, leading to reduced gas exchange between maternal and fetal circulation or preeclampsia.

The associations regarding uPVV, which were only present in the IVF/ICSI group while absent in the naturally conceived pregnancy group, might be explained by differences in gene expression related to IVF/ICSI-procedures in general compared to naturally conceived pregnancies (22). Analysis of genome-wide mRNA expression in placentas of pregnancies conceived after IVF/ICSI compared to naturally conceived pregnancies, showed overexpression of 13 biological pathways, which play an important role in cell cycle control, metabolism, immune response, and the 1-C metabolism (22). However, the exact pathophysiologic explanation for the different expression profiles between naturally and IVF/ICSI pregnancies needs to be elucidated further.

No significant associations between paternal plasma tHcy and placental (vascular) volumes were found. We hypothesized the effect of paternal tHcy to be large, since growth of the placenta is largely driven by paternal genomic imprinting. Previous studies also showed a fetal sex dependent manner of placental development. Animal studies showed that knockout models of paternally imprinted genes resulted in a significant reduction of placental size. The exact role of tHcy in this mechanism is not yet understood, however a high concentration of tHcy is deleterious, because it increases excessive oxidative stress and subsequent global and DNA hypomethylation, and efflux of methionine, necessary for normal methylation. The vasculature in the maternal part of the placenta will be greatly influenced by the local maternal environment, while paternal influences on placental

vasculature are possibly only visible on the fetal side of the placenta. However, due to the small volume of the fetal side during the first trimester, it is not possible to distinguish only the vascular volume of the fetal side by 3D Doppler volume measurements. As a consequence, the paternal influence will initially not outweigh the maternal influence based on the on the total first trimester placenta vascular volume.

Early placentation is clinically important, since abnormal early placental development is associated with miscarriages, fetal growth restriction and hypertensive disorders in pregnancy. However, we did not find an effect of tHcy on placenta-related vascular complications in pregnancy. Previous research investigating the association between tHcy and a placental origin of pregnancy complications is comprised of approximately 8,000 participants and therefore our study was not powered to detect differences regarding pregnancy outcomes. Instead, our study allows for more insight into the early (patho) physiology of the role of tHcy on placental development (8). Although the effect estimates appear to be small, retransformation to the original values showed that placental volume in the third and fourth quartile are 70.9% and 86.7% smaller compared to Q1, which can be clinically relevant, but also asks for further research on the predictive value of PV for adverse placental-related outcome.

Although studies have shown that placental volume is associated with preeclampsia, also other pathophysiological mechanisms are involved such as abnormal spiral artery remodeling and increased placental capillary density. Probably the association between tHcy and placenta related pregnancy outcomes is not only mediated through placental volume but also by other mechanisms not measured in our study.

Plasma tHcy in our study population was lower (median 6.3 µmol/L (IQR 5.4–7.3 µmol/L)) than a previously proposed reference value (12 µmol/L), which was mainly established to determine the risk of spina bifida (23). However, plasma tHcy in our study population is comparable to other studies that investigated the association between plasma tHcy and pregnancy outcomes. In a large population-based cohort, mean plasma tHcy was 6.9 µmol/l, with the lowest quintile <5.8 µmol/L, which is comparable to our cohort (24). tHcy concentrations were higher in men than in women. Studies in general populations in both Asia and Europa showed tHcy concentrations averaging between 14.6 and 15.7 µmol/L in men and 9.6 and 13.1 µmol/L in women . Men in our population had a median concentration of 12.2 µmol/L. We need to take into consideration that tHcy concentrations can vary between different ethnic populations, but also differences in nutrition, internal metabolic parameters and age. Moreover, men in our study were recruited from a tertiary care center, also including men with underlying diseases and medication use, that can interfere with homocysteine metabolism.

Strength and limitations:

The main strengths of our study are the longitudinal early measurements of placental (vascular) development using 3D ultrasound combined with VR. Placental (vascular) volumes were assessed by experienced researchers and these techniques are proven to be valid, with excellent reproducibility (16). Another strength of our study is the early measurement of plasma tHcy in both women and men. We validated the results we found in the Virtual Placenta cohort regarding PV in the larger Rotterdam Periconception cohort with over 600 participants. Limitations of our study are the relatively small sample size (n=190) regarding uPVV, causing a lack of power to investigate the association with pregnancy outcomes. Our study population was recruited from a tertiary hospital, which largely comprises high-risk pregnancies, making the extrapolation of our results to a general population challenging.

Conclusions:

In this manuscript we provide more (patho)physiological insight into the association between plasma tHcy and early placental (vascular) development. As a unique finding, we demonstrate that high maternal periconception tHcy is associated with decreased placental (vascular) volumes in a dose-dependent way with potential reduced maternal and neonatal complication after reaching the viable stage of pregnancy.

Supplemental table 1: Periconception maternal tHcy in quartiles in the Virtual Placenta study and validation in the Predict Study.

	Virtual Placenta Study n=190	Predict Study n=662
Q1 (µmol/L)	3.8–5.4 (n=47)	3.4–5.5 (n =166)
Q2 (µmol/L)	5.4–6.3 (n =48)	5.5–6.3 (n =165)
Q3 (µmol/L)	6.3–7.3 (n=47)	6.3–7.3 (n =165)
Q4 (µmol/L)	7.3–53.7 (n =48)	7.3–53.4 (n =166)

Supplemental table 2: Predict Study: Baseline characteristics of the total study group of women (n=662) and men (n=512) and after stratification for mode of conception.

	Total study population	IVF/ICSI pregnancies	Naturally conceived pregnancies	P-value
Maternal	n=663	n=252	n=410	
Age (years)	32.3 [29.2-35.5]	33.3 [30.8-36.6]	31.4 [28.7-34.9]	0.001
Nulliparous	357 (54)	178 (70.9)	179 (43.8)	0.001
Geographic origin				0.931
Dutch	530 (81.3)	198 (79.8)	331 (82.1)	
Western	31 (4.8)	12 (4.8)	19 (4.7)	
Non-Western	91 (14)	38 (15.3)	53 (13.2)	
Educational level				0.791
Low	49 (7.5)	16 (6.5)	33 (8.2)	
Intermediate	220 (33.8)	88 (35.6)	132 (32.8)	
High	381 (58.6)	143 (57.9)	237 (59.0)	
At the first trimester study entry: BMI, measured (kg/m^2)	24.3 [22.0-28.5]	23.9 [21.7-27.5]	24.7 [22.1-29.0)	0.017
Folic acid supplement use	643 (99.1)	247 (99.6)	395 (98.8)	0.315
Started preconceptionally	535 (83.1)	241 (97.2)	293 (74.2)	0.001
Use of other vitamins	440 (67.8)	168 (67.7)	271 (67.8)	0.964
Alcohol consumption	207 (32.1)	62 (25.0)	145 (36.7)	0.007
Smoking	103 (16.0)	36 (14.5)	67 (16.9)	0.655
tHcy, plasma, uMol/L	6.3 [5.5-7.3]	6.2 [5.5-7.0]	6.4 [5.5-7.5]	0.011
Paternal	n=513	n=192	n=410	
Age (years)	33.9 [31.0-38.3]	35.3 [32.4-39.1]	33.3 [30.1-37.2]	0.001
Geographic origin				0.862
Dutch	419 (85.5)	159 (86.4)	259 (84.9)	
Western	16 (3.3)	4 (2.2)	12 (3.9)	
Non-Western	55 (11.2)	21 (11.4)	34 (11.1)	
Educational level				0.967
Low	63 (12.8)	25 (13.6)	38 (12.4)	
Intermediate	188 (38.3)	72 (39.1)	116 (37.9)	
High	237 (48.3)	86 (46.7)	150 (49.0)	
At the first trimester study entry: BMI, measured (kg/m^2)	25.8 [23.6-28.3]	26.0 [23.7-28.7]	25.7 [23.4-28.2]	0.190
Folic acid supplement use	38 (7.8)	17 (9.3)	21 (6.9)	0.317
Use of vitamins	95 (19.8)	44 (25.1)	50 (16.5)	0.010
Alcohol consumption	358 (75.2)	137 (77.8)	220 (73.6)	0.494
Smoking	152 (31.9)	54 (30.9)	98 (32.7)	0.727
tHcy, plasma, uMol/L	11.5 [9.9-14.1]	11.7 [10.1-14.1]	11.5 [9.8-14.1]	0.517

Data are presented as median [interquartile range (IQR)] or n (%).

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PART V

Discussion and appendices

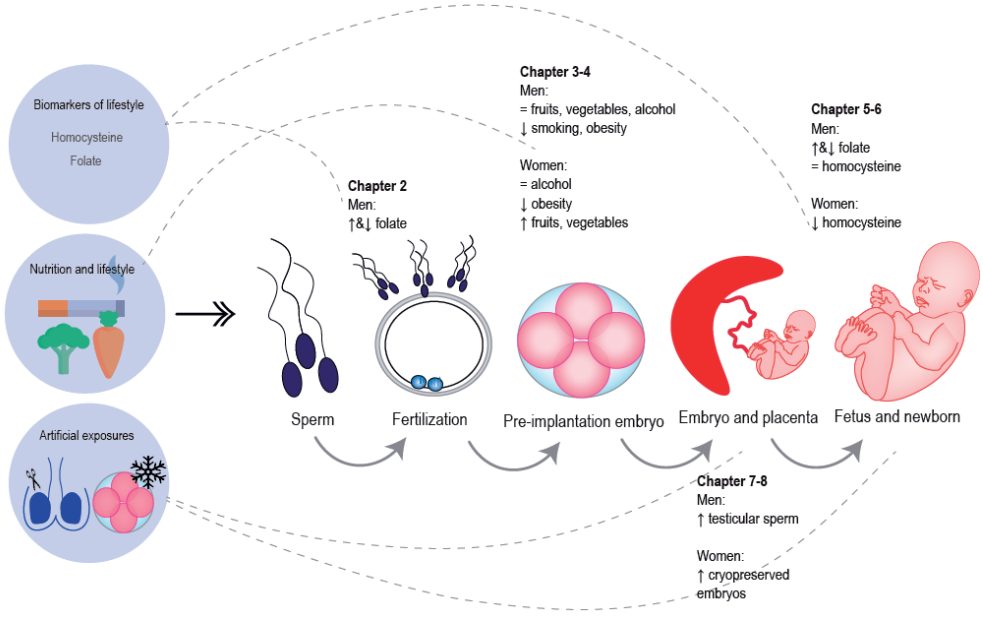
CHAPTER 9

General discussion

Main findings

This thesis shows that in future parents, nutrition, lifestyle and artificial environmental factors can influence not only the very early processes of gametogenesis, but also pre- and postimplantation embryonic and early placental growth (**Figure 1**).

Figure 1: Main findings overview.



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

Preconception care for men

This thesis contributes to the evidence of the importance and relevance of addressing also paternal preconceptional health in research and reproductive care. The first study showing an effect of the relevance of paternal contributions was the importance of social attachment between father and child published in the early 1970s. (1, 2). For decades the role of fathers (to-be) was perceived as sperm-donors, protectors and financial providers for the family, however their role in the ante- and postnatal health of their offspring was not acknowledged. Luckily, more and more research is being performed to investigate the physiological and psychological importance of the father in relation with pregnancy outcome and child health. Most studies performed are initiated in the fields of psychiatry and child development, showing effects of paternal mental health (mainly depression and anxiety) on the father-child attachment (3, 4). These studies eventually resulted in

epidemiological investigations on the impact of multiple environmental factors on sperm quality. However, during recent decades biological (epigenetic) mechanisms showing environmental factors can have an impact on paternal health and the subsequent offspring (5). In rodent models, it is shown that preconceptional paternal psychological stress is associated with altered methylation patterns in offspring's brain. This postpartum altered methylation in the offspring subsequently is associated with depressive and anxiety disorders in later life (6, 7). The unexpected paternal effect on pregnancy and fetal outcome, led to new epidemiological studies and insights showing that general paternal characteristics such as age, ethnicity and education are associated with not only sperm quality, but also pregnancy outcomes (4). Nowadays there is a plethora of studies showing effects of paternal medical history, nutritional status and lifestyle on sperm quality and pregnancy outcome (8, 9).The exact pathophysiological underlying mechanism remains to be elucidated, however causal factors seem to be submitted also through paternal sperm. It has been hypothesized that these "carry over"-effects are caused by alterations in sperm DNA quality and epigenetic alterations during the process of spermatogenesis. Indeed, paternal smoking is associated with reduced pre-implantation embryo quality through increased DNA damage and aneuploidy rates in sperm. The latter being also associated with an increased risk for congenital malformations (10). Recent literature shows that DNA damage and epigenetic information of sperm is also transferred to the embryo (11, 12). The periconceptional epigenome is highly dependent on 1-C moieties, such as folate, methionine, and choline, and plays a key role in molecular biological processes, such as programming of genes and subsequent expression being crucial mechanisms involved in embryonic, fetal and placental growth. Differences in the intake of fruits and vegetables, food groups rich in folate, and folic acid supplement use in combination with differences in genetic variants, greatly influence the availability of 1-C moieties and therewith the epigenetic programming. Recently, a novel potential epigenetic mechanism was identified (13, 14). Sperm cells carry different types of ribonucleic acid (RNA) and also the epididymal epithelium produces exosome vesicles, which are able to transfer RNA molecules to the passing sperm cells (15). In mouse, such RNAs have been shown to be critical for fertilization and pre- and post-implantation embryo development (16, 17). A study in human sperm cells identified the level of expression of a large number of these human sperm RNAs to be responsive to BMI (14).

Also the event of pregnancy influences paternal life course development, leading to an increase in personal growth and identity (5). But becoming a father can also have negative effects, since in approximately 10% postpartum depression is described in fathers in the first month after delivery (18). Presence of men during birth and physically being present in the delivery room was not done for a long period of time due to gender role expectations. Nowadays most of the fathers want to be present during delivery at the birth of their baby. Actively involving future fathers in pre- and periconception health and research is still

challenging. Gender role expectations make men less engaged in the periconception health and some feel they are invading women's personal space during their pregnancy (18). This also leads to less willingness in men compared to women to participate in research during the periconception and pregnancy period, as confirmed in this thesis, which leads to less available information. Paternal involvement is understudied, but the available evidence points towards significant effects on embryo quality, growth and pregnancy outcomes. Therefore fathers-to-be should become more involved in research addressing their role pre- and post-conception and during and immediately after pregnancy. Participation rates might be improved by more stringently pointing out to fathers to be what is known about the impact of their individual lifestyle on the child to be. Moreover, participation could also be improved by offering incentives and may be financial reimbursement of preconceptional counseling costs, since that might be an argument not accompanying their partners. Recent research shows that financial incentives are uncommon in current research projects, with approximately only 9% of all trials offering a financial compensation (19). Interestingly, highest rates of follow-up were seen in trials offering such compensation. Ethical remarks however should be made, since financial incentives could encourage especially socio-economic vulnerable patients to participate, which might influence the unbiased evaluation of the risks and benefits of participating in a trial.

In conclusion, increasing the participation of men in periconception and prenatal care is important. Intrinsic and extrinsic motivation starts with increasing awareness and knowledge on specific health issues. Historically, knowledge is transferred through passive didactic lectures. However, active learning is proven to be more effective in knowledge acquisition and behavioral changes. Currently most patients visiting our clinic have excellent understanding of making use of the online environment with internet, tablets, computers and mobile phones. Serious gaming, an example of active learning, are interactive computer applications that have challenging components and are fun to play. Serious gaming has proven to be effective in improving knowledge and changing behaviours in several fields of medicine, such as asthma, vaccination, rehabilitation and mental health. These active learning components are not yet investigated or implemented in the field of obstetrics and gynecology, which can be of benefit. Mobile health applications in general on the contrary are investigated and implemented, and interestingly are also cost saving (20). Online health platforms should also be offered preconceptionally to men to improve knowledge and thereby possibly also reducing health care costs.

Pathophysiologic mechanisms behind clinical outcomes in women

The relationships between maternal pre- and periconceptional healthy nutrition and lifestyle and the chance to become pregnant and have a healthy pregnancy outcome, have been well established. However the underlying physiological mechanisms have been less studied. Improvement of adherence to the nutritional recommendations of the Dutch Nutrition Centre (covering the intake fruits, vegetables, meat, fish, whole wheat products and fats) resulted in a 65% increase of ongoing pregnancy after IVF/ICSI treatment (21). Multiple hypotheses explaining these results are postulated in current literature, such as increased quality of the oocyte as well as pre-implantation embryo and a healthier, more receptive, endometrial environment (22-24). Plasma total homocysteine (tHcy) is a sensitive biomarker of nutrition, lifestyle, health and disease, and is strongly negatively associated with pregnancy outcome (25-27). Excessive oxidative stress is strongly linked to endothelial dysfunction and atherosclerosis ultimately causing cardiovascular diseases. Hence, tHcy is well known for its associations with cardiovascular diseases, with high levels indicative for endothelial dysfunction resulting in atherosclerosis eventually leading to myocardial infarction. In pregnancy, tHcy is also associated with vascular, metabolic and endocrine problems leading to an increased risk of subfertility, miscarriage, congenital malformations, SGA and pre-eclampsia. This can partially be explained by a disbalance between cell multiplication and apoptosis of the involved vasculature, i.e., utero-placental vasculature, which can lead to abnormal and reduced development of the spiral arteries. The latter might hamper the fetal developmental potential by inadequate supply of oxygen and nutrients resulting in growth restriction and possible fetal demise.

This thesis describes the associations between multiple periconceptional nutrition and lifestyle factors and (pre-implantation) embryonic growth and early placental growth. It is important to realize that many other non-lifestyle factors are associated with early embryonic growth, placental growth and pregnancy outcomes. Maternal age, parity and artificial reproductive techniques are all positively associated with early embryonic growth (28). On the contrary cardiovascular risk factors in women are negatively associated with embryonic growth. Also low socioeconomic status and low-level education are strong risk factors for adverse pregnancy outcome such as gestational diabetes, preeclampsia and preterm delivery (29-31). Importantly many of these factors are also associated with nutrition and lifestyle factors, making nutritional factors important confounders.

Future perspectives for research and clinical practice

Pre-implantation embryo development: It is interesting to investigate the influences of external nutrition and lifestyle exposures on the first embryo cleavage divisions, since these are associated with pregnancy chance. Predicting pregnancy chance based on morphology or morphokinetics in IVF-ICSI cycles has a high priority, since this might aid the selection of the best embryo and thereby increase the chance of pregnancy. It also might be more cost effective in case a successful pregnancy is reached with less number of treatment attempts. Although multiple researchers have tried to develop algorithms to predict pregnancy rates, the highest accuracy of prediction has an area under the ROC-curve of 0.69, which is a fairly poor predictor (32). Ovarian environment and oocyte health, endometrium, maternal age and as investigated in this thesis nutrition and lifestyle can heavily influence these pregnancy chance predictions, but are not taken into account in recent prediction models.

Post-implantation growth: Even though in IVF or IVF-ICSI treatments the morphologically most optimal embryo is transferred into the uterus of the women, there is large drop in the chance to become pregnant between the embryo transfer and the subsequent implantation process leading to the establishment of a successful pregnancy indicating that embryo quality as well as implantation are complex processes that are poorly understood. The window between pre-implantation embryo development and subsequent detection of vital embryonic presence as observed with ultrasound is considered a black box of pregnancy. However, in this period multiple genetic and molecular mechanisms are thought to play a crucial role. Recent animal studies reveal the possibility to investigate embryonic growth in vitro beyond the implantation phase (33). The possibility of human in vitro culturing beyond the pre-implantation stage allows for investigating of human program of lineage diversification, cell-fate specification and morphogenetic movements during this black box time window (33-35). Finding novel key-genes, identifying key features of cross talk between the endometrium and the embryo as well as key regulators of post implantation growth open new areas of understanding patho- and physiological mechanisms. These new insights might also provide opportunities for new diagnostic tools and new treatment options for early pregnancy problems. The first days of postimplantation embryonic growth seems independent of maternal tissue, indicating the large role of the self-organizing embryo and therewith also the possible important role of the paternal genome. These new techniques make it possible to investigate previous unknown territories of embryonic and placental growth.

For many years first trimester embryonic growth was thought to be uniform in all pregnancies. Recent research has proven that this is not the case and that external factors such as nutrition, lifestyle and habits, but also internal factors such as age, ethnicity and educational level can influence and even alter embryonic growth and development (28).

Although all extrinsic and intrinsic factors contribute a relatively small proportion of only one or two days to the measurements of the CRL, this may in the future increase the accuracy of pregnancy dating by new algorithms.

Study design: cohort study vs randomized controlled trial: Randomized controlled trials (RCT's) are considered to be the gold standard in the medical research field. RCT's are comparative, controlled experiments in which effect sizes are determined with less bias than observational studies. Randomization is considered the most powerful experimental design in clinical trials: with other variables being approximately equal between groups, differences in outcome are attributed to the intervention. However, by design, participants usually are not representative of the population as a whole and it is impossible to extract which subset of participants did benefit from the intervention. Usually, large number of participants are needed to achieve significance, which makes RCT's expensive. RCT's should therefore not be the only type of evidence used to make practice evidence based. A wider range of data should be used such as epidemiological data, qualitative data and case series to optimally make use of all evidence. As all designs have their methodological flaws, combining data from different designs gives the most generalizable and strong evidence.

Clinical applicability: We have shown associations between maternal and paternal nutrition and lifestyle as well as artificial environment on pre- and postembryonic, placental and fetal development. These results need to be incorporated into medical training curricula, ensuring that health care providers become properly educated about the effects of parental lifestyle and nutritional behaviours during the life course of themselves and their offspring. Adequate training will allow them to correctly and timely inform and counsel their patients about their lifestyle and their associations. Since paternal smoking is strongly associated with pre-implantation embryo quality it should be advised to actively motivate men to quit smoking before starting any attempt trying to conceive, either naturally or by IVF-ICSI. Since the lifecycle or renewal of sperm only takes up 3 months, paternal lifestyle changes can lead to a rapid response. Similarly, clear associations between maternal and paternal obesity and pre-implantation embryo quality and pregnancy chance have been established. Losing weight, by both parents to be, prior to a pregnancy and before any ART treatment is commenced, is therefore highly mandatory. The first and biggest challenge will be the adoption and maintenance of a healthier lifestyle over a longer period of time. There are multiple ways to achieve this goal, for example by (mental) coaching, dieting, sporting, digital support or any combination of the previous methods. Simultaneously, promotion of vitamin use, specifically folic acid during the periconception period in normal dosages, but also vegetable and fruit intake can already positively influence early embryo quality and placental growth. The periconception period is the most optimal and cost-effective timeframe to improve nutrition and lifestyle, due to the combination that couples with a wish to conceive are most open to changing their lifestyle and nutritional behaviours

and hereby improving the chances to become pregnant and its outcome. Importantly, preconception counseling on lifestyle and nutritional behaviour is most effective when partners actively participate. Periconception counseling, either online using E-health or face-to-face, should be obligatory and be incorporated by all healthcare workers working in the fields related to reproductive medicine. New, innovative methods combining the use of digital provision of information such e-health with physical face-to-face contacts is proven to be effective (36). With the findings of our previous research in mind, it is strongly advised to counsel preconceptionally not only women, but actively involve their partners, using a blended care approach. Hereby, creating the opportunity for the joint couple to increase knowledge and participation, together improve nutrition and lifestyle behaviors and ultimately create healthier individuals with healthier offspring.

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CHAPTER 10

10

Summary
Samenvatting

Summary

The periconception period is defined as the period 14 weeks before conception and 10 weeks thereafter. Within this period, all developmental stages essential for successful fertilization and pregnancy course take place. Examples of these biological developments are spermatogenesis, oogenesis, pre- and postimplantation embryonic development, implantation and placentation. A diversity of maternal and paternal factors can influence the course of these processes, such as genetics and epigenetics. Also nutrition and lifestyle factors are involved, which determine the health of the biological surroundings of the developing pregnancy. The one-carbon (1-C) metabolism is essential in all of the developmental stages since it provides 1-C moieties for various biological processes in order to successfully accomplish optimal embryonic, fetal and placental growth and development. A lot of research is performed to investigate the association between maternal effects of the 1-C metabolism on pregnancy outcomes. The most well-known example is the maternal preconceptional folic acid supplement use, or a folate rich dietary pattern, lowering the risk of neural tube defects in developing embryos during pregnancy. Far less research is performed investigating the association and effects between paternal nutrition and lifestyle factors on early embryo development, pregnancy course and outcome.

Couples suffering from subfertility can accomplish a pregnancy using artificial reproductive technologies. By spontaneous or assisted fertilization of an oocyte by sperm in the laboratory, an embryo is created. Early development of these embryos before transfer to the uterus of the future mother can be studied. This allows for investigation of paternal, and also maternal, effects on embryonic development. Subsequent surplus embryos can be cryopreserved for later use. The use of these artificial techniques, including assisted fertilization, growth in culture medium, freezing followed by thawing, can all potentially induce (epi) genetic differences in the embryonic genome.

The goal of the current thesis was to investigate the effects of parental nutritional and lifestyle and *in vitro* conditions in relation with (preimplantation) embryo and placental development.

Part 1 of the thesis introduces the developmental processes during the periconception period, the relationship with the 1-C metabolism and techniques involved in reproductive medicine.

In **Chapter 1**, the background of all involved developmental processes, such as spermatogenesis, oogenesis, fertilization and embryonic development is described. These

stages are vulnerable to (ir)reversible and acute environmental influences (e.g. folic acid use, smoking, alcohol and nutrition) on molecular processes such as cell multiplication, epigenetic processes, DNA-replication and -repair as well as protein synthesis. Furthermore, we describe the techniques used in this thesis to visualize the developmental processes of the (pre-implantation) embryo and placenta, using time-lapse incubators, two- and three-dimensional ultrasound techniques and virtual reality. The 1-C metabolism, one the most important metabolisms involved in the periconception period is described. We clarify that 1-C metabolism influences the synthesis of nucleotides, crucial for the synthesis of DNA, and proteins and lipids required for most pathways, e.g. biosynthesis of building blocks of membranes, signaling, and inflammation. These pathways also influence the methylation of DNA and associated (histone) proteins. The 1-C metabolism is dependent on intake of folic acid through supplement use, folate through the diet, vitamin B12, methionine and the formation of homocysteine and is therefore strongly associated with daily nutrition and lifestyle, including the use and shortage of vitamins. In conclusion the 1-C metabolism provides essential 1-C moieties and methyl groups for various preconception biological processes in order to successfully conceive and to accomplish optimal embryonic and fetal growth and development.

In **Chapter 2,** we describe the results of a systematic review and meta-analysis investigating the association between periconception paternal folic acid use and folate plasma levels on the effects of paternal reproductive outcome from sperm quality to pregnancy outcomes. Folic acid is essential for biological processes and involved in 1-C metabolism. While the effectiveness of maternal folate in reducing the risk of congenital malformations during pregnancy is well established, the effects of paternal folic acid use and folate plasma levels is scarcely investigated. We describe that both too low and too high levels of folate are associated with decreased sperm parameters, but that only too low levels are associated with increased sperm DNA damage. The paternal folate status seems associated with pregnancy outcomes, but research is limited.

In **part 2** of this thesis we investigated the influences of maternal and paternal nutrition and lifestyle factors on *in vitro* pre-implantation embryo development.

In **Chapter 3** we focused on the relation between nutrition and lifestyle factors and pre-implantation embryo development. The population of 100 women and 40 men undergoing intracytoplasmic sperm injection (ICSI) treatment, were all subscribed to the Smarter Pregnancy program. Smarter pregnancy is a personalized m-health coaching program on the mobile phone, which identifies 5 nutrition and lifestyle habits being vegetables and fruits intake, folic acid use, smoking cigarettes and drinking alcohol. We measured embryo quality using the KIDScore and showed that paternal smoking highly significant negatively influences pre-implantation embryo quality. Furthermore, we also show that low maternal

vegetable and fruit intake is associated with lower quality of the pre-implantation embryos, which is exaggerated in women with obesity.

In **Chapter 4,** the association between paternal obesity on pre-implantation embryo development is investigated. Therefore, we included more than 200 men undergoing IVF with or without ICSI using ejaculated sperm. We showed that paternal obesity was significantly negative associated with the fertilization rate. Paternal obesity is associated with embryos developing faster in the first cleavage divisions of pre-implantation embryo development. However, we could not detect an association between the quality of the embryo as measured by the KIDScore and paternal obesity.

The influences of maternal and paternal nutrition and lifestyle factors on post-implantation embryo development are investigated in **part 3** of this thesis.

In **Chapter 5** we analyzed the association between paternal first trimester serum folate concentrations and the first trimester embryonic growth parameters. Folate levels were measured in red blood cells as indicator of the folate concentrations of the previous three months. Embryonic growth, measured using repeated measurements, of the crown rump length (CRL) and embryonic volume (EV) were performed using three dimensional ultrasound in combination with virtual reality software. We found that in spontaneously conceived pregnancies folate levels in quartile 1, 2 and 4 were associated with reduced embryonic growth as compared to level in quartile 3, indicating that both too low and too high levels are associated with reduced growth. These associations could not be detected in pregnancies after assisted pregnancies using IVF/ICSI. A possible explanation might be that the IVF/ICSI procedure itself has a stronger epigenetic effect than paternal folate concentrations.

Chapter 6 describes the effect of frozen-thawed day 5 embryo transfers on first trimester embryonic growth and pregnancy outcomes. We compared these pregnancies with freshly (non-frozen) transferred embryos and natural pregnancies. The study population consisted of approximately 800 participants. We found that extended culture of frozen-thawed embryo transfer did not alter embryonic growth in the first trimester of pregnancy. We did however find that preterm birth occurred less frequently in the freshly transferred embryo group compared to the frozen-thawed embryo group.

In **part 4** of this thesis we investigated the influences of maternal and paternal nutrition and lifestyle factors on placental development.

In **Chapter 7** associations between the anatomical origin (testicular vs. epididymaal vs. ejaculated) of retrieved sperm and method of collection on embryonic and placental

growth were investigated. We included more than 150 ICSI pregnancies, of which 25 were conceived using surgically retrieved testicular sperm (TESE). We report that TESE sperm is specifically associated with increased placental volume (PV) in the first trimester of pregnancy as compared to freshly ejaculated sperm. TESE seems not to be associated with embryonic growth, while epididymal-originated sperm (which is at a later developmental and anatomical stage) was not associated with either placental or embryonic growth. Numbers in the groups were too small to investigate birth outcomes.

Chapter 8 describes the associations between maternal and paternal first trimester homocysteine concentrations on first trimester placental development as measured by repeated measurements of the utero-placental vascular volume (uPVV) and placental volume (PV). Paternal homocysteine seems not associated with uPVV and PV. We show that especially in pregnancies after IVF/ICSI, maternal plasma total homocysteine concentrations are negatively associated with uPVV. This indicates that high homocysteine concentrations are associated with smaller uPVV's in the first trimester of pregnancy. These findings are in line with other studies showing high homocysteine concentrations to be associated with an increased risk of placenta-related pregnancy complications, such as pre-eclampsia and babies born small for gestational age.

In **Part 4, Chapter 9,** we provide a short summary and general discussion of main findings, clinical and societal implications and directions for future research.

Samenvatting

De periode die 14 weken vóór de conceptie begint en duurt tot 10 weken na de conceptie noemen we de periconceptie periode. In deze periode vinden alle essentiële ontwikkelingen plaats die belangrijk zijn voor een succesvolle fertilisatie en verloop en uitkomst van de zwangerschap. Het gaat dan om biologische ontwikkelingsprocessen zoals spermatogenese, oögenese, (pre- en postimplantatie) embryonale ontwikkeling, embryo-implantatie en placentatie. Verscheidene maternale en paternale factoren kunnen het beloop van deze processen beïnvloeden. Voorbeelden daarvan zijn genetische en epigenetische factoren, maar ook voeding en leefstijlfactoren, die de gezondheid bepalen van de meest directe lichaamsomgeving, waarin de zwangerschap zich ontwikkeld.

Het één-koolstof (1-K) metabolisme is essentieel voor alle ontwikkelingsstadia in de periconceptie periode. Dit metabolisme zorgt voor de productie van essentiële bouwstoffen die leiden tot optimale embryonale, foetale en placentaire groei en ontwikkeling. Er is een aanzienlijke hoeveelheid onderzoek gedaan naar verbanden tussen de maternale effecten van het 1-K metabolisme op zwangerschapsuitkomsten. Het bekendste voorbeeld is dat het gebruik van preconceptioneel maternaal foliumzuur, als supplement of in een foliumzuurrijk voedingspatroon, en een essentieel substraat in het 1-K metabolisme, het risico op neuralebuisdefecten, zoals een spina bifida, verlaagd. Er is echter aanzienlijk minder onderzoek gedaan naar de effecten van het paternale foliumzuur en andere voeding- en leefstijlfactoren op het verloop van de zwangerschap.

Bij koppels met een subfertiliteit probleem kan een zwangerschap bewerkstelligd worden door artificiële reproductieve technieken. Hierbij ontstaat het embryo in het laboratorium door de oocyt en het zaad samen te brengen. Embryo's kunnen daarnaast ook ingevroren worden voor later gebruik. Deze manier van fertilisatie kan ook veranderingen in het (epi) genoom veroorzaken.

Het doel van dit proefschrift is om de periconceptionele voeding en leefstijl van paren en *in vitro* condities te bestuderen in relatie tot de (preïmplantatie) embryonale en placentaire ontwikkeling.

Deel 1 van dit proefschrift introduceert alle biologische ontwikkelingen die plaatsvinden in de periconceptie periode. In het bijzonder wordt ingegaan op de rol van het 1-K metabolisme en *in vitro* technieken die worden gebruikt bij de artificiële fertilisatie in de reproductieve geneeskunde.

Hoofdstuk 1 beschrijft de algemene achtergrond en doelstellingen van het proefschrift. We behandelen alle periconceptionele ontwikkelingsprocessen, zoals spermatogenese, oögenese, fertilisatie en embryonale en placentaire ontwikkeling. Deze stadia zijn gevoelig voor (niet) reversibele omgevingsfactoren (bijvoorbeeld foliumzuurgebruik, roken, alcoholgebruik en voeding). Hierbij worden verschillende moleculaire processen veranderd, zoals celdelingen, epigenetische markeringen, het vermenigvuldigen van DNA en het herstel van DNA schade. Verder leggen we het 1-K metabolisme verder uit, met name waarom het essentieel is voor biologische ontwikkelingsprocessen in de periconceptie periode. Dit metabolisme zorgt voor de productie van nucleotiden, belangrijk voor de opbouw van DNA, proteïnes en lipiden, die belangrijk zijn voor de biosynthese van celmembranen, signalering, metabolisme en inflammatie. Het zorgt daarnaast ook voor de aanmaak van 1-K groepen, o.a. methylgroepen die van belang zijn bij de methylatie van eiwitten, DNA en histonen. Het 1-K metabolisme is afhankelijk van de inname van folaat via voeding, foliumzuur via suppletie, vitamine B12, methionine en de vorming van homocysteïne en is daarmee sterk geassocieerd met dagelijkse voedingsinname.

In dit hoofdstuk beschrijven we ook de verschillende technieken die we gebruiken voor het visualiseren van (pre- en postimplantatie) embryo ontwikkeling en placentaire ontwikkeling. We maken daarbij gebruik van time-lapse incubatoren, twee- en driedimensionaal ultrageluidsonderzoek en virtual reality.

In **hoofdstuk 2** beschrijven we de resultaten van een systematische review en meta-analyse waarin we de associaties tussen periconceptioneel paternaal foliumzuur gebruik en de effecten op paternale reproductieve uitkomsten van zaadkwaliteit tot zwangerschapsuitkomsten onderzoeken. Foliumzuur en folaat zijn essentieel voor het 1-K metabolisme en daarmee voor verschillende biologische processen. De effecten van paternaal foliumzuurgebruik zijn nauwelijks onderzocht, waarbij de meeste studies zijn gericht op de relatie tussen foliumzuurgebruik en de zaadkwaliteit. Wij vonden dat zowel te hoge als te lage folaat waardes in het bloed geassocieerd zijn met verminderde zaadkwaliteit. Alleen te lage folaat waardes in het bloed zijn geassocieerd met een toename van DNA schade in de zaadcellen. De paternale folaat status is in sommige studies geassocieerd met uitkomsten van de zwangerschap, maar de kwaliteit van de studies is te laag om een duidelijke conclusie te kunnen trekken.

In **deel 2** van dit proefschrift onderzoeken we de invloed van maternale en paternale **voeding en leefstijl** op de *in vitro* ontwikkeling van het preïmplantatie embryo.

In **hoofdstuk 3** hebben we onderzocht wat het effect van voeding en leefstijl van zowel de man als de vrouw is op de preïmplantatie embryonale kwaliteit. De studiepopulatie bestond uit 100 vrouwen en 40 mannelijke partners die in ons ziekenhuis een intracytoplasmatische

sperma injectie (ICSI) behandeling hebben ondergaan. Al deze patiënten waren geabonneerd op het Slimmer Zwanger programma, een gepersonaliseerd online coaching programma op de mobiele telefoon. Het programma identificeert 5 voedings- en leefstijlgewoontes, namelijk inname van groente, fruit en foliumzuur en het roken van sigaretten en alcohol gebruik. Wij hebben de preïmplantatie embryo kwaliteit gemeten met de KID-Score en hebben aangetoond dat roken van aanstaande vaders de kwaliteit van het preïmplantatie embryo significant verminderd. Daarnaast laten we ook zien dat weinig inname van groente en fruit door aanstaande moeders geassocieerd is met een lagere embryo kwaliteit, dit effect is nog sterker bij vrouwen met obesitas.

In **hoofdstuk 4** hebben wij de associatie onderzocht tussen paternale obesitas en preïmplantatie embryo ontwikkeling en de uitkomsten van een IVF/ICSI behandeling. De studie populatie bestond uit meer dan 200 mannen die in ons ziekenhuis een IVF of ICSI behandeling met geëjaculeerd zaad hebben ondergaan. We laten zien dat paternale obesitas leidt tot een significant lagere fertilisatie. Daarnaast laten we zien dat hoe hoger het gewicht van de aanstaande vader is, des te sneller de eerste celdelingen verlopen tijdens de preïmplantatie embryo ontwikkeling. Deze snellere deling zorgde niet voor een daling van de embryokwaliteit of slechtere uitkomsten van de IVF/ICSI behandeling.

De invloed van maternale en paternale **voeding en leefstijl** op de ontwikkeling van het postimplantatie embryo onderzoeken wij in **deel 3** van dit proefschrift.

Hoofdstuk 5 beschrijft de resultaten van het onderzoek naar de associatie tussen de paternale folaat concentraties in het bloed, die zijn gemeten in het eerste trimester van de zwangerschap en de embryonale groei in het eerste trimester van de zwangerschap. De folaat concentraties zijn gemeten in de erythrocyten en dit is een goede weergave van de folaat concentratie in de afgelopen drie maanden. Embryonale groei, vastgesteld met herhaalde metingen van de kop-stuit lengte (CRL) en het embryonale volume, hebben wij gemeten met driedimensionaal ultrageluidsonderzoek in combinatie met virtual reality software. We vonden dat in de groep met spontane zwangerschappen folaat levels in de 1^{e}, 2^{e} en 4^{e} kwartiel geassocieerd zijn met verminderde embryonale groei vergeleken met folaat levels in het 3^{e} kwartiel. Dit impliceert dat zowel te hoge als te lage folaat concentraties geassocieerd zijn met verminderde embryonale groei. Wij vonden deze associaties niet in de zwangerschapsgroep na een IVF/ICSI behandeling. Een mogelijke verklaring is dat de IVF/ICSI behandeling een sterker effect heeft dan de paternale folaat status.

In **hoofdstuk 6** beschrijven we het effect van het invriezen van teruggeplaatste embryo's gekweekt tot dag 5, op de eerste trimester embryonale groei en zwangerschapsuitkomsten. We vergeleken deze zwangerschappen met teruggeplaatste verse (niet-ingevroren) embryo's en natuurlijke zwangerschappen. De onderzochte groep bestond uit meer dan

800 patiënten. We vonden dat ingevroren embryo's geen andere embryonale groei hadden in het eerste trimester van de zwangerschap in vergelijking met de verse embryo's. Er werden wel minder prematuur geboren kinderen gezien in de groep verse vergeleken met de ingevroren embryo's.

In **deel 4** van dit proefschrift onderzoeken we de invloed van maternale en paternale **voeding en leefstijl** op de ontwikkeling van de placenta.

Hoofdstuk 7 beschrijft de gevonden associaties in ICSI zwangerschappen tussen de herkomst van de zaadcellen (testiculair vs. epidydimaal vs. geëjaculeerd) en eerste trimester embryonale en placentaire groei en ontwikkeling. We includeerden meer dan 150 ICSI zwangerschappen, waarvan er 25 zijn ontstaan na testiculair verkregen zaad (TESE). We vonden dat TESE geassocieerd is met grotere placentaire groei in het eerste trimester van de zwangerschap in vergelijking met geëjaculeerd zaad. TESE zaad was niet geassocieerd met embryonale groei en ontwikkeling. Epidydimaal zaad was niet geassocieerd met zowel placentaire als embryonale groei en ontwikkeling. De aantallen patiënten in de groepen waren te klein om ook zwangerschapsuitkomsten te bestuderen.

In **hoofdstuk 8** hebben wij de associatie bestudeerd tussen maternale en paternale eerste trimester homocysteïne concentraties en eerste trimester placentaire ontwikkeling. Eerste trimester placenta ontwikkeling hebben wij gemeten door herhaalde metingen uit te voeren van het utero-placentair vasculair volume (uPVV) en placenta volume (PV). Het paternaal homocysteïne is niet geassocieerd met uPVV en PV. We vonden dat vooral in IVF/ICSI zwangerschappen het maternaal homocysteïne negatief geassocieerd is met uPVV en PV. Bovendien blijkt een verhoogd eerste trimester maternaal homocysteïne geassocieerd te zijn met een verminderd placentaire volume. Deze bevindingen passen bij eerdere studies die lieten zien dat een verhoogd maternaal homocysteïne in het eerste trimester geassocieerd is met een hogere kans op placenta-gerelateerde complicaties, zoals pre-eclampsie en foetale groeivertraging.

In **deel 4**, **hoofdstuk 9**, van dit proefschrift geven wij een korte samenvatting van de resultaten. Verder beschrijven wij de algemene discussie van onze resultaten met daarbij ook de methodologische overwegingen, klinische en maatschappelijke implicaties en aanwijzingen voor vervolgonderzoek.

ADDENDUM

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List of abbreviations

1-C	One-carbon
2D	Two-dimensional
3D	Three-dimensional
5-MC	5-methylcytosine
5-MTHF	5-methyl-tetrahydrofolate
AC	Abdominal circumference
AHCY	S-adenosyl-L-homocysteine hydrolase
AnxA5	Annexin A5
AO	Acridine orange
ART	Artificial reproductive techniques
AUC	Area under the curve
BMI	Bodymass index
Cav1	Caveolin 1
CBS	Cystathionine ß-synthase
CI	Confidence interval
CRL	Crown Rump Length
CTH	Cystathionine gamma-lyase
DFI	DNA fragmentation index
DMR	Differentially methylation region
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
DRS	Dietary risk score
EDTA	Ethylenediaminetetraacetic acid
EFW	Estimated fetal weight
Elavl1	Embryonic Lethal, Abnormal Vision, Drosophila-Like 1
Esr1	Estrogen Receptor 1
ET	Embryo transfer
EV	Embryonic volume
EVT	Extravillous trophoblast
FA	Folic acid
FL	Femur length
FOV	Fields of view
FSH	Follicle stimulating hormone
GA	Gestational age
GLMM	Generalized linear mixed model

GnRH	Gonadotropin-releasing hormone
HC	Head circumference
hCG	Human chorionic gonadotropin
HPG	Hypothalamic-pituitary-testes (gonad)
HPO	Hypothalamic-pituitary-ovarian
ICC	Intraclass correlation coefficient
ICSI	Intracytoplasmic sperm injection
IGF2	Insuline like growth factor 2
IQR	Interquartile range
IUI	Intrauterine insemination
IVF	In vitro fertilization
kg	Kilogram
KID	Known implantation data
L	Liter
LGA	Large for gestational age
LH	Luteinizing hormone
LMM	Linear mixed model
LRS	Lifestyel risk score
MEG	Maternally expressed gene
MESA	Microsurgical Epididymal Sperm Aspiration
µg	Microgram
mg	Milligram
mHealth	Mobile health
µm	Micrometer
mm	Millimeteres
mm2	Squared millimeters
mRNA	Messenger ribonucleic acid
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
MTRR	Methionine synthase reductase
n	Number
N/a	Not applicabale
NDN	Necdin
nmol	Nanomol
NNT	Numbers needed tot threat
NOA	Non-obstructive azoospermia
NTD	Neural tube defect
OA	Obstructive azoospermia
OAT	Oligoasthenoteratozoospermia
OHSS	Ovarian hyperstimulation syndrome

OR	Odds ratio
PCOS	Polycystic ovary syndrome
PD	Power Doppler
PE	Preeclampsia
PEG	Paternally Expressed Gene
PGC	Primordial germ cells
PIH	Pregnancy induced hypertension
Pna	Pronuclei appearance
PNf	Pronuclei fading
PTB	Preterm birth
PV	Placental volume
Q	Quartile
RBC	Red blood cell
RCT	Randomised controlled trial
Ref	Reference
ROS	Reactive oxygen species
RR	Relative risk
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SCSA	Sperm chromatin structure assay
Se	standard error
SGA	Small for gestational age
SNP	Single nucleotide polymorphisms
t	Time-point
TESE	Testicular sperm extraction
tHcy	Total plasma homocysteine
THF	Tetrahydrofolate
TRS	Total risk score
uPVV	Utero-placental vascular volume
US	Ultrasound
VCM	Progressively motile sperm
VEGF	Vascular endothelial growth factor
VOCAL	Virtual Organ Computer-aided AnaLysis
VR	Virtual reality
WHO	World health organisation
ZP	Zone pellucida

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PhD portfolio

Name PhD student: Jeffrey Hoek
Department: Obstetrics and Gynaecology
Research school: NIHES
PhD period: 2017-2020
Promotor: Prof. dr. R.P.M. Steegers-Theunissen en Prof. dr. J.S.E. Laven
Copromotor: Dr. S. Schoenmakers

PHD Training	YEAR	ECTS
Courses		
Research master Clinical Research (NIHES, ErasmusMC)	2010-2016	120
e-BROK course (NFU BROK Academy)	2017	1.0
Research integrity in science (ErasmusMC)	2017	0.3
Basic Course on 'R' (MolMed ErasmusMC)	2018	1.8
Motiverende gespreksvoering (Psychodidact)	2019	1.0
Presenting PechaKucha Style (EUR graduate school)	2019	1.0
Attended seminars and workshops		
Annual Wladimiroff award meeting, department O&G	2017-2020	0.5
Weekly research meeting of the department of O&G	2017-2020	3.0
Three monthly research meetings Rotterdam O&G	2017-2020	0.5
Monthly Academic center of Excellence Pregnancy & Childhood	2017-2020	0.5
Symposium de Snoo. Utrecht, the Netherlands.	2019	0,5
Presentations at (inter) national conferences		
9th DOHaD Congres, Rotterdam, The Netherlands. Poster	2017	1.0
65th Annual scientific meeting of the SRI, San Diego, USA. Poster	2018	1.0
Sophia Research Day, Rotterdam, The Netherlands. Oral	2018	1.0
ACE Pregnancy & Childhood day, Rotterdam, The Netherlands. Oral	2018	1.0
54th Gynaecongres, Amersfoort, The Netherlands. Oral	2018	1.0
22e Nederlands-Vlaams doelencongres. Oral	2019	1.0
Sophia Research Day, Rotterdam, The Netherlands. Oral	2019	1.0
66th Annual scientific meeting of the SRI, Paris, France. Poster	2019	1.0
10th DOHaD Congres, Melbourne, Australia. Oral and 2 posters	2019	1.0
67th Annual scientific meeting of the SRI, Vancouver, Canada.	2020	1.0

PHD Training	YEAR	ECTS
Teaching		
Supervising		
Bianca Ringelberg	2019	2.0
Bernice Eggink	2020	2.0
Presentation 'healthy pregnancy' for obstetric nurses in training (3x / year)	2017-2020	2.0
Coordinator of week 2 of the minor 'voeding en leefstijl' including organizing the program and giving a presentation	2019	1.0

About the author

Jeffrey Hoek was born on the 19th of February 1991 in Nieuw-Beijerland (The Netherlands). He grew up in Nieuw-Beijerland and graduated from the Hoeksch Lyceum (VWO Gymnasium) in 2009. In 2009, he started medical school at the Erasmus University Medical Center in Rotterdam (The Netherlands). After obtaining his Bachelor's degree in 2012 he started his research master in Clinical Research (Netherlands Institute for Health Sciences) during which he attended the Summer Institute of Epidemiology and Biostatistics at the Johns Hopkins Bloomberg School of Public Health (Baltimore, United States). After finishing medical school and his research master in 2016 he started working as resident not in training at the department of Obstetrics and Gynaecology in the Amphia hospital, Breda, The Netherlands. In 2017 he started his PhD research in the Erasmus University Medical Center in Rotterdam, at the department of Obstetrics and Gynaecology, subdivision periconception epidemiology, under the supervision of Prof. Régine P.M. Steegers-Theunissen, Prof. Joop. S.E. Laven and Dr. Sam Schoenmakers. In January 2021 he started his clinical training in Obstetrics and Gynaecology at the Amphia hospital, Breda, The Netherlands.

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