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Perinatal outcomes after assisted reproductive technology.
Are adverse perinatal outcomes after assisted reproductive technology affected by mode of treatment or maternal factors?

Doctoral thesis

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Thesis for the degree of
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Norsk sammendrag

Perinatale utfall etter assistert befruktning. Er dårligere perinatal helse etter assistert befruktning et resultat av behandlingsmetode eller faktorer hos mor?

Barn født etter assistert befruktning har høyere risiko for uheldige svangerskapsutfall sammenlignet med naturlig unnfangede barn, selv når de er enkeltfødte. Med ny teknologi har antallet barn født etter fryse-forsøk økt, i tillegg blir stadig flere barn født etter fersk-forsøk. Disse behandlingsmetodene virker å være forbundet med forskjellige uheldige utfall. Om det er faktorer ved mor eller behandlingen som er årsaken til disse dårlige utfallene er usikkert.

I en stor nordisk befolkning undersøkte vi om barn født etter fersk og fryse-behandling hadde høyere risiko for en rekke perinatale utfall sammenlignet med naturlig unnfangede barn. Resultatene viser en tydelig sammenheng mellom fersk behandling og lavere fødselsvekt og høyere risiko for å være for liten ved fødselen i forhold til svangerskapslengde, mens barn født etter fryse-behandling hadde høyere fødselsvekt og høyere risiko for å være født for stor i forhold til svangerskapslengde. Barn født etter begge typer assistert befruktning hadde høyere risiko for prematur fødsel og neonatal død, men samlet sett var det ingen høyere risiko for intrauterin fosterdød blant barna født etter fersk eller fryse-behandling.

Vi inkluderte søskenanalyser som ga ekstra pålitelighet i forsøket på å skille betydningen mors faktorer og behandlingsmetode for vanlige uheldige fødselsutfall. Dette styrket konklusjonene om at forskjellen i fødselsvekt ser ut til å kunne tilskrives behandlingsmetodene, mens risikoen for prematur fødsel ser ut til å påvirkes av både faktorer hos mor og av behandlingsmetode. Da vi undersøkte perinatal død som er både sjelden, men også særlig traumatisk for foreldrene, så vi at dette endret foreldrenes valg, noe som ga stor seleksjonsskjevhet i søskenanalysene, og resultatene ble upålitelige.

Navn kandidat: *Kjersti Westvik-Johari*

Institutt: *Institutt for samfunnsmedisin og sykepleie*

Veiledere: *Signe Opdahl, Liv Bente Romundstad og Siri Eldevik Håberg*

Finansieringskilde: *Norges teknisk- naturvitenskapelige universitet*

Summary

Perinatal outcomes after assisted reproductive technology. Are adverse perinatal outcomes after ART affected by mode of treatment or maternal factors?

Children born after assisted conception have a higher risk of adverse perinatal health compared to naturally conceived, even when they are singletons. New technology has seen a rise in the number of children born after frozen embryo transfer as well as fresh embryo transfer. These treatment modes seem to be associated with different adverse outcomes. Whether these adverse outcomes are caused by factors related to the mother or the assisted reproductive technology is not clear.

We investigated perinatal outcomes among children born after fresh and frozen embryo transfer compared to children conceived naturally in a large Nordic population. Our results show a clear association between fresh embryo transfer and lower birthweights and being born small for gestational age, while children of frozen embryo transfer had higher birthweights and were more likely to be large for gestational age. Children of both assisted conception methods were at higher risk of preterm birth and neonatal death, but overall they were not at higher risk of stillbirth.

Our analytic approach including sibling studies which added robustness to our results when disentangling whether common adverse outcomes are influenced by treatment or maternal factors. This approach strengthened our conclusion that differences in birthweight appear largely driven by treatment factors, whereas risk of preterm birth seems to be influenced by a combination of treatment and maternal factors. However, we also demonstrated the limitations of sibling analyses when investigating perinatal death, a rare but traumatic experience for the parents, which was associated with a change in parental choices and caused substantial selection biases in this sibling comparison.

Name of candidate: *Kjersti Westvik-Johari*

Department: *Department of Public Health and Nursing*

Supervisors: *Signe Opdahl, Liv Bente Romundstad, Siri Eldevik Håberg*

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All the co-authors, including **Anja Pinborg** and **Deborah Lawlor** have contributed with their broad and deep knowledge of assisted conception, epidemiology, and statistics. Collaborating with such highly experienced researchers have been very inspiring and helpful for my project.

I wish to thank my leader at St Olav's Department of Fertility, **Sigrun Kjøtrød** for her patience and support throughout this project. She has been very understanding of the challenges of research life. Special thanks to my colleagues at the department of Obstetrics and Gynaecology, including **Eline, Camilla, Hannah,** and **Maria** for their support and kindness, it is so enjoyable sharing an office with you.

Without my husband **Yasha** I would never have pursued a carrier in gynaecology, and certainly not a PhD- Thank you for your faith in me. Also, thanks for lending me your

illustrative skills. My children: **Marius, Ida** and **Morten** have been a wonderful distraction from work, and I will be ever grateful for their kindness when research-life ate up the days. Also, I must thank my mother **Turid**, who has always been there to “fill in the gaps” over the last ten years- there have been a lot of gaps to fill.

Finally, a special thanks to the founders of the CoNARTaS database, their work and effort has been considerable before achieving this database.

Trondheim, December 2021

Kjersti Westvik-Johari

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Westvik-Johari K, Romundstad LB, Lawlor DA, Bergh C, Gissler M, Henningsen AA, Håberg SE, Wennerholm UB, Tiitinen A, Pinborg A, Opdahl S. ***Perinatal health after fresh and frozen embryo transfer in assisted reproduction - separating parental and treatment contributions. A cohort study with within sib-ship analysis.*** PLoS Med. 2021 Jun 25;18(6): e1003683. doi: 10.1371/journal.pmed.1003683.

Paper II.

Westvik-Johari K, Lawlor DA, Romundstad LB, Bergh C, Wennerholm UB, Gissler M, Henningsen AKA, Håberg SE, Tiitinen A, Spangmose AL, Pinborg A, Opdahl S. ***Stillbirth and neonatal mortality in singletons born after fresh and frozen embryo transfer. Cohort study from the Committee of Nordic Assisted Reproduction Technology and Safety.*** Submitted to Am J Obstet Gynecol.

Paper III.

Westvik-Johari K, Håberg SE, Lawlor DA, Romundstad LB, Bergh C, Wennerholm UB, Gissler M, Henningsen AA, Tiitinen A, Pinborg A, Opdahl S. ***The challenges of selective fertility and carryover effects in within sibship analyses: the effect of assisted reproductive technology on perinatal mortality as an example.*** Will be submitted to Int J Epidemiol.

Abbreviations

AGA	Appropriate for Gestational Age
aOR	Adjusted Odds Ratio
ART	Assisted Reproductive Technology
BMI	Body Mass Index
CI	Confidence Interval
CoNARTaS	Committee of Nordic Assisted Reproductive Technology and Safety
DNA	Deoxyribonucleic acid
ESHRE	European Society of Reproduction and Embryology
Fresh-ET	Fresh embryo transfer
Frozen-ET	Frozen embryo transfer
ICD	International Classification of Diseases
ICSI	Intra Cytoplasmic Sperm Injection
IUI	Intrauterine insemination
IVF	In vitro fertilisation
LGA	Large for Gestational Age
MBR	Medical Birth Registry
NC	Natural Conception
OR	Odds Ratio
pp	percentage points
RD	Risk difference
SD	Standard deviation
SET	Single embryo transfer
SGA	Small for Gestational Age

1. Introduction

Since the beginning of human history fertility has had an important place in society and most cultures and religions have developed fertility rites and marriage ceremonies, including beliefs and remedies on how to cure infertility [1,2]. Motherhood is a valued and desired status, and infertility has been a particular burden to women[3,4]. Involuntary childlessness may lead to prolonged grief, reduced self-worth, and separation [5-7]. Mr. Steptoe and Professor Edwards were the pioneers, who after years of research were able to welcome Louise Brown in 1978, the first baby born after in vitro fertilization (IVF) [8]. In 2010, Professor Edwards was awarded the Nobel Prize of Physiology and Medicine for his lifelong dedication and achievement within the field of reproductive medicine [9]. However, their success was not initially overall cherished; colleagues, religious leaders and the public in general were skeptical to the technology and the inference with reproductive function [1,10]. One of the great concerns was the health of the children conceived by assisted reproduction. Despite the controversy, an overwhelming number of infertile couples volunteered to take part in the early experimental treatment of infertility [1]. Accordingly, medical refinements and technical advances have developed the field into a successful specialty, benefitting infertile couples all over the world. However, new developments including laboratory protocols, culture media, blastocyst transfers, vitrification, intracytoplasmic sperm injection and pre-implantation genetic screening have been implemented without solid evidence to show that new procedures are safe and beneficial to the patient [11,12]. Hence follow-up of pregnancies and the newborns conceived by assisted reproduction technology is of paramount importance to understand whether the conception method has any consequences for their health [13].

2. Background

2.1 ART, the process

ART comprises all methods where fertilisation takes place outside the woman's reproductive organs, with subsequent embryo transfer to the uterus. The process is initiated by controlled ovarian stimulation, achieved by administration of sex hormones to the woman [14]. A range of hormonal medicines and types of protocols are available for follicle growth and maturation of the oocytes [9]. The aim is to stimulate the development of several mature oocytes to improve the chances of good quality embryos which has the potential to produce a successful pregnancy. Regular ultrasound assessments during the treatment ensures oocyte collection at the appropriate time. Fertilisation is completed by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) after oocyte collection. Successfully fertilized oocytes are cultivated in vitro in media for either 2-3 days (cleavage stage embryo) or 5-6 days (blastocyst stage embryo). Following embryo transfer to the uterus, luteal phase hormonal support is given to improve the chances of implantation [15]. Spare embryos may be frozen and stored for thawing and transfer in subsequent cycles, with or without hormonal substitution [16]. The process is illustrated in Figure 1.

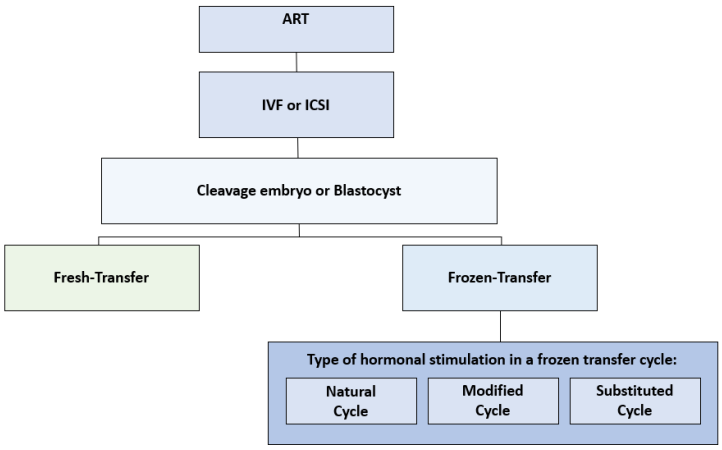


Figure 1. The process of assisted reproductive technology, overview of methods

2.2 Major achievements in ART

The scientific and technological advances in assisted reproduction have been many over the last 40 years, and some of the greatest milestones with important clinical implications are described below.

2.2.1 Method of fertilisation

In vitro fertilization (IVF) is an efficient treatment of female infertility. However, this technique is not sufficient to treat male infertility. A breakthrough for male infertility was the introduction of ICSI in the early 90's [17], though not commonly utilized until a few years later. ICSI is the procedure where a single sperm cell is chosen and injected directly into the oocyte (Figure 2) and is an invaluable method of fertilization the oocyte in cases of reduced sperm quality [18]. Further progress has introduced the possibility of retrieving sperm cells directly from the testicles or the epididymis (testicular sperm aspiration), a procedure mostly utilized where there is an obstruction of the spermatic cord, or extremely reduced sperm production.

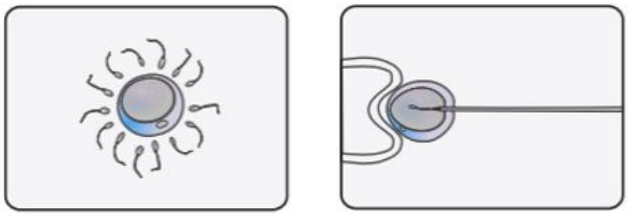


Figure 2. Fertilisation by IVF (left) and ICSI (right)

2.2.2 Freezing and storing of embryos

In the early days of ART, more than one good quality embryos were transferred back to the uterus, both to increase pregnancy rates and because the technology for storing and thawing embryos were poor. This practice led to a high rate of multiple pregnancies, which are high-risk pregnancies associated with more adverse outcomes compared to singleton pregnancies [19-21]. This encouraged the development of freezing techniques, where surplus embryos are stored in a frozen condition for

later utilization. Already in 1983 the first child was born after successful frozen embryo transfer [22], and the initially established freezing technique was called slow freeze. However, the embryo survival rate after thawing was poor, and less than 23% of all transfers in Europe consisted of frozen embryos transfer in 2010 [23].

From around 2008 the vitrification technique was introduced. This technique involves rapid freezing which avoids ice crystals from forming on the surface of the embryo, a common complication of the slow-freeze procedure [24]. Vitrification of embryos quickly overtook the slow-freeze procedure, due to almost 100% survival rate of the thawed embryos and a significantly improved cumulative pregnancy rate [25-27], which is the proportion that achieves pregnancy after a given number of cycles. Frozen embryo transfers have now overtaken fresh- embryo transfers in some countries, including USA and Australia/New Zealand [28]. Figure 3 shows the development in the Nordic countries.

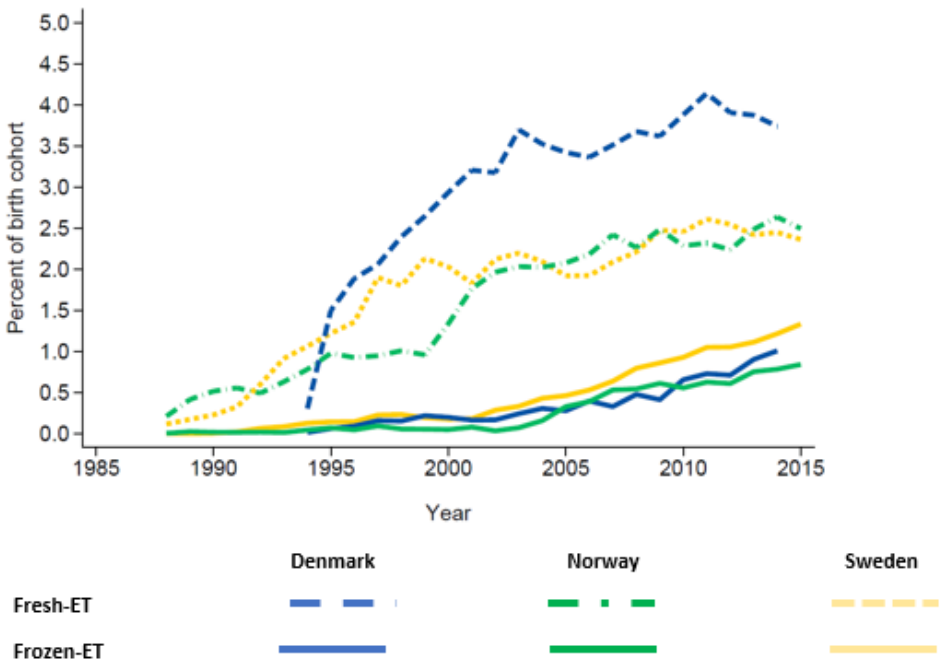


Figure 3. Time trends in Fresh and frozen transfers as proportions of all deliveries. Data from the CoNARTaS cohort.

2.2.3 Single embryo transfer (SET)

Already in 2003, Sweden introduced a single embryo transfer policy in public and private clinics, which achieved an impressive reduction in multiple pregnancies after ART treatment (Figure 4). With the improved vitrification technique other Nordic countries have also reduced their multiple pregnancy rates. A major hesitancy for implementing single embryo transfer (SET) among clinicians was the presumed reduced pregnancy rate and overall success rate. However, many studies have shown that the cumulative live birth rate was maintained despite a SET policy [29-32]. Double embryo transfer is associated with vanishing twin and adverse perinatal outcomes of the surviving twin [33], this may explain why SET is associated with improved perinatal outcomes compared to singletons after double embryo transfer [34,35] including lower risk of preterm birth, low birthweight [36,37] and lower perinatal mortality [38]. In addition, single embryo transfer has been shown to be more cost-effective by reducing the neonatal costs through reduced occurrence of preterm birth and twins [39].

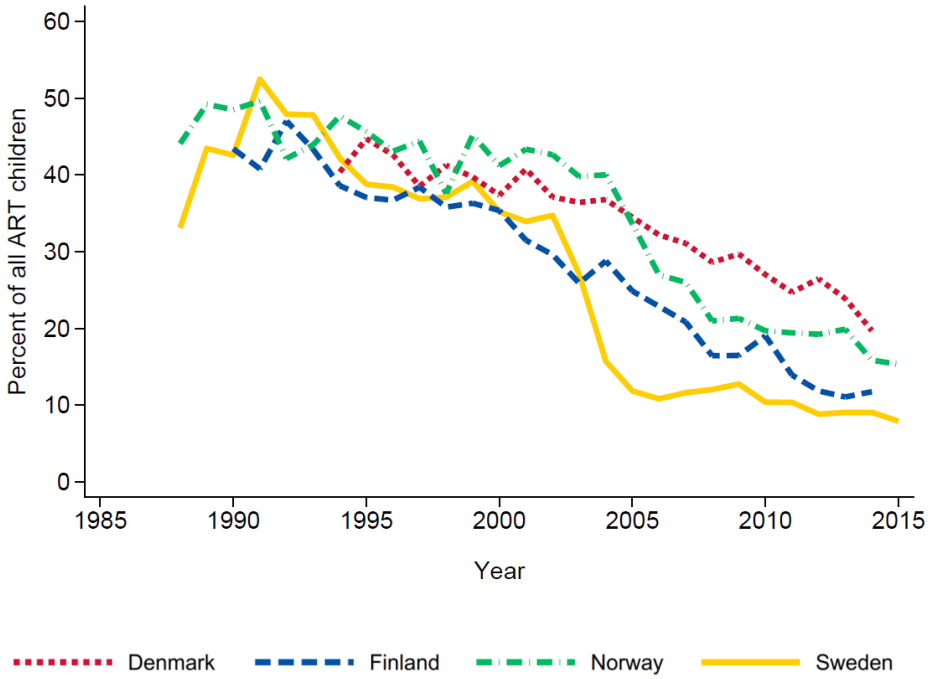


Figure 4. Time trends in proportion of children born from multiple pregnancies after ART conception. Data from the CoNARTaS cohort.

2.2.4 Cleavage stage and blastocyst embryos

With the new vitrification technique culturing of embryos until day 5-6 (blastocyst) rather than day 2-3 (cleavage-stage, figure 5), also grew in popularity [40].

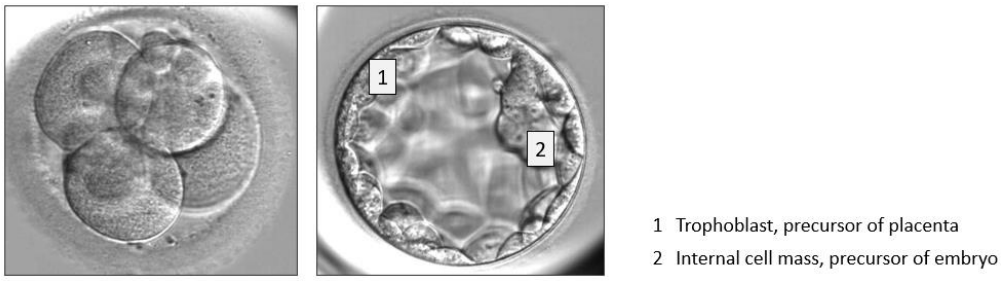


Figure 5. Cleavage stage embryo (left) versus blastocyst embryo (right). Photo Frida Stensen Bakken.

The benefits of culturing embryos to the blastocyst-stage embryos are many. Firstly, the increased observation time gives embryologists more information when scoring and selecting top quality embryos [41]. Secondly, prior to the blastocyst stage some unviable embryos stop developing and a process of self-selection has occurred [42]. Time to pregnancy may hence be shortened by avoiding transfer of such embryos [41,43]. Thirdly, transfer at the blastocyst stage is considered more physiologically appropriate with improved synchronicity between the endometrium and embryo development [44]. Overall, blastocyst transfers are associated with higher pregnancy rates and higher cumulative live birth rate [45].

Despite all these benefits, the prolonged exposure to culture media and laboratory environment associated with blastocyst culture has raised a concern as culture media have been shown to affect the birthweight of the newborn, and with extended culture this impact may be exaggerated [46,47].

2.3 Infertility

2.3.1 Definition and assessment

Infertility is commonly defined as not achieving a pregnancy after 12 months of unprotected intercourse, or 6 months if the woman is >35 years [48,49]. Some degree of fertility issue is common and up to 1/6 couples require some assistance to achieve the family they wish for [50,51].

An infertility evaluation may be offered to couples who are infertile by definition or at high risk of infertility [9]. The evaluation should include a review of the medical history, physical examination and additional tests as indicated. Such tests include assessment of the ovarian reserve, imaging of the reproductive organs including tubal patency and a sperm analysis for a male partner [9,52]. Identifying an underlying cause for a couple's childlessness is important for several reasons. Some causes can be managed without the need of further fertility treatment [53], and sometimes optimizing chances of a successful pregnancy like surgically removing submucosal fibroids, uterine septum and some cases of endometriosis are needed before commencing fertility treatment [54-57]. Hence, diagnostic, and interventional hysteroscopy or laparoscopy are common procedures during the assessment and treatment of infertility [58,59].

2.3.2 Female infertility

Causes of infertility are approximately equally distributed between female factors, male factors, a combination of male of female factors or unknown [9,60]. In the female reproductive system, infertility is commonly classified by (Figure 6):

- Tubal disorders: blockage of the fallopian tubes is a recognized complication of sexually transmitted diseases and previous surgery.
- Uterine disorders: include congenital malformations of the uterus, e.g., septum formations, myomas and adenomyosis causing inflammatory reaction within the uterus.

- Ovarian disorders: includes both “too few” and “too many” oocytes. While reduced ovarian capacity and few available oocytes is typically associated with women at the end of their reproductive age, it can also be a consequence of previous ovarian surgery, chemotherapy, radiation, and premature ovarian failure. In addition, anovulation is mostly caused by polycystic ovarian syndrome, however thyroid function disturbances and severe underweight or obesity may also cause endocrine irregularities of the pituitary gonadal axis.
- Endometriosis: may cause inflammation and adhesion affecting the physiology and functioning of the female reproductive organs.

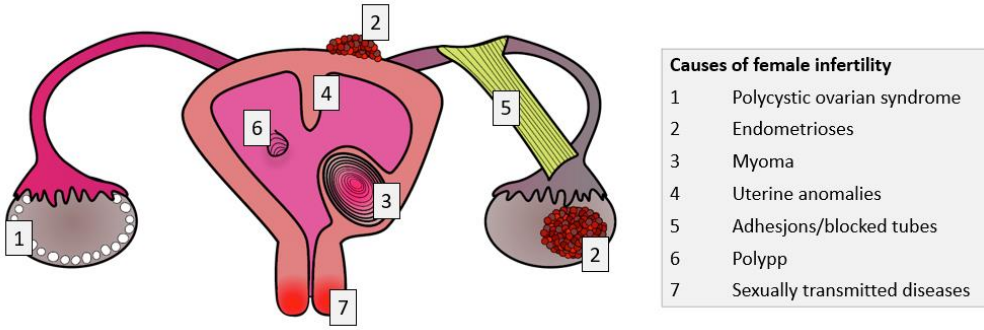


Figure 6: Examples of female infertility

2.3.3 Male infertility

Male fertility evaluation is based on assessing the semen sample, where the volume, sperm concentration and sperm motility is calculated [61]. Some common underlying factors that affect the sperm count and quality includes [62,63]:

- Pre-testicular: endocrine disturbances like hypogonadism and coital disturbances caused by erectile dysfunction

- Testicular: genetic and congenital disorders (Klinefelter's syndrome), vascular diseases, tumors, drugs and medication (chemotherapy and anabolic steroids)
- Post-Testicular: obstruction or absence of vasa deferentia due to conditions such as cystic fibrosis, infections, vasectomy, diabetes.

Nevertheless, male subfertility is most commonly idiopathic [64,65]. In the last decades there has been a worrying trend of declining sperm quality. In a study by Sengupta et al they found a 57% reduction in sperm concentration from 1980 to 2015, where a significant decline was found in North-America, Europe and Africa [66] and though questioned, several recent high quality studies have found similar results [67].The underlying causes of this are debated, but likely to be multifactorial with obesity, diet, environmental toxins, and pollutants accounting for this trend [67-69].

2.3.4 Societal and lifestyle factors

2.3.4.1 Age and fertility

During the last few decades, the average age of parenthood has increased substantially for both women and men in high income countries [70,71], where the mean age of first time mothers in the Nordic countries now is around 30 years compared to 24 years in the early 1980's (Figure7). This trend is complex and caused by several factors. Many women of reproductive age are unaware of the consequences of delaying parenthood [72,73], and others prioritize attaining a higher education and being financially secure before starting a family [74] . Further, work-place barriers and insufficient childcare support, as well as having problems finding a partner, are factors which cause delayed parenthood [73,74]. During the same period, fertility rates have decreased, with the average number of children per woman in the Nordic countries currently at 1.6 compared to 2.3 in the 1980s. However, many European countries have total fertility rates as low as 1.2, which is far below the required rate of 2.1 to maintain a stable population [75]. By the age of 32 the ovarian reserve is significantly in decline while the chances of developing gynecological conditions which may further limit a woman's

natural fertility increases [49,71,76,77]. Both these factors increase the probability of needing reproductive assistance with increasing age.

Whether male infertility decreases with age is more debated. Some studies have shown a marked decrease in sperm quality, including increased DNA mutations and chromosomal aberrations among older men [78,79], but if these changes directly affect male fertility is less clear. However, some studies report a delay in achieving pregnancy among couples where the man is 40 years or older, increased risk of miscarriages and decreased success rates after IVF/ICSI [70,80]. Further, offspring of older men seem to have a higher risk of several disorders and genetic abnormalities, including childhood cancers, and several neuropsychiatric disorders [81,82].

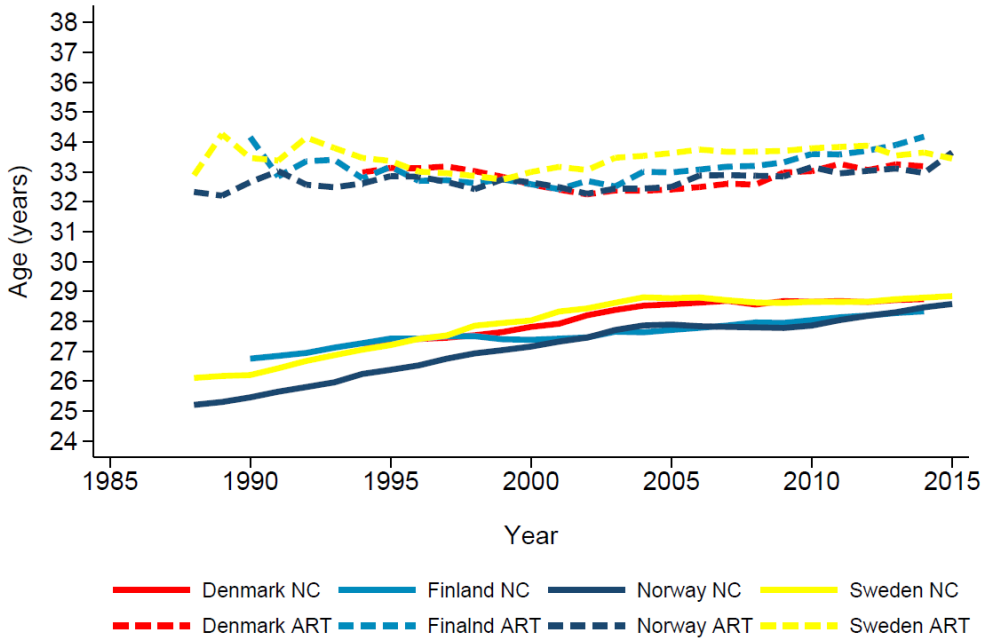


Figure 7. Time trends in mean maternal age at first delivery by country and conception method. Data from the CoNARTaS cohort.

2.3.4.2 Obesity and fertility

The worldwide trend of increasing obesity has been dramatic over the last 4 decades [83-85] , and is particularly well documented in a region in Norway, where obesity (BMI>30 kg/m^2) increased from 13% to 23% among women >20 years of age between 1986 and 2008 [86]. The associated health burden includes impaired reproductive health [87] and studies have found obese women and couples require longer time to pregnancy compared to normal weight women and couples [88,89]. The effect of obesity on fertility is multifactorial, but involves ovulatory problems, where obese women are up to 3-4 times more likely to suffer from ovulatory dysfunction [84,87]. Further, obesity directly affects the maturation of the oocyte, endometrium, and embryo, but the implantation is also affected [84,90]. Even when obese women receive assisted conception, their response is impaired compared to normal weight women, including lower oocyte yield, higher cancellation rates [91] and a lower chance of live birth compared to normal weight women [92]. Pregnancies are further complicated with both higher miscarriage rates [93] and complication rates during pregnancy [94].

Obesity also affects male fecundity, and the declining sperm quality is reported in parallel to the increasing obesity rates [85]. Around 45-50% of couple infertility is caused by male infertility and there is increasing evidence linking male infertility to obesity [95]. The spermatogenesis is sensitive to heat [96] and a stable hormonal environment is essential for optimal function [97]. Hence, obesity can affect the spermatogenesis through a range of mechanisms including the thermal effect from scrotal adiposity [96], hypoestrogenism due to adipose tissue converting testosterone to estrogen (leading to disturbance of the hypothalamic-pituitary-gonadal axis regulating spermatogenesis [85]), diabetes mellitus, sexual dysfunction and sperm epigenetic alterations [97]. There is also evidence that after assisted reproduction, couples where the man is obese have a decreased live birth rate compared to couples where the man is normal weight [95]. However, the association between male obesity and assisted reproductive success is difficult to evaluate as female age and weight may compensate for poor sperm quality [97].

2.3.4.3 Smoking and fertility

Fortunately, smoking is a declining trend among pregnant women [98-100], however it is still a common habit and accounts for a significant proportion of fetal morbidity and mortality [101]. Smoking is associated with higher risk of placental abruption and also with placenta previa and preterm birth [102]. In addition, there is a causal association between smoking and low birthweight, reduced head and abdominal circumference and perinatal mortality [101,103]. Couples in need of ART are less likely to smoke compared couples conceiving naturally [104]. However, smoking impairs female reproductive potential and smokers using ART have higher risk of cycle cancellation, reduced live birth rate, lower clinical pregnancy rate and a higher spontaneous miscarriage rate [102,105,106]. Smoking cessation before commencing ART treatment may improve overall outcomes [102,107].

2.3.5 ART legislation and fertility tourism

While ART started as a therapeutic treatment for women with irreversible causes of tubal infertility, other indications were quickly added, including unexplained infertility and single and same sex couples wanting children [108]. Most countries have now established national legislations regulating the use of ART [108]. With the many sensitive ethical, political, and religious considerations surrounding ART treatment, the framework and availability of ART varies greatly between countries [108]. In most countries, ART is provided by a combination of private and public clinics. In the Nordic countries, health authorities reimburse three cycles of ART to obtain one child, however a deductible fee is required for the medications needed. Oocyte donation has been allowed in Denmark, Finland, and Sweden for several years, but was only recently allowed in Norway (2020). Similarly, the possibility of ART treatment among single women and same sex female couples were established at different times in the Nordic countries (Table 1).

These various legislations directly affect treatment availability. For instance, between 2004 and 2009, Italy introduced conservative restrictions in ART where cryopreservation was forbidden [109] and all fertilized embryos (though not more than 3) had to be transferred, a practice resulting in

high twin and triplet rates, but also an inadequate utilization of expensive treatment [110]. This and other regulatory constraints, including financial costs and waiting lists, are the main sources of fertility tourism, where couples and women seek treatments not available in their own country [111].

	Denmark	Finland	Norway	Sweden
Law	Consolidated ACT 514 of 12/04/2019 on assisted reproduction	Act on Fertility Treatments (1237/2006)	Biotechnology Act, 2020	Genetic Integrity Act (GIA) (1.7.2006/351)
Donation: -Sperm -Oocyte -Embryo	 From 1940's Yes (2006) No	 Yes Yes (1991, 2007) Yes	 Yes Yes (2020) No	 Yes Yes (2003) No
Surrogacy	No	No	No	No
Single women	2006	2006	2021	2018
Same sex women	2006	2006	2009	2005
Age restriction: -Female -Male	 <46 None	 <47 None	 < 46 Reasonable age gap	 Ability to carry out parental responsibility throughout childhood

Table 1. Laws and legislations regulating ART treatment in the Nordic countries

2.4. Knowledge gap and rationale

The health and safety of children conceived by ART is of great importance [112,113] as perinatal health is the foundation for health and morbidity in adulthood [114,115]. The following chapter gives an overview of health outcomes for ART conceived children. Obstetrical complications associated with ART conceived pregnancies are included in addition to perinatal outcomes, as these are closely related.

2.4.1 Obstetrical outcomes after ART

Most large and well conducted studies support an association with adverse obstetrical outcomes such as hypertensive disorders of pregnancy, placenta previa and placenta abruption even among

singletons conceived by ART compared to naturally conceived pregnancies [116-119]. These are potentially catastrophic complications and contribute to a range of serious maternal and fetal morbidities, as well as mortality [119]. The placenta's main function is to provide oxygen and nutrition to the fetus and removal of waste products, though placental dysfunction is common and may appear in early gestation where shallow placentation can initiate growth retardation of the fetus and may also precede the development of hypertensive disorder in pregnancy [120,121].

A large meta-analysis comparing obstetric outcomes between fresh and frozen embryo transfer found a higher risk of hypertensive disorders in pregnancies of frozen transfer compared to fresh transfer(n= 39 501 versus n= 59 155, respectively) [122], which included one Nordic study that showed the same association [123]. In another meta-analysis, transfer of fresh and frozen-thawed blastocysts was compared, showing a higher risk of placental abruption and placenta previa after transfer of fresh blastocysts, while frozen-thawed blastocysts were at higher risk of hypertensive disorders of pregnancy (included up to n= 46 225 fresh blastocysts versus n=205 919 frozen-thawed blastocysts) [124]. These findings were also confirmed in a recent Nordic study by Ernstad et al who compared 3650 vitrified blastocysts to 8121 slow frozen cleavage stage embryos and 4469 fresh blastocysts [125], while Spangmose et al found transfer of fresh blastocysts to be at a considerably higher risk of placenta previa compared to natural conception (OR 9.52, 95% CI 8.10-11.12, n=4601 versus n=2 808 323, respectively) [126].

A Nordic study investigated the time trends in risk of placenta mediated complications in ART versus naturally conceived pregnancies (125 708 versus 6 595 185, respectively) [127]. The risk of hypertensive disorders in pregnancy, placental abruption, and placenta previa were consistently higher following ART and showed a similar declining trend as the background population, apart from placenta previa which was increasing over time among ART-conceived (1988-2015).

With the higher rates of complications in pregnancy, it is not surprising that ART conceived pregnancies are at higher risk of induction of labor and cesarian section, which is observed in most studies of both Nordic and international origin [117,118,125,128]. While we cannot rule out some

selection bias due to concerned women and clinicians, Neuman et al found that pregnancies conceived by fresh and frozen-ET were at higher risk of acute cesarian during active labor, indicating that ART conceived placentas may not tolerate labor as well as naturally conceived [129].

2.4.2 Perinatal outcomes after ART, what is known

While the initially reported adverse perinatal outcomes after ART were thought to be associated with the higher incidence of twins and higher order multiples [130], studies including only singletons also showed worse perinatal outcomes in ART-conceptions compared to naturally conceived pregnancies. ART-conceived had a lower mean birthweight and a shorter gestation, higher risk of preterm birth and small for gestational age [117,131,132]. Singletons born after ART also had a higher risk of congenital malformations [117,133-135] and perinatal death [136,137]. These findings were also consistent in a more recent meta-analysis by Qin et al, including over 50 cohort studies with 161 370 conceived by ART and 2 280 241 conceived naturally [118].

While most of the above studies investigated any ART compared to naturally conceived, most pregnancies were conceived by fresh transfer at that time. As the number of pregnancies from frozen embryo transfers increased, the initial observational studies investigating their perinatal health indicated favorable outcomes compared to children of fresh transfers, including a higher mean birthweight [138] and lower risk of preterm birth [136,138] compared to children born after fresh transfer. However, some studies found children from frozen embryo transfer to be at higher risk of being born large for gestational age compared to children of fresh embryo transfer [139-141], and also compared to naturally conceived [139].

Whether one treatment is more favorable has been debated. Two meta-analyses by, Maheshwari et al [116] and Zhao et al [133] have favored frozen embryo transfer, though the included cohort studies were partly overlapping, they found better perinatal outcomes after frozen embryo transfer, mainly due to their lower risk of preterm birth and low birthweight, and with no difference in perinatal death. However, in a more recent meta-analysis by Maheshwari et al, the authors

recommended that freeze-all should be undertaken at indication [122], due to the higher risk of large for gestational age and hypertensive disorders in pregnancy after frozen embryo transfer. Neither of these included comparisons to naturally conceived children.

Transfer of blastocysts has been shown to improve pregnancy rates [45], however the longer exposure to laboratory environment and culture media compared to cleavage stage embryos may also influence fetal growth and gestational duration, though the findings are not conclusive [142]. Further, pregnancies of fresh and vitrified blastocyst are associated with higher risk of preterm birth, compared to fresh and slow-frozen cleavage stage embryos [125,126].

2.4.3 Causes of infertility and adverse pregnancy and perinatal outcomes

The causes of the adverse outcomes associated with fresh and frozen embryo transfer have been investigated and debated in the literature [112,143,144]. A major concern is whether the IVF techniques themselves could negatively affect the ART offspring [143]. Several studies have found factors such as super-physiological hormonal levels [145,146], culture media [46,47,147], and cryopreservation [148] to be associated with adverse perinatal outcome.

Other studies have found an association between parental factors and poor perinatal outcome [27]. Such factors include reproductive health, and it is known that gynecological disorders like PCOS, endometriosis and myomas are associated with worse perinatal outcomes [149-151], but may also affect a woman's fertility. Supporting this theory are several studies of naturally conceiving, but subfertile women who were found to have worse perinatal outcomes compared to naturally conceiving women without subfertility [143,152,153].

Disentangling the effects of parental and treatment factors is challenging but could be helpful in identifying strategies to improve perinatal health among children born by ART in the future.

2.4.4. Fetal programming and epigenetics

The genome undergoes several phases of epigenetic programming during gametogenesis and early embryo development. Epigenetic modification controls gene activity without changing the DNA sequence [154]. Exposure to the super-physiological hormonal environment, embryo manipulation, embryo culture and media exposure, changes in pH, oxygen concentration and temperatures are all conditions associated with ART that occur under critical times of epigenetic activity and may affect the global programming and activate different epigenetic programming that persist into adulthood [147,155,156].

DNA-methylation is the most studied epigenetic mechanism and is associated with changes in trophoblast migration and invasion. This suggests that epigenetic regulation is critical for proper placentation, and disruption of this regulation by ART could be a mechanism in the disordered placentation associated with preeclampsia, preterm delivery, placenta previa, placental abruption, intrauterine growth restriction and low birth weight [157]. Further, epigenetic changes also affect the genital ridge of the growing embryo, which contains the precursors of gametes for the next generation. Epigenetics may therefore have the potential to change the genome for the current generation but may also directly cause changes to the genome in the next generation[158].

Adverse perinatal outcomes observed after ART, may hence be a consequence from early epigenetic changes caused by ART, which may influence the health of the individual throughout their life as well as their future offspring. The Dutch famine in 1944-1945 is a disastrous real-life example where pregnancies affected by starvation have shown to endure consequences long after they were born [159]. In utero fetal programming was initiated to cope with the harsh environment, which induced changes in later life with higher risk of chronic diseases including cardiovascular and respiratory diseases, cancers and overall mortality compared to those born shortly before or after the famine [160].

2.4.4. Long-term consequences of ART conception

As the oldest birth cohorts after ART are still relatively young adults and younger than the age when chronic diseases and risk factors of such diseases become frequent, long-term consequences of ART are still under investigation. Most previous studies have short follow-up periods and comprise all ART conceived children, where most are born after fresh transfer. Children born after ART may be at increased risk of poor health by at least two mechanisms. Firstly, the phenotype may be altered by epigenetic changes [155,156]. Secondly, ART children are associated with preterm birth, low birth weight and pre-eclampsia, circumstances also known to be associated with higher risk of chronic diseases, cardiovascular morbidity, and mortality in naturally conceived children [114,161].

Current literature comparing children born by ART to naturally conceived have not shown any clear association to psychomotor, language development, cognitive development, school performance, attention deficit hyperactivity disorder, and autism spectrum disease [113,162]. However the results for cerebral palsy are more conflicting [113], though a recent large observational study by Spangmose et al found a decreasing risk of CP with decreasing multiple pregnancy rates and no increased risk of cerebral palsy after ART compared to natural conception in the most recent birth cohorts (2003-2014) [163].

Even with the observed differences in birthweight between ART and naturally conceived, most studies do not indicate different growth during early childhood among children born by ART [164]. However, in a recent study by Norrman et al, a slightly higher risk of obesity was found in ART versus naturally conceived with a mean follow up of 8.6 years [165]. Several studies indicate altered cardiovascular function among children and young adults born after ART [166], including higher blood pressure and unfavorable left ventricular structure [165,167,168]. While no overall higher risk of diabetes type 1 has been shown after ART, there might be a higher risk for children born after frozen cycles [169]. Concerning risk of cancer, most observational studies have been reassuring with no overall higher risk of malignancies after ART conception [170-172]. However, a recent Danish study

comparing risk of cancer after fresh and frozen embryo transfer found a higher risk among children born after frozen transfer, although the number of cases was very limited [173].

2.4.5 Sibling studies

In conventional cohort studies, the population is characterized as belonging to exposed and non-exposed groups, and these groups are compared in analytic models, often with the aim of investigating if a causal effect between exposure and outcome is present [174]. Confounders are defined as common causes of exposure and outcome and are threats to the validity of observational studies if not handled well [175]. Statistical adjustment for measured confounders is one way of tackling confounding [176]. Examples of confounding factors relevant for outcomes after assisted reproduction include maternal age and parity, where women who seek such treatment are likely to be older and more often nulliparous [177]. However, large observational studies often lack data on key confounders, and residual confounding is a persistent weakness of a cohort study [178].

The sibling comparison design is an important epidemiologic tool to control for unmeasured (and unknown) confounding in studies of the causal effect of an exposure on an outcome [179]. Such designs involve comparing siblings with different exposures (e.g., children conceived by natural conception, fresh or frozen embryo transfer), where unexposed sibling(s) will act as the reference for the exposed sibling(s). Between siblings we can assume that confounders at the family level are inherently accounted for as they are similar for all siblings, despite not being known or measured [180]. For instance, maternal genetics, health and life-style choices and socio-economic position may be considered as stable or relatively stable within the small cohort of children with the same mother, and certainly more stable than between unrelated children (with different mothers) in conventional analysis.

Overview and summary characteristics of previous sibling studies on perinatal health after assisted reproductive technology

Study	Country	Study period	Comparison	Pairs (n)	Ref. level[1]	Unadj. estimate (95% CI)	Adj. estimate (95% CI)	Covariates
					Difference in mean birthweight, grams			
Romundstad et al 2008	Norway	1988-2006	ART vs NC	2204	3538	-87 (-125 to -49)	-9 (-36 to 18)	Gestational age, sex, maternal age, parity, birth year, pregnancy interval
Seggers et al 2016	Nether-lands	1999-2007	ART vs NC	1813	3467	-105.0 (-146.0 to -62.8)	-25.3 (-29.4 to 77.8)[2]	Sex, maternal age, parity, ethnicity, socioeconomic status, maternal diabetes, birth year, labour care
Dhalwani et al 2016	USA	2000-2010	ART vs NC	6458	3398	-55.3 (-72.9 to -41.7)	-33.4 (-48.6 to -18.2)	Gestational age, sex, maternal age, parity, birth year, time since last delivery
Goisis et al 2019	Finland	1995-2000	ART vs NC	578	3594	-137 (-189 to -85)	-31 (-85 to 22)	Sex, maternal age, parity, smoking, household income, multiple birth (incl. interaction with ART)
Henningsen et al 2011	Denmark	1994-2006	Fresh-ET vs NC Frozen-ET vs Fresh-ET	3879 358	3556 3443	-114 (-134 to -93) 202 (132 to 271)	-65 (-89 to -41) 167 (90 to 244)	Sex, maternal age, parity, and birth year
Luke et al 2017	USA	2004-2013	Frozen-ET 2nd vs Fresh-ET 1st Frozen-ET 1st vs Fresh-ET 2nd	3371 310	3246 3295	222 (200 to 244) 81 (8 to 154)	- -	Unadjusted, but restricted to siblings with same sex
					Difference in mean gestational age, days			
Romundstad et al 2008	Norway	1988-2006	ART vs NC	2204	278.7	-2.0 (-2.9 to -1.0)	-1.3 (-2.4 to -0.3)	Sex, maternal age, parity, birth year, pregnancy interval
Seggers et al 2016	Nether-lands	1999-2007	ART vs NC	1813	276	-0.85 (-1.9 to 0.2)	-0.12 (-0.08 to 0.32)[2]	Sex, maternal age, parity, ethnicity, socioeconomic status, maternal diabetes, birth year, labour care
Dhalwani et al 2016	USA	2000-2010	ART vs NC	6458	270.3	-0.58 (-0.99 to -0.17)	-0.58 (-1.02 to -0.14)	Sex, maternal age, parity, birth year, time since last delivery
Goisis et al 2019	Finland	1995-2000	ART vs NC	578	278	-2.5 (-3.7 to -1.2)	-1.3 (-2.6 to 0.0)	Sex, maternal age, parity, smoking, household income, multiple birth (incl. interaction with ART)
Henningsen et al 2011	Denmark	1994-2006	Fresh-ET vs NC Frozen-ET vs Fresh-ET	3879 358	277.2 277.6	-0.6 (-1.1 to -0.1) 0.2 (-1.5 to 1.9)	-1.4 (-2.0 to -0.7) 1.5 (-0.3 to 3.3)	Sex, maternal age, parity, and birth year
Luke et al 2017	USA	2004-2013	Frozen-ET 2nd vs Fresh-ET 1st Frozen-ET 1st vs Fresh-ET 2nd	3371 310	267.7 266.2	-0.3 (-0.9 to 0.3) 2.9 (0.7 to 5.1)	- -	Unadjusted, but restricted to siblings with same sex

Abbreviations: Adj. – adjusted, ART – assisted reproductive technology, CI – confidence interval, ET – embryo transfer, Ref. – reference, Unadj. - unadjusted

[1] Crude mean value in the reference category of the sibling group, i.e. the naturally conceived sibling in ART vs natural conception and the Fresh-ET sibling in Frozen-ET vs Fresh-ET.

[2] Note: Asymmetry between point estimate and CI indicates probable error in point estimate and/or CI.

Table 2 Summary of characteristics of previous sibling studies on perinatal health after ART, continuous outcomes only

However, sibship designs also have their limitations and pitfalls [179,181-183]. The strengths and limitations are particularly well illustrated in previous sibship studies [140,153,184-187], consisting of 2004 [184], 3879 [185], 1813 [153], 6458 [187], 3681 [140] and 578 [186] discordant sibling pairs. When investigating birthweight and gestational age, they all found lower mean birthweight and shorter gestation in siblings born after ART compared to their naturally conceived siblings (Table2), indicating that ART contributed to the adverse outcomes after accounting for maternal factors. Even though most individual studies did not show statistically significant associations [153,184,186], they are compatible and collectively point in the direction of lower birthweight and shorter gestation in ART overall. These studies were performed in populations dominated by fresh transfer. No previous sibship studies have compared frozen transfer to natural conception, but two studies that compared frozen transfer to fresh showed larger birthweights after frozen transfer regardless of birth order, whereas the results for gestational age were less clear [140,185].

In contrast, when three of the six previous sibship studies investigated perinatal mortality, they found that ART conceived had a lower risk of death [153,184], indicating an apparently protective effect of ART which is biologically difficult to explain. The findings seemed largely driven by sibships where the naturally conceived sibling preceded the ART conception, and Romundstad et al showed that mothers who experienced a perinatal loss in their first pregnancy were three times more likely to conceive by ART in their next pregnancy. These sibling studies may therefore have been biased by a process where siblings influence each other's exposure levels or outcome (carryover effect or contagion) [183]. Additional distortion of results might be expected if couples who experience perinatal death are more likely to have a subsequent pregnancy compared to couples with a surviving child (selective fertility [188]). Such selection forces would increase the occurrence of sibships that are discordant on exposure and outcome and potentially distort the results of the analysis (Table 3).

Overview and summary characteristics of previous sibling studies on perinatal death after assisted reproductive technology

Study	Country	Study period	Comparison	Pairs (n)	Unadj. estimate (95% CI)	Adj. estimate (95% CI)	Covariates
Romundstad et al 2008	Norway	1988-2006	ART vs NC	2204	0.53 *	0.36 (0.20 to 0.67)	Maternal age, parity, offspring sex, year of birth, time from birth of previous conception and previous perinatal death.
Seggers et al 2016	Netherlands	1999-2007	ART vs NC	1813	0.72 (0.27 to 1.08)	0.54 (0.27 to 1.08)	Parity, maternal age, ethnicity, socioeconomic status, maternal diabetes, neonatal gender, year of birth and level of care at onset of labour.
Henningsen et al 2011	Denmark	1994-2006	NC vs ART	3879	2.4 (1.4 to 4.2)	7.1 (3.0 to 16.7)	Sex, maternal age, parity, and birth year

Abbreviations: Unadj. – unadjusted, Adj. – adjusted, ART – assisted reproductive technology, NC – Natural Conception, CI – confidence interval, Unadj. – unadjusted, vs – versus
*Confidence Interval not estimated

Table 3 Summary of characteristics of previous sibling studies on perinatal death after ART

3. Aims of the thesis

The overall aim of the thesis was to study the impact of ART treatment, specifically fresh and frozen embryo transfer on perinatal outcomes. By including sibship comparisons we hoped to separate the contributions from maternal or treatment factor to the adverse outcomes associated with fresh and frozen-ET pregnancies.

3.1 Aims of study I

The aim was to estimate the effect of fresh-ET, and frozen-ET on birth size and duration of pregnancy compared to naturally conceived children. Included in these outcomes were birthweight, gestational age, z-score, small and large for gestational age and preterm (<37 weeks) and very preterm birth (>32 weeks). Sibship comparisons were included to investigate and disentangle the contribution from maternal and treatment factors.

3.2 Aims of study II

The aim was to investigate whether the risk of stillbirth and neonatal death differs between singletons born after fresh-ET and frozen-ET compared to naturally conceived. In addition, we wanted to explore whether the risk of stillbirth and neonatal death differed between fresh and frozen embryo transfer compared to natural conceived during different gestational weeks and periods.

3.3 Aims of study III

The aim was to compare the associations of perinatal death after ART vs natural conception in population analysis and within sibship analysis, and if these associations differed, to investigate whether within sibship results could be biased from selective fertility and carryover effects.

4. Materials and Methods

4.1 Data sources and The Committee of Nordic ART and Safety (CoNARTaS)

Denmark, Finland, Norway, and Sweden have national Medical Birth Registries where all liveborn and stillborn are registered, according to national criteria. The registries were established in 1973, 1987, 1967 and 1973, respectively [189]. After each delivery a mandatory notification form is sent from the delivery unit to the Medical Birth Registry with details of the mother and child, obstetrical complications, and procedures, with medical conditions coded according to the International Classification of Diseases (ICD). While the information registered in the notification forms has increased over time (e.g., data on smoking habits, height, weight), key parameters on maternal and child health have been recorded throughout the registries' existence.

The countries' ART registration is based on different schemes. In Denmark, a cycle-based registry including all ART treatment cycles in public and private clinics has been in place since 1994 and ART treatment was not registered before this [190]. Finland has registered ART conceived deliveries in the Medical Birth Registry since 1990 through the birth notification form as a dichotomous variable (ART vs no ART). In Norway, all public and private ART clinics notify the Medical Birth Registry after confirming a viable pregnancy by ultrasound scan at 6-7 weeks gestation. In addition, ART conception is reported through a single check box on the birth notification form if the mother informs the midwife about conception method. Lastly, public, and private ART clinics in Sweden initially reported ART conceived deliveries to the National Board of Health and Welfare (1982-2006), but from 2007 all ART cycles were reported to the National Quality Registry of ART [191,192]. Though the nationwide registries are not identical in structure and content, they are of high quality and comparable across the 4 nations [189] and reporting is mandatory for most national health registries [193-196].

The unique personal identity number given at birth or at immigration is used to ensure correct identity when a resident has contact with the public sector including the health services [197]. Linking data between registries are performed at an individual level through the personal identity number securing high level of correctness [189].The unique personal identity number given at birth or at immigration is used to ensure correct identity when a resident has contact with the public sector including the health services [197]. Linking data between registries are performed at an individual level through the personal identity number securing high level of correctness [189]. Due to shared features like tax-funded and public health care systems, similar population-based registries and a personal identity number, these countries provide unique opportunities for joint health registry-based research [189,197].

The Committee of Nordic Assisted Reproductive Technology and Safety (CoNARTaS) was founded in 2008, by members of the European IVF monitoring group in the European Society of Human Reproduction and Embryology (ESHRE) [192,198]. By pooling data from the national health registries and databases in the participating four countries (Denmark, Finland, Norway, and Sweden) they created a large cohort of children born after ART. The first data linkages were performed in 2010-2012 and included all ART conceived deliveries up to 2007 in each country and a matched sample of the background population. A second round of data linkages was performed in 2015-2017 and included all deliveries in Denmark 1994-2014, Finland 1990-2014, Norway 1984-2015, and Sweden 1985-2015. The CoNARTaS cohort is the foundation for all three studies.

4.2 Research ethics and data protection

A shared feature of legislation in all Nordic countries is that informed patient consent is not required for collection data in the national registries, and exemption from the usual requirement to obtain such consent in medical research can be granted for registry data[189]. In Denmark and Finland, ethical approval is not required for scientific projects solely based on registry data. In Norway, ethical approval was given by the Regional Committee for Medical and Health Research Ethics (REK-

Nord, reference numbers 2010/1909-1-24 and 14398). In Sweden approval was obtained from the Ethical committee in Gothenburg (reference numbers Dnr 214–12, T422-12, T516-15, T233-16, T300-17, T1144-17, and T121-18).

All data were de-identified through encryption of the national identity number before being transferred to the researchers and contained no directly identifying information. Data were pooled, stored, and analysed in a secure data platform administered by Statistics Denmark[192]. To prevent re-identification or recognition of individual study participants, number of observations are not presented when combinations of variables result in fewer than three individuals in a cell.

4.3 Study variables

Exposures, outcomes, covariates, and sensitivity analysis for each study are presented in Table 4.

	Study I	Study II	Study III
Exposure	Fresh and frozen embryo transfer compared to natural conceptions	Fresh and frozen embryo transfer compared to natural conceptions	ART versus natural conception
Outcomes	Birthweight, z-score, gestational age, preterm and very preterm birth, small and large for gestational age	Stillbirth and neonatal death	Perinatal death
Variables	Maternal age, parity, year of birth, country BMI, height smoking	Maternal age, parity, year of birth, country BMI, height smoking	Maternal age, parity, year of birth, country
Sensitivity analyses	Full siblings Only siblings Blastocysts only Single embryo transfer Siblings born within 3-year interval	Primiparous Deliveries >28weeks gestation Early neonatal death (0-6d) BMI and smoking	BMI and Smoking
Sibling design	Yes	No	Yes

Figure 4 Overview of variables and exposures in study I, II and III

4.3.1 Exposures

In study I and II, exposures were defined as conceptions from fresh and frozen embryo transfer. Patients conceiving through intrauterine insemination (IUI) were not classified as ART pregnancies. Information on embryo cryopreservation was not available from Finland, hence paper I and II were restricted to participants from Denmark, Norway, and Sweden. In study III participants from Finland were included and any ART-conception was defined as exposure to improve statistical power. Pregnancies not registered as ART-conceptions were considered naturally conceived and non-exposed in all three studies.

Sibships were defined from the maternal and paternal identity codes recorded in the Medical Birth Registries. These identities were available after linkage specific encryption, as separate variables for mothers and father. Maternal identity was available from all four countries, whereas paternal identity was available from Denmark, Norway, and Sweden, for 98% of pregnancies with known maternal identity. For the sibship analyses, we defined siblings as children with the same maternal identity code in the main analyses, and full siblings as children with the same maternal and paternal identity codes in sensitivity analyses.

4.3.2 Outcomes

Birthweight was measured in grams and gestational age in days. For natural conceptions, gestational age was estimated by routine ultrasound examination, performed in week 18-20 of pregnancy in Norway and Sweden, and in late first trimester in Denmark. If this information was missing, the date of last menstrual period was used. For ART conceived pregnancies, gestational age was estimated based on embryo transfer in Sweden, while in Denmark and Norway the first trimester (Denmark) or week 18-20 (Norway) ultrasound screening was used, and only if this was missing, was the date of embryo transfer used. In Finland gestational age is estimated by best clinical estimate, which includes

a combination of ultrasound assessment, last menstrual period, and for ART conceived pregnancies, information on embryo transfer date.

We used Marsal's equations for intrauterine growth to estimate z-scores of birthweights, where one standard deviation was set to 11% of the expected birthweight according to sex and gestational age [199] (Figure 8). Small for gestational age was defined as birthweights <-2 standard deviations and large for gestational age was defined as >+2 standard deviations from expected mean birthweight.

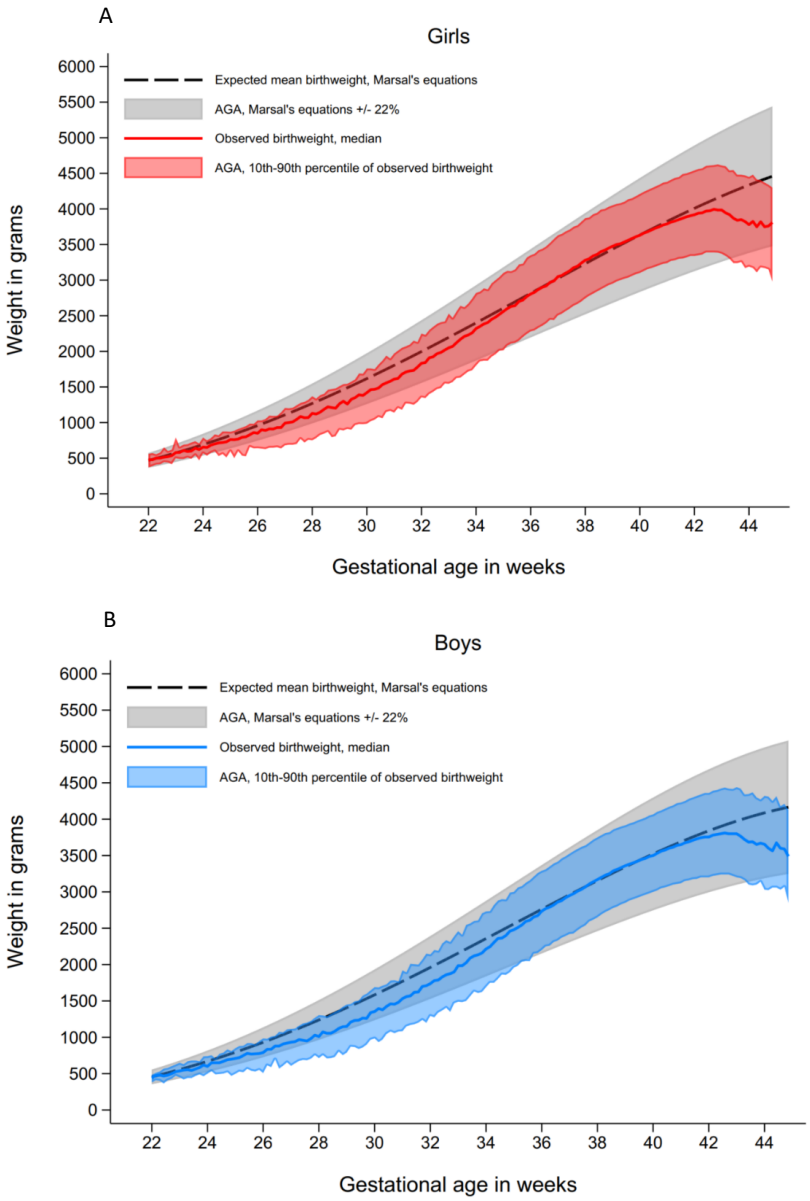


Figure 8. Illustrating the difference in observed and estimated birthweights according to gestational age for liveborn girls (A) and boys (B)

Preterm birth was defined as birth <37 weeks gestation (versus ≥37 weeks) and very preterm birth as birth <32 weeks gestation (versus ≥32 weeks).

In study III, the outcomes were stillbirth and neonatal death, as defined by the Medical Birth Registry in each country. Until April 2004 in Denmark and July 2008 in Sweden, stillbirths were defined as fetal death before or during delivery in deliveries at ≥28+0 weeks' gestation, thereafter the definition was expanded to include deliveries ≥22+0 weeks. In Norway and Finland, the definition of stillbirth included deliveries at ≥22+0 weeks for the whole study period. Live births were registered at any gestational age throughout the study period in all countries. Neonatal death was defined as a liveborn who died 0-27 days after birth [200].

In study III, we combined stillbirth and neonatal death, defined as described above, into perinatal death. The rationale for a joint mortality outcome in study III, was to maximize statistical power and allow for comparison with previous within sibship studies. Further, the suspected bias structure was expected to apply to any early loss [188].

4.3.3 Other study variables

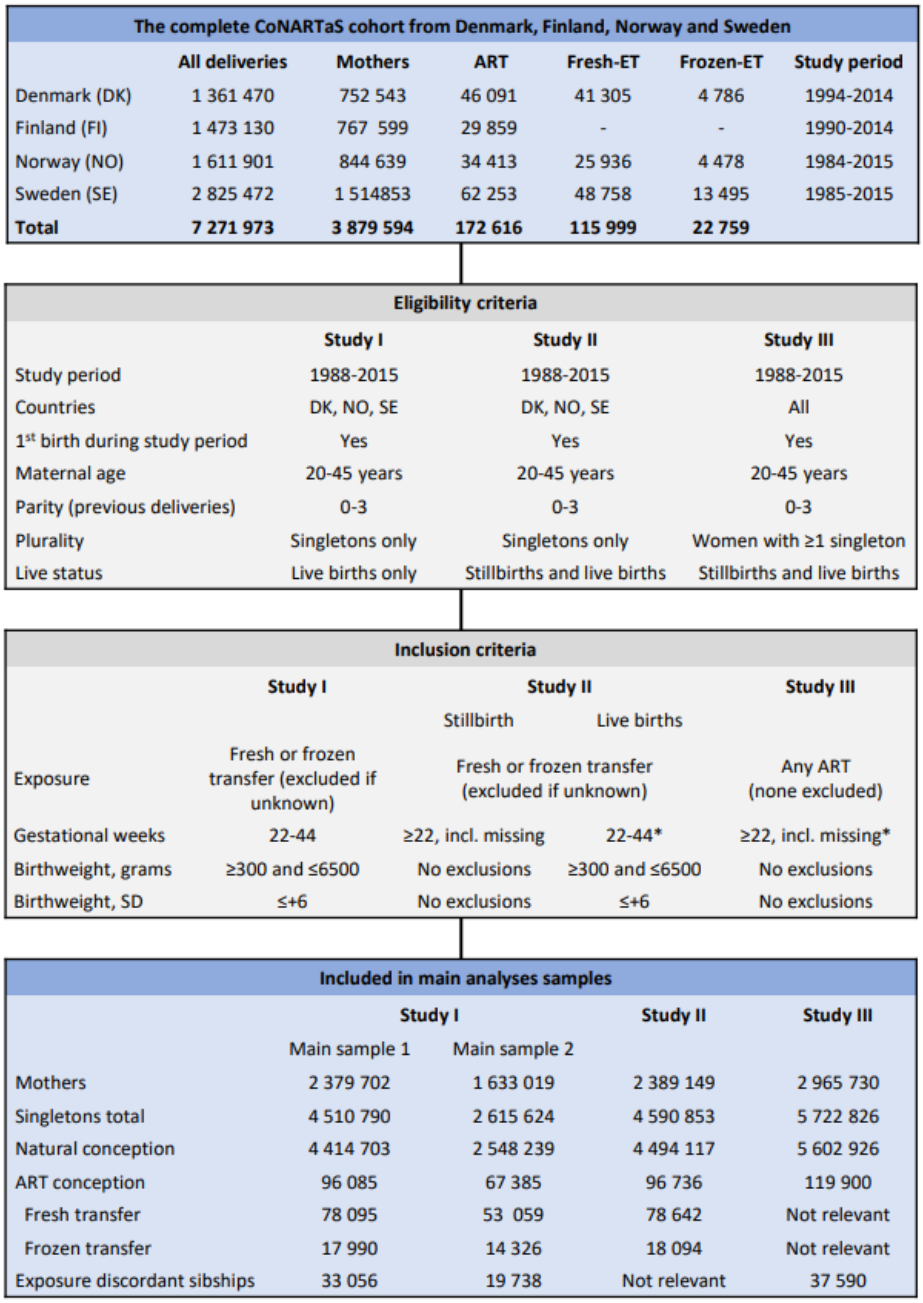
Number of previous deliveries were categorized as 0, 1, 2, and 3. Maternal BMI was calculated as weight in kg divided by height in meters squared, based on pre-pregnancy or first trimester values and categorized as underweight <18.5 kg/m^2, normal 18.5-24.9 kg/m^2, overweight 25-29.9 kg/m^2, and obese ≥30.0 kg/m^2 [201]. Further, we categorized maternal height as <150, 150-159, 160-169, 170-179, ≥180 cm, and smoking as yes or no, where yes was any smoking during pregnancy. Maternal height and weight were registered in 1988-1989 and 1992-2015 in Sweden. Denmark and Finland implemented registration of maternal height and weight from 2004, and Norway from 2007. However, our data has substantial missing data during the first years of registration from all 3 countries. Smoking was registered throughout the study period in Denmark and Sweden and since 1999 in Norway and 2004 in Finland and was harmonised as smoking or non-smoking.

Blastocysts were defined as embryos cultured to day 5 or 6, and cleavage stage embryos were defined as embryos cultured to day 2 or 3. Single embryo transfers included transfer of only one embryo by choice, and cases where only one embryo was available.

4.4 Study population

Figure 9 briefly outlines the eligibility, exclusion and inclusion criteria for the three studies. We defined our study period from 1988, when the first child born after embryo cryopreservation was registered in our data (1994 for Denmark and 1990 for Finland, as data were not available until then), until 2014 (Denmark and Finland) and 2015 (Norway and Sweden). Further, the number of ART conceived pregnancies before 1988 in Norway and Sweden was very limited. Study I and II were restricted to data from Denmark, Norway, and Sweden because no details on ART treatment beyond yes/no were available from Finland. Study III included data from all four countries.

Eligibility was defined as deliveries by women who gave their first birth during the study period and were aged 20-45 years at delivery. Further, parity was restricted to the first four deliveries for each mother. These criteria ensured comparability of maternal age and parity between ART and natural conceptions while maximizing the number of sibships in the analysis samples. We excluded all deliveries with unknown parity, and in study I and II we excluded ART conceived pregnancies with unknown status for fresh or frozen transfer. Most of these were from Norway and had been reported not via the Norwegian clinics, but through directly informing the midwife at the delivery ward, and a high proportion of them may be expected to be conceived abroad.



The complete CoNARTaS cohort from Denmark, Finland, Norway and Sweden						
	All deliveries	**Mothers**	**ART**	**Fresh-ET**	**Frozen-ET**	**Study period**
Denmark (DK)	1 361 470	752 543	46 091	41 305	4 786	1994-2014
Finland (FI)	1 473 130	767 599	29 859	-	-	1990-2014
Norway (NO)	1 611 901	844 639	34 413	25 936	4 478	1984-2015
Sweden (SE)	2 825 472	1 514853	62 253	48 758	13 495	1985-2015
Total	**7 271 973**	**3 879 594**	**172 616**	**115 999**	**22 759**	

Eligibility criteria			
	Study I	**Study II**	**Study III**
Study period	1988-2015	1988-2015	1988-2015
Countries	DK, NO, SE	DK, NO, SE	All
1st birth during study period	Yes	Yes	Yes
Maternal age	20-45 years	20-45 years	20-45 years
Parity (previous deliveries)	0-3	0-3	0-3
Plurality	Singletons only	Singletons only	Women with ≥1 singleton
Live status	Live births only	Stillbirths and live births	Stillbirths and live births

Inclusion criteria				
	Study I	**Study II**		**Study III**
		Stillbirth	Live births	
Exposure	Fresh or frozen transfer (excluded if unknown)	Fresh or frozen transfer (excluded if unknown)		Any ART (none excluded)
Gestational weeks	22-44	≥22, incl. missing	22-44*	≥22, incl. missing*
Birthweight, grams	≥300 and ≤6500	No exclusions	≥300 and ≤6500	No exclusions
Birthweight, SD	≤+6	No exclusions	≤+6	No exclusions

Included in main analyses samples				
	Study I		**Study II**	**Study III**
	Main sample 1	Main sample 2		
Mothers	2 379 702	1 633 019	2 389 149	2 965 730
Singletons total	4 510 790	2 615 624	4 590 853	5 722 826
Natural conception	4 414 703	2 548 239	4 494 117	5 602 926
ART conception	96 085	67 385	96 736	119 900
Fresh transfer	78 095	53 059	78 642	Not relevant
Frozen transfer	17 990	14 326	18 094	Not relevant
Exposure discordant sibships	33 056	19 738	Not relevant	37 590

* Live births excluded from analyses of stillbirth and perinatal death if born in week 22-27 in Denmark before April 2004 or Sweden before July 2008 to account for changes in definitions of stillbirth

Figure 9. Flow of participants into study I, II and III.

In analyses of birth size, duration of pregnancies (study I) and neonatal mortality (study II), only liveborn singletons born between 22-44 weeks and with birthweight 300-6500 g or a z-score <+6 SD, were included. In analyses of stillbirth (study II) and perinatal mortality (study III), the following criteria were applied: Firstly, no weight restrictions were made as stillborn are at risk of extreme weight deviations due to complications preceding the stillbirth, and because the proportion with missing birthweight was high (25.2% of naturally conceived singletons and 35.9% of ART conceived singletons with perinatal death, versus 2.0% and 0.7% of surviving naturally and ART conceived singletons, respectively). Secondly, only stillbirths with gestational ages <22 weeks were excluded. The latter exclusions were mainly deliveries from Norway, where all pregnancies ending after 16 weeks have been registered throughout the study period, regardless of offspring live status at birth. In addition, the definition of stillbirth (and hence, registration) in Denmark and Sweden changed during the study period. Until April 2004 in Denmark and July 2008 in Sweden, stillbirths were defined as deliveries with fetal death ≥28 weeks gestation, whereas pregnancies ending with fetal death <28 weeks were defined as miscarriages and not registered. After the respective time points, all deliveries ≥22 weeks gestation were registered regardless of offspring live status at birth. For Denmark and Sweden, live births were therefore excluded if their gestational age and birth dates implied that they would not have registered in the case of fetal death. In Finland, all deliveries ≥22 weeks gestation were registered for the whole study period, regardless of live status. The proportion with missing data on gestational age was higher for stillbirths (8.9% (NC) and 4.7% (ART)) compared to livebirths (1.8% (NC) and 0.3% (ART)), but gestational age was assumed to be higher than the registration threshold for these deliveries.

Except for one analysis in study III (described in chapter 4.5), all analyses were restricted to singleton pregnancies.

4.5 Statistical analyses

We used multilevel linear and logistic analysis models for all our main analyses, comparing the outcome measures between conception methods, where natural conceptions were the reference group. These multilevel models allow accounting for the clustering of subjects (children or pregnancies) within clusters of higher-level units (mothers) when estimating the association of subject and cluster characteristics with subject outcomes [202]. We used random effects models for the conventional population estimates (study I, II and III) and fixed effects models for within sibship comparisons (study I and III). Precision was estimated by calculating 95% confidence intervals (CIs). To increase interpretability of the odds ratios (ORs), we calculated risk differences (RDs) from the logistic models using postestimation commands. The within sibship estimates were based on siblings who were discordant for conception method.

Potential confounders for all studies were defined as any factors that could plausibly influence the need for ART, and the outcomes for each study [203]. We adjusted for the following measured confounders: country, year of birth, maternal age, and parity (number of previous deliveries: 0, 1, 2, or 3). Maternal age and offspring year of birth were used as continuous variables. Maternal BMI, height, and smoking were used as additional covariates in smaller populations for each study. Height was included as a separate variable in addition to BMI as height is an independent confounder for adverse perinatal outcomes even within the same BMI level [204]. The sibling comparisons were adjusted for the same covariates except country and height, which are stable within mothers. In addition to these observed confounders, we considered parental socioeconomic position and cause of infertility to be key confounders, but we did not have data on individual's income or education.

4.5.1 Additional analyses in study II

To investigate whether conception method modified the impact of gestational age on risk of stillbirth and neonatal death, we repeated analyses within categories of gestational age. For stillbirth, we used

'ongoing pregnancies' as the denominator (i.e. all pregnancies at risk of stillbirth at the start of a given gestational age interval), with multilevel logistic models for categorical estimates and survival analysis for continuous gestational age. For neonatal death, the denominator was singletons born alive during the given interval, with multilevel logistic models for categories and single weeks of gestational age.

4.5.3 Additional analyses in study III

To investigate if the results from within sibship analyses (as described above) could be biased, we examined whether selective fertility and carryover effects were present.

As a first step, we performed a "bidirectional analysis" of, where effect measure modification by order of exposure within sibships is examined, has been suggested as a method to identify some certain types of carryover effects [183], such as the type where the outcome for the first sibling influences exposure in subsequent sibling(s). In this analysis, we compared perinatal mortality in the mothers' first two (consecutive) singleton deliveries for women with natural conception followed by ART (NC-ART) and women with ART followed by natural conception (ART-NC), using random effects models with interaction terms between order of birth and conception method. For comparison, we also included estimates for women with only natural conception (NC-NC) or only ART-conception (ART-ART) in both pregnancies.

Selective fertility [188] was estimated by comparing the proportions of women with a firstborn singleton who had a second delivery from either conception method, according to conception method and perinatal death in the first pregnancy. The proportions that had a second delivery were calculated over the full study period, and within 5 years after first delivery, to account for the fact that more deliveries from ART-conception took place during the later years of the study period. The proportions who continued with a second delivery included both singleton or with a multiple deliveries (as separate proportions) for a more complete overview of the selection into the within sibship models.

Crosswise associations were examined in the presence of selective fertility, by comparing the probabilities of having a second singleton by each conception method, for women with perinatal death or survival in the first pregnancy.

Finally, we compared perinatal mortality of the second singleton for ART vs natural conception among women who had the same conception method and outcome in the first pregnancy. The purpose of this analysis was to control bias from selective fertility, assuming that a potential contribution from ART-conception to perinatal mortality in the second pregnancy would be independent of the specific combinations in the first pregnancy.

4.5.4 Sensitivity analyses

For each study we carefully considered sensitivity analysis which could add information and robustness to our analysis[205] (Table 4).

In study I we included the following sensitivity analysis:

1) Investigating the contribution from constant paternal factors by only including siblings of same mother and father.
2) Restricting analysis to siblings born within a three-year interval as both family background and parental health is likely to be more constant among siblings born within a short timeframe.
3) Including only infants who had siblings in the study population (i.e., excluding all infants from mothers who had only one delivery in the main analysis sample). This enabled us to explore whether any differences between population and sibling results might be driven by families with only one child being different to those with two or more.
4) Single embryo transfers
5) Blastocysts

The latter two analyses account for changes in practice over the study period which could potentially influence our results [125,126].

In study II we included the following sensitivity analysis:

1) Subpopulation with known BMI, height and smoking to enable adjustment for these confounders.
2) Primiparous women only, as primiparity is more common among those who give birth after ART conception.
3) For stillbirths, we repeated the analyses in a sample restricted to deliveries ≥28 weeks to examine the potential impact of different definitions of stillbirth between the countries during the study period.
4) To facilitate comparison with other studies [136], we also analyzed early neonatal deaths, defined as live born children who died 0-6 days after birth, though some studies have not clarified which definition utilized [206].

In study III we only included one sensitivity analysis where we repeated the population level and within sibship analysis in the population with known maternal height, BMI, and smoking status to allow adjustment for these confounders.

5. Results

5.1 Characteristics of the population

Although the study populations of the three studies were different in size and inclusion criteria, the characteristics showed similar patterns across all studies and can be summarized collectively.

Women who gave birth after ART treatment were older than women who gave birth after conceiving naturally, and women who gave birth after frozen transfer were the oldest. Mean maternal BMI was similar (24.2 kg/m^2) for all conceptions methods, but higher in pregnancies resulting in offspring death for all conception methods. Smoking was more common among mothers who conceived naturally compared to mothers who conceived by fresh and frozen embryo transfer, but for all conception modes the proportion of smoking mothers was higher in pregnancies ending with offspring death.

The utilization of single embryo transfer was quite high in the study period overall, at 47% for fresh and even higher for frozen transfer, 64%. Mode of fertilization was similar for both ART methods, where 40% of oocytes were fertilized by ICSI. Blastocyst culture was not commonly used during the study period, comprising 5.7% of fresh and 20.8% of frozen transfers, however the practice became more common towards the end of the study period.

Though the risk of preterm birth was higher after fresh and frozen transfer compared to naturally conceived, this was substantially higher for singletons who died.

5.2 Main results of study I

5.2.1 Birthweight

In population analyses, children from fresh-ET were on average lighter, and those born after frozen-ET were on average heavier, at birth compared to naturally conceived children, after adjustment for

all observed confounders. Analyses of birthweight z-score according to gestational age and sex showed similar trends. Children born after fresh-ET had a higher risk of being born small for gestational age and lower risk of being large for gestational age compared to naturally conceived children, whereas the opposite was true for children conceived via frozen-ET. The sibship comparisons showed the same patterns with clear differences in mean birthweight, and risk of being small and large for gestational age between children born after fresh-ET and frozen-ET, compared to their siblings who were naturally conceived. The magnitude of associations was similar between sibship and population level analyses, indicating that unmeasured maternal level confounding was limited. The sibling results were consistent across order of conception method and results of all sensitivity analyses were consistent with the findings from the main analyses samples.

5.2.2 Duration of pregnancy

In both main samples, mean gestational age was shorter after fresh and frozen transfer compared to natural conception in population analyses. In the corresponding sibling comparisons, children of fresh transfer had mean gestational ages closer to, but still shorter than, their naturally conceived siblings, while children of frozen transfer had a slightly longer mean gestational age compared to their naturally conceived siblings. In population analysis, in both main samples, children conceived by fresh and frozen transfer had substantially higher odds of preterm and very preterm birth compared to naturally conceived children. For preterm birth, there was some attenuation within sibships compared to population analyses, particularly for frozen transfers in the minimized selection sample. In sibship analyses of very preterm birth, there was more marked attenuation than for preterm birth, with point estimates close to the null value, though with wide confidence intervals.

Risk of preterm birth according to combinations of conception methods in consecutive sibling pairs showed that combinations involving ART had higher risk compared to the naturally conceived sibling pairs. However, there were no systematic differences between treatment types (fresh and frozen transfer) in risk of preterm birth.

Sensitivity analyses were overall in line with the findings from the minimized confounding sample. One exception was the sibship comparison where ART treatment was restricted to blastocyst transfers, which may indicate an increased risk of both preterm and very preterm birth after both fresh and frozen transfers compared to their naturally conceived siblings. However, these estimates were imprecise due to small sample sizes.

5.3 Main results of study II

We found no clear association between conception method and the overall risk of stillbirth, while neonatal mortality was higher after both fresh and frozen transfers compared to natural conception. Results from sensitivity analyses supported those from the main analyses.

In analyses according to gestational age, the absolute risk of stillbirth was highest in term (37-41 weeks) gestations, when most deliveries occurred. For children conceived by fresh transfer, risk of stillbirth varied with gestational age, being higher than for natural conception before 28 weeks gestation, and thereafter similar or slightly lower, although with wide confidence intervals. Risk in pregnancies after frozen transfer did not clearly differ from that in natural conceptions at any gestational ages, but precision was low. For all conception methods risk of neonatal death was highest for births before 28 weeks and declined steeply with increasing gestational age to the lowest observed risk for term births (37-41 weeks). However, for each gestational age, neonatal mortality was similar for fresh and frozen transfers compared to natural conceptions, apart from a higher risk post term for fresh transfer compared to natural conception, although with wide confidence intervals.

5.4 Main results of study III

At the population level, perinatal mortality was higher after ART compared to natural conception, but within sibships, we found a markedly lower perinatal mortality for ART-conceived singletons

compared to their naturally conceived siblings. Adjustment for available confounders had little influence on the associations.

Bi-directional analysis (i.e., interaction with order of conception method) showed that the within sibship association was driven mainly by women with natural conception before ART-conception (Figure 2)**,** who had the highest perinatal mortality of all in the first delivery and the steepest decline in perinatal mortality from first to second delivery. This heterogeneity suggested that a carryover effect could be present.

Women with a perinatal loss in their first pregnancy were more likely to give birth again than women with a child who survived the perinatal period (selective fertility). Among women with natural conception in their first pregnancy, 70% of those with a surviving child proceeded with a second delivery, compared to 82.4% of women with a perinatal loss. For women with ART conception in their first pregnancy, the corresponding percentages were 46.4% and 63.7%, respectively. Women who lost their naturally conceived firstborn were much more likely to continue with an ART-conceived singleton pregnancy (1.8%) than women with a naturally conceived firstborn surviving child (0.43%). This indicated a strong influence of the outcome in the first pregnancy on exposure in the next (carryover effect). A similar influence was seen for women with ART-conception in their first pregnancy, who were also more likely to have a second ART-conceived singleton if the firstborn died (35.1% vs 18.7%). This suggested that carryover effects were present regardless of which conception method had been used first, and that in both cases, these increased the selection into the double discordant sibship sample of sibships where the naturally conceived sibling had died, and the ART conceived sibling had survived.

In an attempt to avoid the influence of these selection biases, we estimated perinatal mortality in the second singleton pregnancy, according to conception method and outcome of the first pregnancy. Estimates were very imprecise, but indicated no consistent pattern across the groups: for ART vs NC in second pregnancy, perinatal mortality was higher when the first pregnancy was naturally conceived, and lower when the first pregnancy was conceived by ART.

6. Discussion

6.1 Summary of findings

The aim of our studies was to contribute from an epidemiological perspective to a deeper understanding of the association between conception method and risk of adverse perinatal outcomes, by disentangling the contributions from maternal and treatment factors to the adverse outcomes. Our principal findings were:

Study I:

- Children born after ART were at higher risk of adverse perinatal outcomes compared to natural conception.
- Fresh transfer was associated with lower mean birthweight and a higher risk of being small for gestational age, whereas frozen transfer was associated with higher mean birthweight and a higher risk of being large for gestational age, compared to naturally conceived children. These findings were similar at the population level and within sibships, indicating little contribution from unmeasured maternal factors.
- Fresh-ET was associated with a shorter mean gestational age, and both treatments were associated with higher risk of preterm and very birth. Within sibships, the associations with preterm birth attenuated somewhat, whereas associations with very preterm birth attenuated substantially compared to population level results. This indicated that unmeasured confounding by maternal factors contributed to the associations for preterm birth in addition to contribution from treatment factors.

Study II:

- Singletons conceived by fresh and frozen transfers had an overall similar risk of stillbirth, but a higher risk of neonatal death, compared to natural conceptions.

- Apart from a higher risk of stillbirth in pregnancies after fresh-ET during week 22-27, we found no clear differences in associations for fresh and frozen-ET according to gestational age.
- The higher risk of neonatal death after both fresh and frozen-ET might be attributed to a higher risk of all degrees of preterm birth in ART conceived pregnancies.

Study III:

- Perinatal mortality was higher in singleton pregnancies conceived by ART compared to natural conception at the population level, but much lower within sibships.
- Further investigation revealed strong selection mechanisms into the double discordant sibship group, where women with perinatal loss were more likely to conceive again (selective fertility) and to conceive by ART in their second pregnancy compared to women with a surviving child (carryover effect).
- When accounting for these biases by comparing perinatal mortality in second pregnancy among women with similar experience in first pregnancy, ART conception was not consistently associated with risk, but precision was very limited.
- These findings illustrate that selection biases in a sibship design may require special awareness in situations where knowledge of the outcome for one sibling may influence parental behavior and/or clinical management in a subsequent pregnancy.

6.2 Methodological considerations

Error in estimation is the difference between the estimated value and the true value, accurate estimates are hence dependent on little error [176]. Errors in the estimation process are usually classified as random or systematic [174] and the strengths and limitations of the studies included will be discussed within this framework .

6.2.1 Precision (lack of random error)

Precision is the lack of random error [176]. In any empirical study there will be an element of random error, which is defined as variability of measurements that cannot be explained [174]. Sources of random error include sampling variation and measurements error. Statistical precision may be measured in confidence intervals, which in our studies was set to a level of 95%. This should be interpretated as follows: the 95% confidence interval will include the true value 95% of the time if the study had been repeated numerous times and was free of bias. A narrow confidence interval implies little random error[176].

Sampling variation may be reduced by increasing the study size, and all our study samples were large compared to those from previous studies. Precision also depends on the frequency of exposures and outcomes, and in our studies, both were relatively uncommon, in particular the mortality outcomes in study II and III. This was reflected in the precision, with relatively large confidence intervals in study II and III compared to study I, where some outcomes were continuous and available for almost all pregnancies. For all sibling comparisons (study I and III), with considerably fewer participants compared to the population level analyses, the precision was still high in most comparisons, although lower than in population analyses. This may be explained by a reduced random variance between siblings and less residual confounding. The multilevel model approach allowed different sizes of sibships (clusters), which made it possible to include more than one sibling of either conception method, which also improved precision compared to previous sibship studies. In all sensitivity analyses the samples were smaller, which was also reflected by a larger confidence interval.

6.2.2 Validity (lack of systematic error)

Validity is the lack of systematic error [176]. Unlike random error, systematic error is not affected by sampling size [174]. The presence of systematic error may lead to incorrect results, or the results may not describe the research question they intended to [207]. Assessment of a study's validity relies

mainly on the researcher's prior knowledge and validity cannot be measured directly. Systematic error may be introduced by the way study participants are selected, by the measures used to assess their characteristics and by analytical approaches [176]. Figure 10 illustrates the differences between precision and validity. The main categories of systematic errors are selection bias, information bias and confounding.

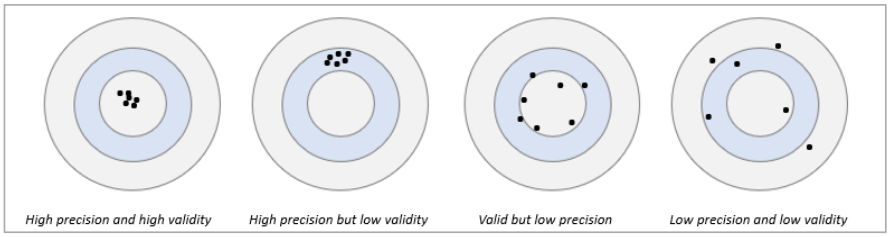


Figure 10. Illustration of accuracy as a combination of precision and validity

6.2.3 Selection Bias

Selection biases occur when the participants of the study sample are not representative of the population intended to be analysed [176]. Consequently, the association between the exposure and outcome of those selected for analysis may differ from the association among those eligible. To reduce the risk of selection bias we had strict criteria for eligibility and exclusions in all three studies, to include as much of the source populations as possible. A major strength of registry-based research is the opportunity to include close to complete populations, with recruitment based on residency rather than health or health related factors. Further, we carried out a range of sensitivity analyses which confirmed that results were consistent across various subpopulations and did not depend on changes in treatment practice (single embryo transfer and culture duration) and outcome definition (stillbirth, study II and III).

Despite our efforts to reduce the risk of selection bias, we discovered a major source of selection bias in the sibship results of offspring mortality (study III), where we found a substantially reduced risk of perinatal death in siblings conceived by ART compared to their naturally conceived

siblings. While the intended benefit of a sibship design is to adjust for unmeasured confounding and improve the validity of the results [180,208,209], previous sibship studies indicated that bias could be a possible explanation, and a biological explanation seemed unlikely, given that previous studies support an increased risk of preterm birth and placental complications in ART pregnancies, also within sibships [210] . We subsequently identified biases from selective fertility [188] and carryover effects [183] and concluded that though the within sibship results were precise, they were not valid due to these large biases distorting the results. These selection biases cannot be avoided through increased statistical power, but the large study sample allowed us to set up an alternative sibship comparison, where we were able to identify the source of selection bias.

6.2.4 Information bias (misclassification)

Information bias result from errors in measurement, reporting or classification of the study variables [176]. Misclassification can be divided in differential (dependent on other study factors) of non-differential misclassification (independent of other study factors). Because most variables were recorded as part of routine clinical care by health professionals, we expect some misclassification in registration from typos and measurement error to be present in our data, though mostly non-differential and hence affecting all modes of pregnancies equally and causing bias towards the null. Further, outlying observations were excluded in the definition of the study populations.

Our decision to use intrauterine growth curves to estimate expected birthweight according to gestational age, was based on the fact that preterm birth is associated with pregnancy complications which may also affect the growth of the fetus [211]. Therefore, the observed (preterm) birthweights are not representative of the normal birthweight distribution for healthy pregnancies at a given gestational age, a difference we have calculated from our own data (Figure 9A girls and 9B boys). Although the growth curves we used are based on few observations, they have been shown to correspond well with subsequent measurements in larger populations [212], and should improve

classification of small and large for gestational age compared to using observed birthweights as the standard.

Unfortunately, we had no numbers or details on women from the Nordic countries that conceive through intrauterine insemination or travel abroad for fertility treatment. In our data they would all be registered as natural conceptions, and even if fertilization took place inside the female body, some level of medical intervention was performed. However, compared to the substantial number of truly natural conceptions in the population, they will have minimal effect on the effect measure in our studies. The latter is also an example of non-differential misclassification, because it does not depend on other study variables, such as outcomes or confounders

Self-reported information about health, morbidity and lifestyle are particularly prone to misclassification [213]. Smoking was a self-reported variable and categorised by us as smoking or not smoking during pregnancy. However, this variable is known for being underreported among pregnant women [214,215] and an unknown proportion of smokers from all exposed and non-exposed groups were likely to be misclassified into the non-smoking group. [174].

Among the included outcomes, gestational age (and hence, preterm birth) might be more prone to misclassification than birthweight and death. Such misclassification could be differential if awareness of the conception method leads to adjustment of the expected delivery date based on ultrasound measures in natural conception, but not in ART conception because transfer date is considered more valid.

6.2.5 Confounding

A confounder is defined as a common cause of the exposure and outcome, but is not a consequence of any of these [174]. Confounding can lead to a bias of the measured effect between exposure and the outcome if not dealt with by the researcher [176]. For confounding to appear, the confounder must also be differentially distributed among exposed and unexposed [176]. Confounders are identified by the knowledge of the researcher and may be separated from mediators (intermediate

variables conveying some or all the effect of exposure and the outcome) and colliders (common consequences of exposure and outcome) by utilizing graphical presentation such as Directed Acyclic Graphs [176]. Figure 11 illustrates some expected confounders, mediators, and colliders in the association of ART and preterm birth.

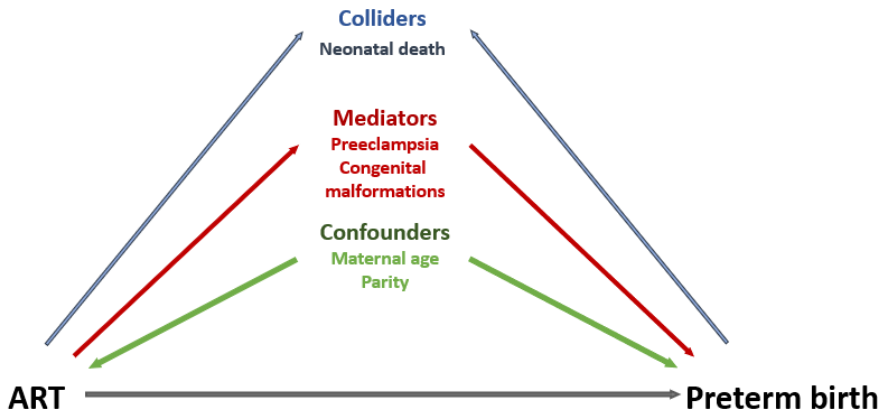


Figure 11. Simplified example of how to identify confounders, mediators, and colliders by using a Directed Acyclic Graph.

To deal with confounding we adjusted our main analysis for a range of potential confounders. In study I we additionally included a second main population of participants with known BMI and smoking. In study I and II we performed sensitivity analyses to reduce residual confounding from parental or treatment characteristics [216]. Still, a cohort study is always prone to residual confounding [217], and to account for this we included sibship comparison to our analytical approach [180,182,209,218]. Siblings conceived by different methods, but born by the same mother, will share many of the unmeasured maternal or family level confounders, like socioeconomic position, underlying health, and infertility status as well as other factors that are difficult to measure like genetics [209]. The intention was to reduce residual confounding from all shared unmeasured and

unknown confounders. This appeared to work well for birth size and duration of pregnancy, which are continuous outcomes that we also dichotomized at clinically relevant cut points. However, when studying offspring death, the dramatic outcome had consequences for the next pregnancy, and though reducing residual confounding, we also introduced selection bias which distorted the results, as illustrated in Figure 12.

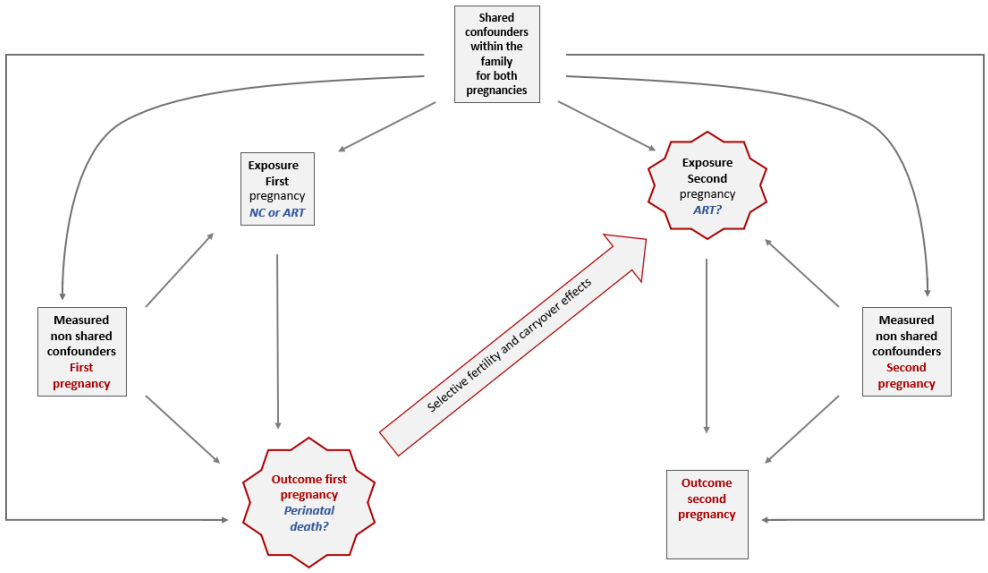


Figure 12. Illustration of collider bias by selective fertility and carryover effects in a sibship analysis.

6.2.6 Interaction, mediation, and risk of collider bias

Because gestational age is an intermediate factor between ART-conception and neonatal death, and not a confounder, interpretation of our analyses in study II of neonatal death according to gestational age requires careful consideration [175]. On one hand, our results indicate that conception method did not modify the association between preterm birth and neonatal mortality (i.e. no interaction), suggesting that infants conceived after fresh- or frozen-ET are equally vulnerable to the impact of preterm birth as naturally conceived infants. On the other hand, they suggest that the higher risk of neonatal death might be attributed to (mediated by) the higher risk of preterm birth in ART-conceived

pregnancies. However, this interpretation depends on the assumption of no unmeasured confounding between the intermediate factor (gestational age) and the outcome (neonatal death) [219]. This assumption is likely not met as both fetal malformations and placental complications could be common causes of gestational age and neonatal death, but also affected by ART-conception.

In all analyses, we restricted our study populations to singleton deliveries. Since ART treatment increases the risk of multiple pregnancies due to frequent transfer of more than one embryo [28], this restriction may also be seen as conditioning on an intermediate variable. However, including multiples, with multiple perinatal outcomes per delivery, would have required a different analytical strategy. Moreover, elective single embryo transfer is increasingly used to avoid multiple pregnancies in ART [28], and restriction to singletons may therefore be considered as more relevant for contemporary clinical practice.

6.2.7 Missing data

All research is vulnerable to missing data [220], though data collected as part of routine clinical care is far more likely to be afflicted even on a prospective basis [221]. Missing data can be categorised as missing not at random, missing at random and missing completely at random[221], the former is most prone to biasing the results.

There was great variation in the proportion of missingness for the variables in our data. Birthweight and gestational age were only missing for a small proportion of the eligible study population, but to be able to calculate the main outcomes of study I, the participants with missing birthweight and gestational age were excluded. In study II and III stillborn, without a recorded birthweight were not excluded from the analysis [222]. Still, we expect these data to be missing at random, meaning that bias should be limited [221].

Our population had a considerable sub-population with known maternal BMI, height, and smoking status, even though the missingness was great. Mostly this missingness was due to changes

in reporting practices during the study period, and therefore missing data at random and not bias the results but reduce the precision of the estimate. We did not impute missing data for maternal BMI or smoking because we had limited information available that could predict these factors.

In addition, we had small proportion of missing data concerning the number of embryos transferred as well as their culture duration. We therefore performed sensitivity analysis in defined subpopulations based on complete cases [205,221]. When investigating this missingness (number of embryos transferred and culture duration), it appeared that almost all missing data exclusively originated from one country (Norway), and we expect this to be a consequence of lack of reporting from a few clinics. Hence, the data may be considered as missing at random, because we do not expect the fertility clinic to influence outcomes beyond what is captured by factors included in the models.

6.3. Generalizability

A prerequisite for external validity is that the internal validity is high. Still, the extent to which our findings can be generalized to other populations needs to be addressed. The inclusion of all women who gave birth after ART or natural conception across four nations, implies that our results are relevant for a general population of ART treated. They have a similar age and parity as in other international studies perinatal outcomes after ART, supporting that they are representative of infertile women from other countries. The Nordic countries have populations that are similar in lifestyle, income, education, access to fertility treatment and health care, including free of charge ante-natal visits. These are all factors which are important for good health, perinatal care, and perinatal outcome. However, our findings are not necessarily directly comparable to other populations, as these underlying factors may differ between countries, including other high-income countries. For example, the occurrence of preterm birth and stillbirth are lower in the Nordic countries than in the UK and USA [223,224]. Still, a contribution from treatment factors to the associations between conception method and perinatal outcome is likely to be present across various societal factors, and as such generalizable, though with variation in association strength.

6.4 Comparison to other studies

6.4.1 Birthweight and gestational age

We present results of the largest study investigating birthweight and gestational duration after fresh and frozen-ET compared to naturally conceived. In line with previous observational studies with a conventional approach, our findings also confirm that fresh-ET is associated with low birthweight and high risk of preterm and very preterm birth [133,225], while frozen-ET has been associated with higher birthweights and some studies indicate a lower risk of preterm birth compared to fresh-ET [133,225].

Only a few previous studies have included a sibship design, though with a considerable variation in the covariates [140,153,184-187], table 2 shows a summary of characteristics of these studies. While we decided to define birthweight for gestational age by intrauterine growth curves [199], previous studies have used measured birthweight and different criteria for large and small for gestational age [140,153,184,186,187].

Still, the results from the sibship analysis are broadly consistent with ours. In a Danish study from 1994-2006 (total sibling pairs = 3879) fresh-ET singletons were associated with lower mean birthweight and higher risk of preterm birth, compared to their naturally conceived siblings, but no difference in very preterm birth[185]. They also showed that frozen-ET siblings had a higher risk of being large for gestational age, but a similar duration of pregnancy compared to their siblings born by fresh-ET (358 pairs). A US study included ART-singletons from 2003-2013 and compared fresh to frozen-ET within sibships (total 3681 discordant sibling pairs) and found siblings of frozen-ET to be of higher risk of large for gestational age compared to their fresh-ET siblings, though duration of pregnancy was similar between the siblings[140].

In four previous studies comparing ART-conceived children to their naturally conceived siblings, conclusions were less consistent [153,184,186,187]. Still, direction of association was similar across the studies. Different results and conclusions may reflect their different sample sizes and power

to estimate associations with sufficient precision. A study by Romundstad et al including deliveries in Norway from 1988-2006 (2204 sibling pairs), a study by Goisis et al on deliveries from Finland 1995-2000 (578 sibling pairs) and a study by Seggers et al on deliveries in the Netherlands 1999-2007 (1813 sibling pairs) all showed direction of association toward lower birthweight and gestational age in ART conceived compared to their naturally conceived siblings, though they lacked statistical precision. Further, none of these studies presented separate results from fresh and frozen-ET, however fresh-ET was the most common treatment method during these study periods [153,184,186]. A later study on deliveries in the USA from 2000-2010, with a larger sibling cohort (6458 sibling pairs) showed lower birthweight with a stronger statistical support, but again did not separate fresh from frozen-ET[140].

6.4.2 Offspring death

Previous studies investigating the risk of stillbirth after conception with ART have shown conflicting results. A population-based study from the Netherlands found a nearly doubled risk of stillbirth among ART-conceived (n=19 896) compared to naturally conceived (n=999 050) singletons (OR 1.94, 95% CI 1.54-2.44) [226]. A larger meta-analysis comparing 68 274 ART-conceived and 3 570 990 naturally conceived singleton pregnancies, mainly from cohort studies, also found higher odds of stillbirth after ART (OR 1.41, 95%CI 1.20-1.65) [227]. However, in line with our results is a study from the first CoNARTaS data linkage, that overlaps our study population with deliveries from 1988-2007) and also found a similar risk of stillbirth for frozen-ET (n=6 647) and fresh-ET (n= 42 242) compared to natural conceptions (n=288 542) [136]. The underlying causes of these conflicting results may be several. Stillbirth may be defined differently in different countries [228], and the occurrence also varies between populations [229], which may reflect different factors of the health care systems as well as general health and lifestyle factors of the population.

We found an higher risk of neonatal death in our study, which is consistent with the limited number of previous studies on conception method and neonatal mortality, showing higher neonatal

mortality after any ART [136,226,230], but do not support previous observations of higher neonatal mortality after frozen compared to fresh transfer [136,231,232].

We are not aware of previous studies assessing whether pregnancies after fresh and frozen transfer are more vulnerable to stillbirth or neonatal death at specific gestational ages. An Australian study compared pregnancies after any ART (n=15 416) to natural conception (n=391 952) and found lower risk of perinatal death (stillbirth or neonatal death) <32 weeks [137]. However, they did not use an 'ongoing pregnancies' approach and further differed from our study by including all births ≥20 weeks gestation, including stillbirths and late terminations. A Danish study which investigated stillbirth at term, found a higher risk after ART compared to natural conception, but included only uncomplicated pregnancies [233], thereby increasing the risk of selection bias by conditioning on a range of intermediate factors. Rather our findings support a previous Nordic study, using data from the first CoNARTaS linkage, where a higher risk of stillbirth after any ART compared to natural conception was found between 22-27 gestational weeks. Our study indicates that the apparent vulnerability during the period of pregnancy may only apply to fresh-ET.

Combining stillbirth and neonatal death into perinatal death, our results from study III are in accordance with previous sibship studies showing associations of opposite directions at the population level and within sibships [153,184,185]. We also confirm, using a study population close to 10 times larger than the largest of these studies, that the opposing results were largely driven by the subgroups with natural conception before ART. Compared to these previous studies, we show more explicitly how selection into the double discordant sibship group is increased for sibships with perinatal death after natural conception and survival after ART conception.

6.5 Implications

6.5.1 Clinical implications

We have clearly exposed the perinatal risks associated with fresh transfer, including lower mean birthweight and being small for gestational age, while frozen embryo transfer is associated with higher mean birthweight and higher risk of being born large for gestational age. However, both fresh and frozen-ET are associated with higher risk of preterm, very preterm birth and neonatal death, while stillbirth is not associated with either treatment mode. Based on our results we cannot advice on which treatment is most favorable, neither that a freeze-all approach will improve perinatal outcomes overall. Still, these findings should be highlighted for clinicians dealing with women pregnant after ART and couples receiving or considering assisted conception. By informing expecting couples of the risks associated with an ART pregnancy they may seek medical attention directly when needed, while clinicians and midwifes may also identify complications in pregnancy earlier. In the Nordic countries, ART conceived pregnancies are followed through the general antenatal program and not targeted specifically, hence patient education and awareness may be important in managing pregnancies after fresh and frozen embryo transfer.

6.5.2 Research implications

The results from our studies raise several important questions for further research. Firstly, it is vital to identify which factors of the treatment might be responsible for the adverse effects seen after both fresh and frozen embryo transfer. Such factors may be possible to avoid or change to improve the perinatal outcomes without interfering with success rates. Further, preterm birth poses a substantial risk after both fresh and frozen embryo transfer, however the underlying causes are expected to be multifactorial and closely entangled with maternal underlying health and obstetrical complications in

pregnancy. Further research might help identify women at high risk, and whether changes in antenatal monitoring or management could reduce the risk.

As fetal programming and epigenetic changes may be the source of some adverse outcomes seen after fresh and frozen embryo transfer, it is critical to investigate whether these adverse perinatal outcomes are associated with poor health and higher risk of chronic diseases in later life also among ART conceived. Follow up studies of long-term consequences for the children born after any ART, should also examine fresh and frozen embryo transfers separately, as the type of risk may differ between these treatments. A range of chronic diseases may be preventable with appropriate management and may be a key incentive to maintain a healthy lifestyle into later adulthood.

In our third paper we demonstrated a real-life example of strong biases in sibship comparison, created by carryover effects where the outcome in one sibling affects the exposure in the next sibling. This bias was further strengthened by the selective fertility seen when a couple experienced perinatal death. Although our alternative sibling comparison could control these biases by conditioning on the experience from the first pregnancy, statistical power was limited and results inconclusive. Even larger studies or using alternative family designs for triangulation (such as comparing sisters with and without ART treatment), might be helpful to further disentangle the contribution from ART treatment and maternal factors. Similar biases may also occur in other situations where a dramatic event in the perinatal period will affect the parents' drive for another child and may also contribute to parents changing their lifestyle before the next conception. This emphasizes the need to carefully assess whether sibship analyses are subject to bias and compare with results from population level analyses.

7. Conclusion

In our studies we have demonstrated that fresh embryo transfer is associated with lower birthweight and higher risk of being small for gestational age, while frozen embryo transfer is associated with higher birthweight and a higher risk of being large for gestational age. Both conception methods and maternal factors are associated with higher risk of preterm birth in ART conceived pregnancies. We found no higher risk of stillbirth overall for fresh and frozen embryo transfer compared to naturally conceived, but a higher risk of early stillbirths for fresh transfer. Both ART methods were associated with a higher risk of neonatal death compared to natural conceptions, possibly mediated through their higher risk of preterm birth.

Collectively, our studies also demonstrate the strengths and weaknesses of sibship analyses. On one hand, they provide a valuable approach to control for unmeasured maternal or family level confounding, thereby contributing to disentangling whether adverse effects are associated with treatment and maternal factor. On the other hand, they require large study populations and may be subject to substantial biases from selection into the double discordant sibships through various mechanisms.

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reviews. They have no conflicts of interest related to commercial funding. Professor Farquhar is a

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Paper I

PLOS MEDICINE

RESEARCH ARTICLE

Separating parental and treatment contributions to perinatal health after fresh and frozen embryo transfer in assisted reproduction: A cohort study with within-sibship analysis

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Abstract

Background

Compared to naturally conceived children, adverse perinatal outcomes are more common among children born after assisted reproductive technology with fresh embryo transfer (fresh-ET) or frozen embryo transfer (frozen-ET). However, most previous studies could not adequately control for family confounding factors such as subfertility. We compared birth size and duration of pregnancy among infants born after fresh-ET or frozen-ET versus natural conception, using a within-sibship design to account for confounding by maternal factors.

Methods and findings

This registry-based cohort study with nationwide data from Denmark (1994–2014), Norway (1988–2015), and Sweden (1988–2015) consisted of 4,510,790 live-born singletons, 4,414,703 from natural conception, 78,095 from fresh-ET, and 17,990 from frozen-ET. We identified 33,056 offspring sibling groups with the same mother, conceived by at least 2 different conception methods. Outcomes were mean birthweight, small and large for gestational age, mean gestational age, preterm (<37 weeks, versus ≥37), and very preterm birth (<32 weeks, versus ≥32). Singletons born after fresh-ET had lower mean birthweight (−51 g, 95% CI −58 to −45, $p < 0.001$) and increased odds of small for gestational age (odds

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Abbreviations: ART, assisted reproductive technology; CoNARTaS, Committee of Nordic Assisted Reproductive Technology and Safety; fresh-ET, fresh embryo transfer; frozen-ET, frozen embryo transfer; OR, odds ratio.

ratio [OR] 1.20, 95% CI 1.08 to 1.34, $p < 0.001$), while those born after frozen-ET had higher mean birthweight (82 g, 95% CI 70 to 94, $p < 0.001$) and increased odds of large for gestational age (OR 1.84, 95% CI 1.56 to 2.17, $p < 0.001$), compared to naturally conceived siblings. Conventional population analyses gave similar results. Compared to naturally conceived siblings, mean gestational age was lower after fresh-ET (−1.0 days, 95% CI −1.2 to −0.8, $p < 0.001$), but not after frozen-ET (0.3 days, 95% CI 0.0 to 0.6, $p = 0.028$). There were increased odds of preterm birth after fresh-ET (OR 1.27, 95% CI 1.17 to 1.37, $p < 0.001$), and in most models after frozen-ET, versus naturally conceived siblings, with somewhat stronger associations in population analyses. For very preterm birth, population analyses showed increased odds for both fresh-ET (OR 2.03, 95% CI 1.90 to 2.12, $p < 0.001$) and frozen-ET (OR 1.66, 95% CI 1.42 to 1.94, $p < 0.001$) compared with natural conception, but results were notably attenuated within siblings (OR 1.18, 95% CI 1.0 to 1.41, $p = 0.059$, and OR 0.92, 95% CI 0.67 to 1.27, $p = 0.6$, for fresh-ET and frozen-ET, respectively). Sensitivity analyses in full siblings, in siblings born within 3-year interval, by birth order, and restricting to single embryo transfers and blastocyst transfers were consistent with the main analyses. Main limitations were high proportions of missing data on maternal body mass index and smoking.

Conclusions

We found that infants conceived by fresh-ET had lower birthweight and increased odds of small for gestational age, and those conceived by frozen-ET had higher birthweight and increased odds of large for gestational age. Conception by either fresh-ET or frozen-ET was associated with increased odds of preterm birth. That these findings were observed within siblings, as well as in conventional multivariable population analyses, reduces the likelihood that they are explained by confounding or selection bias.

Trial registration

ClinicalTrials.gov ISRCTN11780826.

Author summary

Why was this study done?

- Children born after assisted reproductive technology have more adverse perinatal outcomes than naturally conceived children, which differ according to treatment method.
- It is unknown to what extent these associations result from the fertility treatment or from confounding by underlying maternal or family factors.

What did the researchers do and find?

- Using health registry data from Denmark, Norway, and Sweden, we compared perinatal health after fresh embryo transfer (fresh-ET) or frozen embryo transfer (frozen-ET) to that after natural conception, in a cohort of 4,606,875 newborns. In addition, we

compared siblings conceived by different methods to account for family confounding (n = 33,056 sibling groups).

- We found that children conceived by frozen-ET have a higher birthweight and higher risk of large for gestational age, whereas children conceived by fresh-ET have a lower birthweight and higher risk of small for gestational, compared to naturally conceived children, both in the population and within siblings.
- Within sibships, children conceived by fresh-ET and frozen-ET had increased risks of preterm birth (<37 weeks) of similar magnitude, while neither fresh-ET nor frozen-ET was associated with risk of very preterm birth (<32 weeks), despite strong associations for both outcomes in population analyses.

What do these findings mean?

- Fresh-ET and frozen-ET showed opposite associations with birthweight, but similar associations with preterm birth, after controlling for measured and unmeasured family-level confounding.
- Both treatments are associated with adverse perinatal outcomes, in comparison to natural conception. Our findings provide important information that can be used by couples and their clinicians in making decisions about which type of ART to undertake.

Introduction

The use of assisted reproductive technology (ART) is increasing worldwide, and children born after ART now comprise more than 7% of births in some countries [1–4]. The number of children born after fresh embryo transfer (fresh-ET) has increased steadily over 3 decades, and the number of children born after frozen embryo transfer (frozen-ET) has increased sharply during the last decade [1,2,5]. Whilst elective single embryo transfer has reduced multiple pregnancy and adverse outcomes associated with that [5,6], singleton ART newborns still have worse perinatal outcomes compared with naturally conceived newborns [7]. Meta-analyses show lower birthweight, lower gestational age, higher risk of small for gestational age, and higher risk of preterm birth among newborns after fresh-ET compared to naturally conceived newborns [8,9]. In contrast, newborns after frozen-ET have lower risk of small for gestational age and preterm birth compared to newborns after fresh-ET [10,11], but higher mean birthweight and higher risk of large for gestational age compared to naturally conceived newborns [8,12–14]. Most previous studies have not adequately controlled for family confounding factors, such as maternal health and socioeconomic position [8,14,15]. Subfertile couples who conceive while awaiting ART treatment have suboptimal perinatal outcomes compared to fertile couples, indicating that parental factors contribute to the adverse events [3]. Without attempts to control for potential family confounding, it is unclear whether these associations are attributable to treatment.

Comparing siblings born after different conception methods offers an alternative approach to conventional multivariable analyses in unrelated children, and may help disentangle the contributions from ART treatment, shared genetics, parental health factors, and confounding

from, for example, background family socioeconomic position [16,17]. Four previous studies with a sibling design compared any ART conception with natural conception, and all reported lower birthweight and shorter gestational duration in infants conceived by ART, though for some outcomes, wide confidence intervals included the null [18–21]. A Danish study could differentiate between fresh and frozen transfer and found lower birthweight and shorter gestation for fresh-ET compared to naturally conceived siblings (3,879 sibling pairs) and higher birthweight for frozen-ET compared to fresh-ET siblings (358 sibling pairs) [22,23]. An American study included only children conceived after ART and found that children conceived by frozen-ET had higher birthweights and higher risk of large for gestational age than their fresh-ET siblings (3,681 pairs) [22]. None of the previous studies compared children conceived by frozen-ET to naturally conceived siblings, which is a necessary comparison to understand whether the higher birthweights and increased risk of large for gestational age associated with frozen-ET simply reflect the observed lower birthweight for fresh-ET compared with natural conception.

The aim of this study was to determine the associations of fresh-ET and frozen-ET, compared to natural conception, with birth size and duration of pregnancy. We used nationwide data from 3 countries that provided a sufficiently large sample size to precisely estimate associations using a within-sibship design. The within-sibship analysis assumes that most confounders are at the family level and that there is very little individual-level confounding. Specifically, in this study we assume that in the within-sibship analyses we can control for unmeasured confounding by shared family factors, such as socioeconomic position, underlying maternal health, and health behaviors [16,17,24].

Methods

Data sources

This cohort study is based on the Committee of Nordic Assisted Reproductive Technology and Safety (CoNARTaS) cohort [5], which includes data on all births registered in the nationwide medical birth registries in Denmark (1994–2014), Norway (1984–2015), and Sweden (1985–2015). Children born after ART were identified through data linkage with the national ART registries and databases, using the unique national identity number assigned to each resident. The registration of ART pregnancies was initiated at different times in each country. In Denmark, all ART cycles from both public and private clinics have been registered in the national ART registry since 1994, resulting in almost 100% completeness [25]. Since 1984, Norwegian public and private ART clinics send notifications to the Medical Birth Registry for all ART cycles that result in pregnancy verified by ultrasound in gestational week 6–7. In Sweden, deliveries after ART were reported to the National Board of Health and Welfare from 1982 to 2006. Since 2007, all ART cycles in Sweden are reported to the National Quality Registry for Assisted Reproduction.

Exposures, outcomes, and covariates

Exposures were ART conception with fresh-ET or frozen-ET versus natural conception (the reference group). Fresh-ET and frozen-ET were defined based on treatment entries in the ART registries/databases. Frozen-ET included both first embryo transfer (i.e., when a "freeze-all" treatment was undertaken) as well as those with a subsequent transfer after an initial fresh transfer. Natural conceptions were defined based on any registered pregnancy with no registration of ART conception.

We defined perinatal health outcomes as birth size (birthweight, small for gestational age, and large for gestational age) and duration of pregnancy (gestational age at birth, preterm

birth, and very preterm birth). Birthweight was measured in grams. We used Marsal's equations for intrauterine growth to estimate *z*-scores of birthweights, where 1 standard deviation was set to 11% of the expected birthweight according to sex and gestational age [26]. Small for gestational age was defined as birthweight < -2 standard deviations, and large for gestational age was defined as birthweight $> +2$ standard deviations from expected mean birthweight. For natural conceptions, gestational age was reported in days and estimated by routine ultrasound examination, performed in week 18–20 of pregnancy in Norway and Sweden, and in late first trimester in Denmark. If this information was missing, the date of last menstrual period was used to calculate gestational age. For ART pregnancies, gestational age was estimated based on embryo transfer in Sweden, while in Norway and Denmark the first trimester (Denmark) or week 18–20 (Norway) ultrasound screening was used, and only if this was missing was the date of embryo transfer used. Preterm birth was defined as birth before 37 weeks of gestation, versus at $\geq$37 weeks, and very preterm birth as birth before 32 weeks of gestation. Maternal and paternal identity codes were recorded in the medical birth registries, with paternal identity available for 98% of newborns. For our main analyses, we identified siblings as children with the same mother from the maternal identity code. In sensitivity analyses, we repeated analyses using full siblings (same mother and father) identified using the maternal and paternal identity codes.

Potential confounders were defined as any factor that could plausibly influence the need for ART, birthweight, or gestational age; these were identified based on previous literature. We adjusted for the following observed confounders: country, year of birth, and maternal age, parity, BMI, height, and smoking. Maternal BMI was calculated as weight in kilograms divided by height in square meters, based on pre-pregnancy or first trimester values and categorized as underweight ($<$18.5 kg/m^2), normal (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), and obese ($\geq$30.0 kg/m^2). Further, we categorized maternal height ($<$150, 150–159, 160–169, 170–179, or $\geq$180 cm), smoking (yes or no, where yes was any smoking during pregnancy), and parity (number of previous deliveries: 0, 1, 2, or 3). Maternal age and offspring year of birth were used as continuous variables. Smoking was registered throughout the study period in Denmark and Sweden and since 1999 in Norway. Maternal height and weight were registered in 1988–1989 and 1992–2015 in Sweden, with substantial missing data in the early years. In Denmark and Norway, registration of maternal height and weight was implemented from 2004 and 2007, respectively, also with substantial missing data during the first years of registration. In addition to these observed confounders, we considered parental socioeconomic position to be a key confounder, but we did not have data on individual income or education. However, the sibship analysis approach controls for this family-level confounding on the assumption that parental socioeconomic position is likely to be very similar between siblings.

Study population

Fig 1 shows the flow of participants into the main analysis and sensitivity analysis datasets. We defined our study period as being from 1988, when the first child born after embryo cryopreservation was registered in our data (from 1994 for Denmark, as data were not available until then), until 2014 (Denmark) and 2015 (Norway and Sweden). Eligibility was defined as live-born singletons whose mothers delivered their first child within the study period and were age 20 years or older at their first delivery (4,617,121 infants with 2,390,386 mothers). These criteria ensured comparability of maternal age between ART and natural conceptions while maximizing the number of sibling groups in the analysis sample. We excluded all singletons with unknown parity in pregnancies after the first birth, maternal age $\geq$ 45 years, and parity $\geq$ 4, as there were very few ART births to mothers with 4 or more deliveries. We further excluded

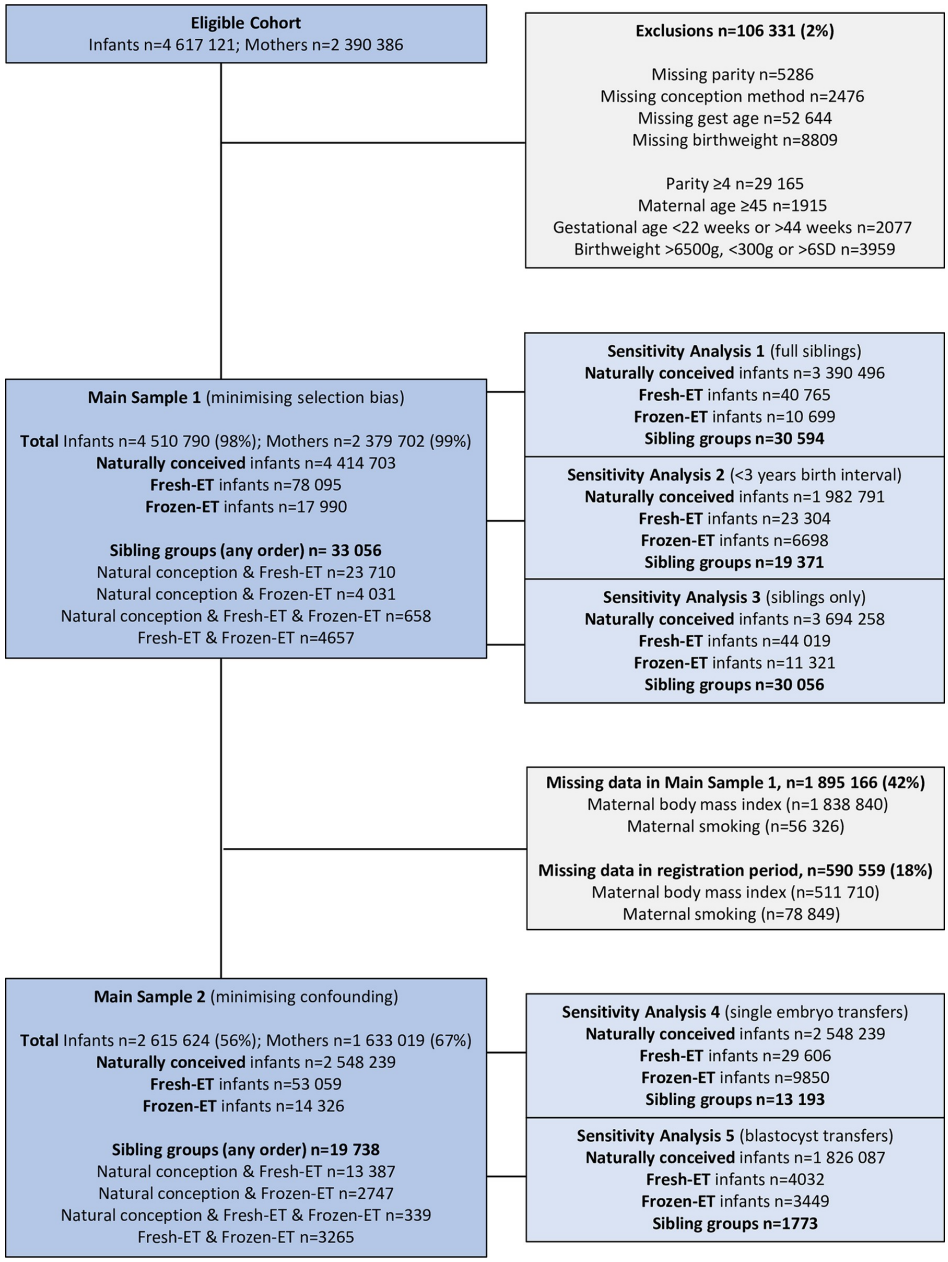

Fig 1. Flow chart of the study population. If not otherwise specified, sibling groups refer to maternal offspring siblings conceived through at least 2 of the 3 different conception methods. Fresh-ET, fresh embryo transfer; Frozen-ET, frozen embryo transfer; gest, gestational.

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singletons with unknown conception method, gestational age, or birthweight, as well as singletons with extreme values of gestational age (<22 weeks or >44 weeks) and birthweight (>6,500 g, <300 g, or >6 SD). After these exclusions, our main sample 1 (largest sample, with lowest risk of selection bias) comprised 4,510,790 infants with 2,379,702 mothers, where 78,095 were born from fresh-ET and 17,990 were born from frozen-ET. In this sample there were 33,056 sibling groups with at least 2 of the 3 different conception methods, including 24,368 sibling groups with both fresh-ET and natural conception and 4,689 sibling groups with both frozen-ET and natural conception. For main sample 2 (smallest sample, with maximum confounder adjustment), we restricted main sample 1 to deliveries with complete data on maternal BMI and smoking (58% of main sample 1). Corresponding numbers for main sample 2 were 2,615,624 infants with 1,633,019 mothers, including 53,059 born after fresh-ET and 14,326 born after frozen-ET. In main sample 2, there were 20,227 sibling groups with at least 2 of the 3 conception methods, including 13,869 sibling groups with both fresh-ET and natural conception and 3,168 sibling groups with both frozen-ET and natural conception. To explore whether the results were driven by specific subgroups or whether the associations were influenced by which conception method occurred first, we identified each mother's 2 first consecutive deliveries and categorized them by order of conception method. In main sample 2, this gave a total of 698,990 offspring sibling groups that belonged to 1 of 9 possible sibling combinations.

Statistical analysis

We used multilevel linear and logistic models to compare outcomes across conception methods with children as one level and mothers as another. We used random effects models for conventional population estimates and fixed effects models for sibship comparisons (i.e., comparisons within sibships). Precision was estimated by 95% confidence intervals (CIs). To increase interpretability of the odds ratios (ORs), we used post-estimation commands to obtain absolute risks and risk differences. The within-sibling estimates were based on siblings who were discordant for conception method. Population estimates in main sample 1 were adjusted for year of birth, country, maternal age, and parity. In main sample 2 we additionally adjusted for height, pre-pregnancy or first trimester BMI, and smoking status during pregnancy. The sibling comparisons were adjusted for the same covariates except country and height, which are stable within mothers.

We performed several sensitivity analyses to test the robustness of our findings (Fig 1). First, we explored the importance of constant paternal factors by repeating analyses on full siblings only (same mother and father). Second, we restricted analyses to siblings born within a 3-year interval as both family background and health are likely to be more constant among siblings born within a short timeframe. Third, we restricted the population-level analyses to siblings (excluding all infants where the mother had only 1 child in the sample). This enabled us to explore whether any differences between population and sibling results might be driven by families with only 1 child being different to those with 2 or more. Finally, we restricted the ART population to single embryo transfers and to blastocyst transfers (i.e., culture duration 5–6 days) to account for changes in practice over the study period that could potentially influence our results [5,27,28]. Single, compared with double, embryo transfer is associated with higher [29] and eliminates vanishing twin syndrome, which may also influence birthweight and gestational duration [30]. Blastocysts are exposed to culture medium and other in vitro conditions for a longer period than cleavage stage embryos (5–6 versus 2–3 days), and this may also influence fetal growth and gestational duration.

This study is reported as per the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline (S1 STROBE Checklist). Our analyses were planned in

advance of the research team accessing any data, and our study protocol is provided (S1 Study Protocol). The CoNARTaS project is also registered in the ISRCTN registry (ISRCTN11780826).

Ethical approval

In Denmark, ethical approval is not required for scientific projects based solely on registry data. In Norway, ethical approval was given by the Regional Committee for Medical and Health Research Ethics (REK-Nord, 2010/1909). In Sweden approval was obtained from the ethical committee in Gothenburg (Dnr214-12, T422-12, T516-15, T233-16, T300-17, T1144-17, and T121-18).

Patient and public involvement

This study was a secondary data analysis and was done without patient involvement. Patients were not involved in setting the research question or the outcome measures, nor were they involved in developing plans for the design or implementation of the study.

Results

Baseline characteristics

Mothers who conceived by ART were older and more commonly primiparous than mothers of naturally conceived children (Table 1). While the naturally conceived children were evenly distributed through the study period, more than 80% of ART children were born after 2002. Mean maternal height and BMI were comparable between all conception groups. Fewer ART mothers were underweight and obese, but there was a higher proportion in the overweight category. Among children born after fresh-ET, about 42% of the children were conceived by intracytoplasmic sperm injection, 47.4% were born after single embryo transfer, and only 5.7% were born after blastocyst transfer. Similar proportions were found in children born after frozen-ET, with 40.2% conceived by intracytoplasmic sperm injection, 64.4% born after single embryo transfer, though 20.8% born after blastocyst transfer.

Birthweight

In population analyses in both samples, children from fresh-ET were on average lighter, and those born after frozen-ET were on average heavier, at birth compared to naturally conceived children, after adjustment for all observed confounders (Tables 2 and 3). Analyses of birthweight *z*-score according to gestational age and sex showed similar patterns. Children born after fresh-ET had a higher risk of small for gestational age and lower risk of large for gestational age compared to naturally conceived children, whereas the opposite was true for children conceived via frozen-ET (Fig 2A; Tables A and B in S1 Text). The sibship comparisons showed the same patterns, with clear differences in mean birthweight and small and large for gestational age between children born after fresh-ET and frozen-ET, compared to their siblings who were naturally conceived. The magnitudes of association were also similar between sibship and population-level analyses (Fig 2A).

Duration of pregnancy

In both main samples, gestational age was shorter for children of fresh-ET and frozen-ET in population analyses (Tables 2 and 3). In the corresponding sibling comparisons, children of fresh-ET had gestational ages closer to their naturally conceived siblings, while children of frozen-ET had a longer gestational age compared to their naturally conceived siblings. In

Table 1. Characteristics of the 4,510,790 live-born singletons in main sample 1.

Characteristic	Natural conception		Fresh embryo transfer		Frozen embryo transfer	
	n or mean	Percent or SD	*n* or mean	Percent or SD	*n* or mean	Percent or SD
Participants (*n*, %)	4,414,703	97.9	78,095	1.7	17,990	0.4
Country (*n*, %)						
Denmark	977,754	22.2	25,041	32.1	3,347	18.6
Norway	1,193,617	27.0	16,551	21.2	3,283	18.3
Sweden	2,243,334	50.8	36,503	46.7	11,360	63.2
Birth year (*n*, %)						
1988–1996	1,020,394	23.1	5,762	7.4	494	2.8
1997–2001	806,469	18.3	11,190	14.3	1,098	6.1
2002–2006	909,995	20.6	17,727	22.7	2,541	14.1
2007–2011	965,027	21.9	24,346	31.2	6,499	36.1
2012–2015	712,820	16.2	19,070	24.4	7,358	40.1
Parity (*n*, %)						
0	2,258,213	51.2	58,739	75.2	10,413	57.9
1	1,585,604	35.9	16,977	21.7	6,539	36.5
2	476,823	10.8	2,039	2.6	920	5.1
3	94,065	2.1	340	0.4	118	0.7
Maternal age, in years (mean, SD)	29.6	4.8	33.8	4.2	34.3	4.1
Sex (*n*, %)						
Boys	2,269,179	51.4	39,914	51.1	9,200	51.1
Girls	2,145,526	48.6	38,181	48.9	8,790	48.9
Smoking						
Yes	447,967	10.2	4,040	5.2	540	3.0
Missing (%)		15.4		9.4		6.1
Maternal height, in cm (mean, SD)	166.8	6.3	167.7	6.4	167.5	6.5
Missing (%)		35.5		27.9		2.8
Maternal BMI, in kg/m^2 (mean, SD)	24.2	4.5	24.2	4.1	24.2	4.0
Missing (%)		41.0		30.8		19.3
Maternal BMI, in kg/m^2 (*n*, %)						
<18.5	80,471	3.2	1,256	2.4	310	2.2
18.5–24.9	1,627,235	63.9	33,733	63.6	9,096	63.5
25.0–29.9	575,551	22.6	12,938	28.8	3,541	24.7
≥30.0	264,982	10.4	5,133	9.7	1,379	9.6
Fertilization method (*n*, %)						
IVF	—	—	44,474	58.0	9,818	59.8
ICSI	—	—	32,164	42.0	6,597	40.2
Embryos transferred (*n*, %)						
1	—	—	36,992	47.4	11,577	64.4
2	—	—	29,915	38.3	4,197	23.3
3	—	—	1,880	2.4	128	0.7
Unknown			9,308	11.9	2,088	11.6
Embryo culture duration, in days (*n*, %)						
2–3	—	—	61,654	79.0	11,695	65.0
5–6	—	—	4,437	5.7	3,748	20.8
Unknown			12,004	15.4	2,547	14.2

BMI, body mass index; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; SD, standard deviation.

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Table 2. Birthweight and gestational age by conception method: Population estimates and within-sibship estimates in main sample 1 (minimizing selection).

Outcome and conception method	Population estimates (random effects)					Within-sibship estimates (fixed effects)				
	Number	Mean[1]	Mean difference[1]	Adj. mean difference[2]	95% CI	Number[3]	Mean[1]	Mean difference[1]	Adj. mean difference[2]	95% CI
Birthweight, grams										
Natural conception	4,414,703	3,541	0	0	Ref.	33,889	3,540	0	0	Ref.
Fresh-ET	78,095	3,410	−127	−71	−75 to −67	30,167	3,424	−116.3	−51	−58 to −45
Frozen-ET	17,990	3,581	51	66	59 to 74	9,589	3,623	83	82	70 to 94
Birthweight, *z*-score										
Natural conception	4,414,703	−0.01	0	0	Ref.	33,889	−0.01	0	0	Ref.
Fresh-ET	78,095	−0.19	−0.18	−0.05	−0.06 to −0.04	30,167	−0.23	−0.22	−0.06	−0.78 to −0.05
Frozen-ET	17,990	0.16	0.18	0.21	0.18 to 0.21	9,589	0.2	0.21	0.19	0.17 to 0.22
Gestational age, days										
Natural conception	4,414,703	279.1	0	0	Ref.	33,889	279.0	0	0	Ref.
Fresh-ET	78,095	276.6	−2.3	−2.1	−2.2 to −2.0	30,167	277.9	−1.1	−1.0	−1.2 to −0.8
Frozen-ET	17,990	278.1	−0.8	−0.6	−0.8 to −0.4	9,589	279.2	0.2	0.3	0.0 to 0.6

Adj., adjusted; CI, confidence interval; fresh-ET, fresh embryo transfer; frozen-ET, frozen embryo transfer; Ref., reference.

[1]Unadjusted.

[2]Adjusted for maternal age, parity, and year of birth. Random effects are additionally adjusted for country.

[3]Number of children that are part of a sibling group with at least 2 different conception methods within the group.

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population analysis, in both main sample 1 and main sample 2, children conceived with fresh-ET and frozen-ET had substantially higher odds of preterm and very preterm birth than naturally conceived children. For preterm birth, there was some attenuation in sibling compared to population analyses (Fig 2; Tables A and B in S1 Text), particularly for frozen-ET in main sample 1. In sibship analyses of very preterm birth, there was more marked attenuation than seen in analyses of preterm birth, with point estimates close to the null value, though with wide confidence intervals.

Fig 3D shows the risk of preterm birth according to birth order for the different combinations of conception methods in consecutive sibling pairs. All sibling groups with 1 or 2 children of ART were at higher risk compared to the naturally conceived sibling pairs, and there were no clear differences between treatment types (fresh-ET and frozen-ET) in risk of preterm birth.

Sensitivity analyses

For the birthweight outcomes (mean birthweight, small for gestational age, and large for gestational age), the results of all sensitivity analyses (Tables C–L in S1 Text) were consistent with the findings from the main analysis. Concerning duration of pregnancy (mean gestational age, preterm birth, and very preterm birth), the sensitivity analyses were overall in line with the findings from main sample 2. One exception was the sibship comparison where ART treatment was restricted to blastocyst transfers, which may indicate an increased risk of both preterm and very preterm birth among children born after fresh-ET frozen-ET compared to their naturally conceived siblings (Table L in S1 Text). However, these estimates were imprecise due to small sample sizes.

Table 3. Birthweight and gestational age by conception method: Population estimates and within-sibship estimates in main sample 2 (minimizing confounding).

Outcome and conception method	Population estimates (random effects)					Within-sibship estimates (fixed effects)				
	Number	Mean[1]	Mean difference[1]	Adj. mean difference[2]	95% CI	Number[3]	Mean[1]	Mean difference[1]	Adj. mean difference[2]	95% CI
Birthweight, grams										
Natural conception	2,548,239	3,547	0	0	Ref.	19,656	3,547	0	0	Ref.
Fresh-ET	53,059	3,413	−134	−83	−87 to −78	17,631	3,415	−132	−52	−61 to −44
Frozen-ET	14,326	3,583	42	56	48 to 65	6,538	3,610	63	75	61 to 89
Birthweight, *z*-score										
Natural conception	2,548,239	0.01	0	0	Ref.	19,656	0.01	0	0	Ref.
Fresh-ET	53,059	−0.20	−0.22	−0.08	−0.09 to −0.07	17,631	−0.28	−0.28	−0.08	−0.10 to −0.06
Frozen-ET	14,326	0.14	0.15	0.19	0.18 to 0.21	6,538	0.15	0.14	0.17	0.15 to 0.20
Gestational age, days										
Natural conception	2,548,239	279.0	0	0	Ref.	19,656	279.0	0	0	Ref.
Fresh-ET	53,059	276.9	−2.0	−2.1	−2.2 to −2.0	17,631	278.2	−0.8	−0.8	−1.0 to −0.6
Frozen-ET	14,326	278.4	−0.5	−0.7	−0.9 to −0.5	6,538	279.3	0.4	0.4	−0.0 to 0.7

Adj., adjusted; CI, confidence interval; fresh-ET, fresh embryo transfer; frozen-ET, frozen embryo transfer; Ref., reference.

[1]Unadjusted.

[2]Adjusted for maternal age, parity, year of birth, maternal pre-pregnancy or first trimester body mass index, and maternal smoking during pregnancy. Random effects are additionally adjusted for country and maternal height.

[3]Number of children that are part of a sibling group with at least 2 different conception methods within the group.

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Discussion

Summary of findings

We found evidence that children born after ART were at higher risk of adverse perinatal outcomes compared to the background population. Given the consistency of findings across conventional population and sibship analyses, in 2 samples (one minimizing selection bias and the other minimizing confounding) and multiple sensitivity analyses, our findings indicate that conception through fresh-ET was associated with lower mean birthweight and a higher risk of small for gestational age, whereas conception with frozen-ET was associated with higher mean birthweight and a higher risk of large for gestational age, compared to natural conception. Further, fresh-ET was associated with a shorter mean gestational age, and both fresh-ET and frozen-ET were associated with higher odds of preterm birth. Whilst population analyses suggested increased odds of very preterm birth in children conceived by either fresh-ET or frozen-ET, this was markedly attenuated in sibship analyses, though statistical power was limited in these analyses and confidence intervals were wide. The stronger associations at the population level for mean duration, preterm birth, and very preterm birth suggest that unmeasured maternal factors contribute to gestational duration in addition to the contribution of conception by either fresh-ET or frozen-ET.

Strengths and limitations

Our study involved 2 main samples, both with detailed maternal data, including information on previous deliveries and conception method. While main sample 1 was less prone to selection bias because it consisted of an unselected and larger population, main sample 2 provided

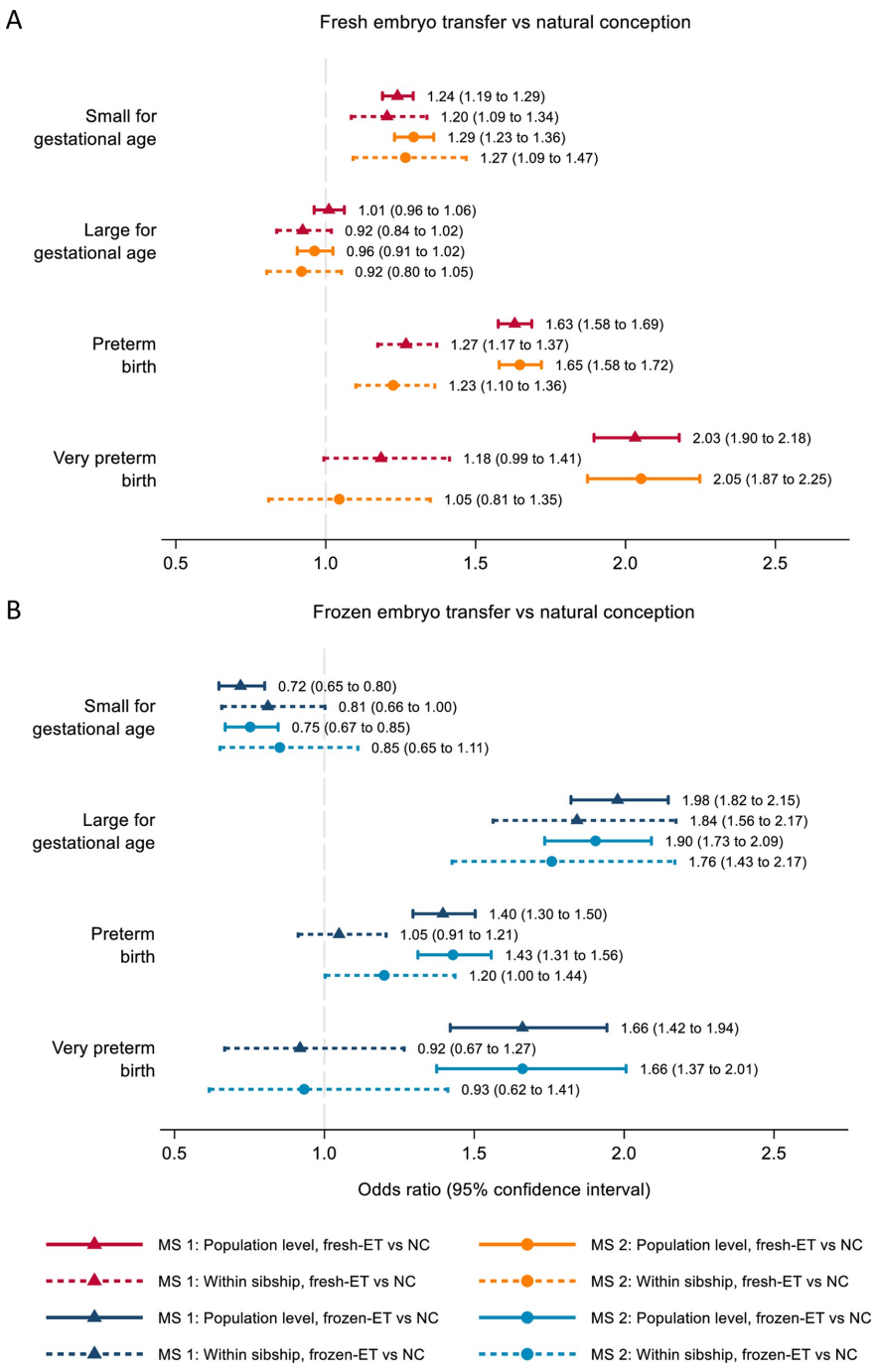
A
Fresh embryo transfer vs natural conception
Small for gestational age
1.24 (1.19 to 1.29)
1.20 (1.09 to 1.34)
1.29 (1.23 to 1.36)
1.27 (1.09 to 1.47)
Large for gestational age
1.01 (0.96 to 1.06)
0.92 (0.84 to 1.02)
0.96 (0.91 to 1.02)
0.92 (0.80 to 1.05)
Preterm birth
1.63 (1.58 to 1.69)
1.27 (1.17 to 1.37)
1.65 (1.58 to 1.72)
1.23 (1.10 to 1.36)
Very preterm birth
2.03 (1.90 to 2.18)
1.18 (0.99 to 1.41)
2.05 (1.87 to 2.25)
1.05 (0.81 to 1.35)
0.5
1.0
1.5
2.0
2.5
B
Frozen embryo transfer vs natural conception
Small for gestational age
0.72 (0.65 to 0.80)
0.81 (0.66 to 1.00)
0.75 (0.67 to 0.85)
0.85 (0.65 to 1.11)
Large for gestational age
1.98 (1.82 to 2.15)
1.84 (1.56 to 2.17)
1.90 (1.73 to 2.09)
1.76 (1.43 to 2.17)
Preterm birth
1.40 (1.30 to 1.50)
1.05 (0.91 to 1.21)
1.43 (1.31 to 1.56)
1.20 (1.00 to 1.44)
Very preterm birth
1.66 (1.42 to 1.94)
0.92 (0.67 to 1.27)
1.66 (1.37 to 2.01)
0.93 (0.62 to 1.41)
0.5
1.0
1.5
2.0
2.5
Odds ratio (95% confidence interval)
MS 1: Population level, fresh-ET vs NC
MS 1: Within sibship, fresh-ET vs NC
MS 1: Population level, frozen-ET vs NC
MS 1: Within sibship, frozen-ET vs NC
MS 2: Population level, fresh-ET vs NC
MS 2: Within sibship, fresh-ET vs NC
MS 2: Population level, frozen-ET vs NC
MS 2: Within sibship, frozen-ET vs NC

Fig 2. Adverse perinatal outcomes according to conception method: Population estimates and offspring sibling comparison. Odds ratios with 95% confidence intervals for fresh embryo transfer (fresh-ET) versus natural conception (NC) (A) and frozen embryo transfer (frozen-ET) versus natural conception (B). Main sample 1 (MS 1) estimates are adjusted for maternal age, parity, offspring birth year, and country (population level only) and minimize selection bias. Main sample 2 (MS 2) estimates are additionally adjusted for maternal body mass index, smoking status, and height (population level only) and minimize confounding. Fig 3A–3C shows the mean birthweights and risks of small and large for gestational age for a given birth order among sibling pairs with different combinations of conception methods. Overall, mean birthweight and risk of large for gestational age were greater, and risk of small for gestational age lower, in second-born compared to first-born siblings in all groups. Infants born after fresh-ET had the lowest birthweights and highest risk of small for gestational age for their birth order, while children born after frozen-ET had the highest birthweights and highest risk of large for gestational age, regardless of the conception method of their respective siblings.

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results after more confounder adjustments, including for maternal BMI and smoking, both strongly associated with adverse perinatal outcomes [31–33] and largely missing from similar previous studies [20–23]. As reporting of BMI was introduced and improved during the study period, the children in main sample 2 comprise a more recent study population, reflecting contemporary treatment practice. Our sample was considerably larger than any previous population and included over 7-fold the number of discordant siblings compared with the 2 previous sibling studies directly comparing fresh-ET to frozen-ET.

A major strength was the comparison of siblings born after different conception methods. While the results from conventional population analyses are prone to residual confounding from unmeasured maternal and family characteristics, such as maternal health and family socioeconomic position, we expect the sibship analysis to account for many of these confounders as they are highly likely to be the same or very similar for siblings. Even if some characteristics may change between a woman's pregnancies, they are more likely to be similar within women than between women, and, therefore, the within-sibship analyses provide extra control for these characteristics. In addition, the large sample size supported analyses comparing the risk of outcomes according to order of conception methods used, as well as several sensitivity analyses that accounted for possible differences between maternal and full siblings, greater differences in maternal or family characteristics between siblings born more than 3 years apart, and the use of single embryo transfer and blastocyst culture. We found similar results for birthweight outcomes in all our approaches and populations, strengthening the evidence that type of ART treatment influences birthweight outcomes. For duration of pregnancy, results were also broadly consistent, and collectively they support that both ART treatments increase the risk of preterm birth, without clearly influencing risk of very preterm birth.

All birth institutions and ART clinics in the study countries adhere to a policy of mandatory reporting, ensuring valid and exhaustive data collection. Even so, women who receive cross-border reproductive care are likely to be misclassified as having natural conceptions in our study because they do not appear in the national ART registries. These will be a small group compared to the large group of correctly classified naturally conceiving women [34,35], and are therefore unlikely to substantially bias the results. Smoking was self-reported and could only be harmonized across all countries as a dichotomous variable. Further, smoking is commonly underreported among pregnant women [36] and is a source of residual confounding that we expect to be considerably worse in the population than within the sibship analyses. Estimation of gestational age in comparisons of natural and ART conception is challenging because fetuses from both fresh-ET and frozen-ET may have a greater estimated fetal size by ultrasound in both the first and second trimester compared to naturally conceived fetuses [37]. ART-conceived pregnancies may therefore be expected to have a higher gestational age when estimated from ultrasound measurements than from transfer date. Whether clinicians took this into consideration when determining gestational age is not known in our data. Our data from Denmark and Norway allowed comparison of the 2 methods of determining gestational

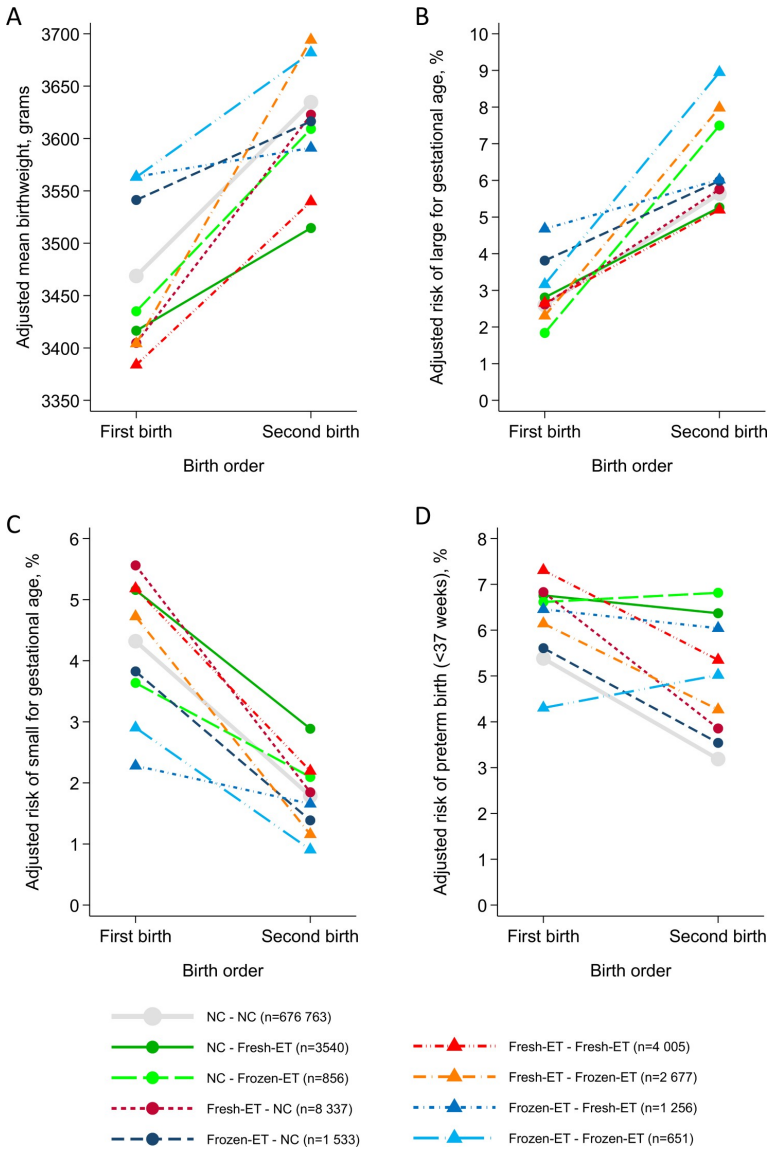

Fig 3. Perinatal outcomes in consecutive offspring sibling pairs according to birth order and conception methods. Means and absolute risks are estimated in main sample 2, using random effects logistic models with post-estimation commands. Adjusted for maternal age, offspring birth year, country, maternal body mass index, smoking status, and height. Fresh-ET, fresh embryo transfer; Frozen-ET, frozen embryo transfer; NC, natural conception.

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age and indicated very similar distributions. As a result, we decided to use the ultrasound measurements since these are used in clinical management. Another limitation is the lack of information on embryo culture medium, which has been shown to affect perinatal outcomes [38].

Even if culture medium differed between clinics and over time, this should not specifically differ between fresh-ET and frozen-ET, which makes it less likely that our results are confounded by culture medium.

We pooled data from Denmark, Sweden, and Norway, and assume that results are consistent across these 3 countries. We believe this is a reasonable assumption given that they are all high-income Nordic countries with accessible and affordable healthcare systems that provide similar fertility treatment and perinatal care [5]. However, this may limit the generalizability to other populations.

Comparison with other studies

We are not aware of previous studies of this size where perinatal outcomes of children born after fresh-ET or frozen-ET conception are compared to those of naturally conceived siblings as well as being explored using conventional population analyses. Previous studies that used within-sibship analysis to explore the associations of ART treatments with birth size and pregnancy duration had different designs and varied considerably in the covariates included [18–23]. While we defined birthweight for gestational age based on intrauterine growth curves [26], previous studies used observed birthweight and different criteria for large and small for gestational age [18–22]. As preterm birth often results from pregnancy complications that can affect fetal growth [39], the observed birthweights in preterm deliveries are not representative of the normal fetal weight distribution for healthy pregnancies at a given gestational age. Despite these differences, the results are broadly consistent (see Table M in S1 Text for a summary of study characteristics and estimates). In a Danish study of singletons born in 1994–2006, the results were largely consistent with ours, with lower mean birthweight and higher odds of low birthweight (<2,500 g) and preterm birth for children born after fresh-ET compared to their naturally conceived siblings (3,879 pairs), but no difference for very preterm birth [23]. Furthermore, infants born after frozen-ET had higher mean birthweights and lower odds of preterm birth than their siblings born after fresh-ET (358 pairs). A US study of ART-conceived singletons born in 2004–2013 compared fresh-ET to frozen-ET within sibships (3,681 discordant pairs) and found that siblings conceived with frozen-ET had greater odds of large for gestational age than those conceived with fresh-ET, but similar duration of pregnancy [22].

In 4 previous studies comparing ART-conceived infants to their naturally conceived siblings, conclusions were conflicting [18–20,23]. However, directions of associations were similar across these studies and magnitudes similar in several. Different conclusions may therefore reflect their different sample sizes and associated variation in power to detect statistical evidence. A Norwegian study of 2,204 sibling pairs born in 1988–2006 [18], a Dutch study of 1,813 sibling pairs born in 1999–2007 [20], and a Finnish study of 578 sibling groups born in 1995–2000 [19] showed no strong statistical support for associations, but associations in all 3 studies were in the direction of lower birthweight and gestational age in infants conceived after ART (either fresh-ET or frozen-ET) compared to their naturally conceived siblings. Although these studies did not provide separate estimates for fresh-ET and frozen-ET, their results would be expected to mainly reflect fresh-ET, which was by far the more common treatment during the study periods. A US study including 6,458 discordant sibling pairs born in 2000–2010 showed lower birthweight and gestational age after ART compared to natural conception, with stronger statistical support than the other studies, but, as with those studies, did not separate fresh-ET and frozen-ET [21].

We could not distinguish "freeze-all" cycles, a strategy to prevent ovarian hyperstimulation syndrome [40], from frozen-ET after an initial fresh transfer. However, in a recent study by Smith et al. [41], perinatal outcomes after a planned freeze-all cycle were similar to those after

frozen-ET in the conventional setting. This is in accordance with our study, where order of conception method was not associated with the perinatal outcomes.

In addition to the small number of previous within-sibship analyses described above, we also find some consistency with previous conventional observational studies, in which fresh-ET was associated with low birthweight and high risk of preterm and very preterm birth [8,9]. Frozen-ET, on the other hand, has been consistently associated with high birthweights, and some reports also indicate a lower risk of preterm birth compared to fresh-ET [8,9].

Implications of findings and conclusion

We provide important evidence on the likely impact of fresh-ET compared with natural conception and of frozen-ET compared with natural conception. Infants born large for gestational age have a higher risk of delivery complications, and being born small for gestational age, large for gestational age, and preterm are all associated with increased perinatal morbidity and mortality [42,43]. They are also associated with long-term adverse outcomes [43–45]. Small for gestational age and preterm birth are associated with increased risk of cardiovascular diseases, mental health disorders, and social difficulties [44,45], and large for gestational age is associated with a higher risk of obesity and obesity-related adverse outcomes [45]. To ensure informed decision-making for infertile couples, and couples who are considering postponing childbearing, knowledge about adverse perinatal outcomes and their potential long-term consequences should be balanced against couples' desire to have a family at a time that suits them. Future studies should address whether close antenatal monitoring beyond present guidelines may improve perinatal outcomes in ART-conceived pregnancies.

The increased risk of large for gestational age and higher mean birthweight seen after frozen-ET has potential implications for the recent increase in freeze-all approaches [46], in particular when evidence from a recent large cohort study and a randomized trial suggests no benefit from freezing all embryos compared with an initial fresh transfer with respect to the cumulative live birth rate [41,47]. It has been suggested that the freeze-all approach should be limited to couples with a clinical indication, such as where the risk of maternal ovarian hyperstimulation syndrome is high [46,47]. Our findings add to the debate about the role of freeze-all strategies, by providing indirect evidence that it may not reduce adverse perinatal outcomes compared to fresh-ET followed by frozen-ET.

In this study we found that frozen-ET was associated with increased birthweight and risk of large for gestational age, whereas fresh-ET was associated with the opposite. Furthermore, sibship comparisons indicated that both fresh-ET and frozen-ET were associated with increased risk of preterm birth but not with risk of very preterm birth, despite strong associations in conventional population analyses. These findings should contribute to the ongoing discussions on the role of emerging ART approaches, such as the freeze-all approach, and to informed decision-making by couples and their healthcare providers. They should prompt studies to identify possible mechanisms and preventive measures to improve perinatal health in ART-conceived children.

Supporting information

S1 STROBE Checklist. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline.
(DOCX)

S1 Study Protocol. The prospective analysis plan.
(PDF)

S1 Text. Tables of main (Tables A and B) and sensitivity analyses (Tables C–L), and summary of previous sibship studies (Table M).
(DOCX)

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Transparency

The first and last author (KWJ and SO) have had full access to the data and affirm that this paper is an honest, transparent, and accurate account of the study and that no important aspects of the study have been omitted

Dissemination to participants and related patient and public communities

It will not be possible to send the results to the study participants, but we plan to disseminate the findings to the public through media channels, our institutions' websites, and the CoNARTaS website (http://www.conartas.com).

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SUPPORTING INFORMATION

Table A. Risk of adverse neonatal outcomes by conception method: population estimates and within sibship estimates in Main Sample 1 (minimizing selection).

Main Sample1	Population estimates (Random effects)								Within sibship estimates (Fixed effects)				
	Numbers	Risk [1], %	RD [1], *pp*	Adj. RD (95% CI) [2]		OR [1]	Adj. OR (95% CI) [2]		Numbers [3]	Risk [1], %	OR [1]	Adj. OR (95% CI) [2]	
SGA													
Natural Conception	4 414 703	3.6	0	0	Ref.	1	1	Ref.	33 889	3.0	1	1	Ref.
Fresh-ET	78 095	5.3	1.6	0.76	(0.61 to 0.92)	1.60	1.24	(1.19 to 1.29)	30 167	4.5	1.64	1.20	(1.08 to 1.34)
Frozen-ET	17 990	2.9	-0.8	-0.66	(-0.91 to 0.41)	0.75	0.72	(0.65 to 0.80)	9 589	2.2	0.74	0.81	(0.66 to 1.00)
LGA													
Natural Conception	4 414 703	4.5	0	0	Ref.	1	1	Ref.	33 889	5.3	1	1	Ref.
Fresh-ET	78 095	3.6	-0,9	-0.14	(-0.30 to 0.05)	0.75	1.01	(0.96 to 1.06)	30 167	3.9	0.65	0.92	(0.84 to 1.02)
Frozen-ET	17 990	6.5	2.1	2.4	(2.0 to 2.8)	1.70	1.98	(1.82 to 2.15)	9 589	7.3	1.88	1.84	(1.56 to 2.17)
Preterm birth <37 weeks													
Natural Conception	4 414 703	4.8		0	Ref.	1	1	Ref.	33 889	5.3	1	1	Ref.
Fresh-ET	78 095	8.0	3.1	2.2	(2.0 to 2.4)	1.92	1.63	(1.58 to 1.69)	30 167	7.2	1.55	1.27	(1.17 to 1.37)
Frozen-ET	17 990	6.5	1.7	1.4	(1.1 to 1.8)	1.46	1.40	(1.30 to 1.50)	9 589	5.4	1.05	1.05	(0.91 to 1.20)
Preterm birth <32 weeks													
Natural Conception	4 414 703	0.6	0	0	Ref.	1	1	Ref.	33 889	0.9	1	1	Ref.
Fresh-ET	78 095	1.5	0.9	0.57	(0.50 to 0.64)	2.60	2.03	(1.90 to 2.12)	30 167	1.2	1.46	1.18	(1.0 to 1.41)
Frozen-ET	17 990	1.2	0.51	0.37	(0.23 to 0.50)	1.91	1.66	(1.42 to 1.94)	9 589	0.9	0.93	0.92	(0.67 to 1.27)

Abbreviations: Adj. – adjusted, CI – confidence interval, LGA – large for gestational age, OR – odds ratio, *pp* – percentage points, RD – risk difference, Ref. – reference, SGA – small for gestational age

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth. Random effects are additionally adjusted for country. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table B. Risk of adverse neonatal outcomes by conception method: population estimates and within sibship estimates in Main Sample 2 (minimizing confounding).

	Population estimates (Random effects)								Within sibship estimates (Fixed effects)				
	Numbers	Risk [1], %	RD [1], *pp*	Adj. RD (95% CI) [2]		OR [1]	Adj. OR (95% CI) [2]		Numbers [3]	Risk [1], %	OR [1]	Adj. OR (95% CI) [2]	
SGA													
Natural Conception	2 548 239	3.3	0	0	Ref.	1	1	Ref.	19 656	2.5	1	1	Ref.
Fresh-ET	53 059	4.9	1.8	0.73	(0.61 to 0.92)	1.73	1.29	(1.23 to 1.36)	17 631	4.3	2.07	1.27	(1.09 to 1.47)
Frozen-ET	14 326	2.8	-0.4	-0.67	(-0.91 to 0.41)	0.87	0.75	(0.67 to 0.85)	6538	2.1	0.93	0.85	(0.65 to 1.11)
LGA													
Natural Conception	2 548 239	4.4	0	0	Ref.	1	1	Ref.	19 656	5.4	1	1	Ref.
Fresh-ET	53 059	3.5	-1.0	-0.12	(-0.30 to 0.07)	0.72	0.96	(0.90 to 1.02)	17 631	3.7	0.59	0.92	(0.80 to 1.05)
Frozen-ET	14 326	6.3	1.9	2.4	(2.0 to 2.8)	1.65	1.90	(1.73 to 2.09)	6538	7.2	1.67	1.76	(1.43 to 2.17)
Preterm birth <37 weeks													
Natural Conception	2 548 239	4.5	0	0	Ref.	1	1	Ref.	19 656	4.7	1	1	Ref.
Fresh-ET	53 059	7.6	3.0	2.1	(1.9 to 2.3)	1.96	1.65	(1.58 to 1.72)	17 631	6.7	1.62	1.23	(1.11 to 1.36)
Frozen-ET	14 326	6.2	1.7	1.4	(1.0 to 1.8)	1.52	1.43	(1.31 to 1.56)	6538	5.2	1.21	1.20	(1.00 to 1.44)
Preterm birth <32 weeks													
Natural Conception	2 548 239	0.5	0	0	Ref.	1	1	Ref.	19 656	0.7	1	1	Ref.
Fresh-ET	53 059	1.3	0.76	0.50	(0.41 to 0.57)	2.67	2.05	(1.87 to 2.25)	17 631	1.0	1.36	1.05	(0.81 to 1.35)
Frozen-ET	14 326	1.0	0.45	0.32	(0.17 to 0.47)	1.96	1.66	(1.37 to 2.00)	6538	0.8	1.0	0.93	(0.62 to 1.41)

Abbreviations: Adj. – adjusted, CI – confidence interval, LGA – large for gestational age, OR – odds ratio, *pp* – percentage points, RD – risk difference, Ref. – reference, SGA – small for gestational age

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth, maternal pre-pregnancy or first trimester body mass index, maternal smoking during pregnancy. Random effects are additionally adjusted for country & maternal height. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table C. Birthweight and gestational age by conception method: population estimates and within sibship estimates. Full siblings in Main Sample 1 (minimizing selection).

	Population estimates (Random effects)					Within sibship estimates (Fixed effects)				
	Numbers	Mean [1]	Mean difference [1]	Adj. mean difference (95% CI) [2]		Numbers [3]	Mean [1]	Mean difference [1]	Adj. mean difference (95% CI) [2]	
Birthweight, grams										
Natural Conception	3 390 496	3567	0	0	Ref.	30 639	3576	0	0	Ref.
Fresh-ET	40 765	3450	-117	-69	(-74 to -64)	27 318	3434	-129	-50	(-57 to -43)
Frozen-ET	10 699	3634	67	60	(51 to 70)	9 005	3630	67	78	(66 to 90)
Birthweight, z-score										
Natural Conception	3 390 496	0.04	0	0	Ref.	30 639	0.07	0	0	Ref.
Fresh-ET	40 765	-0.15	-0.19	-0.07	(-0.08 to -0.06)	27 318	-0.24	-0.27	-0.07	(-0.08 to -0.05)
Frozen-ET	10 699	0.25	0.21	0.19	(0.17 to 0.21)	9 005	0.19	0.16	0.18	(0.16 to 0.21)
Gestational age, days										
Natural Conception	3 390 496	279.3	0	0	Ref.	30 639	279.2	0	0	Ref.
Fresh-ET	40 765	277.5	-1.8	-1.8	(-1.9 to -1.6)	27 318	278.3	-0.9	-0.8	(-1.0 to -0.6)
Frozen-ET	10 699	278.6	-0.6	-0.6	(-0.8 to -0.4)	9 005	279.6	0.3	0.4	(0.1 to 0.7)

Abbreviations: Adj. – adjusted, CI – confidence interval, – reference

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth. Random effects are additionally adjusted for country. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table D. Risk of adverse neonatal outcomes by conception method: population estimates and within sibship estimates. Full siblings in Main Sample 1 (minimizing selection).

	Population estimates (Random effects)								Within sibship estimates (Fixed effects)				
	Numbers	Risk [1], %	RD [1], *pp*	Adj. RD (95% CI) [2]		OR [1]	Adj. OR (95% CI) [2]		Numbers [3]	Risk [1], %	OR [1]	Adj. OR (95% CI) [2]	
SGA													
Natural Conception	3 390 496	3.1	0	0	Ref.	1	1	Ref.	30 639	2.7	1	1	Ref.
Fresh-ET	40 765	4.3	1.2	0.76	(0.55 to 1.0)	1.51	1.36	(1.26 to 1.47)	27 318	4.5	1.86	1.31	(1.1 to 1.5)
Frozen-ET	10 699	2.2	-0.8	-0.4	(-0.77 to -0.12)	0.70	0.80	(0.68 to 0.96)	9 005	2.3	0.86	0.91	(0.7 to 1.2)
LGA													
Natural Conception	3 390 496	4.8	0	0	Ref.	1	1	Ref.	30 639	5.5	1	1	Ref.
Fresh-ET	40 765	4.0	-1.0	-0.30	(-0.5 to 0.03)	0.74	0.91	(0.84 to 1.00)	27 318	3.8	0.58	0.91	(0.79 to 1.05)
Frozen-ET	10 699	7.2	2.5	2.30	(1.8 to 2.7)	1.79	1.81	(1.61 to 2.04)	9 005	7.2	1.70	1.77	(1.42 to 2.21)
Preterm birth <37 weeks													
Natural Conception	3 390 496	4.4	0	0	Ref.	1	1	Ref.	30 639	5.0	1	1	Ref.
Fresh-ET	40 765	6.7	2.3	1.7	(1.5 to 2.0)	1.72	1.58	(1.49 to 1.69)	27 318	7.0	1.58	1.20	(1.07 to 1.34)
Frozen-ET	10 699	5.3	0.9	1.2	(0.7 to 1.6)	1.26	1.38	(1.22 to 1.56)	9 005	5.3	1.09	1.23	(1.02 to 1.49)
Preterm birth <32 weeks													
Natural Conception	3 390 496	0.5	0	0	Ref.	1	1	Ref.	30 639	0.8	1	1	Ref.
Fresh-ET	40 765	1.1	0.58	0.4	(0.29 to 0.50)	2.32	2.07	(1.80 to 2.40)	27 318	1.1	1.50	1.12	(0.85 to 1.49)
Frozen-ET	10 699	0.8	0.26	0.26	(0.08 to 0.43)	1.58	1.67	(1.26 to 2.22)	9 005	0.8	0.94	0.98	(0.62 to 1.54)

Abbreviations: Adj. – adjusted, CI – confidence interval, LGA – large for gestational age, OR – odds ratio, *pp* – percentage points, RD – risk difference, Ref. – reference, SGA – small for gestational age

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth. Random effects are additionally adjusted for country. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table E. Birthweight and gestational age by conception method: population estimates and within sibship estimates. Restricted to participants with < 3-year interval between siblings in Main Sample 1 (minimizing selection).

	Population estimates (Random effects)					Within sibship estimates (Fixed effects)				
	Numbers [1]	Mean [2]	Mean difference [2]	Adj. mean difference (95% CI) [3]		Numbers [4]	Mean [2]	Mean difference [2]	Adj. mean difference (95% CI) [3]	
Birthweight, grams										
Natural Conception	1 982 791	3557	0	0	Ref.	16 674	3558	0	0	Ref.
Fresh-ET	23 304	3426	-131	-63	(-70 to 56)	16 602	3378	-180	-38	(-47 to -29)
Frozen-ET	6 698	3621	64	67	(55 to 79)	5 580	3580	22	91	(76 to 107)
Birthweight, z-score										
Natural Conception	1 982 791	0.024	0	0	Ref.	16 674	0.02	0	0	Ref.
Fresh-ET	23 304	-0.23	-0.25	-0.06	(-0.8 to -0.05)	16 602	-0.42	-0.44	-0.07	(-0.09 to -0.05)
Frozen-ET	6 698	0.2	0.18	0.20	(0.18 to 0.22)	5 580	.03	0.01	0.19	(0.16 to 0.22)
Gestational age, days										
Natural Conception	1 982 791	279.2	0	0	Ref.	16 674	279.2	0	0	Ref.
Fresh-ET	23 304	277.6	-1.54	-1.71	(-1.87 to -1.55)	16 602	278.9	-0.28	-0.43	(-0.68 to -0.19)
Frozen-ET	6 698	278.8	-0.41	-0.49	(-0.78 to -0.20)	5 580	280.0	0.84	0.72	(0.31 to 1.13)

Abbreviations: Adj. – adjusted, CI – confidence interval, Ref. – reference,

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth. Random effects are additionally adjusted for country. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table F. Risk of adverse neonatal outcomes by conception method: population estimates and within sibship estimates. Restricted to participants with < 3-year interval between siblings from Main Sample 1 (minimizing selection).

	Population estimates (Random effects)								Within sibship estimates (Fixed effects)				
	Numbers [1]	Risk [2], %	RD [2], *pp*	Adj. RD (95% CI) [3]		OR [2]	Adj. OR (95% CI) [3]		Numbers [4]	Risk [2], %	OR [2]	Adj. OR (95% CI) [3]	
SGA													
Natural Conception	1 982 791	3.1	0	0	Ref.	1	1	Ref.	16 674	2.3	1	1	Ref.
Fresh-ET	23 304	4.4	1.4	0.65	(0.42 to 0.90)	1.58	1.28	(1.18 to 1.39)	16 602	4.7	2.90	1.20	(1.01 to 1.41)
Frozen-ET	6 698	2.1	-0.9	-0.65	(-1.04 to -0.25)	0.66	0.75	(0.61 to 0.91)	5 580	2.2	1.30	0.89	(0.66 to 1.20)
LGA													
Natural Conception	1 982 791	4.5	0	0	Ref.	1	1	Ref.	16 674	5.8	1	1	Ref.
Fresh-ET	23 304	3.7	-1.1	0.01	(-0.5 to 0.02)	0.70	1.0	(0.91 to 1.1)	16 602	3.4	0.40	0.98	(0.85 to 1.15)
Frozen-ET	6 698	6.6	2.1	2.2	(1.58 to 2.76)	1.72	1.78	(1.56 to 2.04)	5 580	6.7	1.12	1.83	(1.45 to 2.31)
Preterm birth <37 weeks													
Natural Conception	1 982 791	4.4	0	0	Ref.	1	1	Ref.	16 674	4.6	1	1	Ref.
Fresh-ET	23 304	6.8	2.40	1.85	(2.10 to 2.72)	1.75	1.58	(1.48 to 1.69)	16 602	6.9	1.77	1.13	(1.00 to 1.27)
Frozen-ET	6 698	5.0	0.60	0.89	(0.33 to 1.44)	1.18	1.27	(1.11 to 1.45)	5 580	5.0	1.18	0.97	(0.79 to 1.18)
Preterm birth <32 weeks													
Natural Conception	1 982 791	0.5	0	0	Ref.	1	1	Ref.	16 674	0.9	1	1	Ref.
Fresh-ET	23 304	1.2	0.70	0.53	(0.40 to 0.66)	2.42	2.15	(1.87 to 2.47)	16 602	1.2	1.47	1.04	(0.80 to 1.35)
Frozen-ET	6 698	0.8	0.19	0.21	(-0.004 to 0.41)	1.40	1.42	(1.04 to 1.94)	5 580	0.8	0.88	0.78	(0.48 to 1.27)

Abbreviations: Adj. – adjusted, CI – confidence interval, LGA – large for gestational age, OR – odds ratio, *pp* – percentage points, RD – risk difference, Ref. – reference, SGA – small for gestational age

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth. Random effects are additionally adjusted for country. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table G. Birthweight and gestational age by conception method: population estimates and within sibship estimates. Restricted to mothers with 2-4 children in Main Sample 1 (minimizing selection).

	Population estimates (Random effects)					Within sibship estimates (Fixed effects)				
	Numbers [1]	Mean [2]	Mean difference [2]	Adj. mean difference (95% CI) [3]		Numbers [4]	Mean [2]	Mean difference [2]	Adj. mean difference (95% CI) [3]	
Birthweight, grams										
Natural Conception	3 694 258	3563	0	0	Ref.	33 889	3540	0	0	Ref.
Fresh-ET	44 019	3492	-111	-71	(-76 to -66)	30 167	3424	-116.3	-51	(-58 to -45)
Frozen-ET	11 321	3628	75	65	(55 to 74)	9 589	3623	83	82	(70 to 94)
Birthweight, z-score										
Natural Conception	3 694 258	0.03	0	0	Ref.	33 889	-0.01	0	0	Ref.
Fresh-ET	44 019	-0.14	-0.17	-0.07	(-0.08 to -0.06)	30 167	-0.23	-0.22	-0.06	(-0.78 to -0.05)
Frozen-ET	11 321	0.26	0.23	0.20	(0.18 to 0.22)	9 589	0.2	0.21	0.19	(0.17 to 0.22)
Gestational age, days										
Natural Conception	3 694 258	279.2	0	0	Ref.	33 889	279.0	0	0	Ref.
Fresh-ET	44 019	277.3	-1.90	-1.86	(-1.98 to -1.74)	30 167	277.9	-1.1	-1.0	(-1.2 to -0.84)
Frozen-ET	11 321	278.6	-0.62	-0.49	(-0.72 to -0.27)	9 589	279.2	0.2	0.3	(0.0 to 0.6)

Abbreviations: Adj. – adjusted, CI – confidence interval, Ref. – reference

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth. Random effects are additionally adjusted for country. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table H. Risk of adverse neonatal outcomes by conception method: population estimates and within sibship estimates. Restricted to mothers with 2-4 children in Main Sample 1 (minimizing selection).

	Population estimates (Random effects)								Within sibship estimates (Fixed effects)				
	Numbers	Risk [1], %	RD [2], *pp*	Adj. RD (95% CI) [2]		OR [1]	Adj. OR (95% CI) [2]		Numbers [4]	Risk [2], %	OR [2]	Adj. OR (95% CI) [3]	
SGA													
Natural conception	3 694 258	3.2	0	0	Ref.	1	1	Ref.	33 889	3.0	1	1	Ref.
Fresh ET	44 019	4.3	1.1	0.70	(0.072 to 1.2)	1.43	1.29	(1.21 to 1.36)	30 167	4.5	1.64	1.20	(1.08 to 1.34)
Frozen ET	11 321	2.2	-1.0	-0.70	(-0.95 to -0.33)	0.64	0.76	(0.65 to 0.88)	9 589	2.2	0.74	0.81	(0.66 to 1.00)
LGA													
Natural conception	3 694 258	4.8	0	0	Ref.	1	1	Ref.	33 889	5.3	1	1	Ref.
Fresh ET	44 019	4.0	-0.9	0.30	(-0.50 to -0.03)	0.74	0.91	(0.84 to 0.99)	30 167	3.9	0.65	0.92	(0.84 to 1.02)
Frozen ET	11 321	7.2	2.5	2.34	(1.80 to 2.90)	1.80	1.81	(1.61 to 2.04)	9 589	7.3	1.88	1.84	(1.56 to 2.17)
Preterm birth <37 weeks													
Natural conception	3 694 258	4.5	0	0	Ref.	1	1	Ref.	33 889	5.3	1	1	Ref.
Fresh ET	44 019	6.9	2.4	2.0	(1.75 to 2.21)	1.72	1.60	(1.53 to 1.68)	30 167	7.2	1.55	1.27	(1.17 to 1.37)
Frozen ET	11 321	5.3	0.8	1.0	(0.60 to 1.56)	1.22	1.30	(1.18 to 1.44)	9 589	5.4	1.05	1.05	0.91 to 1.20)
Preterm birth <32 weeks													
Natural conception	3 694 258	0.56	0	0	Ref.	1	1	Ref.	33 889	0.9	1	1	Ref.
Fresh ET	44 019	1.21	0.63	0.52	(0.43 to 0.63)	2.31	2.10	(1.90 to 2.32)	30 167	1.2	1.46	1.18	(1.0 to 1.41)
Frozen ET	11 321	0.86	0.28	0.29	(0.12 to 0.46)	1.56	1.58	(1.27 to 1.98)	9 589	0.9	0.93	0.92	(0.67 to 1.27)

Abbreviations: Adj. – adjusted, CI – confidence interval, LGA – large for gestational age, OR – odds ratio, *pp* – percentage points, RD – risk difference, Ref. – reference, SGA – small for gestational age

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth. Random effects are additionally adjusted for country. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table I. Birthweight and gestational age by conception method: population estimates and within sibship estimates. Children conceived by assisted reproduction are restricted to single embryo transfers in Main Sample 2 (minimizing confounding).

	Population estimates (Random effects)					Within sibship estimates (Fixed effects)				
	Numbers	Mean [1]	Mean difference [1]	Adj. mean difference (95% CI) [2]		Numbers [3]	Mean [1]	Mean difference [1]	Adj. mean difference (95% CI) [2]	
Birthweight, grams										
Natural Conception	2 548 239	3538	0	0	Ref.	19 656	3547	0	0	Ref.
Fresh-ET	29 606	3403	-135	-83	(-89 to -77)	10 684	3410	-137	-61	(-72 to -49)
Frozen-ET	9 850	3587	48	59	(49 to 69)	4 461	3622	76	74	(56 to 92)
Birthweight, z-score										
Natural Conception	2 548 239	-0.01	0	0	Ref.	19 656	.007	0	0	Ref.
Fresh-ET	29 606	-0.23	-0.22	-0.07	(-0.09 to -0.06)	10 684	-0.27	-0.27	-0.08	(-0.10 to 0.06)
Frozen-ET	9 850	0.15	0.16	0.20	(0.18 to 0.22)	4 461	-0.20	0.19	0.19	(0.15 to 0.22)
Gestational age, days										
Natural Conception	2 548 239	279.0	0	0	Ref.	19 656	279.0	0	0	Ref.
Fresh-ET	29 606	277.0	-2.0	-2.1	(-2.3 to 2.0)	10 684	277.9	-1.1	-1.1	(-1.4 to -0.9)
Frozen-ET	9 850	278.4	-0.6	-0.7	(-1.0 to -0.5)	4 461	279.0	0.05	0.06	(-0.4 to 0.5)

Abbreviations: Adj. – adjusted, CI – confidence interval, LGA – large for gestational age, OR – odds ratio, *pp* – percentage points, RD – risk difference, Ref. – reference, SGA – small for gestational age.

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth, maternal pre-pregnancy or first trimester body mass index, maternal smoking during pregnancy. Random effects are additionally adjusted for country & maternal height. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table J. Risk of adverse neonatal outcomes by conception method: population estimates and within sibship estimates. Children conceived by assisted reproduction are restricted to single embryo transfers in Main Sample 2 (minimizing confounding)

	Population estimates (Random effects)								Within sibship estimates (Fixed effects)				
	Numbers	Risk [1], %	RD [1], *pp*	Adj. RD (95% CI) [2]		OR [1]	Adj. OR (95% CI) [2]		Numbers [3]	Risk [1], %	OR [1]	Adj. OR (95% CI) [2]	
SGA													
Natural Conception	2 548 239	3.3	0	0	Ref.	1	1	Ref.	19 656	2.5	1	1	Ref.
Fresh-ET	29 606	4.9	1.67	0.70	(0.50 to 0.90)	1.67	1.28	(1.20 to 1.37)	10 684	4.2	2.24	1.44	(1.18 to 1.76)
Frozen-ET	9 850	2.8	-0.50	-0.73	(-1.0 to -0.43)	0.82	0.73	(0.63 to 0.84)	4 461	2.1	0.90	0.90	(0.64 to 1.25)
LGA													
Natural Conception	2 548 239	4.4	0	0	Ref.	1	1	Ref.	19 656	5.4	1	1	Ref.
Fresh-ET	29 606	3.3	-1.0	-0.01	(-0.27 to 0.24)	0.71	1.0	(0.92 to 1.08)	10 684	3.8	0.63	0.97	(0.81 to 1.15)
Frozen-ET	9 850	6.4	2.1	2.6	(2.05 to 3.05)	1.72	1.96	(1.75 to 2.20)	4 461	7.5	1.93	2.05	(1.57 to 2.66)
Preterm birth <37 weeks													
Natural Conception	2 548 239	4.5	0	0	Ref.	1	1	Ref.	19 656	4.6	1	1	Ref.
Fresh-ET	29 606	7.4	2.90	2.08	(1.80 to 2.35)	1.91	1.64	(1.55 to 1.74)	10 684	6.6	1.75	1.35	(1.17 to 1.57)
Frozen-ET	9 850	6.1	1.57	1.37	(0.91 to 1.83)	1.46	1.41	(1.27 to 1.56)	4 461	4.9	1.18	1.22	(0.97 to 1.54)
Preterm birth <32 weeks													
Natural Conception	2 548 239	0.54	0	0	Ref.	1	1	Ref.	19 656	0.7	1	1	Ref.
Fresh-ET	29 606	1.2	0.70	0.46	(0.35 to 0.56)	2.52	2.00	(1.76 to 2.25)	10 684	0.9	1.20	0.94	(0.67 to 1.32)
Frozen-ET	9 850	0.96	0.43	0.30	(0.13 to 0.47)	1.91	1.63	(1.29 to 2.05)	4 461	0.7	0.90	0.89	(0.52 to 1.51)

Abbreviations: Adj. – adjusted, CI – confidence interval, LGA – large for gestational age, OR – odds ratio, *pp* – percentage points, RD – risk difference, Ref. – reference, SGA – small for gestational age

[1] Unadjusted. [2] Adjusted for maternal age, parity, year of birth, maternal pre-pregnancy or first trimester body mass index, maternal smoking during pregnancy. Random effects are additionally adjusted for country & maternal height. [3] Numbers refer to children that are part of a sibling groups with at least two different conceptions methods within the group.

Table K. Birthweight and gestational age by conception method: population estimates and within sibship estimates. Children conceived by assisted reproduction are restricted to blastocyst transfers in Main Sample 2 (minimizing confounding).

	Population estimates (Random effects)					Within sibship estimates (Fixed effects)				
	Numbers [1]	Mean [2]	Mean difference [2]	Adj. mean difference (95% CI) [3]		Numbers [4]	Mean [2]	Mean difference [2]	Adj. mean difference (95% CI) [3]	
Birthweight, grams										
Natural Conception	1 826 087	3544	0	0	Ref.	1696	3544	0	0	Ref.
Fresh-ET	4032	3430	-110	-77	(-93 to -61)	1190	3460	-85	-53	(-85 to -22)
Frozen-ET	3449	3593	57	81	(63 to 98)	880	3653	109	93	(56 to 130)
Birthweight, z-score										
Natural Conception	1 826 087	0.004	0	0	Ref.	1696	.004	0	0	Ref.
Fresh-ET	4032	-0.12	-0.13	-0.03	(-0.06 to -0.01)	1190	-.12	-0.12	-0.03	(-0.10 to 0.03)
Frozen-ET	3449	0.18	0.20	0.26	(0.24 to 0.31)	880	.34	0.34	0.30	(0.23 to 0.38)
Gestational age, days										
Natural Conception	1 826 087	278.9	0	0	Ref.	1696	278.9	0	0	Ref.
Fresh-ET	4032	276.4	-2.40	-2.68	(-3.05 to -2.31)	1190	277.4	-1.49	-1.58	(-2.36 to -0.80)
Frozen-ET	3449	278.2	-0.68	-0.95	(-1.35 to -0.55)	880	278.1	-0.81	-0.87	(-1.78 to 0.5)

Abbreviations: Adj. – adjusted, CI – confidence interval, Ref. – reference

[1] Population restricted to singletons born from 1997 in Denmark, 2002 in Sweden and 2010 in Norway, when blastocyst transfer was implemented in fertility clinics in the respective countries. [2] Unadjusted. [3] Adjusted for maternal age, parity, year of birth, maternal pre-pregnancy or first trimester body mass index, maternal smoking during pregnancy. Random effects are additionally adjusted for country & maternal height. [4] Numbers refer to children that are part of a full sibling group with at least two different conceptions methods within the group.

Table L. Risk of adverse neonatal outcomes by conception method: population estimates and within sibship estimates. Children conceived by assisted reproduction are restricted to blastocyst transfers in Main Sample 2 (minimizing confounding).

	Population estimates (Random effects)								Within sibship estimates (Fixed effects)				
	Numbers [1]	Risk [2], %	RD [2], *pp*	Adj. RD (95% CI) [3]		OR [2]	Adj. OR (95% CI) [3]		Numbers [4]	Risk [2], %	OR [2]	Adj. OR (95% CI) [3]	
SGA													
Natural Conception	1 826 087	3.2	0	0	Ref.	1	1	Ref.	1696	2.5	1	1	Ref.
Fresh-ET	4032	4.5	1.29	0.62	(0.072 to 1.2)	1.52	1.26	(1.04 to 1.52)	1190	4.3	1.89	1.86	(1.07 to 3.22)
Frozen-ET	3449	2.6	-0.72	-1.07	(-1.5 to -0.62)	0.74	0.61	(0.47 to 0.78)	880	2.1	0.61	0.75	(0.36 to 1.56)
LGA													
Natural Conception	1 826 087	4.4	0	0	Ref.	1	1	Ref.	1 696	4.7	1	1	Ref.
Fresh-ET	4032	3.9	-0.47	0.49	(-0.22 to 1.12)	0.86	1.16	(0.94 to 1.43)	1190	4.5	1.03	1.43	(0.90 to 2.30)
Frozen-ET	3449	6.5	2.24	3.37	(2.44 to 4.29)	1.77	2.34	(1.94 to 2.83)	880	7.5	2.08	2.15	(1.29 to 3.57)
Preterm birth <37 weeks													
Natural Conception	1 826 087	4.5	0	0	Ref.	1	1	Ref.	1696	4.4	1	1	Ref.
Fresh-ET	4032	7.9	3.37	2.92	(2.14 to 3.71)	2.10	1.96	(1.69 to 2.28)	1190	6.6	1.66	1.65	(1.01 to 2.47)
Frozen-ET	3449	6.8	2.31	2.04	(1.23 to 2.86)	1.72	1.64	(1.39 to 1.95)	880	7.0	2.05	2.43	(1.48 to 3.98)
Preterm birth <32 weeks													
Natural Conception	1 826 087	0.6	0	0	Ref.	1	1	Ref.	1696	0.7	1	1	Ref.
Fresh-ET	4032	1.4	0.83	0.68	(0.36 to 1.00)	2.85	2.51	(1.84 to 3.42)	1190	1.1	1.70	1.58	(0.62 to 4.02)
Frozen-ET	3449	0.9	0.32	0.22	(-0.06 to 0.50)	1.67	1.47	(0.97 to 2.21)	880	0.7	1.17	1.29	(0.38 to 4.43)

Abbreviations: Adj. – adjusted, CI – confidence interval, LGA – large for gestational age, OR – odds ratio, *pp* – percentage points, RD – risk difference, Ref. – reference, SGA – small for gestational age

[1] Population restricted to singletons born from 1997 in Denmark, 2002 in Sweden and 2010 in Norway, when blastocyst transfer was implemented in fertility clinics in the respective countries. [2] Unadjusted. [3] Adjusted for maternal age, parity, year of birth, maternal pre-pregnancy or first trimester body mass index, maternal smoking during pregnancy. Random effects are additionally adjusted for country & maternal height. [4] Numbers refer to children that are part of a sibling group with at least two different conceptions methods within the group.

Table M. Overview and summary characteristics of previous sibling studies on perinatal health after assisted reproductive technology

Study	Country	Study period	Comparison	Pairs (n)	Ref. level[1]	Unadj. estimate (95% CI)	Adj. estimate (95% CI)	Covariates
					Difference in mean birthweight, grams			
Romundstad et al 2008 [2]	Norway	1988-2006	ART vs NC	2204	3538	-87 (-125 to -49)	-9 (-36 to 18)	Gestational age, sex, maternal age, parity, birth year, pregnancy interval
Seggers et al 2016 [3]	Nether-lands	1999-2007	ART vs NC	1813	3467	-105.0 (-146.0 to -62.8)	-25.3 (-29.4 to 77.8)[8]	Sex, maternal age, parity, ethnicity, socioeconomic status, maternal diabetes, birth year, labour care
Dhalwani et al 2016 [4]	USA	2000-2010	ART vs NC	6458	3398	-55.3 (-72.9 to -41.7)	-33.4 (-48.6 to -18.2)	Gestational age, sex, maternal age, parity, birth year, time since last delivery
Goisis et al 2019 [5]	Finland	1995-2000	ART vs NC	578	3594	-137 (-189 to -85)	-31 (-85 to 22)	Sex, maternal age, parity, smoking, household income, multiple birth (incl. interaction with ART)
Henningsen et al 2011[6]	Denmark	1994-2006	Fresh-ET vs NC Frozen-ET vs Fresh-ET	3879 358	3556 3443	-114 (-134 to -93) 202 (132 to 271)	-65 (-89 to -41) 167 (90 to 244)	Sex, maternal age, parity, and birth year
Luke et al 2017 [7]	USA	2004-2013	Frozen-ET 2nd vs Fresh-ET 1st Frozen-ET 1st vs Fresh-ET 2nd	3371 310	3246 3295	222 (200 to 244) 81 (8 to 154)	- -	Unadjusted, but restricted to siblings with same sex
					Difference in mean gestational age, days			
Romundstad et al 2008 [2]	Norway	1988-2006	ART vs NC	2204	278.7	-2.0 (-2.9 to -1.0)	-1.3 (-2.4 to -0.3)	Sex, maternal age, parity, birth year, pregnancy interval
Seggers et al 2016 [3]	Nether-lands	1999-2007	ART vs NC	1813	276	-0.85 (-1.9 to 0.2)	-0.12 (-0.08 to 0.32)[8]	Sex, maternal age, parity, ethnicity, socioeconomic status, maternal diabetes, birth year, labour care
Dhalwani et al 2016 [4]	USA	2000-2010	ART vs NC	6458	270.3	-0.58 (-0.99 to -0.17)	-0.58 (-1.02 to -0.14)	Sex, maternal age, parity, birth year, time since last delivery
Goisis et al 2019 [5]	Finland	1995-2000	ART vs NC	578	278	-2.5 (-3.7 to -1.2)	-1.3 (-2.6 to 0.0)	Sex, maternal age, parity, smoking, household income, multiple birth (incl. interaction with ART)
Henningsen et al 2011[6]	Denmark	1994-2006	Fresh-ET vs NC Frozen-ET vs Fresh-ET	3879 358	277.2 277.6	-0.6 (-1.1 to -0.1) 0.2 (-1.5 to 1.9)	-1.4 (-2.0 to -0.7) 1.5 (-0.3 to 3.3)	Sex, maternal age, parity, and birth year
Luke et al 2017 [7]	USA	2004-2013	Frozen-ET 2nd vs Fresh-ET 1st Frozen-ET 1st vs Fresh-ET 2nd	3371 310	267.7 266.2	-0.3 (-0.9 to 0.3) 2.9 (0.7 to 5.1)	- -	Unadjusted, but restricted to siblings with same sex

Abbreviations: Adj. – adjusted, ART – assisted reproductive technology, CI – confidence interval, ET – embryo transfer, Ref. – reference, Unadj. - unadjusted

[1] Crude mean value in the reference category of the sibling group, i.e. the naturally conceived sibling in ART vs natural conception and the Fresh-ET sibling in Frozen-ET vs Fresh-ET.

[2] Romundstad LB, Romundstad PR, Sunde A, von Düring V, Skjaerven R, Gunnell D, et al. Effects of technology or maternal factors on perinatal outcome after assisted fertilisation: a population-based cohort study. Lancet. 2008;372(9640):737-43. [3] Seggers J, Pontesilli M, Ravelli ACJ, Painter RC, Hadders-Algra M, Heineman MJ, et al. Effects of in vitro fertilization and maternal characteristics on perinatal outcomes: a population-based study using siblings. Fertil Steril. 2016;105(3):590-8. [4] Dhalwani NN, Boulet SL, Kissin DM, Zhang Y, McKane P, Bailey MA, et al. Assisted reproductive technology and perinatal outcomes: conventional versus discordant-sibling design. Fertil Steril. 2016;106(3):710-6. [5] Goisis A, Remes H, Martikainen P, Klemetti R, Myrskylä M. Medically assisted reproduction and birth outcomes: a within-family analysis using Finnish population registers. Lancet. 2019;393(10177):1225-32. [6] Henningsen AK, Pinborg A, Lidegaard Ø, Vestergaard C, Forman JL, Andersen AN. Perinatal outcome of singleton siblings born after assisted reproductive technology and spontaneous conception: Danish national sibling-cohort study. Fertil Steril. 2011;95(3):959-63. [7] Luke B, Brown MB, Wantman E, Stern JE, Toner JP, Coddington CC, 3rd. Increased risk of large-for-gestational age birthweight in singleton siblings conceived with in vitro fertilization in frozen versus fresh cycles. J Assist Reprod Genet. 2017;34(2):191-200. [8] Note: Asymmetry between point estimate and CI indicates probable error in point estimate and/or CI.

Paper II

This paper is awaiting publication and is therefore not included.

Paper III

This paper is awaiting submission and is therefore not included.