Health of children born to subfertile couples
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Chapter 1

Introduction
The introduction of the oral contraceptive pill in the early 1960s enabled women to become emancipated and engage with their careers by reliably managing voluntary infertility. More and more women in Western society postponed child-bearing and, as a consequence, the mean age at which Dutch women give birth to their first child has increased from 25.6 years in 1980 to 29.4 years in 2013 (Statistics Netherlands 2014). It is generally known that the trend of delaying motherhood has a price. Increased female age is associated with fertility problems and unwanted childlessness. Between 10% and 20% of European couples are subfertile: they fail to achieve a successful pregnancy within 12 months of unprotected intercourse (Juul et al. 1999). Simultaneously, treatment possibilities for women with involuntary infertility increased rapidly in the latter half of the twentieth century. In 1978, Louise Brown, world’s first ‘test-tube baby’ conceived through in vitro fertilization (IVF) was born (Steptoe and Edwards 1978). Since this Nobel prize-winning breakthrough by Steptoe and Edwards, approximately 5 million children have been born following assisted reproductive techniques (ART) (Ferraretti et al. 2013). Since 1992, ART also includes intracytoplasmic sperm injection (ICSI) (Palermo et al. 1992).

Concerns about the consequences of IVF and ICSI for the resulting offspring were expressed shortly after the introduction of these techniques. As the use of ART is still steadily increasing, it is of paramount importance to carefully assess the health of ART offspring. This thesis focuses on the health of children born following controlled ovarian hyperstimulation IVF/ICSI (COH-IVF), modified natural cycle IVF/ICSI (MNC-IVF), preimplantation genetic screening (PGS), and children born to subfertile parents who eventually conceived naturally. In this introduction, first the techniques will be briefly addressed. Second, a short overview of the literature on the health outcomes of ART singletons will be given; short-term outcomes and long-term outcomes will be discussed separately. Third, the health of children born after PGS will be discussed. Finally, the aim and outline of this thesis will be presented.

**Assisted reproductive techniques (ART)**

There are several definitions for ART. In this thesis we use the following definition: fertility treatments in which both oocytes and sperm are handled outside the body. This means that we have not considered fertility treatments like ovulation induction and intra-uterine insemination (IUI) as falling under ART.
Controlled ovarian hyperstimulation IVF/ICSI (COH-IVF)

The conventional IVF procedure starts with controlled ovarian hyperstimulation (COH) by injecting a high dose of follicle stimulating hormone (FSH). This induces the growth of multiple follicles. To prevent premature ovulation, either gonadotropin-releasing hormone (GnRH)-agonists or antagonists are given. This suppresses the endogenous gonadotropin production of the pituitary gland. Regular ultrasound measurements are performed to monitor the growth of the follicles closely. When the follicles reach a diameter of 18-20 mm, human chorionic gonadotropin (hCG) or luteinising hormone (LH) is injected and 34-36 hours later transvaginal ultrasound-guided follicle aspiration is performed, just before the expected ovulation. The obtained oocytes are preserved in a special medium while 'semen work-up' takes place. The fertilization of the oocytes can either occur by adding a selected semen sample (i.e. conventional IVF), or by applying ICSI, a procedure that involves the selection of a single sperm cell and manually injecting it into the ovum with a fine needle while viewing it under a microscope. ICSI is mainly used to treat severe male subfertility. If actual fertilization occurs, the pronuclei fuse 20 hours after insemination and the first mitotic division is seen. The resulting embryo is cultured for 2 to 3 days to form a cleavage stage embryo (6-8 cells) or sometimes longer to form a blastocyst (70-100 cells). On the basis of morphology, one or two embryos are selected for transfer to the uterus. The number of embryos transferred is positively correlated with pregnancy rates, but also with the risk of a multiple pregnancy. Surplus embryos may be cryopreserved for future transfer (Zeilmaker et al. 1984). Ovarian hyperstimulation syndrome is the most serious complication of COH-IVF and manifests as severe disease in 0.1% to 2% of IVF cycles (Delvigne and Rozenberg 2002).

Modified natural cycle IVF/ICSI (MNC-IVF)

In contrast to COH-IVF, ovarian hyperstimulation is not performed in modified natural cycle (MNC)-IVF and the one follicle that naturally develops to dominance is used. In MNC-IVF, follicle growth is also closely monitored, and GnRH-antagonists and low dose FSH are started but not before the late follicular phase (when the lead follicle reaches a diameter of approximately 14 mm) (Pelinck et al. 2005). The amount of medication used is low compared to COH-IVF and the natural selection of the dominant follicle is not bypassed. MNC-IVF has a lower success rate per cycle, but the risk of ovarian hyperstimulation syndrome and a multiple pregnancy is negligible (Pelinck et al. 2007, Pelinck et al. 2008).
Preimplantation genetic screening (PGS)

Pregnancy rates after ART are lower than desired: the cumulative success rate after three COH-IVF cycles is around 50%. Pregnancy rates in ART also decrease with maternal age. In order to enhance the efficiency of ART, new techniques such as pre-implantation genetic screening (PGS) have been developed. With PGS, the embryo is screened for aneuploidies and embryos with a normal chromosomal constitution are selected for transfer to the uterus (Wilton 2002). The rationale for PGS comes from the following three facts:

1. Aneuploidies are common in clinical miscarriages of women of advanced age.
2. Aneuploidies are also found in preimplantation embryos of these women after IVF.
3. Aneuploidies are mostly incompatible with life.

In PGS, the zona pellucida of the embryo is opened with a laser or by using chemicals, followed by the aspiration of one or two blastomeres. Subsequently, all chromosomes or a selected set of chromosomes in the blastomeres are analysed, for instance with fluorescence in situ hybridisation (FISH). In theory, PSG should result in higher pregnancy rates. However, randomized controlled trials have indicated reduced pregnancy rates after PGS with FISH in a selected set of chromosomes in women of advanced maternal age (Hardarson et al. 2008, Mastenbroek et al. 2007). As a consequence, PGS is no longer recommended until the running of randomized controlled trials (RCTs) studying newer techniques for PGS have been completed. Regardless of the outcomes of these RCTs, the effects of embryo biopsy on the resulting offspring are important, since embryo biopsy is increasingly being used for preimplantation genetic diagnosis (PGD). embryo selection in PGD is not based on chromosomal content but on the exclusion of specific, heritable diseases. Couples who are significantly at risk of having a child with an inherited genetic disorder can opt for PGD.

Health outcome of ART offspring

Is there a price to pay for the miracle of assisted conception? And if so, are the adverse effects on child health attributable to the ART procedures or to the parental characteristics involved in the subfertility? ART-related procedures that may be involved in reduced child health are ovarian hyperstimulation and the in vitro procedure. Ovarian hyperstimulation results in the growth of multiple follicles and therefore may bypass the natural selection of one oocyte. Furthermore, ovarian hyperstimulation influences endometrial receptivity with potential consequences for implantation and placentation (Jovanovic and Kramer 2010, Rackow et al. 2008). The in vitro procedure involves the aspiration of the oocytes, in some cases the selection of a sin-
gle spermatozoon for ICSI, the early maturation of the embryo in a culture medium, and the selection of the morphologically ‘best looking’ embryo by an embryologist. In theory, all these ART-related procedures could interfere with developmental processes of the embryo, but factors associated with the underlying parental subfertility may also be involved. The potential consequences of PGS – a procedure involving embryo biopsy – are also still not well known.

**Short-term outcomes**

**Perinatal outcomes**

The first studies addressing the health of children born following ART focussed on perinatal outcomes and reported alarming results. Part of the adverse outcomes could be explained by the initially high rates of multiple pregnancies, but singleton pregnancies too were associated with perinatal adversities such as prematurity and lower birthweights (Helmerhorst et al. 2004, Jackson et al. 2004). More recent studies tried to identify the relative contributions of the ART procedure and the underlying subfertility to perinatal adversities. There is an increasing body of evidence to suggest that parental subfertility is associated with worse perinatal outcomes. Subfertile couples who eventually conceived naturally were also at increased risk for preterm labour and low birthweight in their offspring (Basso and Baird 2003, Jaques et al. 2010). In addition, a longer time to pregnancy (TTP) - reflecting more severe subfertility - was also associated with less optimal perinatal outcomes (Raatikainen et al. 2010). However, studies using the so-called ‘sibling-ship’ approach suggested an additional role for the ART procedure in causing perinatal adversities. In a sibling-ship, one child is conceived naturally and the other child after ART. As the children share the same mother, and often the same father, confounding by parental characteristics is minimized and differences can mainly be attributed to the ART procedure. A meta-analysis on the two papers by Romundstad et al. (2008) and Henningsen et al. (2011), which used this approach, indicated that ART is involved in perinatal adversities (pooled estimate adjusted odds ratio (aOR): 1.27 (95% confidence interval (95%CI): 1.08-1.49) for preterm birth) (Pinborg et al. 2012). In addition, the study by Pelinck et al. reported a difference of 134 grams in birthweight between children born following COH-IVF and MNC-IVF after correcting for confounders, suggesting that ovarian hyperstimulation may be a causative factor in the occurrence of low birthweight after standard IVF (Pelinck et al. 2010). In conclusion, it seems that both the underlying subfertility as well as ART negatively influence perinatal outcomes, but it is not known how much both factors contribute.
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**Congenital abnormalities**

Many large, register-based studies have been performed in order to estimate the risk for congenital abnormalities after ART. As the definition of a congenital abnormality differed per study, and as some studies included minor abnormalities and others did not, the risk increase of congenital abnormalities after ART has been the topic of long debate. A recent meta-analysis reported that singletons born after ART (n = 92,671) had a relative risk of 1.36 (95%CI: 1.30-1.43) for a birth defect compared to singletons born after natural conception (n = 3,870,760) (Hansen et al. 2013). Fortunately, this large relative risk increase only represents a modest absolute risk increase: considering that congenital abnormalities occur in approximately 5% of individuals in the normal population, the risk after ART constitutes 6.8%. For major birth defects, the risk increases from 2% to 2.7%. A meta-analysis in which 46,890 children born following IVF were compared with nearly 27,754 children born following ICSI showed no difference in risk for a congenital abnormality between these two treatments (Wen et al. 2012).

Whether the increased risk for congenital abnormalities is the result of the ART procedure or the underlying subfertility is not clear. The underlying subfertility was first suggested to play a role by Ghazi et al. who found higher rates of congenital abnormalities in children from couples who experienced subfertility of at least four years and finally conceived without ART (Ghazi et al. 1991). Zhu et al. and Davies et al. confirmed that a history of subfertility without ART was associated with congenital abnormalities (Davies et al. 2012b, Zhu et al. 2006). In addition, Zhu et al. found that a longer time to pregnancy was associated with a higher prevalence of congenital abnormalities, while Davies et al. also used the sibling-ship approach and found that children born after ART more often had a congenital abnormality than their naturally conceived siblings (OR: 1.50, 95%CI: 1.08-2.09), suggesting that the ART procedure played a role in addition to the parental subfertility in causing congenital abnormalities (Davies et al. 2012a).

The increase in the overall rate of congenital abnormalities after ART has been studied thoroughly. Some studies also documented the occurrence of larger subgroups of defects after ART and reported an increased risk of cardiovascular anomalies, neural tube defects, urogenital anomalies, gastrointestinal anomalies, clefts, musculoskeletal anomalies and limb anomalies (Hansen et al. 2012, Reefhuis et al. 2009, Wen et al. 2012). However, as these large subgroups of congenital abnormalities are of heterogeneous origin, it is difficult to assess the meaning or biological plausibility of the associations found. It is unclear whether ART and a history of subfertility are associated with specific, pathogenetically similar, congenital abnormalities.

Furthermore, it has been suggested that the risk for congenital abnormalities after ART has decreased over the past few years (Kallen et al. 2010b). It could be that
subfertile couples are being treated more quickly, meaning that ART is offered to less ‘re-productively ill’ couples than a decade ago. If the adverse effect of ART on congenital abnormalities was mediated through the underlying subfertility, this effect will be reduced by treating less subfertile couples. Better birth outcomes over the years could also be the result of improved medication regimes and culture conditions, or changing policies that involve transferring only one embryo into the uterus instead of two. The vanishing twin phenomenon is far less common after the transfer of one embryo and it is known that singleton survivors of a vanished co-twin have worse health outcomes than ‘true’ singletons (Pinborg et al. 2005, Pinborg et al. 2007, Sullivan et al. 2012).

**Imprinting disorders**

Little is known on the effects of ART on the epigenome. Humans possess diploid cells that all have 22 pairs of autosomes and one pair of sex chromosomes (one set is inherited from the father and one set from the mother). According to classic Mendelian genetics, each pair of autosomes is equivalent. However, in a small subset of genes, one allele is silenced by imprinting: the gene is expressed only from either the paternally or maternally inherited copy. Disruption of normal imprinting can lead to dysregulated growth and development of the embryo and is associated with clinical disorders such as Beckwith-Wiedemann syndrome (BWS), Prader-Willi syndrome, Angelman syndrome and Silver-Russell syndrome. Several studies demonstrated an association between ART and imprinting disorders (DeBaun et al. 2003, Gicquel et al. 2003, Ludwig et al. 2005, Maher et al. 2003, Sutcliffe et al. 2006). In theory, ART could indeed easily disturb the imprinting process as oocytes complete the process of erasing old imprints and establishing new imprints just before ovulation, when the follicles are aspirated for fertility treatment. Animal studies have provided a considerable amount of evidence for adverse effects of ovarian hyperstimulation and culture media on imprinting status (Doherty et al. 2000, Sato et al. 2007, Shi and Haaf 2002, Zaitseva et al. 2007). One finding supporting this suggestion is the Large Offspring Syndrome (LOS) in cattle, a phenotype characterized by high birthweight, neonatal respiratory distress, organ overgrowth and skeletal anomalies; it has been described in calves born after in vitro culture (Young et al. 1998). After changing the culture media, LOS no longer occurred (Young et al. 2001).

In humans, a weighted relative risk of 5.2 (95%CI: 1.6-7.4) for BWS after ART was recently calculated from eight studies (Vermeiden and Bernardus 2013). Only one of these studies adjusted the ART effect for a history of subfertility, and by doing so, the association initially found between ART and BWS disappeared (Doornbos et al. 2007). Another study demonstrated that a time to pregnancy of more than 2 years was associated with Angelman syndrome (Horsthemke and Ludwig 2005). As these couples did not receive fertility treatment, it was suggested that a history
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of subfertility is associated with imprinting disorders. As the incidence of imprinting disorders is rare, the risk remains difficult to define and no firm statements about the association between ART, the underlying subfertility, and imprinting disorders in humans can be made yet.

LONG-TERM OUTCOMES

Compared to perinatal outcomes and congenital abnormalities, much less is known about long-term health outcomes after ART. The main reason that we are not well informed on long-term outcomes is that good quality follow-up is a methodological challenge. Many studies on health outcomes after ART have all-too-familiar shortcomings: they are cross-sectional, retrospective, numbers are small, and follow-up rates poor. Reliable follow-up preferably starts with prenatal inclusion. Furthermore, assessors should be blinded to mode of conception. This type of research is both time-intensive and expensive. A retrospective design could introduce bias as parents could be less, or more, willing to cooperate in a study based on their child’s health. An alternative approach for evaluation of rare events is offered by registry-based studies. However, registers are often incomplete or do not contain data of, for example, detailed neurological development. Furthermore, when interpreting data from registries, it is important to take the many confounding factors that may be present after ART into consideration. It is almost universal across the literature that couples requesting ART have a better socioeconomic background: they are older, have smaller families, and their children are more often firstborn. Furthermore, females undergoing ART tend to smoke less and have higher mean body mass indexes (BMIs) than females who do not undergo ART (Sutcliffe and Ludwig 2007). Most registers, however, do not have these data available to make corrections in multivariable analyses. The long-term health outcomes that will be briefly discussed below are the neurological, cognitive and behavioural development and cardiometabolic health of ART offspring.

NEUROLOGICAL DEVELOPMENT

A higher prevalence of cerebral palsy after ART has been reported, but as a direct consequence of the higher prevalence of preterm births in the ART group. ART itself has not been directly associated with adverse neurodevelopment during the first postnatal years (Middelburg et al. 2008). There are, however, some suggestions of an adverse effect of subfertility on neurodevelopment in the offspring: Zhu et al. found a mild psychomotor delay in 18-month-old children (Zhu et al. 2009) and a modest increased risk for developmental coordination disorder (DCD) in 7-year-old children born to subfertile couples compared to children of fertile couples (Zhu et al. 2009).
A meta-analysis on neurological outcome after ART could not be performed due to different methodologies and child assessment at different ages. Furthermore, many of the studies assessed the children at relatively young ages. Studies that had a longer follow-up, like those by Ludwig et al. (5.5 years of age) and Leunens et al. (8 years and 10 years of age) did not find any adverse effects of ART, but faced high attrition rates (Leunens et al. 2006, Leunens et al. 2008, Ludwig et al. 2009a).

The Groningen ART cohort was conducted by our research group in order to evaluate neurodevelopmental status in a precise and age-specific manner in children born following COH-IVF and MNC-IVF. The control group for this cohort consists of children born to subfertile couples who eventually conceived naturally (the Sub-NC group). The findings – up to the age of 2 years – were described in the thesis of my predecessor, Dr. K.J. Middelburg (University of Groningen 2011). The children were assessed at the ages of 2 weeks, and 3, 4, 10, 18 and 24 months. No association between mode of conception and the quality of general movements was found at the age of 2 weeks and 3 months. General movements are spontaneous movements of the fetus and young infant that are involuntary and involve all body parts. The quality of these movements is considered to reflect the condition of the central nervous system (Prechtl 1990). At the age of 3 months, the babies born to subfertile couples (n = 215) showed a reduced quality of their general movements compared to a reference population (n = 450), suggesting an adverse effect of factors associated with subfertility on early neurodevelopmental outcome (Middelburg et al. 2010).

At the ages of 4 and 10 months, neurodevelopment was assessed using the Touwen Infant Neurological Examination (TINE) (Touwen 1976), and at 18 months using the neurological examination according to Hempel (Hempel 1993a). The examinations differ as age-dependent changes occur in neuromotor behaviour, but both examinations aim to assess ‘minor neurological dysfunction’ (MND). Two forms of MND can be distinguished: simple and complex MND. Simple MND implies the presence of a suboptimal, yet normal form of brain function. Complex MND represents the clinically relevant form of MND, which is associated with learning and behavioural disorders (Batstra et al. 2003, Hadders-Algra 2002). In addition, a neurological optimality score (NOS) and fluency score were calculated at 18 months. The items of the neurological examination have a predefined optimal range and the number of items scored as optimal determine the NOS (range 0-58), with higher scores representing better performance. As the range for optimal behaviour is narrower than for normal behaviour, the NOS is able to evaluate subtle differences in neurological outcome (Prechtl 1980). A sub-score of the NOS is the fluency score (range 0-13), which evaluates the fluency of motor behaviour. No differences between the COH-IVF, MNC-IVF and Sub-NC group were found using these highly sensitive measures of neurodevelopment.
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At 2 years of age, the children in the Groningen ART cohort were reassessed using the neurological examination according to Hempel (Hempel 1993a). The NOS, fluency score and prevalence of MND were comparable amongst COH-IVF, MNC-IVF and Sub-NC children, indicating no adverse effect of ovarian hyperstimulation or the in vitro procedure. In order to evaluate an effect of the underlying subfertility, a new fertile reference group was retrospectively recruited at child welfare clinics in the region around Groningen, the Netherlands. Surprisingly, the children born to fertile parents showed lower (worse) NOS and fluency scores and more often had MND than the children in the Groningen ART cohort who were born to subfertile couples. Perhaps the parents who volunteered to participate in the fertile control group were worried about the development of their child and were therefore more willing to participate in our study than parents who did not wish to participate. As the manner of recruiting this control group could have introduced bias, the study could not draw firm conclusions on the absence of an effect of subfertility on neurodevelopment. It is clear from the above that the question of whether ART affects long-term neurological development has not yet been answered satisfactorily, but thus far, the results are reassuring. As neurodevelopmental disorders may emerge as the children grow older, follow-up should continue.

Cognitive development

One of the first studies with a follow-up of 5 years concluded that the mental development of ICSI, IVF and naturally conceived children was similar across groups. A low level of maternal education was the only predictor of a below average intelligence quotient (IQ) (Leslie et al. 2003). This reassuring study was followed by other studies reporting no adverse effects of ART on cognitive outcome (Leunens et al. 2008, Ludwig et al. 2009a, Wagenaar et al. 2009b). A Dutch study found no differences in the results of a national test for primary school performance (the so-called CITO test) or in the educational grouping in secondary education. Also, the need for educational support was comparable between ART and naturally conceived children born to subfertile parents (Wagenaar et al. 2008a). Some studies found better mental outcomes after ART compared to naturally conceived children (Leunens et al. 2006, Mains et al. 2010). Leunens et al., who reported higher IQ levels at 8 years of age, reassessed their cohort two years later. The IQ of the children in their cohort had approximated the IQ of the spontaneously conceived children. The authors hypothesized that the positive influence of maternal educational level diminished over the years (Leunens et al. 2008). The study by Mains et al. found that ART-conceived children performed better on standard testing, but the authors could not correct for socioeconomic background, parental age or educational level. The better mental outcomes after ART could therefore be the result of more favourable parental characteristics in this group compared to the control group (Mains et al. 2010).
In contrast, the study by Knoester et al. reported worse cognitive outcomes in ICSI children (n = 83) than in naturally conceived children (n = 85; mean difference 7.1, 95%CI: 1.7-12.5). The study also included a group of children born following IVF (n = 83). The difference in IQ between ICSI and IVF children was not statically significant (mean difference 3.6 points lower after ICSI, 95%CI: -0.8 ; 8.0) (Knoester et al. 2008). The study by Goldbeck et al., however, did find differences in IQ between IVF and ICSI children: mean IQ was 94 (standard deviation (SD) 14) in the ICSI children (n = 35) and 102 (SD 9) in the IVF children (n = 34) (Goldbeck et al. 2009). A population-based study that included 30,959 children born following ART reported an increased risk of mental retardation (RR: 1.18, 95%CI: 1.01-1.36), but when the analyses were restricted to singletons the risk of mental retardation was no longer statistically significant (Sandin et al. 2013).

Our research group also studied mental development after ART. At the age of 2 years, we found no differences in mental abilities in the children of the Groningen ART cohort. As mental development was similar between COH-IVF, MNC-IVF and Sub-NC children, this suggests that ovarian hyperstimulation and the in vitro procedure do not affect cognitive development. In addition, comparing the three subfertile groups with a newly recruited fertile reference group did not indicate adverse effects of a history of subfertility on the mental development (Jongbloed-Pereboom et al. 2011). Overall, children born following ART seem to have a normal mental health (Hart and Norman 2013a).

Behavioural development

When studying the behaviour of children born following ART, it is difficult to control for parental attitudes and their expectations for their children. Theoretically, parental stress accompanying ART treatment might affect adaptation to the parental role. In addition, parent-child relationships could be different after a strong and long-standing child wish. The children’s subsequent social and emotional development could therefore be different from that of children conceived naturally (Sutcliffe and Ludwig 2007). A review by Wagenaar et al. reported no differences in the incidence of behavioural and socio-emotional problems up to the age of 8 years between ART and naturally conceived children (Wagenaar et al. 2008b). A year later, Wagenaar and colleagues found that 9 to 18-year-old children born following ART more often showed withdrawn or depressed behaviour than their spontaneously conceived counterparts. The scores were based on parental and teacher observations (Wagenaar et al. 2009a). Beydoun et al. sent a questionnaire to ART offspring with a mean age of 21 years and reported a slightly increased risk of depression (15.6% instead of the expected 12.7% (lifetime prevalence up to the age of 25 years)). In addition, this study reported a significantly increased prevalence of female binge drinking (55%)
in the ART group compared to a control group (37%). It should be noted that only 173 (31%) of the 560 eligible young adults completed the questionnaire, so the results could have been biased (Beydoun et al. 2010).

Several studies evaluated the relation between IVF and attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD). IVF is associated with risk factors for ADHD and ASD, including advanced maternal age, a period of unwanted childlessness, a high BMI, preterm delivery, low birthweight and growth restriction (Hvidtjorn et al. 2011, Kallen et al. 2011). Most studies did not find an increased risk for ADHD after ART (Mains et al. 2010, Wagenaar et al. 2009b), with the exception of the previously mentioned study by Beydoun et al. that performed a cross-sectional evaluation of self-reported ADHD in young adults (Beydoun et al. 2010). A population-based study in Denmark found no differences in the prevalence of ASD after ART and ovulation induction (0.68%, n = 33,139) compared to naturally conceived children (0.68%, n = 555,828) after adjusting for maternal age, educational level, parity, smoking during pregnancy, birthweight and multiplicity (Hvidtjorn et al. 2011). Another population-based study that included 2.5 million Swedish children, of which 30,959 were born following ART, also did not demonstrate an association between ART and ASD in general (RR: 1.14, 95%CI: 0.94-1.39). However, after ICSI using frozen embryos, a small increase in the relative risk for ASD in singletons was reported (RR: 2.36, 95%CI: 1.04-5.36) (Sandin et al. 2013).

Our research group studied the behavioural development of 2-year-old children in the Groningen ART Cohort Study by means of a parental questionnaire: the Achenbach Child Behaviour Checklist (CBCL) for children aged 1.5 to 5 years. No differences were found between the COH-IVF, MNC-IVF and Sub-NC group, suggesting no adverse effects of ovarian hyperstimulation or the in vitro procedure. However, the children in the Groningen ART cohort more often displayed anxious-depressed behaviour than the children in the fertile reference group (Jongbloed-Pereboom et al. 2011).

Cardiometabolic outcomes

The interest in cardiometabolic health after ART originates from the ‘developmental origins of health and disease hypothesis’ (DOHaD). This hypothesis, formerly known as the ‘Barker hypothesis,’ states that adaptive responses to environmental changes during critical time windows in early life may have long-term consequences due to reprogramming of metabolic and endocrine key systems (Barker 1995). Support for this hypothesis is delivered by the so-called ‘Hunger winter studies’ that, amongst others, demonstrated that humans prenatally exposed to the Dutch Famine in 1944 were at increased risk for coronary artery disease later in life (Roseboom et al. 2000). Noting
that the early environment shapes an individual's health later in life makes it plausible that ART-related procedures compromise the environment of the early embryo (Watkins and Fleming 2009).

Indeed several reports have evoked concerns that ART is associated with sub-optimal cardiometabolic health in the offspring (Belva et al. 2007, Belva et al. 2012a, Ceelen et al. 2007, Ceelen et al. 2008b, Hart and Norman 2013b, Sakka et al. 2010, Scherrer et al. 2012, Yeung and Druschel 2013). Thus far, one study reported a more favourable lipid profile in pre-pubertal ART children (Miles et al. 2007), whereas four studies reported increased blood pressure (BP) levels in children born following ART (Belva et al. 2007, Ceelen et al. 2008b, Sakka et al. 2010, Valenzuela-Alcaraz et al. 2013). The first of these four studies reported higher BP levels in 8-year-old ICSI children \((n = 137, \text{ median systolic BP (SBP) } 100 \text{ mmHg, range 80-125 mmHg; median diastolic BP (DBP) } 60 \text{ mmHg, range 45-75 mmHg})\) than in children from a fertile reference group \((n = 143, \text{ median SBP 95 mmHg, range 70-120 mmHg; median DBP 55 mmHg, range 35-85 mmHg})\) (Belva et al. 2007). At the age of 14 years, a larger part of the eligible ICSI cohort was reassessed \((n = 217)\) and compared to naturally conceived controls \((n = 223)\). At this time, no differences in BP levels were detected (Belva et al. 2012b). The authors hypothesized that the difference in BP could be temporarily absent as most children were assessed during puberty and the pubertal growth phase may disturb the tracking of BP (Lever and Harrap 1992). The authors did find that girls born after ICSI had a significantly higher sum of peripheral, central and total sum of skinfolds, a significantly higher mean mid-upper arm circumference and waist circumference, a higher BMI, and a higher percentage body fat mass in comparison with girls from the reference group (difference between ICSI and the naturally conceived reference group after correction for confounders: 2.8 mm, 3.1 mm, 6.3 mm, 1.5 cm, 2.1 cm, 1.1 kg/m\(^2\) and 1.8% respectively; all p-values < 0.05). No differences in these outcome measures were found in boys, except for thicker peripheral skinfolds in boys with more advanced pubertal stages (difference 3.5 mm compared to naturally conceived boys). When the authors limited the analyses to girls with more advanced pubertal stages, the differences in body fat measurements between ICSI and naturally conceived girls became even more pronounced (Belva et al. 2012a).

The second study that reported higher BP levels after ART is that of Ceelen et al. who compared 225 Dutch ART children aged 8 to 18 years with 225 age and gender-matched control children who were born to subfertile couples who eventually conceived naturally. SBP and DBP levels were higher in ART children than in controls \((109 \pm 11 \text{ mmHg vs. } 105 \pm 10 \text{ mmHg and } 61 \pm 7 \text{ mmHg vs. } 59 \pm 7 \text{ mmHg respectively})\) (Ceelen et al. 2008b). In addition, ART offspring had a higher sum of skinfolds \((41 \pm 20 \text{ mm vs. } 37 \pm 18 \text{ mm})\) and higher fasting glucose levels \((5.0 \pm 0.4 \text{ mmol/l vs.} ...
4.5 ± 0.4 mmol/l). No differences were found in height, weight, BMI, fasting insulin concentrations, and insulin resistance measures between ART children and controls (Ceelen et al. 2007, Ceelen et al. 2008b).

In the third study reporting higher BP levels in human ART offspring, 106 children aged 4 to 14 years born following ART had higher BP levels than 68 age-matched control children (Sakka et al. 2010). The mean SBP and DBP standard deviation scores were 0.3 (SD 0.9) and 0.7 (SD 0.8) after ART, and -0.3 (SD 1.0) and 0.2 (SD 1.0) after natural conception, respectively. In addition, ART children had higher levels of triglycerides (mean 59.5 mg/dL, SD 25.1 mg/dL) compared to controls (mean 52.4 mg/dL, SD 23.3 mg/dL). Still, SBP and DBP standard deviation scores and triglyceride concentrations were within the normal range for age and sex after ART. No significant differences between ART and naturally conceived children were found in BMI standard deviation scores, waist-to-hip ratio, fasting glucose-to-insulin ratio, total cholesterol, high-density lipoprotein, low-density lipoprotein, uric acid, apolipoprotein-A1, apolipoprotein-B, lipoprotein(a) values, circulating concentrations of leptin and adiponectin, and the markers of chronic inflammation (hsIL-6 and hsCRP) (Sakka et al. 2010).

The fourth study that reported higher BP levels in ART children also found that cardiovascular remodelling was already present in utero (Valenzuela-Alcaraz et al. 2013). The authors included 100 fetuses conceived through ART and 100 control pregnancies matched for maternal age in a prospective cohort and performed ultrasonographic examinations at 28-30 weeks of gestation. Fetuses conceived through ART showed a more globular heart with thicker myocardial walls, decreased longitudinal function (tricuspid ring displacement), impaired relaxation and dilated atria. Even though cardiac output was similar among the two groups, ART fetuses had a lower left ejection fraction (63% (range 57-68%) vs. 69% (range 63-73%). When the children were assessed at 1 and 6 months after birth, most cardiac changes had persisted. One month after birth, systolic BP (SBP) was similar between the two groups while median diastolic BP (DBP) centiles were significantly higher after ART than after natural conception (71st centile (range 44-91) vs. 55th centile (range 21-85)). Six months after birth, SBP was significantly increased (83 mmHg (range 75-94 mmHg) vs. 74 mmHg (range 67-83 mmHg)) after ART and so was aortic intima-media thickness (0.64 mm (0.62-0.67 mm) vs. 0.52 mm (range 0.45-0.56 mm)) compared to controls. Cardiac output and anthropometrics were similar between the two groups, but ART infants showed increased right atrial size, thicker right ventricular walls, decreased shortening fraction, and increased heart rates (Valenzuela-Alcaraz et al. 2013).

A study from Switzerland also extensively assessed multiple parameters of vascular function in ART children (n = 65) at a mean age of 12 years and found similar BP levels compared to naturally conceived controls (n = 57). BMI, heart
rate, low-density lipoprotein, and high-density lipoprotein were also comparable between ART and naturally conceived children. Yet the ART children displayed generalized vascular dysfunction: flow-mediated dilation of the brachial artery was 25% smaller (6.7 ± 1.6% vs. 8.6 ± 1.7%), carotid-femoral pulse-wave velocity was faster (7.8 ± 2.4 m/s vs. 6.5 ± 1.3 m/s) and carotid intima-media thickness was greater after ART compared to control children (410 ± 30 vs. 370 ± 20 µm). Furthermore, the systolic pulmonary artery pressure at high altitude (3450 m) was 30% higher in ART than in control children (39 ± 11 mmHg vs. 30 ± 9 mmHg) (Scherrer et al. 2012). The authors attempted to disentangle the effects of ovarian hyper-stimulation from that of the *in vitro* procedure by including a small group of children born following ovulation induction. Scherrer *et al.* concluded that ovarian hyperstimulation is not associated with vascular dysfunction as the 16 children born following hormonal stimulation without ART had a normal vascular function. However, in ovulation induction low doses of hormones are applied in order to stimulate the growth of only a few follicles, while in COH-IVF higher doses of hormones are applied to stimulate the growth of multiple follicles. This implies that it is too early to refute a potentially adverse effect of ovarian hyperstimulation, as applied in COH-IVF, on vascular function.

Recently published work demonstrated decreased insulin sensitivity and adverse effects of overfeeding on SBP levels in 20-year-old ART offspring (Chen *et al.* 2014). Thirty-four individuals born following ART who had a normal birthweight were recruited and matched by gender and BMI to naturally conceived individuals. First, participants underwent metabolic baseline testing after a three-day energy-balanced diet based on estimated energy requirements per individual. Second, individuals were switched to an overfeeding diet (1250 kcal/day extra) for three days. After three days of overfeeding, the metabolic assessments were repeated. At the baseline measurement no differences were found with respect to BMI, fat mass (%), BP, cholesterol levels, fasting glucose or fasting insulin. However, peripheral insulin sensitivity was significantly lower in ART than in naturally conceived individuals. Furthermore, in response to the overfeeding, a greater increase in SBP levels was noticed in the ART group than in the naturally conceived control group (+3 mmHg in the ART group, -2 mmHg in the control group) (Chen *et al.* 2014). In the same study, in parallel, the metabolic status of adult male mice born following ART was assessed and compared with that of naturally conceived mice. Both conception groups were divided in two subgroups and fed a standard rodent chow diet or a high-fat diet for eight weeks. The mice conceived through ART displayed higher fasting glucose levels independently of their diet. In addition, impaired glucose tolerance and hepatic insulin resistance at both normal and high body weight were found in ART mice (Chen *et al.* 2014).

Poorer cardiometabolic outcome after ART can be expected as such conceptions more often result in preterm birth and low birthweight (Helmerhorst *et al.*
2004), which are risk factors for hypertension and adiposity (Wells et al. 2007). In the above mentioned studies, the poorer cardiometabolic outcomes in ART offspring could not be explained by these factors alone (Belva et al. 2007, Belva et al. 2012a, Ceelen et al. 2007, Ceelen et al. 2008b, Sakka et al. 2010, Scherrer et al. 2012). The topic ‘imprinting disorders’ has already been mentioned. Several studies suggested that not only clear imprinting disorders are associated with ART, but that more subtle epigenetic changes can also occur. These changes can cause less dramatic, but still potentially relevant changes in phenotypically normal children (Batcheller et al. 2011). The epigenetic modifications that may potentially have arisen due to adaptive responses to early environmental changes related to the ART procedure may increase the risk for adverse cardiometabolic outcomes.

**Health after preimplantation genetic screening (PGS)**

As mentioned earlier, preimplantation genetic screening (PGS) was originally developed in order to enhance the efficiency of ART by selecting embryos with a normal chromosomal constitution (Wilton 2002). As lower pregnancy rates were reported after PGS in women of advanced maternal age (Hardarson et al. 2008, Mastenbroek et al. 2007), PGS with FISH in a selected set of chromosomes is no longer recommended. The lack of success of this type of PGS may be due to embryo mosaicism at the cleavage stage (about three days after fertilization) when the embryo is biopsied and because only eight chromosomes are tested for aneuploidies instead of 23 pairs of chromosomes. Currently, newer techniques that may decrease the problems associated with mosaicism are applied, such as polar body (a byproduct of the meiotic cell cycle) biopsy or trophectoderm (precursor of the placenta) biopsy at the blastocyst stage (about five days after fertilization) (Brezina et al. 2012, Ly et al. 2011). Examining the health of children born following PGS with FISH in a selected set of chromosomes is still important as it may reveal valuable information on the consequences of embryo biopsy. Embryo biopsy is now being increasingly performed when couples are significantly at risk of having a child with an inherited genetic disorder and request preimplantation genetic diagnosis (PGD).

Despite the invasiveness of the embryo biopsy inherent to PGS, few studies have addressed the health and development of children born following PGS. At 2 years of age, mental, motor, socio-emotional and language development were comparable for PGS/PGD-children, ICSI-children and naturally conceived children (Nekkebroeck et al. 2008a, Nekkebroeck et al. 2008b). Another study, however, reported less optimal locomotor development after PGS/PGD (Banerjee et al. 2008). Furthermore, blastomere biopsy of preimplantation mice embryos has been sug-
gested to affect behaviour and body weight in male mice (Sampino et al. 2014). Child health in terms of anthropometrics and received medical care after PGS/PGD was evaluated by three cohort studies, reporting reassuring results (Banerjee et al. 2008, Desmyttere et al. 2009a, Desmyttere et al. 2009b). It should be noted that studies addressing cardiovascular health in PGS offspring are lacking.

Our research group initiated the PGS Follow-Up Study: a prospective, assessor-blinded, follow-up study of children born to women who were randomly assigned to IVF with PGS or to IVF without PGS (Mastenbroek et al. 2007). There were no significant differences between the two groups regarding dysmorphic features: the percentage of children with ≥ 1 major (clinically relevant or irrelevant) abnormality was 28% in the group with PGS (PGS+) and 35% in the group without PGS (PGS-) (difference -7%, 95%CI: -23 ; 10) (Beukers et al. 2013). Furthermore, PGS had no effect on parental distress or anxiety (Beukers et al. 2012). Neurodevelopmental outcome up to 2 years of age in children born following IVF with PGS was largely similar to that of children born following IVF without PGS (Middelburg et al. 2010, Middelburg et al. 2011). Nevertheless, application of the neurological optimality score (NOS) indicated that 2-year-old children born following ART with PGS had a somewhat less optimal neurological condition than children born following ART without PGS (Middelburg et al. 2011). It is clear that knowledge of the long-term consequences of embryo biopsy on the health and development of offspring is scarce and that detailed follow-up should continue as embryo biopsy is increasingly being applied in the form of PGD.

**Aim and outline of the thesis**

The above review of the literature underscores the need for the careful and precise monitoring of the health of ART offspring. Nowadays, up to 5% of newborns in Europe are born following ART (Ferraretti et al. 2013). As a result of these large numbers, even subtle changes in the health of children conceived with ART may contribute to the burden of health care. Furthermore, studying the health of ART children may improve clinical practice. If, for example, a negative effect of ovarian hyperstimulation on child health is found, this may result in the adjustment and improvement of the ART procedure. The aim of this thesis is to study the effects of ovarian hyperstimulation, the *in vitro* procedure, the underlying subfertility, and PGS on one or more of the following health parameters: perinatal outcomes, dysmorphic features, neurodevelopment, blood pressure (BP) and anthropometrics in the resulting offspring. Four projects were conducted to realize this aim: the Eurocat Subfertility Project, the Netherlands Perinatal Registry Sibling Project, the Groningen ART Cohort Study, and the PGS Follow-Up Study. In the Groningen ART Cohort and
PGS Follow-Up Study, we performed a subproject, in which the relationship between dysmorphic features and child development was studied in order to shed light on aetiological pathways of neurodevelopment. First, these four projects will be described below and then the outline of the thesis will be presented.

**The Eurocat Subfertility Project**

The aim of the Eurocat Subfertility Project was to explore whether we could identify specific congenital anomalies, imprinting disorders, or syndromal disorders with unknown aetiology associated with parental subfertility or the application of ART. We used data from the Eurocat Northern Netherlands (NNL) population-based birth defects registry. Eurocat NNL registers live births and still births diagnosed with a congenital abnormality, and pregnancies terminated due to congenital anomalies in the following cases: if the mother lived in the registration area at the time of birth and if the child was under 10 years at the time of registration. Only information on malformed children is collected. The registration covers approximately 19,000 births per year. Approximately 80% of the parents sign the informed consent and agree to participate. Of these couples, another 80% fills in a questionnaire about their own health, life style, fertility and pregnancy. For the Eurocat Subfertility project we identified three fertility groups:

1. **Fertile parents**
2. **Subfertile parents who eventually conceived naturally**
3. **Subfertile parents who conceived through IVF or ICSI**

We searched the medical records of fertility clinics for all couples with fertility problems who reported eventually conceiving spontaneously or after ART. Couples were defined as subfertile after a time to pregnancy of at least 12 months of unprotected intercourse. Cases were excluded from the analyses if the subfertility could not be confirmed (e.g. no fertility files available or a time to pregnancy < 12 months), or if any treatment other than IVF or ICSI was used. Cases with a known underlying cause of their anomaly, including chromosomal and monogenic disorders and defects resulting from congenital infections or exposure to teratogens, were excluded. As an exception, we did include all imprinting disorders as several studies have reported an increased risk of imprinting disorders after ART and we wanted to explore this association in the Eurocat NNL data.
The Netherlands Perinatal Registry Sibling Project

The aim of the Netherlands Perinatal Registry (PRN) Sibling Project was to disentangle the effects of the maternal characteristics including subfertility from that of the ART treatment on perinatal outcomes. In order to do so, we adopted two approaches: the ‘inter-sibling approach’ and the ‘sibling-ship approach’ (Figure 1). The inter-sibling approach \( (n = 514,442 \text{ children}) \) involves comparing the children born to four groups of mothers, each giving birth to two consecutive singletons:

1. Mothers A \( (n = 254,721) \) conceived both children naturally
2. Mothers B \( (n = 1342) \) first conceived through ART and thereafter naturally
3. Mothers C \( (n = 471) \) first conceived naturally and thereafter through ART
4. Mothers D \( (n = 687) \) conceived both children through ART

The following comparisons were made: B1 vs. A1 was compared with B2 vs. A2, C1 vs. A1 was compared with C2 vs. A2 and D1 vs. A1 was compared with D2 vs. A2. The outcomes of the two comparisons (for example A1 vs. B1 and A2 vs. B2) were placed next to each other to interpret the effects of maternal characteristics and ART separately.

Children born to mothers B and C were also used for the sibling-ship analyses, in which one of the two consecutively born singletons is conceived through ART \( (n = 1,813) \) and the other following natural conception \( (n = 1,813) \) and their perinatal outcomes are compared. An advantage of the sibling-ship approach is that confounding by maternal characteristics is strongly minimized and the differences can mainly be attributed to the ART treatment. Limitations of the sibling-ship approach are, however, that parity is an important confounder (even when adjusting for parity) and that it does not provide information about the effects of maternal characteristics on perinatal outcomes. These limitations are not present when an inter-sibling design is used. Considering these advantages and disadvantages we adopted both approaches.

A limited set of outcome parameters was selected based on the literature and/or in case an underlying pathophysiological mechanisms was biologically plausible. The outcome measures included gestational age in days, spontaneous preterm delivery \( (<37 \text{ weeks}) \), iatrogenic preterm delivery \( (<37 \text{ weeks}) \), birthweight in grams, fetal growth retardation, perinatal mortality, any congenital abnormality, and maternal hypertensive disease.
Figure 1. Graphic depiction of the comparisons made for the inter-sibling approach and the sibling-ship approach.
The Groningen ART Cohort Study

The third and largest project described in this thesis is the Groningen ART Cohort Study. The aim of this prospective, assessor-blinded, study was to examine the effect of ovarian hyperstimulation, the *in vitro* procedure and a combination of these two on dysmorphic features, neurological, cognitive and behavioural development and cardiometabolic health. Subfertile couples with a term date between March 2005 and December 2006 were recruited at the Department of Reproductive Medicine of the University Medical Center Groningen, during the third trimester of pregnancy. The singletons born to the couples formed three groups:

1. Children born following controlled ovarian hyperstimulation IVF/ICSI (COH-IVF)
2. Children born following modified natural cycle IVF/ICSI (MNC-IVF)
3. Children born to subfertile couples (i.e. subfertile-naturally conceived: Sub-NC)

Placement in one of the first two groups depended on the presence (COH-IVF) or absence (MNC-IVF) of ovarian hyperstimulation prior to the *in vitro* procedure. The third group consisted of naturally conceived singletons born to subfertile couples who were waiting for fertility evaluation or treatment. This control group was chosen as we assumed that parity, age, and possibly other unknown factors closely resemble the characteristics of couples requesting ART. We excluded multiple pregnancies and pregnancies after the transfer of cryopreserved or donated oocytes or embryos.

The composition of the study groups allowed us to make three comparisons, shown in Figure 2. Differences between the COH-IVF group and the MNC-IVF group can be mainly attributed to ovarian hyperstimulation, differences between the MNC-IVF group and the Sub-NC group can be mainly attributed to the *in vitro* procedure, and by comparing the COH-IVF group with the Sub-NC group, a combination of these two factors was studied.

Figure 2. The three fertility groups in the Groningen ART Cohort Study.

*This figure is adapted from Middelburg et al. 2010 with permission from the authors.*
The children in the Groningen ART Cohort were neurologically assessed at 2 weeks, and then at age 3 months, 4 months, 10 months and 18 months. As mentioned before, we found no adverse effects of ovarian hyperstimulation or the \textit{in vitro} procedure on neurodevelopment (Middelburg \textit{et al.} 2009, Middelburg \textit{et al.} 2010).

This thesis focuses on the assessments at the ages of 2 and 4 years, when examination of dysmorphic features, BP and anthropometrics was added to the neurodevelopmental follow-up. Figure 3 shows a flow chart of the participants in the Groningen ART cohort up to the age of 4 years.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{flowchart.png}
\caption{Flow chart of the Groningen ART cohort.}
\end{figure}

This figure is adapted from Schendelaar \textit{et al.} 2014 with permission from the authors.
The PGS Follow-Up Study

The PGS Follow-Up Study is a prospective, multicentre, assessor-blinded, follow-up study of children born to women who were randomly assigned to ART with PGS, or to ART without PGS (controls) (Mastenbroek et al. 2007). Originally, this randomized, controlled trial (RCT) was performed to assess the efficiency of PGS in improving ongoing pregnancy rates. The aim of the follow-up of the RCT was to examine the effect of embryo biopsy on neurological, cognitive and behavioural development and BP, anthropometrics and the medical care received. Inclusion criteria for the RCT were: female age 35 to 41 years, no preceding failed ART, and no objections to a double-embryo transfer. Between May 2003 and November 2005, 408 women were randomized. Randomization was performed centrally, with minimization for female age (35-37 or 38-41 years) and the type of ART (IVF or ICSI), and with stratification for the study centre (University Medical Center Groningen (UMCG), Groningen, the Netherlands or Academic Medical Centre (AMC), Amsterdam, the Netherlands). It is conceivable that PGS, which includes more extensive embryo manipulation than conventional ART, introduces extra risks for the health of offspring. The health and development of the children conceived in the UMCG in the PGS Follow-Up Study has been assessed at various ages during infancy: 2 weeks, 3 months, 4 months, 10 months, 18 months, and 2 years. The children conceived in the AMC were only assessed at the age of 2 years. Our research group found that neurodevelopmental outcome up to 2 years of age was largely similar between children born following ART with or without PGS (Middelburg et al. 2009, Middelburg et al. 2011). However, as mentioned above, when using the ‘neurological optimality score (NOS),’ which is a more sensitive outcome measure, children born following ART with PGS had a less optimal neurological condition at 2 years than children born following ART without PGS (Middelburg et al. 2011). In this thesis, the assessment at the age of 4 years was used, this included measurements of the children’s anthropometrics and BP.
Introduction

**OUTLINE OF THIS THESIS**

This thesis addresses the health of children born to subfertile parents and describes the results of 4 main projects and 1 subproject (parts 1 to 5).

**PART 1: THE EUROCAT SUBFERTILITY PROJECT**

*Chapter 2* describes a registry-based study performed in the Northern Netherlands and addresses associations between ART and a history of subfertility with specific types of congenital abnormalities.

**PART 2: THE NETHERLANDS PERINATAL REGISTRY SIBLING PROJECT**

*Chapter 3* describes a Dutch registry-based study performed in order to disentangle the effects of ART from those of maternal characteristics on perinatal outcomes, by using the so-called ‘sibling-ship approach’ and the new ‘inter-sibling approach’.

**PART 3: THE GRONINGEN ART COHORT STUDY**

*Chapter 4* describes the prevalence of dysmorphic features, including minor anomalies, in the Groningen ART Cohort Study. Special attention was paid to the putative relation between a longer time to pregnancy and dysmorphic features in 2-year-olds.

*Chapter 5* describes the relation between a longer time to pregnancy and the neurodevelopmental status of 2-year-old children in the Groningen ART cohort.

*Chapter 6* focuses on cardiometabolic outcome in the Groningen ART cohort and tries to answer the question whether ovarian hyperstimulation or the *in vitro* procedure is associated with higher blood pressure and thicker skinfolds in 4-year-old offspring.

*Chapter 7* describes an explorative, causal inference approach used to answer the question whether ovarian hyperstimulation is involved in elevating blood pressures and increasing skinfold thickness of 4-year-old children in the Groningen ART cohort.

**PART 4: THE PGS FOLLOW-UP STUDY**

*Chapter 8* evaluates the blood pressure and anthropometrics of 4-year-old children in the PGS Follow-Up Study.
PART 5: AETIOLOGICAL PATHWAYS OF NEURODEVELOPMENT

Chapter 9 describes the relation between dysmorphic features and developmental outcome of 2-year-old children in the Groningen ART Cohort Study and PGS Follow-Up Study.

DISCUSSION, FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Chapter 10 discusses the clinical implications of the main findings of this research, provides an overview of changes in fertility practice and its consequences for follow-up research, gives some methodological considerations and suggestions for future research, and offers my concluding remarks.

SUMMARY

Chapter 11 provides a summary of the research in English and in Dutch.
Part 1

The Eurocat Subfertility Project