CHAPTER 1
General introduction
Women’s role in society has changed considerably over the last decades. In modern society women are educated equivalent to men and more often pursue a successful career. As a consequence of the increasing economic independence, women postpone having children. The latter is also attributable to the development and availability of better contraceptive methods. Nearly three decades ago two-thirds of Dutch women gave birth below the age of 30 years; nowadays, only one-third of women does.¹ The mean maternal age at first birth has increased by about four years between 1970 and 2011 in the United States (from 21.4 years to 25.6 years) and is even higher in developed European countries, with the Netherlands as one of the outliers with an average maternal age of 29.4 years.¹² The shift towards a higher maternal age at child conception has its impact on the population’s fertility. Fertility clearly declines after the age of thirty, with a more progressive decline in the late thirties.³⁵ Moreover, older women who conceive are at greater risk of pregnancy complications.⁶,⁷

While the women’s emancipation has taken its effort during the second half of the twentieth century, medical science has gained momentum as well. In 1976 the gynaecologist Patrick Steptoe and the physiologist Robert Edwards took the first steps in the development of assisted conception by achieving the first pregnancy after transfer and implantation of a human embryo which was fertilized in vitro.⁸ In 1978 they gained international attention by reporting the first birth of a baby after in vitro fertilization (IVF), named Louise Brown.⁹ Since that date, up to 5% of newborns in Europe.¹⁰ and 1% of newborns in the United States¹¹ are born following assisted reproductive techniques (ART). In the Netherlands, up to 3% of children are born following ART.¹²

With the introduction of ART, the responsibility follows to carefully monitor and warrant its safety. The health and development of ART offspring is of general interest, since these children nowadays represent a substantial part of the world’s population.

In this thesis the neurodevelopmental outcome of children born following ART is evaluated up until 4 years of age by exploring the influence of specific factors involved in assisted conception, such as ovarian hyperstimulation, the in vitro laboratory procedures and subfertility-related aspects. Prior to the content of the thesis, the applied fertility treatment techniques are introduced briefly and a general and concise overview of the literature available on child health and developmental outcome is given.

Assisted reproductive techniques
Over the years, many assisted reproductive techniques have been developed, with a wide variation in medical indications and invasiveness of the procedures. IVF is considered to be the mainstay of technologies in the field of assisted conception. The techniques that were applied in the studies of this thesis will be outlined briefly. No specific attention will be paid to fertility treatments such as intra-uterine insemination (IUI) and ovulation induction (OI).
Conventional controlled ovarian hyperstimulation IVF

In vitro fertilization is the process of fertilizing oocytes outside the human body in the laboratory after which one or more embryos are transferred into the uterine cavity. The conventional procedure, controlled ovarian hyperstimulation IVF (COH-IVF), consists of several phases. It starts with ovarian hyperstimulation, in which the ovaries are stimulated by means of daily injections of exogenous follicle stimulating hormone (FSH) to induce growth of multiple follicles. These injections are combined with the supplementation of either gonadotropin-releasing hormone (GnRH)-agonists or GnRH-antagonists to prevent the endogenous luteinizing hormone (LH) surge and thus, premature ovulation. Next, follicle growth is carefully monitored by ultrasound. In case of a follicle diameter of approximately 18 to 20 mm, either human chorionic gonadotrophin (hCG) or LH is administered to facilitate follicle maturation. About 34 to 36 hours later, one or more oocytes are retrieved from the ovarian follicles by means of transvaginal ultrasound-guided aspiration. Subsequently, the retrieved oocytes are mixed with spermatozoa in a small volume of culture medium, or a single spermatozoon is directly injected into the cytoplasm of the oocyte. The latter technique is known as intracytoplasmic sperm injection (ICSI). ICSI is mostly applied in case of male subfertility. After fertilization, the resulting zygotes are maintained in culture for two to three days to develop into a cleavage stage embryo. Approximately two to five days after fertilization, one or two morphologically good embryos are transferred into the uterine cavity (single or double embryo transfer, respectively SET and DET). Prior to the transfer, progesterone or hCG may be administered to support the endometrium. The embryos of good quality that are not transferred to the uterus can be cryopreserved for future transfer in subsequent cycles.

Modified natural cycle IVF

A disadvantage of COH-IVF is the increased chance of having a multiple pregnancy, which in turn is associated with obstetric and perinatal adversities. Another disadvantage of COH-IVF is the chance of developing ovarian hyperstimulation syndrome (OHSS), a rare but potentially life-threatening complication of ovarian hyperstimulation. In OHSS a combination of ovarian enlargement occurs, due to multiple ovarian cysts and an acute fluid shift out of the intravascular space. To reduce the risk of such complications, an alternative for conventional IVF was developed: modified natural cycle IVF (MNC-IVF). In contrast to COH-IVF, in MNC-IVF the one follicle that naturally develops as dominant is used for conception. Again, follicle growth is carefully monitored by means of ultrasound. However, in the late follicular phase of the natural cycle, when the leading follicle has reached a diameter of approximately 14 mm, GnRH antagonists and FSH are administered, in most cases resulting in the maturation of a single follicle. Benefits of MNC-IVF are the maintenance of the natural selection of the follicle and the minimal use of hormonal medication. A drawback, however, is the low chance of an ongoing pregnancy per IVF treatment cycle of approximately 8%.
Preimplantation Genetic Screening

Preimplantation genetic screening (PGS) was introduced to enhance efficiency of assisted conception. The success rate of IVF is relatively low, with approximately 50% chance of an ongoing pregnancy after three IVF treatment cycles. In PGS, embryonic cells are screened for aneuploidy and only embryos with euploid cells are selected for transfer to the uterus. Aneuploid cells contain an abnormal number of chromosomes: the cell either contains additional chromosomes or is missing chromosomes, a phenomenon that occurs during cell division when the chromosomes do not separate properly. In order to screen for aneuploidy, one or two blastomeres (i.e. cells produced by division in a fertilized ovum) are aspirated from a cleavage stage embryo. To this end, an opening is created in the embryos zona pellucida by enzymatic digestion or by laser. After removal of one or two blastomeres fluorescent in situ hybridization (FISH) is applied to identify the copy numbers of several sets of chromosomes in the cell.

In theory, PGS results in improved implantation, increased ongoing pregnancies, and live-birth rates, as it is assumed that about half of all embryos obtained through IVF are aneuploid. Unfortunately, PGS was less effective in improving ongoing pregnancy rates than expected. In fact, PGS even significantly reduced the live-birth rate for women of advanced maternal age. As a consequence, PGS as described in the present thesis is no longer applied. The European Society of Human Reproduction and Embryology PGD Consortium recommends applying PGS only in the context of properly constructed trials.

Embryo biopsy is currently still applied in the form of preimplantation genetic diagnosis (PGD). In PGD, embryos of couples with a significant risk of a genetic disorder are analysed for inheritance. The application of PGD is steadily increasing, underlining the importance of evaluating the effects of embryo biopsy.

Assisted reproduction – the potential interference with child development in general

The idea of potential influences of assisted conception on child development can be traced to the 'developmental origin of health and disease paradigm', formerly known as the 'Barker hypothesis', which states that the early development of an organism serves as a template for its future health, or, in other words: 'the early environment shapes an individual's health later in life'. During critical phases of an organism's early development, functional and structural organic changes take place depending on the environmental circumstances in which the organism lives. Such physiologic and metabolic alterations may predispose organisms to increased susceptibility to disease and developmental disturbances later in life. A number of animal studies have indeed demonstrated that the periconceptional environment is decisive for the individual's later quality of life.

ART may interfere with early human development during several steps in human reproduction. First, ovarian hyperstimulation causes a down-regulation of the pituitary
function and stimulates the ovaries to produce an increased number of follicles. As a result of the growth of multiple follicles, natural selection of the dominant follicle may be bypassed. Other points of concern are the fertilization and maturation of the oocyte and the embryo in vitro and the manipulation of the oocyte and embryo both mechanically and chemically by means of the use of cultured media. Several animal studies demonstrated that in vitro culture impairs embryonic development, pregnancy establishment and outcome. Cultured media have been linked to alterations in gene imprinting in mice and abnormal fetal outcome, including high and low birthweight. The scarce literature on humans shows conflicting results. A study by Dumoulin et al. showed that culture conditions can affect birthweight of newborns. The authors compared perinatal outcome of children born following IVF in which either one of two widely used, commercially available culture media were used (Vitrolife AB media, Göteborg, Sweden; Cook media, Brisbane, Australia). They found significantly higher pregnancy rates and clinical pregnancy rates in the Vitrolife culture group and a significant higher birthweight in the Vitrolife culture group (mean birthweight: Vitrolife: 3453; Cook: 3208) than in the control group exposed to the Cook medium. Later on, the authors found similar results in a study using frozen embryos and the same culture media (adjusted mean difference birthweight: 122 g, \( P = 0.03 \)). However, they were not able to find an effect of culture media on gestational age. Other groups were not able to find associations between cultured media types and birthweight and gestational age. In addition, Lin et al. did not find significant associations between birthweight and gestational age and the type of culture media (G5 TM, Global and Quinn’s advantage media) in 1201 singletons and 445 sets of twins.

Another source of potential developmental interference of IVF may be the endometrium, which is subjected to an altered endocrinological environment due to hormonal stimulation. Normal preimplantation development and establishment and maintenance of a pregnancy rely on mutual communication between the young embryo and adjacent uterine epithelial cells that coordinate embryonic development with consecutive changes in uterine function. Several animal studies demonstrated that ovarian hyperstimulation is associated with alterations in hormonal levels and disturbances in the endometrium due to, among other things, a non-optimal expression of endometrial growth factors and reduced endometrial receptivity.

An increasing body of evidence suggests that ART is associated with epigenetic changes, i.e. modification of chromosomes that do not alter the nucleotide base sequences but alter the expression of genes. Disruptions in genomic imprinting is one of epigenetic processes that seems to be associated with ART. Genomic imprinting is an epigenetic process of the differential expression of genetic material depending on whether it was inherited from the mother or father, with the silent allele based on the parent-of-origin. Theoretically, ART could interfere with genomic imprinting through ovarian hyperstimulation and follicle aspiration. Germ cells have the ability to erase the imprinting marks during their development in embryonic life. After resetting, differentiating germ cells re-establish de novo imprinting marks according to their sex. The latter occurs for both
sexes in late fetal stages and continues after birth. In oocytes, the process is not completed until just before each ovulation, whereas spermatozoa complete the process of re-establishing paternal imprinting marks earlier in the gametogenesis. Both mouse and human oocytes from stimulated cycles appear to have altered methylation status of several imprinted genes compared to oocytes from non-stimulated cycles.\textsuperscript{45} Several epidemiological studies indicate an increase in the incidence of imprinting disorders in children born following ART compared to naturally conceived (NC) children, such as Beckwith-Wiedemann syndrome and Angelman’s syndrome.\textsuperscript{43} Moreover, not only ART aspects seem to interfere with genomic imprinting. Some studies reported associations between a history of subfertility and a higher prevalence of imprinting disorders.\textsuperscript{46,47} Although interesting, imprinting disorders are beyond the scope of this thesis on child neurodevelopment.

The following paragraphs focus on the health and development of children born to subfertile couples, with or without the application of ART. First, the general health and development will be described, from birth until puberty, in terms of congenital abnormalities, cardiometabolic and cardiovascular health, and growth and pubertal development. Thereafter, a shift is made towards the main focus of this thesis: neurodevelopment after ART and subfertility. The literature available on child neurological development will be outlined in terms of neuromotor, cognitive and behavioural development.

\textbf{Assisted reproduction – child health and developmental outcome in general}

Over time, concerns have been raised about the safety of ART with regard to child outcome. ART is known to be associated with multiple gestations, which is in turn known to be associated with an increased risk for obstetric and perinatal adversities, in particular, preterm delivery and low birthweight. The latter are associated with increased perinatal mortality and short-term and long-term morbidity.\textsuperscript{15,16} In addition, multiple gestation may also be associated with the early loss and absorption of one twin, known as the vanishing twin syndrome.\textsuperscript{48} A little more than 10\% of IVF singletons originates from a twin gestation early in pregnancy,\textsuperscript{49} which may contribute to adverse developmental outcome after IVF.\textsuperscript{50,51}

Despite the fact that ART is inextricably linked to the increased number of twins, it is well established that the numerous adverse perinatal outcomes, which are associated with ART, cannot be ascribed only to the effects of multiple gestation. Also singletons born following ART are two times more often born preterm and more often have low birthweight compared to NC peers.\textsuperscript{52-56} Moreover, ART singletons are three times more often very preterm and have very low birthweight, even when factors such as maternal age at conception and parity were taken into account.\textsuperscript{52-56} Moreover, ART singletons are more often born following Caesarean section and more often referred to the neonatal intensive care unit (NICU). The risk of perinatal mortality among ART singletons is almost twofold compared to NC peers.\textsuperscript{53-56} Evidence is accumulating that not only the ART aspects interfere with child outcome, but inherent factors of the treated couples, such as increased maternal...
age and a history of subfertility may also contribute to unfavourable developmental outcome. Subfertility is known to be associated with obstetrical problems such as the need of Caesarean section, pregnancy-induced hypertension or preeclampsia, placenta praevia and placental abruption and increased perinatal adversities such as preterm birth, low birthweight, and perinatal deaths.

**Congenital abnormalities**

Another point of concern regarding health outcome after assisted conception is the fact that children born following ART are at increased risk for congenital abnormalities compared to their NC peers. Hansen et al. pooled the estimates derived from the meta-analyses of six systematic reviews on birth defects in ART children compared to NC peers. The pooled odds ratios (ORs) show a 30 to 70% increase in birth defects (OR: 1.3 to 1.7), with slightly lower pooled ORs for studies examining singletons and multiples combined (OR: 1.3 to 1.4). A recent population-wide cohort study by Davies et al. demonstrated an increased risk of congenital abnormalities in children born following IVF with ICSI and not in those born following IVF per se. The increased risk persisted after adjustment for maternal age and several other risk factors.

Whether the increased risk of congenital abnormalities can be attributed to ART aspects or the underlying parental subfertility is unclear; the literature shows conflicting results. Davies et al. found a higher prevalence of congenital abnormalities among children born following ART compared to their NC siblings, suggesting that ART-related aspects may be the decisive factor. Zhu et al. on the other hand, found that severe subfertility was associated with a higher prevalence of congenital abnormalities.

**Cardiometabolic and cardiovascular health**

Recent evidence suggests that, to some extent, ART children are presumably more prone to develop cardiometabolic diseases due to the increased risk of perinatal adversities, which in turn are risk factors for type 2 diabetes, hypertension, and cardiovascular disease. However, the increased risk may also be related to epigenetic modifications as a result of the ART procedure per se or the underlying parental subfertility. Several animal studies have shown that in vitro embryo culture contributes to significantly heavier offspring than spontaneous conception and that ART may be associated with impaired glucose metabolism and elevated blood pressure levels in offspring, adversities that were found in human ART children as well. It has been suggested that ART children display a level of vascular dysfunction similar to that seen in children of mothers with preeclampsia. For example, Scherrer et al. found an indication for generalized vascular dysfunction in healthy children conceived by ART. With the study of Scherrer et al. included, only a handful of studies have reported worse cardiometabolic health in children born following ART. Several studies reported subtle, yet significantly elevated blood pressure levels in ART children compared to NC peers born to fertile or subfertile couples, independent of early life factors and parental characteristics. One study even reported elevated blood
pressure levels and increased aortic intima-media thickness after ART in utero. However, Belva et al. reassessed their ART children at older age, resulting in the disappearance of the previously found difference in blood pressure levels, presumably because of the influence of puberty. Nevertheless, the authors did find higher amounts of total body fat, particularly in ICSI girls compared to NC peers. Also Miles et al. could not detect a disadvantageous effect of ART and reported an even favourable lipid profile in pre-pubertal ART children. On the contrary, Ceelen et al. reported, besides the aforementioned elevated blood pressure levels, thicker skinfolds and higher fasting glucose levels in ART children. However, no differences were found in BMI or fasting insulin concentrations and insulin resistance measures compared to NC peers born to subfertile parents. Given the scarcity of literature available on cardiometabolic health among ART children, further research needs to be done, especially given the long-term consequences of cardiometabolic and cardiovascular risks.

Growth and pubertal development
Several cohort studies from Western-European countries have studied long-term growth and physical development of children and have not found clear differences between ART and NC children, both in singletons and multiples. The weight, height, and head circumference of IVF and ICSI children at various ages, varying from 1 to 8 years, have been reported to be normal. Considering the fact that ART children are at risk for perinatal adversities such as low birthweight and prematurity, the disappearance of impaired growth with increasing age implies catch-up growth. Indeed, several of the former reported studies reported the occurrence of catch-up growth in ART children. However, one study observed sustained growth retardation in both IVF singletons and multiples compared to natural conceived controls at the age of 3 years, presumably due to insufficient catch-up growth after a period of poor perinatal outcome. Catch-up growth seems positive, but it may have consequences for later life. In SGA born children it has been linked to early onset obesity with the subsequent emergence of (components of) the metabolic syndrome. The higher prevalence of perinatal adversities in ART children increases their chance of experiencing catch-up growth. Consequently, the ART population may indirectly be more prone to develop health problems later in life.

As much is known about birthweight and growth of ART children, so little is known about the later pubertal development. The majority of people who were born following ART have not reached adulthood until now and only a small group of offspring has reached the age of 30 and beyond. According to some studies, ART does not appear to affect the onset of puberty. However, there are some suggestions that ART may interfere at a different level in puberty. For instance, Belva et al reported delayed breast development in 14-year-old ICSI girls, however, the onset of the pubarche and menarche was similar to that of NC peers. Ceelen et al. reported an advanced bone age in pubertal IVF girls, but not for IVF boys, compared to NC peers. Moreover, the authors found higher serum levels of dehydroepiandrosterone sulphate (DHEAS, a steroid hormone, that functions as a
metabolic intermediate in the biosynthesis of the androgen and oestrogen) and LH in IVF girls. The clinical relevance of these higher values need to be elucidated, but according to the authors the elevated DHEAS and LH levels could be due to perinatal growth restriction and the high variation in pulsatile LH secretion in the girls, respectively. A study by Mau Kai et al. reported disadvantageous effects of ART for boys, in terms of lower serum testosterone concentrations and a higher LH/testosterone ratio in ICSI boys compared to NC boys. No differences were found between IVF boys and NC boys. According to the authors, the ICSI boys may have inherited the subtle impairment of Leydig cell from their fathers.

Assisted reproduction – neurodevelopmental outcome of the child

Neurodevelopment is a broad term and generally used as an umbrella term for the growth and development of the brain and central nervous system. Terms such as impaired neurodevelopment or neurodevelopmental disorders or disabilities are used to refer to brain dysfunction that affects motor function, emotion and behaviour, learning ability, self-control and memory.

A thoroughly performed systematic review by Middelburg et al. in 2008 indicated that ART has not been associated with adverse neurodevelopmental outcome during the first postnatal years. It also revealed that in 2008, surprisingly little was known on long-term outcome due to the relative paucity of long-term follow-up studies of good methodological quality. Early development is only a moderate predictor of long-term development, meaning that reassuring findings on short-term neurodevelopmental outcome do not preclude an effect of ART or subfertility on long-term neurological development, as it takes time for some disorders to emerge.

After 2008, various studies on long-term neurological development after assisted conception, beyond the age of 18 months, have been performed. However, consensus on whether or not ART has an impact on long-term neurodevelopmental outcome is still lacking. The results of the studies vary due to methodological issues such as diversity in study outcome and instruments used to assess outcome, the lack of a suitable proper control group, high rates of attrition, the variety in age at which the subjects were studied and the type of study subjects that were studied, such as singletons, multiples or both. An overview of the current literature on long-term neurodevelopmental outcome, i.e. outcome beyond the age of 18 months, is presented in the following paragraphs.

Neuromotor development

There are some concerns about the potential risk of cerebral palsy (CP) in children born following ART. CP is an umbrella term for a group of permanent clinical disorders that are characterized by motor and postural dysfunction. These conditions, which range in severity, are due to non-progressive lesions or abnormalities of the developing brain resulting from a variety of causes, of which the appearance may change over time as the brain matures.

A systematic review and meta-analysis on the association between CP and fertility treatment were performed by Hvidtjorn et al. on 41 published studies. The authors found
an increased risk for CP in IVF children compared to NC peers, which may be attributable to prematurity, multiplicity, and the vanishing twin phenomenon in early pregnancy. Soon after, Hvidtjorn et al. performed a population based follow-up study including nearly 600,000 children to assess the risk of CP in IVF children in comparison to NC children.94 The authors found that children born after IVF had an increased risk of a CP diagnosis (hazard ratio (HR) [95% confidence interval (CI)]: 2.34 [1.81-3.01]) compared to NC children, a significant finding that disappeared after adjustment for multiplicity and gestational age. Hereafter, a publication by Zhu et al., followed, using register-based data. The authors found that children born after ART had an increased risk of CP, even after adjustment for prematurity and multiplicity (HR [95% CI]: 2.30 [1.12-4.73]).95 The authors did not find a significant association between time to pregnancy (TTP) and the risk of CP in children conceived spontaneously.95

Various studies reported on the less severe but more common neurodevelopmental disorders in ART children. The studies, however, vary considerably in the nature of ART, assessment method, age at follow-up and in study outcome. Some studies reported worse neuromotor outcome in ART children,96-98 whereas others concluded that outcome of ART children was similar to that of NC children.78,99-106 Few studies addressed neurological development beyond the age of 18 months. Zhu et al. found a delay for most milestones, especially motor milestones, in 18-month-old ICSI children.97 On the other hand, Ludwig et al. reported that neuromotor skills of 5.5-year-old ICSI singletons were similar to those of NC peers.105 Similar results were found by Leunens et al. in 8 and 10-year-olds.107,108 Both latter studies, however, suffered from a high attrition rate. Knoester et al., who assessed minor neurological dysfunction (MND, see page 29) at 5 to 8 years, reported that the neurological condition of children born after IVF was similar to that of NC children.104 However, ICSI children had a higher prevalence of MND than the NC children. Finally, Levy-Shiff et al. performed a general paediatric neurological examination in 9 to 10-year-old children and found no differences between IVF/ICSI children and NC children.100

Thus, the effect of ART on long-term neuromotor is still unclear. The picture is complicated by a potential effect of subfertility. As mentioned before, suggestions have been made for the effect of factors inherent to treated couples on neurodevelopment in the offspring, such as increased maternal age,7,57,58 and a history of subfertility.59-62 For instance, Zhu et al. found a modest increased risk for developmental coordination disorder (DCD) in 7-year-old children born to subfertile couples. The majority of studies does not properly distinguish between potential ART-related effects and subfertility aspects on child neurodevelopmental outcome due to the design of the study groups. They compare ART outcomes with those of fertile populations rather than to the results of a subfertile population conceiving spontaneously and, consequently, overestimate the potential effect of the fertility treatment. A more proper control group consists of naturally conceived children born to subfertile couples, as it resembles an ART population more in terms of parity and parental age.
Cognitive development

The majority of long-term follow-up studies on cognitive outcome in ART children reported similar cognitive functioning compared to NC peers or even better cognitive functioning. The better cognitive functioning found in the latter two studies in 8-year-old ICSI children and 8 to 17-year-old IVF children in comparison to NC children was presumably the result of more favourable parental characteristics in the ART population than in the spontaneously conceived controls. In contrast, Knoester et al. reported that cognitive function of 5 to 8-year-old singleton ICSI children was worse than that of NC children matched for gender, socioeconomic status, the presence of prematurity and maternal age at time of conception and date of birth. Goldbeck et al. reported lower IQ scores in 5 to 10-year-old ICSI singletons (mean IQ = 94.1, standard deviation [SD] = 13.8) compared to IVF peers (mean IQ = 102.0, SD = 9.1; P = 0.005). Moreover, they reported that 23.5% of ICSI children, but only 2.9% IVF children had at least borderline delayed cognitive development. In a study on language assessment in 5-year-old singletons and twins by Gucuyener et al., it was demonstrated that twins performed worse on the Stanford-Binet Intelligence Scale Form and the Peabody Picture Vocabulary test, especially when the twins were born after IVF. Two studies paid attention on the effect of a history of subfertility on the child’s cognitive outcome. Zhu et al. reported that a longer TTP may be associated with a delay in achieving certain milestones, in particular cognitive and language development. It appeared that maternal age and parity were the most consistent factors associated with infertility or fertility treatment. Carson et al. performed a prospective population based cohort study and investigated whether pregnancy planning, TTP and ART treatment influenced cognitive development in 3 and 5-year-old children. In contrast to the findings of Zhu et al., the authors found that these factors did not adversely affect the child’s cognitive development at age 3 or 5 and that the slight differences found in the unadjusted statistical analyses – findings in favour of the ART children – were entirely attributable to socioeconomic differences among the participants.

Behavioural development

As already mentioned, ART is associated with perinatal adversities such as low birthweight and preterm delivery. These perinatal adversities, in turn, are, together with other ART-related risk factors such as advanced maternal age, unwanted childlessness, and obesity, associated with neurodevelopmental disorders, such as attention deficit hyperactive disorder (ADHD) and autism spectrum disorders (ASD). Therefore, the main focus of studies on behavioural development in ART children is on ADHD and ASD. The review by Middelburg et al. concluded that ART is not associated with adverse behavioural development during the first postnatal years. But again, early development is not a good predictor for the development of the aforementioned minor developmental problems. A review by Wagenaar et al. reported no differences in the prevalence of behavioural and socio-emotional problems in ART and NC children up to the age of 8. Hereafter, several studies reported additional reassuring findings on long-term behavioural outcome, with
similar behavioural outcome in ART children compared to NC children. However, some unfavourable findings were reported as well in IVF children, varying from more vocalization and higher energy levels, withdrawn and depressive behaviour and behavioural problems, particularly in boys. A pilot study by Zachor and Itzcak on the association between assisted conception and the risk of ASD in 8 months to 18-year-old children demonstrated that ART appears to be a significant independent risk factor for ASD. Beydoun et al. evaluated the quality of life and susceptibility for chronic disease development in 21-year-old ART children by means of self-administered questionnaires. The authors reported a slightly increased risk of depression (15.6% compared to the expected 12.7% lifetime prevalence up to age 25) and a significantly increased prevalence of binge drinking in the ART group compared to a control group (55% versus 37%). However, this may not be fully representative for the ART population as less than one-third of participants completed the questionnaire.

The overview indicates that we are not well informed about the long-term consequences of assisted conception and subfertility-related aspects on a child’s neurodevelopment. We only have sufficient information on short-term neurodevelopmental outcome. However, as indicated before, early development is only a moderate predictor of long-term development, as children may grow into neurodevelopmental deficits at older age. In order to understand the latter concept, I will now briefly summarize brain development.

**Brain development**

The human nervous system already starts to develop when the embryo is merely 2 weeks old. Around 3 to 4 weeks of embryonic life a hollow cylindrical structure, the neural tube, is formed, originating from the ectodermic neural plate, the source of the majority of neurons and glia cells in the mature human. The tube sinks under the surface of the skin, the forward end enlarges and differentiates into the forebrain, midbrain, and spinal cord. The fluid-filled cavity within the neural tube becomes the central canal of the spinal cord and the four ventricles of the brain, with the fluid being the cerebrospinal fluid. Its interaction with the surrounding mesoderm gives rise to the dura, the cranium, and the vertebrae. A process of neuronal proliferation, migration, organization and myelination follows, events that span a period from 9 weeks to adult life. Brain mass develops by the production of new cells, i.e. cell proliferation, from particular regions of the expanding neural tube, mainly between 8 to 16 weeks. Some cells continue to divide and redivide as stem cells, whereas other cells differentiate into neurons and glia cells, the main cellular components of the brain. Mainly at 12 to 20 weeks, the newly generated neurons and glia cells migrate towards particular areas of the outer layer of the neural tube by following specific chemical paths of immunoglobulins and chemokines, eventually forming the cortex and subcortical nuclei. By 20 to 24 weeks, the human cerebral cortex essentially has its full complement of neurons. Hereafter, a delicate process of organizational events occur, mainly from 20 weeks to several years after birth. Once the neurons have reached their final destination in the outer
layer, they extend axons and dendrites and connect them to synapses. The resulting white matter represents the cortical connectivity. Dendritic development accelerates from the third trimester onwards to the end of the first postnatal year and on a lower level till about the age of 5.\textsuperscript{131} Like all neurodevelopmental processes, the progress of the dendritic differentiation depends on the establishment of afferent input and presumably synaptic activity. Synaptic formation differs considerably among brain regions. The synaptogenesis starts early during fetal life with a peak at 34 to 36 weeks and some decline after birth.\textsuperscript{128-130} Initially, synapses make unspecified functional connections, resulting in an overproduction of synapses. Some of the synaptic contacts are utilised in emerging neuronal circuits, whereas those that remain unspecified are eliminated by means of programmed cell death, i.e. apoptosis.\textsuperscript{128} Not only synapses are eliminated. Selective elimination of other neuronal processes co-occur. The phase of such regressive events is critical to eliminate abnormal projections and to match the number of incoming axons to the number of receiving cells.\textsuperscript{129,130} Even though apoptosis depends on endogenous programmed processes, animal experiments indicate that enriched behavioural experience during the period of programmed cell death results in an increased level of neural cells in adult life.\textsuperscript{131} Next to the synaptic reorganisation, myelination is another long-lasting human neurodevelopmental event, that starts in the second pregnancy trimester and continues throughout a major part of adult life.\textsuperscript{128-130} Myelination involves the producing of insulating fatty sheaths around axons by glia cells in order to accelerate transmission.

Thus, it becomes clear that the development of the human nervous system is a delicate, dynamic and ongoing process that already starts early in gestation and continues into adulthood, with different brain areas maturing at different times and rates.\textsuperscript{128,131} Between birth and adulthood the human brain increases in size from around 100 grams at birth to 1000 grams by the end of the first year, close to the adult weight of 1200 to 1500 grams.\textsuperscript{128-130} The resulting continuous synaptic reorganisation forms the basis of neural development and brain plasticity. Experience modifies development: within limits, the human brain has evolved the ability to remodel itself by reorganizing neural pathways in response to experience. The continuous functional and structural changes during pre- and postnatal life have important clinical consequences and implications for the prediction of functional outcome.\textsuperscript{90,128,131,132} The age-specific brain of the child determines the way in which neural (dys)function is expressed. Children can only display (minor) deviations in functions which belong to their age-specific abilities. In addition, specific neurodevelopmental events result in specific vulnerability for impaired neurodevelopmental outcome, depending on the maturation and development of the brain at the time of the event. Circumstances that would almost certainly lead to severe neurological dysfunction in an adult can have quite different consequences for children. Moreover, children that initially have normal neurological function at an early age may grow into a functional neurological deficit, as with increasing age the complexity of neural functions increases. A simple example of such a ‘growing in a deficit’ phenomenon may be dyslexia, a disorder that only comes to light when children start learning reading and writing. Neurodevelopmental
changes may result in the opposite as well, meaning that early dysfunction may disappear at later age. The latter is seen during puberty, which is related to neurological improvement, presumably due hormonal influences. Adolescence is a sensitive period for steroid-dependent brain organization. The elevated levels of gonadal steroid hormones trigger the (re)organisation of neural circuits and increase the white matter integrity.

Aim of the thesis

The general aim of this thesis is to evaluate the effects of assisted conception and subfertility-related aspects on neurodevelopmental outcome in preschool-aged children, ranging from 4 months to 4 years of age. Data of two parallel running projects, the Groningen ART cohort study and the PGS study, are used. Both projects will be outlined below. The specific research questions addressed in this thesis are:

- Do ART-related aspects such as ovarian hyperstimulation, the in vitro laboratory procedures, or a combination of both affect neurodevelopmental outcome
  - in terms of movement variation in children of 4, 10 and 18 months of age?
  - in terms of neuromotor function in children of 2 and 4 years of age?
  - in terms of cognition and behaviour in children of 4 years of age?
- Do subfertility-related aspects such as the presence of a history of subfertility, the underlying cause of subfertility and the severity of subfertility affect neurodevelopmental outcome in terms of neuromotor function, cognition and behaviour in children of 2 and 4 years of age?
- Does PGS in addition to conventional IVF or ICSI affect neurodevelopmental outcome in terms of neuromotor function, cognition and behaviour in children of 4 years of age?

The Groningen ART cohort study

The Groningen ART cohort study is a prospective assessor-blinded longitudinal follow-up study that primarily aims to evaluate the effects of ovarian hyperstimulation, the in vitro procedure, and the combination of both on neurodevelopmental outcome in children born following ART, including both IVF and ICSI. Pregnant 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 (UMCG). All couples who achieved a singleton pregnancy following IVF or ICSI were invited to participate in the study. This resulted in a group of children born following controlled ovarian hyperstimulation IVF or ICSI (COH-IVF) and a group of children born following IVF or ICSI in a modified natural cycle (MNC-IVF), in which no ovarian hyperstimulation was applied and the follicle that developed naturally to dominance was used for assisted conception (Figure I and II). All oocytes were cultured in human tubal fluid (HTF) medium (Lonza, Verviers, Belgium) and supplemented with 10% plasma solution (Sanquin, Amsterdam, The Netherlands). Couples that were treated with cryopreserved or donated oocytes or embryos to achieve pregnancy were excluded. A third group was formed by naturally conceived children born to subfertile parents (Sub-NC). These couples had tried to conceive
General introduction

for at least one year and eventually conceived naturally while on the waiting list for fertility evaluation or treatment (Figure I and II). Potential differences in neurodevelopmental outcome of COH-IVF and MNC-IVF children may largely be attributed to ovarian hyperstimulation, whereas potential differences in MNC-IVF and Sub-NC children may largely be attributed to the in vitro procedure (Figure I).

The secondary aim of the Groningen ART cohort study is the evaluation of the effect of three aspects of subfertility on neurodevelopmental outcome in children born following ART: the presence of subfertility, the underlying cause of subfertility and the duration of subfertility.

In order to evaluate the effect of the presence of a history of subfertility, a reference group was retrospectively recruited at the assessment ages of 2 and 4 years, consisting of children born to fertile parents who conceived naturally (Figure I and II). The reference groups were recruited at six child welfare centres in and around Groningen between February and October 2009 for the 2-year assessment and December 2009 and February 2012 for the 4-year assessment. All parents of the children who visited the child welfare clinics for routine general health care were invited to participate. Children of parents who had tried to achieve a pregnancy for more than one year or who achieved a pregnancy by any form of assisted conception were excluded.

The underlying cause of subfertility and the duration of subfertility were studied in the three ART study groups. The causes of subfertility were classified as 'tuba pathology' in case of abnormalities of the fallopian tubes, as 'male factor' in case of male infertility, as 'other causes' in case of endometriosis, cervical factor or hormonal cause and as 'unknown cause' in case of lack of a specific cause for subfertility. The duration of subfertility was expressed in terms of time to pregnancy (TTP) as a proxy for the severity of subfertility. The notion that TTP can be used as a proxy for the severity of subfertility is based on models that have shown that TTP is an important predictor of the chance of pregnancy in subfertile couples. TTP was defined as the interval between the start of timed unprotected intercourse or a previous pregnancy and conception, recorded in years and months and finally converted into decimal years.

So far, the Groningen ART cohort study revealed that ART has not been associated with adverse neurodevelopmental outcome up until the age of 18 months, i.e. at the ages of 2 weeks, and 3, 4, 10 and 18 months. At the assessment age of 3 months it was possible to compare developmental outcome of the Groningen ART cohort children with that of a reference group. This group consisted of a representative sample of the general Dutch population, as the infants had been assessed as a part of a general health check-up provided for all infants. The data showed that neurological condition of the Groningen ART cohort children at 3 months of age was less favourable than that of 3-month-olds of the general population, suggesting that neurological sub-optimality at 3 months in children born after ART may rather be related to factors associated with subfertility than to ART techniques per se.
FIGURE I. The Groningen ART cohort study. The effects of ovarian hyperstimulation, the IVF laboratory procedure, the combination of both and a history of subfertility are studied by four different comparisons. The groups controlled ovarian hyperstimulation IVF (COH-IVF), modified natural cycle IVF (MNC-IVF) and natural conception in subfertile couples (Sub-NC) were pooled to form the subfertile group.
The PGS study

The PGS study is a multicentre double-blind randomized clinical trial performed in the UMCG and Academic Medical Center (AMC), originally elaborated to evaluate the effectiveness of PGS by comparing ongoing-pregnancy rates after IVF with and without PGS in women of advanced maternal age (‘PGS for aneuploidies in IVF’, ZonMw project number 945-03-013). Inclusion criteria for participating women were a conception age ranging from 35 to 41 years, having no previously failed IVF-cycles and having no objections against a possible double embryo transfer. Randomization of women was performed centrally, using a computer program, with minimization for age (35-37 or 38-41 years) and reproductive technology (IVF or ICSI), and with stratification according to study centre prior to the start of the IVF procedures. Group status was revealed to participating parents after 12 weeks of gestation.

Linked to this study, a follow-up study of children born after PGS was started in which all children born to women included in the double-blind randomized PGS-trial were examined.
for the physical and mental health during childhood (Figure III). Prior to inclusion and randomization in the original PGS trial, couples were informed about the follow-up evaluation as part of the PGS trial. For practical reasons, children born after treatment in the UMCG were invited to participate in an extensive follow-up program similar to that of the Groningen ART cohort at the ages of 2 weeks, 3, 4, 10, 18 months, 2 and 4 years, whereas children born after treatment in the AMC were only invited for assessments at the age of 2 and 4 years.

So far, the PGS study revealed that neurodevelopmental outcome up until 2 years of age in children born following IVF with PGS was largely similar to that of children born following IVF without PGS.\textsuperscript{141,142} However, application of the detailed neurological optimality score revealed that children born after IVF with PGS had a somewhat less optimal neurological condition at 2 years than children born after IVF without PGS. This may suggest a less favourable neurological development in children born following PGS.

**Neurodevelopmental assessments and outcome parameters**

In children, it is important to perform an age-specific assessment, since the young brain is continuously subjected to developmental changes. The following assessments were applied in the studies in this thesis at different ages.

**Infant Motor Profile (IMP)**

The IMP is a video-based instrument that evaluates neuromotor condition in terms of the quality of spontaneous motor behaviour in infants aged 3 to 18 months.\textsuperscript{143} Its 80 items are organized in five domains, three traditional neuromotor domains (symmetry, fluency and performance) and two novel domains, variation and variability, that are based on the Neuronal Group Selection Theory (NGST).\textsuperscript{144,145} The first novel domain, variation, denotes the size of a child’s movement repertoire. Evidence is accumulating that movement variation is a better predictor of developmental outcome than the traditional neurological examination.\textsuperscript{146} Reduced variation is associated with early lesions of the periventricular white matter.\textsuperscript{145,147-149} and, more generally, may reflect the integrity of cortical connectivity.\textsuperscript{150} Moreover, reduced variation during infancy may be associated with neurodevelopmental disorders later in life\textsuperscript{145,151} and reduced intelligence at school age.\textsuperscript{152} The second novel domain, variability, reflects the ability to select adaptive motor strategies out of the repertoire. Variability in motor behaviour emerges during the first 18 postnatal months and is often deviant in children with a developmental disorder, regardless of the absence or presence of overt brain injury.\textsuperscript{145} The limited ability to select the best strategy for a specific situation is primarily due to deficiencies in the processing of sensory information associated with the child’s motor actions.\textsuperscript{145,147}

In infants aged 3 to 6 months the total IMP score consists of the mean of four domain scores, i.e. all domains except the variability domain. In infants older than 6 months the total IMP score consists of the mean of all five domain scores, i.e. it includes also the variability domain. Prior and up to 6 months the infant’s limited variability precludes
inclusion of the variability domain into the total score. Total IMP scores and domain scores were expressed in percentages, varying from 0 to 100%. The reliability of the IMP is good and the construct validity is satisfactory.

The neurological examination according to Hempel
At 2 and 4 years, the neurological examination according to Hempel was applied. This is a standardized, precise and age-specific tool to assess minor neurological dysfunction (MND) at preschool age by means of five domains of functions: fine motor function, gross motor function, posture and muscle tone, reflexes and visuomotor function. Each of the domains can be scored as typical or deviant.

Children are classified as being neurologically normal, having simple MND, having complex MND, or being neurologically abnormal. Neurologically normal implies the absence of neurological dysfunction; it implies the absence of deviant domains or the isolated presence of dysfunction in the domain of reflexes. Simple MND indicates the presence of one deviant domain (except the domain of reflexes) and is regarded as a non-optimal but normal form of brain function. Complex MND indicates the presence of more than one domain of dysfunction. It represents the clinically relevant form of MND, as it is associated with prematurity and other perinatal adversities, and learning and behavioural disorders at later age. Neurologically abnormal implies the presence of a distinct neurological syndrome such as CP.

The outcome of the Hempel assessment was also expressed in a neurological optimality score (NOS). The NOS consists of 58 items (range 0 – 58) for 2-year-olds and 56 items (range 0 – 56) for 4-year-olds, for which an optimal condition is defined. The total score results from the sum of the items which fulfil the criteria for optimality. Higher scores represent better performance. Note that the range for optimal behaviour is narrower than for normal behaviour. The application of the optimality concept turns the NOS into a sensitive tool to evaluate neurological integrity. The fluency score (2-year-olds: range 0 – 13, 4-year-olds: 0 – 15) is a subscore of the NOS that evaluates the fluency of motor behaviour. The fluency score is a sensitive measure to detect subtle changes in neuromotor development, as minor dysfunction of the nervous system already results in a reduction of fluency of motion. The inter-rater reliability of the Hempel assessment is satisfactory (κ = 0.62 – 1.00 [mean 0.93]) and its construct validity is good.

The Kaufman Assessment Battery for Children, second edition (K-ABC-II)
The K-ABC-II is an individually administered standardized clinical instrument that measures cognitive and processing abilities in children and adolescents in the age of 3 to 18 years. Cognitive and processing abilities are expressed in a total intelligence quotient (IQ) score (i.e. Fluid-Crystallized Index) and four IQ scale scores: 1) a sequential processing IQ, reflecting short-term memory, 2) a simultaneous processing IQ, reflecting spatial aptitude, 3) a learning ability IQ, reflecting long-term memory capacity and 4) a knowledge IQ, reflecting general knowledge. Raw test scores are normalized into global scores with a
mean of 100 (SD: 15). The K-ABC-II has been used for children with different social backgrounds or ethnic differences without critical effects on test-scores. Reliability and validity of the K-ABC-II are good. The original American norms were applied as Dutch norms are lacking.

The Child Behavior CheckList (CBCL)
The CBCL is a parental questionnaire which is used to identify emotional and behavioural problems in children aged 1.5 to 5 years. The questions on the CBCL are classified into the following problem scales: emotionally reactive, anxious/depressed, somatic complaints, withdrawn, sleep problems, attention problems and aggressive behaviour. The first four scales together form the internalizing scale; the latter two scales form the externalizing scale. The sum of all questions results in the total problem scale. Raw test scores are normalized into T-scores with a mean of 50 (SD: 10). Higher T-scores represent more problematic behaviour: T-scores < 60 are in the normal range of behaviour, T-scores between 60 and 63 represent borderline behaviour, and T-scores > 63 are in the clinically abnormal range of behaviour. In the papers of this thesis, the T-scores were used in the analyses. The reliability and validity of the CBCL are good. In this thesis, the validated Dutch version of the CBCL was used.

Outline of the thesis
The evaluation of the potential effects of ART and subfertility-related aspects on neurodevelopmental outcome in preschool-aged children is divided in two sections, related to the aforementioned research questions.

Part I: The Groningen ART cohort study
Chapter 2 describes the neuromotor development in terms of movement variation in the children of the Groningen ART cohort at the age of 4, 10 and 18 months.
Chapter 3 describes the neurological condition of the children of the Groningen ART cohort at the age of 2 years in terms of neurological optimality (fluency score, NOS) and MND. Potential effects of the presence and the underlying cause of subfertility on child neurological condition are evaluated.
Chapter 4 describes the effect of the duration of subfertility (in terms of TTP, a proxy for the severity of subfertility) on the neurological condition of the children of the Groningen ART cohort at 2 years, again, in terms of neurological optimality and MND.
Chapter 5 describes the effect of both ART-related aspects such as ovarian hyperstimulation and the in vitro laboratory procedures, and subfertility-related aspects, such as the presence, the underlying cause, and the severity of subfertility on neurological condition of the children of the Groningen ART cohort at the age of 4 years, in terms of neurological optimality and complex MND.
Chapter 6 describes an explorative approach on the effect of both ART-related aspects such as ovarian hyperstimulation and the in vitro laboratory procedures, and subfertility-related
aspects, such as the presence, the underlying cause and the severity of subfertility on cognitive and behavioural development of the children of the Groningen ART cohort at the age of 4 years. Causal inference search algorithms and structural equation modelling were applied as statistical tools in order to unravel underlying causal mechanisms and to distinguish in intermediate and confounding effects.

Part II: The Groningen PGS study

Chapter 7 describes the effect of preimplantation genetic screening in addition to IVF on neurodevelopmental outcome, in terms of neuromotor, cognitive and behavioural development in children of the PGS study at the age of 4 years.

Chapter 8 consists of a general discussion of the findings, the final conclusions, the strengths and limitations of the studies in this thesis, and future perspectives. Chapter 9 and 10 summarize the content of this thesis in English and Dutch respectively.