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Published in:
Reproductive Biomedicine Online

DOI:
10.1016/j.rbmo.2016.08.021

Reproductive BioMedicine Online (2016) 33, 752–762

www.sciencedirect.com
www.rbmonline.com

PERICONCEPTION, PREGNANCY AND CHILD OUTCOMES

ARTICLE

Subfertility factors rather than assisted conception factors affect cognitive and behavioural development of 4-year-old singletons

Pamela Schendelaar [a], Sacha La Bastide-Van Gemert [b], Maas Jan Heineman [c], Karin J Middelburg [d], Jorien Seggers [a], Edwin R Van den Heuvel [d], Mijna Hadders-Algra [a,*]

[a] *University Medical Center Groningen, Department of Paediatrics, Division of Developmental Neurology, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands;* [b] *University Medical Center Groningen, Department of Epidemiology, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands;* [c] *Department of Obstetrics and Gynaecology, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands;* [d] *Department of Department of Mathematics and Computer Science, Division of Stochastics W&I, Eindhoven University of Technology, De Rondom 70, 5612 AP Eindhoven, The Netherlands*

* Corresponding author. *E-mail address:* m.hadders-algra@umcg.nl (M Hadders-Algra).

Pamela Schendelaar is a medical doctor and researcher at the Institute of Developmental Neurology, Department of Paediatrics, The University Medical Center Groningen. In February 2015, she obtained her PhD at the University of Groningen with the thesis 'Offspring of subfertile couples: neurodevelopmental outcome at preschool age'.

Abstract Research on cognitive and behavioural development of children born after assisted conception is inconsistent. This prospective study aimed to explore underlying causal relationships between ovarian stimulation, in-vitro procedures, subfertility components and child cognition and behaviour. Participants were singletons born to subfertile couples after ovarian stimulation IVF (n = 63), modified natural cycle IVF (n = 53), natural conception (n = 79) and singletons born to fertile couples (reference group) (n = 98). At 4 years, cognition (Kaufmann-ABC-II; total IQ) and behaviour (Child Behavior Checklist; total problem T-score) were assessed. Causal inference search algorithms and structural equation modelling was applied to unravel causal mechanisms. Most children had typical cognitive and behavioural scores. No underlying causal effect was found between ovarian stimulation and the in-vitro procedure and outcome. Direct negative causal effects were found between severity of subfertility (time to pregnancy) and cognition and presence of subfertility and behaviour. Maternal age and maternal education acted as confounders. The study concludes

http://dx.doi.org/10.1016/j.rbmo.2016.08.021

that no causal effects were found between ovarian stimulation or in-vitro procedures and cognition and behaviour in children aged 4 years born to subfertile couples. Subfertility, especially severe subfertility, however, was associated with worse cognition and behaviour.

KEYWORDS: assisted reproduction techniques, causal inference search algorithm, cognitive and behavioural outcome, ovarian stimulation, subfertility

Introduction

Up to 5% of European newborns are born after assisted reproduction techniques (Kupka et al., 2016). Consequently, their development and health is of general significance. Assisted reproduction techniques are associated with perinatal adversities (Pandey et al., 2012; Sutcliffe and Ludwig, 2007). These perinatal adversities, in turn, are associated with neurodevelopmental disorders, such as attention deficit hyperactive disorder and learning disabilities (Bhutta et al., 2002; De Jong et al., 2012b; Murray et al., 2016). Yet, it seems that assisted reproduction techniques are not associated with adverse cognitive and behavioural development during the first postnatal years (Middelburg et al., 2008; Yeung et al., 2016). Although reassuring, it does not preclude an association with impaired development at later age. Although only subtle signs or even no symptoms are present at early childhood, children may still grow into neurodevelopmental deficits at older age, as it may take time for developmental disorders to emerge (Hadders-Algra, 2002). In addition, over time, genetic effects on intelligence and behaviour may get increasingly expressed owing to the long-lasting interaction with social conditions (Bates et al., 2013; Brendgen et al., 2015).

Various factors related to assisted reproduction techniques could potentially interfere with development, such as ovarian stimulation (Griesinger et al., 2008), in-vitro procedures (Olivennes et al., 1993), the effect of underlying subfertility on time to pregnancy (TTP), (Basso and Baird, 2003; Draper et al., 1999; Jaques et al., 2010; Raatikainen et al., 2012) and the effect of parental characteristics on educational level (Jolly et al., 2000; Salem Yaniv et al., 2010; Tornqvist et al., 2010).

Results of long-term studies on cognitive and behavioural development in children born via assisted reproduction techniques vary, partly owing to difficulties in distinguishing relationships between assisted reproduction techniques and underlying characteristics of subfertility, parents and child. Other factors that may explain the diversity in study outcomes may be the age at which children were studied (varying from 5–26 years), total sample sizes (varying from 69–45.557 children), sex ratios (the proportion of females varying from 37.5–52%) and the instruments used to assess outcome (among others the Teacher's Report Form, Stanford-Binet Intelligence Scale, Revised Amsterdam Child Intelligence Test, Iowa Tests of Basic Skills/Educational Development, Wechsler Intelligence Scale for Children-Revised). As a consequence, cognitive and behavioural outcome of children born via assisted reproduction techniques has been reported as similar (Leslie et al., 2003; Leunens et al., 2008; Ludwig et al., 2009; Wagenaar et al., 2009b; Zhu et al., 2011), worse (Knoester et al., 2007, 2008; Goldbeck et al., 2009; Wagenaar et al., 2009a; Zhu et al., 2009; Beydoun et al., 2010; Gucuyener et al., 2011) or better (Leunens et al., 2006; Mains et al., 2010) than that of naturally conceived children.

To investigate the influence of specific factors involving assisted conception on developmental outcome, we composed the Groningen assisted reproduction technique cohort. It consists of three groups of singletons born to subfertile couples: children born after conventional ovarian stimulation with IVF and intracytoplasmic sperm injection (ICSI), children born after modified natural cycle IVF–ICSI (MNC–IVF), and children born after natural conception (Sub–NC) (Middelburg et al., 2010). We previously reported on neurodevelopmental and cardiometabolic outcome of the Groningen assisted reproduction technique cohort until 4 years of age. Developmental outcome of the groups was similar up to the age of 2 years (Middelburg et al., 2009, 2010; Schendelaar et al., 2011, 2013). A negative association was found between the duration of subfertility (TTP) and neurological condition at 2 and 4 years, suggesting that the severity of subfertility rather than its presence or the assisted reproduction technique components affect neurological outcome (Schendelaar et al., 2014; Seggers et al., 2013). Cognitive and behavioural development within the Groningen assisted reproduction technique cohort was studied up to the age of 2 years. We reported no differences in cognitive and behavioural outcome between the three study groups. Children of the three subfertile study groups, however, had higher scores on anxious and depressed behaviour than the children from a reference group born to fertile parents (Jongbloed-Pereboom et al., 2011).

In a previous study at 4 years, we applied a causal inference approach to evaluate factors affecting anthropometrics and cardiovascular health. The study indicated direct positive effects of children born after OS–IVF and not of children born after MNC–IVF on outcomes, suggesting that ovarian stimulation was involved in worse cardiometabolic health (La Bastide-Van Gemert et al., 2014; Seggers et al., 2014).

Unlike traditional statistics, a causal inference approach is able to unravel underlying causal mechanisms and distinguish between confounders and intermediate effects. This makes it an appropriate tool to explore relationships between assisted reproduduction techniques and underlying characteristics of subfertility, parents and child. The primary aim of this study is to explore underlying causal relationships between ovarian stimulation, the in-vitro procedure and the combination of both and cognition and behaviour in 4-year-old singletons. Second, we aim to explore the underlying causal relationships between two aspects of subfertility and cognition and behaviour. We addressed the contribution of the presence of a history of subfertility by including a reference group of 4-year-old singletons born to fertile parents, and the duration of subfertility in terms of TTP, as a proxy for the severity of subfertility. We have chosen cognitive outcome as our primary outcome parameter, as cognition has a stronger neurobiological basis than behaviour (De Jong et al., 2012a; Hadders-Algra and Touwen, 1992).

Marterials and methods

Participants

Couples who achieved a singleton pregnancy after IVF–ICSI 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) (Middelburg et al., 2010). This resulted in two groups: OS–IVF and MNC–IVF, in which medication use was minimal and in which the follicle that develops naturally to dominance is used for assisted conception (for details see Pelinck et al., 2007, 2008). Couples who were treated with donated or cryopreserved oocytes or embryos to achieve pregnancy were excluded. A third group (Sub–NC) was formed by naturally conceived children born to subfertile couples. These couples had tried to conceive for at least 1 year and eventually conceived naturally while on the waiting list for fertility evaluation or treatment at the UMCG. For details of the Groningen assisted reproduction technique cohort study see **Figure 1** (Middelburg et al., 2010; Schendelaar et al., 2014).

For the present study, a new retrospective reference group was recruited between December 2009 and February 2012 at six child-welfare centres in and around Groningen. All parents of 4-year-old singletons who visited the child welfare clinic for routine general health care were invited to participate. Children of couples who had tried to achieve pregnancy for more than 1 year or achieved pregnancy by any form of assisted conception were excluded.

Setting

Prenatal, perinatal and demographic information was gathered by means of standardized charts during the first follow-up assessment at about 2 weeks after term (Middelburg et al., 2010). Information on the causes and treatment of infertility was retrieved from medical records. The causes of subfertility were classified as 'tubal 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 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. Note that, in case of spontaneous abortion, a new onset of TTP was defined. This explains why some participating subfertile couples in the present study had a TTP of less than 1 year (**Table 1**). In the fertile reference group, TTP was not recorded in months but in one-half-year periods (<0.5 year or 0.5–1.0 year).

Follow-up assessments were carried out by trained assessors supervised by a neurodevelopmental expert (MH-A), who

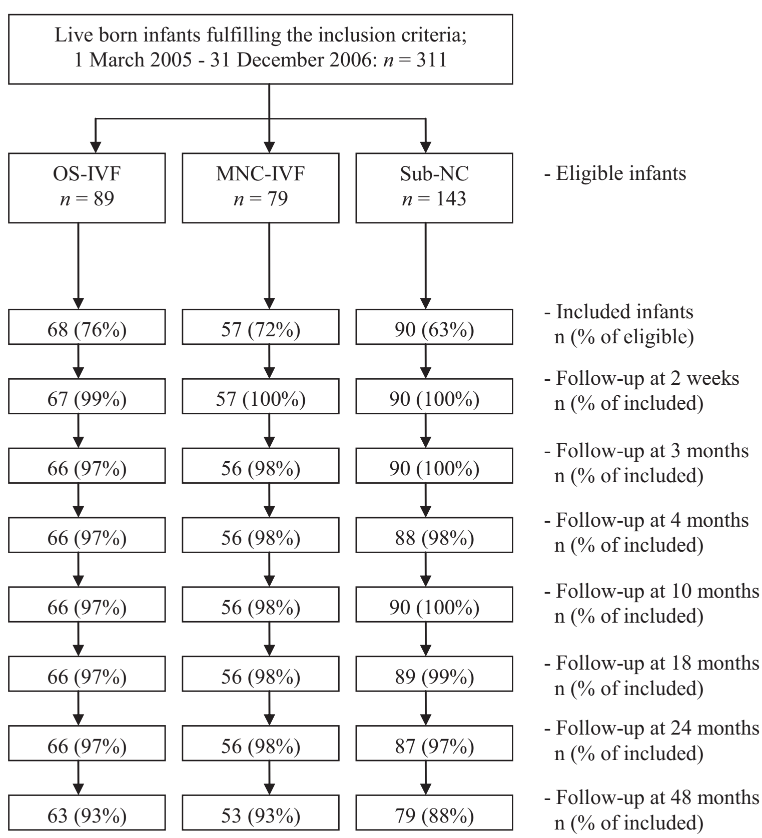

Figure 1 Flow chart of the Groningen assisted reproduction technique cohort.

Table 1 Characteristics of participating parents and children.

Characteristics	*OS–IVF*	*MNC–IVF*	*Sub–NC*	*Subfertile group*	*Reference group*
	n = *63*	n = *53*	n = *79*	n = *195*	n = *98*
Child characteristics					
Male gender, *n* (%)	34 (54.0)	26 (49.1)	41 (51.9)	101 (51.8)	54 (55.1)
First born, *n* (%)	43 (68.3)	37 (69.8)	49 (62.0)	129 (66.2)**	46 (46.9)**
Corrected age at examination at 4 years of age (months), median (range)	50 (47.5–60.1)	48.9 (48.0–52.5)	48.9 (47.9–56.4)	48.9 (47.5–60.1)	49.1 (48.0–54.6)
Birth characteristics					
Gestational age (weeks)[e], median (range)	39.4 (33.4–42.3)*	40.1 (34.6–42.6)	40.0 (30.1–42.7)*	40.0 (30.1–42.7)	40.1 (32.0–42.4)
Preterm birth (<37 weeks)[e], *n* (%)	7 (11.1)	6 (11.3)	5 (6.3)	18 (9.2)	3 (3.1)
Birthweight (g)[e], mean (sd)	3393.1 (563.2)*	3384.4 (585.7)*	3577.9 (519.4)*/*	3465.6 (557.2)*	3599.7 (507.0)*
Low birthweight (<2500 gram)[e], *n* (%)	3 (4.8)	4 (7.5)	3 (3.8)	10 (5.1)	2 (2.1)
Small for gestational age[a,e], *n* (%)	0 (0)	3 (5.7)	1 (1.3)	4 (2.1)	4 (4.3)
Signs of fetal distress[b], *n* (%)	19 (30.2)	15 (28.3)	34 (43.0)	68 (34.9)	29 (29.6)
Neonatal characteristics					
Apgar score 5 min <7[e], *n* (%)	0 (0)	0 (0)	1 (1.3)	1 (0.5)	3 (4.2)
Neonatal intensive care admission, *n* (%)	1 (1.6)	2 (3.8)	5 (6.3)	8 (4.1)	10 (10.2)
Parental characteristics					
Maternal age at conception in years[e], median (range)	32.3 (26.3–40.9)	32.8 (25.3–37.5)	33.0 (22.2–40.3)	32.8 (22.2–40.9)***	30.4 (18.8–40.5)***
Paternal age at conception in years[e], median (range)	35.4 (27.5–56.1)*	34.0 (28.3–47.8)*	35.0 (25.5–48.7)	35 (25.5–56.1)***	32.6 (22.5–45.1)***
Education level mother (high[c]), *n* (%)	20 (31.7)	20 (37.7)	37 (46.8)	77 (39.5)*	52 (53.1)*
Education level father (high[c])[e], *n* (%)	28 (46.7)	17 (32.7)	29 (36.7)	74 (38.7)**	53 (56.4)**
Smoking during pregnancy, *n* (%)	7 (11.1)	7 (13.2)	9 (11.4)	23 (11.8)	5 (5.1)
Alcohol consumption during pregnancy, *n* (%)	3 (4.8)	0 (0)	2 (2.5)	5 (2.6)	4 (4.1)
Divorced, *n* (%)	1 (1.6)	1 (1.9)	3 (3.8)	5 (2.6)**	11 (11.2)**
Fertility parameters					
Time to pregnancy in years;[d,e] median (range)	4.0 (0.0–13.5)***	4.0 (0.5–13.5)***	2.5 (0.5–11.5)***/***	3.5 (0.0–13.5)***	0.5 (0.5–1.0)***
Type of infertility (primary), *n* (%)	35 (55.6)	32 (60.4)	41 (51.9)	108 (55.4)	NA
ICSI performed; *n* (%)	41 (65.1)	26 (49.1)	NA	NA	NA

ICSI, intracytoplasmic sperm injection; MNC–IVF, infants born after modified natural cycle IVF or ICSI; OS–IVF: infants born after ovarian hyperstimulation IVF or ICSI; Sub-NC, naturally conceived infants born to subfertile parents. In the subfertile group COH–IVF, MNC–IVF and Sub–NC groups are taken together. TTP, time to pregnancy.

Mann–Whitney U tests, Student's t-test or Fisher's exact tests were carried out to investigate differences between groups.

**P* < 0.05; ***P* < 0.01; ****P* < 0.001. [a]Birth weight for gestational age is less than −2 SD compared with the Dutch reference population (Dutch reference tables, perinatal Registration Netherlands). [b]Signs of fetal distress denoted by meconium stained amniotic fluid, cardiotocographic signs, acidosis or all three. [c]University education or vocational colleges. [d]TTP was recorded in one-half-years. In case of a spontaneous abortion, the onset of TTP restarted, therefore TTP may be shorter than 1 year. [e]Overall missing data: gestational age (*n* = 1), preterm (*n* = 1), birthweight (*n* = 4), low birthweight (*n* = 3) , small for gestational age (*n* = 1) , Apgar score 5 min <7 (*n* = 31), maternal age at conception (*n* = 1), paternal age at conception (*n* = 8), education level father (*n* = 8), time to pregnancy (*n* = 1).

were blind to the mode of conception. Blinding was not possible for the reference group, as this group was recruited separately from the subfertile groups.

Measurements

Cognitive development was evaluated using the Kaufman Assessment Battery for Children, second edition (K-ABC-II, Kaufman and Kaufman, 2004). This standardized instrument measures in about 45 min cognitive and processing abilities in children aged between 3 and 18 years. Outcome is expressed in a total intelligence quotient (IQ) score and four IQ scale scores. In the present paper, only the total IQ score is used. Raw test scores are normalized into global scores (mean: 100; SD: 15). Reliability and validity of the K-ABC-II are good (Kaufman and Kaufman, 2004). The original American norms were applied as Dutch norms are lacking.

Behavioural development was evaluated using the validated Dutch version of the Child Behavior Checklist (CBCL) (Achenbach and Rescorla, 2000; Verhulst et al., 2000). The CBCL is a parental questionnaire to identify emotional and behavioural problems in children aged 1.5 years to 5 years, classified into problem scales. The sum of all questions results in the total problem scale score. Raw test scores are normalized into T-scores (mean: 50, SD: 10). Higher T-scores represent more problematic behaviour. The reliability and validity of the CBCL are good (Achenbach and Rescorla, 2000; Verhulst et al., 2000).

Statistical analyses

Fisher's exact tests, Mann–Whitney U-tests and Student's t-tests were carried out to investigate differences between groups using the IBM Statistical Package for the Social Sciences (SPSS), version 20 (IBM Corp., USA). $P < 5\%$ were considered statistically significant for differences between the subfertile group and the reference group. Bonferroni correction was applied for the comparisons between the assisted reproduciton technqiue study group, meaning that $P < 1.7\%$ was considered statistically significant.

Two separate sets of explorative analyses, each consisting of a dozen of explorations, using causal inference search algorithms were carried out. The first focused on assisted reproduction technique treatment effects (ovarian stimulation; in-vitro procedure; combination of both) on cognitive and behavioural outcome on the basis of the children of the subfertile couples of the Groningen assisted reproduction technique cohort. The second focused on subfertility effects (presence and severity of subfertility, defined as TTP >1 year and TTP as a continuous variable, recorded in one-half years, respectively) on outcome at 4 years on the basis of the assisted reproduction technique cohort children and the reference children born to fertile couples. The modelling takes into account causal relations of the 'focus' factors and other parental and child aspects.

Causal inference search algorithms result in causal models that are found to be compatible with the data, based on the theory of causal graphs. A causal model consists of a statistical model and a graph, in which the latter describes the causal relation between the variables and consists of vertices (the variables), connected by edges, which can be oriented by arrows. An oriented edge represents a direct causal effect between two connecting vertices. Conservative Peter-Clark (CPC), Greedy Equivalency Search (GES) and Conservative Fast Causal Inference (CFCI) algorithms were applied. For a concise overview about the terminology and theory of causal models and causal inference search algorithms, including references, please see refer to Supplementary material.

The resulting graphs *per se* represent an observationally equivalent class of causal models, i.e., other graphs from that class are equally likely to have generated the data. Model fit was calculated and compared between the found classes of models, by choosing one directed acyclic graph, representing such a class. We used the chi-square test, chi-squared test/degrees of freedom ratio and the Bayesian information criterion to compare models. A ratio of less than 2 suggests a good model fit. Additionally, structural equation modelling was applied to a graph that represents one of the better fitted classes of models to estimate effect size.

By comparing the various graphs resulting from the performed search algorithms, the most prominent and consistent direct effects could be distinguished. Additionally, effects that are consistently not found can be considered an indication of the absence of a causal effect. The explorative analyses were conducted using the freeware program TETRAD, version 403.10-6 (The Tetrad Project, 2010) and the sem library in R, version 2.15.0 (R Development Core Team, 2012).

Ethical approval

The Medical Ethical Commission of the UMCG approved the study design on 7 July 2009 (reference number M09.074824). Parents provided written informed consent for study participation of their child.

Results

Participation and demographic characteristics

At the age of 4 years, 20 (9.3%) children were lost to follow-up (**Figure 1**). Intra-group characteristics for participants and non-participants were similar, except for a longer TTP in participating MNC-parents compared with non-participating MNC-parents ($P = 0.021$). Demographic characteristics of the three groups are listed in **Table 1**.

Parents of 215 eligible singleton reference children were invited to participate in the present follow-up study. Ninety-eight (46%) parents allowed their child to participate. Demographic characteristics such as sex, gestational age, first-born, maternal age and parental educational levels were similar for participants and non-participants (data not provided). Demographic characteristics of participants of the subfertile and reference groups are listed in **Table 1**.

Effect of ovarian stimulation and in-vitro procedure

Most of the children in the three study groups ($n = 189$ [98%]) had an IQ within the normal range (scores between 85 and

115), except for two OS–IVF children (IQ: 82 and 79) and two MNC–IVF children (IQ: 79 and 77). Most children in the assisted reproduction technique study groups (n = 186 [97%]) also had total problem T-scores within the normal range, except for two OS–IVF children and four Sub–NC children. The mean IQ and total problem T-scores of the three assisted reproduciton technique study groups are presented in **Table 2**. Univariable analyses indicated no differences between the assisted reproduction technique study groups in mean IQ and total problem T-scores (**Table 1**) (Bonferroni correction $P < 0.017$). Moreover, no differences were found between the groups on the secondary outcome parameters mean IQ subscale scores and internalizing and externalizing behaviour. The same accounts for other behavioural parameters measured with the CBCL (emotionally reactive, anxious/depressed, somatic complaints, withdrawn behaviour, sleep problems, attention problems and aggressive behaviour; data not shown). Also, ICSI and specific causes of subfertility were not associated with IQ and behaviour (data not shown).

The causal graph that had the best model fit was the causal model found as a result of running the GES algorithm with a penalty discount of 0.5 (χ^2 = 98.5, df = 94, P = 0.356, chi-squares/df = 1.048, suggesting a good model fit). This model indicated the absence of direct effects of OS–IVF or IVF on cognitive or behavioural outcome given the other variables in the model. A direct effect of OS–IVF or IVF was also absent in causal graphs resulting from other searches (data not provided).

Presence and severity of subfertility

Most of the children of the subfertile group (n = 189 [98%]) had an IQ within the normal range, except for the four children mentioned above. None of the reference children had IQ scores below average (n = 97 [100%]). Most of the children in both groups (subfertile group n = 186 [97%]; reference children n = 92 [96%]) also had total problem T-scores within the normal range, except for six children of the subfertile group and four reference children. The mean IQ and total problem T-scores of the subfertile group and the reference group are listed in **Table 2**. Univariable analyses indicated that children of the subfertile group had significantly lower mean total IQ scores compared with children of the reference group ($P < 0.05$ (**Table 2**). No differences were found between the groups in problem T-scores. Moreover, no differences were found between the groups on the secondary outcome parameters mean IQ subscale scores and internalizing and externalizing behaviour. The same accounts for other behavioural parameters measured with the CBCL (data not shown).

The causal graph that presented the class of found models with the best model fit as a result of the causal effect search algorithms is represented in **Figure 2** (chi-squared = 84.7, df = 72, P = 0.145, chi-square/df = 1.176, suggesting a good model fit). It indicated that the presence of subfertility did not have a direct or indirect effect on the total IQ score. Similar results were found in resulting causal graphs from other searches (data not provided). The causal graph suggests that the total IQ score was directly affected by two factors: TTP and maternal smoking during pregnancy. A longer TTP was associated directly with a lower total IQ score. This effect was confounded by high maternal education through various paths: one path ran from maternal education via maternal and paternal age, via TTP to total IQ, whereas another path ran from maternal education via smoking during pregnancy to total IQ. The other direct effect on the child's total IQ score was the negative effect of maternal smoking during pregnancy, an effect that again was confounded by maternal education. Taking this complexity of the pathways into account and correcting for maternal education level, the direct, unconfounded effect of TTP on total IQ score is estimated at −0.712 (**Figure 2**).

The causal graph suggests that the presence of subfertility had a direct adverse effect on the total problem T-score of behaviour (**Figure 2**). As such, subfertility status acted as mediator on three different paths running from high maternal educational level on total problem T-score. These paths ran either via maternal age at child conception, or via TTP, or via a combination of both, including an effect of paternal age at child conception (**Figure 2**). It also indicates that maternal age directly affected the total problem T-score: a higher maternal age was associated with less child behavioural problems. Also in this path, maternal age mediated the effect of high maternal educational level on total problem T-score. Subsequently, maternal age at conception and high maternal educational level are considered to be confounders for the effect of subfertility status on the total problem T-score. The unconfounded, direct effect of the presence of subfertility on the total problem T-score is estimated as follows: conditionally on maternal age at conception, high maternal educational level, TTP, or both, the direct effect of subfertility status is estimated to be 3.003 for less optimal behaviour.

The causal graph also suggests a relationship between the outcome parameters, cognition and behaviour: an increase in total IQ score of one point was associated with a decrease in total problem T-score with 0.120 points, indicating better behaviour in case of a more optimal cognitive development (**Figure 2**). According to the causal implications, TTP has an indirect (mediated by subfertility status and total IQ score) effect on more problematic behaviour (**Figure 2**).

Discussion

The present study indicates that in 4-year-old singletons born to subfertile couples direct or indirect causal effects between ovarian stimulation or the in-vitro procedure on the one hand and cognitive and behavioural outcome on the other hand are absent. A direct causal relationship, however, was found between the severity of subfertility (TTP) and cognition at age 4 years. Also, a direct negative causal relationship between the presence of subfertility and behaviour was found. Both effects were confounded by maternal age at child conception and maternal educational level. The TTP had a direct negative effect on the child's IQ score, whereas the presence of subfertility had a direct adverse effect on behaviour. Given that cognition and behaviour are directly related to one another (with behaviour being influenced by cognition), our results suggest that the severity of subfertility has an indirect, through subfertility status as well as through IQ, effect on the child's behavioural scores, i.e., was associated with more behavioural problems. The association between a higher IQ and less behavioural problems corresponds to the findings

Table 2 Group differences in IQ and CBCL scores of participating children.

	Reference mean (SE)	*Mean difference [95% CI]*		*Reference mean (SE)*	*Mean difference [95% CI]*
Cognitive development	*Sub–NC*	*MNC–IVF*	*OS–IVF*	*Fertile reference group*	*Subfertile group*
	(n = 77)	*(n = 53)*	*(n = 63)*	*(n = 97)*	*(n = 193)*
Total IQ	108.9 (1.22)	–3.7 [–7.8 ; 0.5]	–2.8 [–6.8; 1.2]	110.4 (1.10)*	–3.5 [–6.3; –0.7]*
Sequential IQ	95.9 (1.31)	1.2 [–3.0; 5.4]	0.5 [–3.5; 4.5]	99.6 (1.18)	–3.2 [–6.0; –0.3]
Simultaneous IQ	115.5 (1.59)	–1.5 [–6.3; 3.4]	–2.4 [–7.0; 2.2]	117.8 (1.46)	–3.5 [–6.9; –0.0]
Learning IQ	98.3 (1.26)	–2.6 [–6.7; 1.5]	–1.5 [–5.4; 2.4]	99.6 (1.02)	–2.4 [–5.2; 0.3]
Knowledge IQ	112.7 (1.12)	–6.0 [–10.3; –1.8]	–4.4 [–8.5; –0.4]	111.3 (1.26)	–1.7 [–4.7; 1.4]
Behavioral development	*Sub–NC*	*MNC–IVF*	*OS–IVF*	*Fertile reference group*	*Subfertile group*
	(n = 78)	*(n = 52)*	*(n = 62)*	*(n = 96)*	*(n = 192)*
CBCL total problem T-score	47.8 (1.06)	–1.6 [–4.8; 1.5]	–0.5 [–3.4; 2.4]	44.4 (0.99)	2.2 [–0.0; 4.5]
CBCL internalizing T-score	48.0 (1.14)	–1.3 [–4.8; 2.3]	–3.0 [–6.3; 0.4]	45.0 (1.04)	1.8 [–0.7; 4.2]
CBCL externalizing T-score	48.7 (1.01)	–1.2 [–4.2; 1.9]	–0.5 [–3.4; 2.4]	46.2 (0.95)	2.0 [–0.2; 4.2]

MNC–IVF, infants born after modified natural cycle IVF or ICSI; OS–IVF, infants born after ovarian hyperstimulation IVF or ICSI; Sub-NC, naturally conceived infants born to subfertile parents. In the subfertile group OS–IVF, MNC–IVF and Sub–NC groups are taken together. CBCL, Child Behaviour Checklist.

Two children of the subfertile group (2 Sub–NC) and one reference child had missing data on the K-ABC-II. Three children of the subfertile group (1 OS–IVF, 1 MNC–IVF and 1 Sub–NC) and two reference children had missing data on the CBCL questionnaire.

*Significant mean differences in outcomes: $P < 0.05$ for differences between the subfertile group and the fertile reference group and $P < 0.017$ for differences between the ART study groups (Bonferroni correction applied).

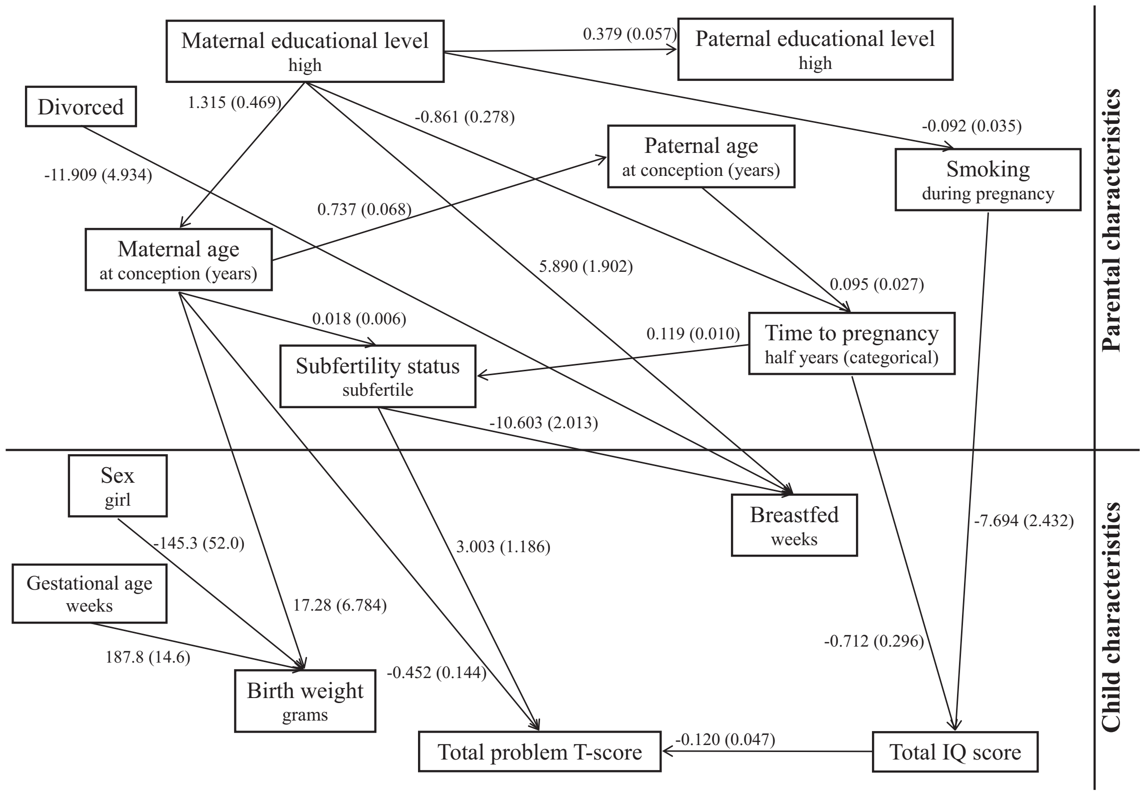

Figure 2 The underlying causal mechanism as found in the subfertile (OS–IVF, MNC–IVF and Sub–NC groups pooled) and fertile reference group regarding presence and severity of subfertility and cognitive and behavioural development. The causal graph representing the model with the best model fit of the causal inference search algorithms (Greedy Equivalency Search algorithm, penalty discount: 0.5; model fit: chi-squared = 84.7, df = 72, $P = 0.145$, chi-square/df = 1.176 and Bayesian information criterion score = −317.0). Each arrow in the graph is accompanied by two numbers, reflecting the size of the causal effect per unit change. The first number is the estimated regression weight and the second number between brackets is its corresponding standard error. A positive estimated regression weight in the graph means that higher values of the variable are associated with an increase in the value of the variable to which the arrow is pointing. Likewise, a negative estimated regression weight in the graph means that higher values of the variable are associated with a decrease in the value of the variable to which the arrow is pointing. Time to pregnancy was recorded in one-half-years. High educational level indicates university education or vocational colleges.

of Flouri et al. (2013) that a higher IQ may protect a child to develop behavioural problems in response to life stress events.

Our findings strengthen the impression that aspects of subfertility rather than components of assisted reproduction techniques are involved in determining cognitive and behavioural development of children born after IVF–ICSI. Our statistical approach also revealed important confounders for these effects, especially maternal age and education. In turn, the effect of the presence of subfertility on behavioural outcome was confounded by TTP. Our results imply that suffering from subfertility *per se*, especially from more severe subfertility, which in turn is affected by a higher maternal age and higher educational level, may negatively affect the child's cognitive and behavioural development. More studies, however, are needed to test the effects.

The fact that already known and generally expected mechanisms were also detected (**Figure 2**), underlines the general robustness and validity of our results. The graph with the best model fit of the GES search algorithm and also other graphs resulting from the GES searches with a good model fit showed similar mechanisms concerning the above-mentioned effects, indicating a certain consistency of the associaitons found between the variables. Similar effects of maternal age, maternal educational level and TTP on the outcomes were also found with the CPC search algorithm (model fit indices for the CPC-result with the best model fit: alpha value 0.15; model fit: chi-square = 72.1, df = 68, $P = 0.344$, chi-square/df = 1.060). The fact that similar mechanisms are revealed by two different algorithms in search approach and data assumptions, underline the validity of the found mechanisms. One might have expected to find direct effects of birth weight and gestational age on cognitive and behavioural development; however, our analyses did not result in such effects (**Figure 2**).

Birth weight is indeed associated with the total problem T-score via maternal age at conception, but this association disappeared after correction for maternal age. This is in line with our expectations: our groups contain few preterm and low birth weight infants. Therefore, it seems more probable that our analyses resulted in effects of subfertility and maternal factors on developmental outcome, rather than in effects of birth weight and gestational age.

The participating children in both the subfertile and the reference group had a relatively high IQ, which may be explained by the relatively large proportion of highly educated participating parents. The well-known positive association of maternal age and maternal education with child cognitive and behavioural development (Chapman et al., 2002) is also present in our material. The graphs illustrate the complexity of the relationships: on the one hand higher maternal age and better maternal education are associated with higher IQ-scores and lower behavioural problem scores, but on the other hand they are also associated with subfertility and prolonged TTP which, in turn, are associated with lower IQ-scores and higher behavioural problem scores.

To the best of our knowledge, this is the first study in the context of neurodevelopment in children born via assisted reproduction techniques using causal inference search algorithms combined with structural equation modelling. It allowed us to distinguish between direct and indirect causal effects and to detect and correctly adjust for confounders at the same time. Our findings that the severity of subfertility plays a role in cognitive development is in line with Zhu et al. (2009), who reported that longer TTP may be associated with a delay in achieving certain milestones, in particular those involved in cognitive and language development. In another study by Zhu et al. (2011), however, a subfertility effect on child behaviour could not be demonstrated.

To intelligibly answer our research questions, we conducted two separate sets of explorative analyses: one focusing on the effects of assisted reproduction technqiue treatment, the other focusing on the effects of presence and severity of subfertility on developmental outcome. The first exploration can only be translated to its particular (smaller) subfertile population and its findings do not extend to the different population that was used for the second exploration. In the latter analysis a reference group was added, allowing for the in concert evaluation of the effect of the presence of subfertility and that of its severity.

The search algorithm strategy was an important strength of the study. Additional strengths of the study are the attrition rate of 9.3%, blinding of assessors to the mode of conception and its prospective design with three subfertile groups.

It must be realized that the statistical tools used in our study are explorative in nature and especially serve as indications for new research hypotheses, meaning that our results need to be interpreted with appropriate caution. This caution, however, is not restricted to search algorithms alone: applying multiple sets of multivariable regression analyses is in fact also explorative in nature. Because of the explorative nature of our analyses a post-hoc power calculation is irrelevant. Previously group sizes of the Groningen assisted reproduction technique cohort were based on neurological outcome at 18 months (Hadders-Algra and Touwen, 1992). As a general rule for a structural equation model, 10 or more observations for each variable may be considered reliable, a criterion that we have met.

A more general limitation of the study is related to the reference group. The group was recruited retrospectively, and consequently, we were not blinded to group status of the children. In addition, only about one-half of the eligible families agreed to participate. This is a well-known phenomenon in the field of child development, also in the field of assisted reproduction techniques (among others Knoester et al., 2008; Leunens et al., 2008; Ludwig et al., 2009 but unfortunately rarely reported. Previous studies reported that parents who are concerned about their child's health in general are more likely to participate in developmental studies (Groen et al., 2005). This means, however, that the associations reported in the present study may have been underestimated. The relatively low participation rate in the reference group may be an indicator for selection bias. An additional limitation is that we used American norms instead of Dutch norms. It is difficult to know how this may have confounded our results. We assume, however, that this effect is minor compared with the effect of parental education, which we took into account.

In conclusion, our study implies that suffering from subfertility *per se*, and especially from more severe subfertility, which by itself is affected by higher age and high educational level of the mother, is associated with less favourable cognitive and behavioural outcome of the child. Long-term follow-up of development and growth of children born from subfertile couples, in particular when assisted reproduction technqiues is applied, remains important, given the steadily increasing prevalence of subfertility in modern society.

Acknowledgement

We thank participating parents and children for their cooperation and enthusiasm during the assessments; Arend F Bos, MD, PhD, for his help in initiating the study and Maaike Haadsma, MD, PhD, for her help in including the participants; Rosan Aapkes, MD, Elise Bennink, MSc, Hanneke Geut, MD, Marjolein Jongbloed-Pereboom, MSc and Bertine de Vries, MSc, for support in collecting and organizing the data; Michiel Schrier, MSc, Linze Dijkstra BSc, and Loes de Weerd for technical assistance. The study was financially supported by the University Medical Center Groningen, grant number: 754510, the Junior Scientific Masterclass, the Postgraduate School Behavioural and Cognitive Neurosciences and the Cornelia Foundation, Groningen, The Netherlands.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2016.08.021.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 13 February 2016; refereed 21 August 2016; accepted 23 August 2016.