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PROTOCOL

Health and fertility of ICSI-conceived young men: study protocol

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STUDY QUESTIONS: What are the long-term health and reproductive outcomes for young men conceived using ICSI whose fathers had spermatogenic failure (STF)? Are there epigenetic consequences of ICSI conception?

WHAT IS KNOWN ALREADY: Currently, little is known about the health of ICSI-conceived adults, and in particular the health and reproductive potential of ICSI-conceived men whose fathers had STF. Only one group to date has assessed semen parameters and reproductive hormones in ICSI-conceived men and suggested higher rates of impaired semen quality compared to spontaneously conceived (SC) peers. Metabolic parameters in this same cohort of men were mostly comparable. No study has yet evaluated other aspects of adult health.

STUDY DESIGN, SIZE, DURATION: This cohort study aims to evaluate the general health and development (aim 1), fertility and metabolic parameters (aim 2) and epigenetic signatures (aim 3) of ICSI-conceived sons whose fathers had STF (ICSI study group). There are three age-matched control groups: ICSI-conceived sons whose fathers had obstructive azoospermia (OAZ) and who will be recruited in this study, as well as IVF sons and SC sons, recruited from other studies. Of 1112 ICSI parents including fathers with STF and OAZ, 78% (n = 867) of mothers and 74% (n = 823) of fathers were traced and contacted. Recruitment of ICSI sons started in March 2017 and will finish in July 2020. Based on preliminary participation rates, we estimate the following sample size will be achieved for the ICSI study group: mothers n = 275, fathers n = 225, sons n = 115. Per aim, the sample sizes of OAZ-ICSI (estimated), IVF and SC controls are: Aim 1—OAZ-ICSI: 28 (maternal surveys)/12 (son surveys), IVF: 352 (maternal surveys)/244 (son surveys), SC: 428 (maternal surveys)/255 (son surveys); Aim 2—OAZ-ICSI: 12, IVF: 72 (metabolic data), SC: 391 (metabolic data)/365 (reproductive data); Aim 3—OAZ-ICSI: 12, IVF: 71, SC: 292.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Eligible parents are those who underwent ICSI at one of two major infertility treatment centres in Victoria, Australia and gave birth to one or more males between January 1994 and January 2000. Eligible sons are those aged 18 years or older, whose fathers had STF or OAZ, and whose parents allow researchers to approach sons. IVF and SC controls are age-matched men derived from previous studies, some from the same source population. Participating ICSI parents and sons complete a questionnaire, the latter also undergoing a clinical assessment. Outcome measures include validated survey questions, physical examination (testicular volumes, BMI and resting blood pressure), reproductive hormones (testosterone, sex hormone-binding globulin, FSH, LH), serum metabolic parameters (fasting glucose, insulin, lipid profile, highly sensitive C-reactive protein) and semen analysis. For epigenetic and future genetic analyses, ICSI sons provide specimens of blood, saliva, sperm and seminal fluid while their parents provide a saliva sample. The primary outcomes of interest are the number of mother-reported hospitalisations of the son; son-reported quality of life; prevalence of moderate-severe oligozoospermia (sperm concentration <5 million/ml) and DNA methylation profile. For each outcome, differences between the ICSI study group and each control group will be investigated using multivariable linear and logistic regression for continuous and binary outcomes, respectively. Results will be presented as adjusted odds ratios and 95% CIs.

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WHAT DOES THIS MEAN FOR PATIENTS?

Intracytoplasmic sperm injection (ICSI) is a technique where a single sperm is injected into an egg to achieve fertilisation. It was introduced in 1992 to overcome poor semen quality (male factor infertility) and now represents the main type of assisted reproductive treatment worldwide. There is concern that using poor quality sperm, or the technique itself, may have a negative impact on the development and long-term health of children conceived using ICSI. Only one group so far has looked at the reproductive and metabolic health of adults born following ICSI.

We are inviting men conceived using ICSI in Australia (aged 18–25 years) to take part in a study about their general and reproductive health. These men had fathers who had either a problem with sperm production or a blockage to the sperm ducts. Parents are first invited to complete a questionnaire about themselves, and their sons' physical and psychological health, and educational and social development. With parental agreement, sons are then invited to complete a questionnaire and clinical assessment. We will compare our findings with two other groups of men of similar age, including men conceived by *in vitro* fertilisation and men conceived naturally.

There is likely to be a genetic cause of many types of male infertility. Therefore, we anticipate there will be some transmission of infertility to young men conceived using ICSI whose fathers had impaired sperm production. The wide range of other health outcomes to be examined will provide important information to couples and clinicians about the safety of ICSI, and identify areas in need of further study.

Introduction

ICSI has been in clinical use since 1992 for the management of male-factor infertility (Palermo *et al.*, 1992). Its use for all causes of infertility has escalated globally, although evidence indicates no benefit compared with conventional IVF in cases of non-male factor infertility (Bhattacharya *et al.*, 2001; Boulet *et al.*, 2015; Li *et al.*, 2018; Drakopoulos *et al.*, 2019). The long-term effects of underlying paternal infertility, and those of the technique itself, on offspring health and future fertility are not clear. In addition, the origin of male infertility is frequently unknown. Genetic factors, such as chromosomal abnormalities, account for at least 15% of male infertility and the prevalence increases to 25% among men with azoospermia (Yoshida *et al.*, 1997; Foresta *et al.*, 2001; Krausz and Riera-Escamilla, 2018). Infertile men have also higher rates of sperm aneuploidy (Rubio *et al.*, 2001), sperm DNA damage (Rex *et al.*, 2017) and epigenetic alterations (Marques *et al.*, 2004, 2010; Camprubi *et al.*, 2016; Sujit *et al.*, 2018) compared to their fertile counterparts. Such changes to the sequence and structure of DNA may have detrimental long-term effects on offspring health and development. In addition, the ICSI technique itself may disrupt normal epigenetic phenomena, as evidenced by DNA methylation variation in human studies of ART conception (Amor and Halliday, 2008; Lazaraviciute *et al.*, 2014; Novakovic *et al.*, 2019), with potential consequences for development and long-term health.

Compared with spontaneous conception, both IVF and ICSI are associated with an increased risk of neonatal and obstetric complications, congenital malformations and imprinting disorders (Hansen *et al.*, 2005; Pandey *et al.*, 2012; Wen *et al.*, 2012; Lazaraviciute *et al.*, 2014; Qin *et al.*, 2015). Most studies of neurodevelopment demonstrate that ICSI-conceived children are comparable to their spontaneously conceived (SC) and IVF-conceived peers (Catford *et al.*, 2017, 2018). Data suggest no differences in growth and physical health between ICSI- and IVF-conceived children (Pinborg *et al.*, 2004a; Bonduelle *et al.*, 2005; Kai *et al.*, 2006; Knoester *et al.*, 2008; Basatemur *et al.*, 2010; Woldringh *et al.*, 2011), although evidence is mixed on neurodevelopmental disorders (Pinborg *et al.*, 2004b; Sandin *et al.*, 2013; Kissin *et al.*, 2015). Rates of autism, cancer risk, growth, vision and hearing of ICSI and SC offspring appear comparable (Kai *et al.*, 2006; Wikstrand *et al.*, 2006; Basatemur *et al.*, 2010; Ludwig *et al.*, 2010; Woldringh *et al.*, 2011; Ackerman *et al.*, 2014; Williams *et al.*, 2018). However, some data suggest that ICSI offspring may have worse physical health than SC offspring, indicated by increased surgical interventions, childhood illnesses and hospitalisations (Bonduelle *et al.*, 2004, 2005; Ludwig *et al.*, 2009). More recent studies suggest ICSI offspring may experience poorer reproductive and metabolic health compared to their SC peers (Belva *et al.*, 2012, 2016, 2017, 2018a,b). The clinical significance of many findings, however, remains unclear.

In men aged between 18 and 22 years, conceived by ICSI using ejaculated sperm, data from a Belgian cohort found increased rates of impaired spermatogenesis, as indicated by poorer semen quality and a tendency for higher FSH and lower inhibin B levels, compared to age-matched SC controls (Belva *et al.*, 2016, 2017). This study was limited by small sample size, lack of information on aetiology of paternal infertility and likely participation bias in the SC controls. Furthermore, it was not possible to differentiate the effect of the ICSI procedure from the background risk of paternal infertility, as only one control group of SC men were included. Further data from this cohort that focused on cardiometabolic parameters and adiposity revealed comparable results, with the exception of lower high-density lipoprotein cholesterol concentrations (Belva *et al.*, 2018a) and a higher peripheral fat deposition in ICSI-conceived men (Belva *et al.*, 2018b).

As the first generation of ICSI-conceived children have recently entered adulthood, there is a critical need to determine if there are any paternally inherited, or technique-associated, adverse effects on the health and fertility of the ICSI-conceived young men in particular. Given the prevalence of male infertility and the widespread use of ICSI, understanding these potential long-term effects is an important clinical and public health issue.

Aims

(1) To investigate the long-term physical and psychological health, and educational and social development of young men conceived using ICSI whose fathers had spermatogenic failure (STF) (ICSI study group) and compare to those of:
- Young men conceived using ICSI whose fathers had obstructive azoospermia (OAZ), including a subgroup of men with vasectomy
- Young men conceived using IVF (derived from the *IVF Young Adult Study* (IYAS)) (Halliday *et al.*, 2014)
- Young men conceived spontaneously (Halliday *et al.*, 2014)

(2) To investigate the long-term measures of reproductive and metabolic health of young men conceived using ICSI whose fathers had STF and compare to those of:
- Young men conceived using ICSI whose fathers had OAZ, including a subgroup of men with vasectomy
- Young men conceived using IVF (derived from the *Clinical review of the Health of 22–33 years old conceived with and without ART* (CHART study)) (metabolic data only) (Halliday *et al.*, 2019)
- Young men conceived spontaneously (derived from the Western Australian Pregnancy (Raine) study (reproductive and metabolic data) and CHART study (metabolic data only)) (Hart *et al.*, 2015; Halliday *et al.*, 2019)

(3) To identify epigenetic consequences of ICSI conception by comparing blood DNA methylation profiles of young men conceived using ICSI whose fathers had STF to:
- Young men conceived using ICSI whose fathers had OAZ, including a subgroup of men with vasectomy
- Young men conceived using IVF (derived from the CHART study) (Novakovic *et al.*, 2019)
- Young men conceived spontaneously (derived from the CHART and Raine study) (Novakovic *et al.*, 2019; Rauschert *et al.*, 2019)

These aims are summarised in Table I.

Outcomes

The primary outcomes are: the number of mother-reported hospitalisations of sons and son-reported quality of life, derived from questionnaires; the prevalence of moderate-severe oligozoospermia in sons (sperm concentration <5 million/ml), assessed by semen analysis according to World Health Organization (WHO) criteria and DNA methylation profiles of sons measured from blood. Secondary outcomes are listed in Table II.

Mothers are asked to report on the number, reason and total length in days for any hospital admissions their son required during his first year of life, pre-school period and primary school and secondary school period (up to 18 years). An International Classification of Diseases, 10th revision (ICD10) code will be assigned to the condition causing the admission (WHO, 2010a,b). The proportion with one or more admissions during a particular period of life will be calculated. The Australian World Health Organization Quality of Life Instruments (WHOQol-Bref) is used to measure quality of life (WHOQoL Group, 1998). This validated tool contains 26 items, including two general questions and four domains where the physical health, psychosocial, social and environment domains have seven, six, three and eight items, respectively. Each domain will be coded, summed and scored to create a raw domain score, according to described methods (Murphy *et al.*, 2000). Unknown values within each domain (do not know, refused, missing) will be imputed using horizontal mean imputation provided there are less than three missing items, following recommendations of Hawthorne (Hawthorne and Elliott, 2005). Raw domain scores will then be transformed to percentage scores.

Materials and Methods

Study design

This is a cohort study involving retrospective data collection from medical records, participant questionnaires and a contemporaneous clinical evaluation of sons.

Study setting

Monash IVF and Melbourne IVF infertility treatment centres located in Victoria, Australia. These services were the primary ART treatment centres in Victoria during the period 1994–2000.

Ethics approval

Ethics approval was granted by Human Research Ethics Committees at Monash Health (Project 16316A) and Melbourne IVF (Project 56/17). Governance approval was obtained from The Royal Children's Hospital (Project 36256B) and Monash University (Project 0464).

Table I Aims of study on the health and fertility of ICSI-conceived young men, showing the ICSI study group and control groups.

	ICSI study group	Control groups					
		ICSI control group	IYAS study		Raine study	CHART study	
	ICSI sons (STF)	ICSI sons (OAZ)	IVF sons	SC sons	SC sons	IVF sons	SC sons
(1) Health and development (questionnaire data)	✓	✓	✓	✓			
Sample size (n)	*275 (maternal reports)* *115 (son reports)*	*28 (maternal reports)* *12 (son reports)*	352 (maternal reports) 244 (son reports)	428 (maternal reports) 255 (son reports)			
(1) Reproductive and metabolic health (clinical data)	✓	✓			✓	✓	✓
Sample size (n)	*115*	*12*			365	72[a]	26[a]
(1) Epigenetic analysis	✓	✓			✓	✓	✓
Sample size (n)	*115*	*12*			268	71	24

[a]Metabolic data only.
CHART, *C*linical review of the *H*ealth of 22–33 years old conceived with and without *ART*; IYAS, *IVF Y*oung *A*dult *S*tudy; OAZ, obstructive azoospermia; SC, spontaneously conceived; STF, spermatogenic failure.

Table II Secondary outcomes of the study.

Maternal questionnaire	• Son child special health care needs • Son chronic health conditions
Son questionnaire	• Growth and development • Pubertal development • Educational achievement • Chronic health conditions • Hospitalisations • Attachment (quality of parental relationships)
Son clinical review	• Total sperm count • Total sperm motility • Progressive sperm motility • Normal sperm morphology • Serum FSH and LH • Serum testosterone • Testicular volumes • BMI • Resting systolic and diastolic blood pressure • Serum metabolic parameters (insulin, glucose, cholesterol profile, hsCRP) • HOMA-IR index

HOMA-IR, homeostasis model assessment-estimated insulin resistance; hsCRP, highly sensitive C-reactive protein.

Study populations

Mothers and fathers of ICSI-conceived young men.

Eligibility criteria: Potential participants are couples who underwent ICSI at Monash or Melbourne IVF who gave birth to one or more males between January 1994 and January 2000, and provided consent for future contact. Singleton and multiple births are included from a stimulated cycle, using fresh or frozen embryos and fertilised with ejaculated, testicular or epididymal sperm. Exclusion criteria: Deceased or their child has died, live overseas, cannot be traced or used donor sperm.

ICSI-conceived young men of fathers with STF (ICSI study group).

Eligibility criteria: Potential participants are ICSI-conceived young men aged 18 years or older, whose fathers had quantitative and/or qualitative defects of spermatogenesis from any cause, and whose mother and/or father gives permission for researchers to approach them.

ICSI-conceived young men of fathers with OAZ (ICSI control group).

Eligibility criteria: Potential participants are ICSI-conceived young men aged 18 years or older, whose fathers had OAZ (e.g. vasectomy, congenital bilateral absence of the vas deferens) and whose mother and/or father gives permission for researchers to approach them.

IVF-conceived young men (control group).

Participants are IVF-conceived young men aged 18 years or older (born between 1982 and 1992), who initially took part in the IYAS study (Halliday *et al.*, 2014). This study compared the health and development of IVF-conceived young adults to SC age-matched peers using questionnaire data from mothers and the young adults themselves. Only data concerning sons will be retrieved and depending on the outcome will be sourced from maternal surveys (n = 352) or son surveys (n = 244). The CHART study approached the same individuals several years later, and involved a clinical review and epigenome-wide DNA methylation analysis (Halliday *et al.*, 2019; Novakovic *et al.*, 2019). For the purpose of this study, only data regarding sons on metabolic (n = 72) and epigenetic (n = 71) outcomes of interest will be retrieved. IVF was performed at Monash or Melbourne IVF infertility treatment centres located in Victoria, Australia, in a stimulated cycle with own or donor eggs and using fresh or frozen embryos.

SC young men (control group).

Depending on the aim, SC controls derive from three prior studies including the IYAS (Halliday *et al.*, 2014), CHART (Halliday *et al.*,

2019; Novakovic *et al.*, 2019) and Raine (Hart *el al.*, 2015; Rauschert *et al.*, 2019) studies. In the IYAS study, SC young adults were those of mothers who were selected through random digit dialling of households within Victoria, Australia, i.e. the same source population as the IVF- and ICSI-conceived young adults. As above, a portion of these young adults also participated in the follow-up CHART study. SC young men aged 18 years or older (born between 1982 and 1992) who participated in the IYAS and CHART studies will serve as controls for questionnaire (maternal surveys n = 428; son surveys n = 255), and metabolic (n = 26) and epigenetic (n = 24) data, respectively (Halliday *et al.*, 2014, 2019; Novakovic *et al.*, 2019).

The Raine Study was formed from a pregnancy cohort study (https://www.rainestudy.org.au). A total of 2900 women were enrolled by the 18th week of gestation from antenatal booking clinics between 1989 and 1992. The resulting 2868 children born to 2804 mothers were retained to form the Raine cohort, to investigate the role of perinatal events on subsequent childhood and adult health (Straker *et al.*, 2017). This cohort of children has been followed until 22 years of age with a current retention rate of over 70% and is representative of the population (White *et al.*, 2017). Data will be retrieved regarding SC young men aged 18 years or older on reproductive and metabolic (n = 365), and epigenetic (n = 268) outcomes of interest to allow comparison with other groups (Hart *el al.*, 2015; Rauschert *et al.*, 2019). Data regarding perinatal events will be obtained from participating mothers of sons.

Recruitment of ICSI study and control groups

Tracing of eligible ICSI parents was achieved using the Victorian and Australian Electoral Rolls, and yielded a current address for 78% of mothers and 74% of fathers. A tracing letter is first sent by registered mail to eligible mothers and fathers to introduce the research study. If no feedback is received, a formal invitation package is sent to them individually 3 weeks later by registered mail. This contains an invitation letter from either their treating fertility specialist or fertility centre, a summary of the study, a participant information and consent form, a non-participation form, a 'consent to contact' form for the son(s), and a reply-paid envelope. Parents may participate without involving their son(s). If there is no response, a reminder letter is sent 3 weeks later and a final reminder letter is sent in another 3 weeks. Sons are invited only with parental permission. Sons can either sign the 'consent to contact' form or give verbal permission recorded on the parent's consent form. A similar invitation package is then sent. The consent form allows sons the opportunity to be involved in some or all parts of the study. They are all offered a gift voucher for participation. Reminder invitation letters are sent following the same protocol as parents.

Study requirements

A summary of the study requirements of parents and sons in the ICSI study and control groups is shown in Table III.

Review of medical records.

Information about cause of infertility, medical history, semen parameters, source of sperm, use of fresh or cryopreserved embryos, and obstetric and perinatal outcomes will be collected from the medical records of eligible ICSI parents. Data are collected from hard copy and electronic data records using a standardised case report form.

Table III Study requirements of mothers, fathers and sons in ICSI study and control groups.

	Mothers	Fathers	Sons
Review of medical records	✓	✓	✓
Questionnaire	✓	✓	✓
Clinical review			
Physical examination			✓
Fasting blood sample			✓
Semen analysis			✓
Epigenetic analysis			✓
Tissue samples for storage[a]			
Saliva	✓	✓	✓
Blood			✓
Semen			✓

[a]Ethics approval granted to store samples for future genetic and epigenetic analysis.

Questionnaire.

Mothers, fathers and sons are each invited to complete a health questionnaire (summary shown in Table IV; full details in Supplementary Data). The mother and son questionnaires for the ICSI groups are derived from those used in the IYAS study of IVF-conceived young adults (Wilson *et al.*, 2013; Halliday *et al.*, 2014). This will allow direct comparison, but with some additional questions included to meet the objectives of this study. Questions were drawn from validated questionnaires and assessment modules used in established youth health cohorts (refer to Table IV). Given the present study is focused on male fertility, fathers of ICSI-conceived sons are also asked to complete a questionnaire about their health and fertility. Questionnaires may be completed online, on hard copy or by phone interview.

Clinical review.

Physical examination.

- Genital examination: testicular volumes are measured by Prader orchidometer (Andrology Australia, Melbourne, Australia). The presence of varicocoele and vas deferens, and Tanner pubertal stage is also recorded. To ensure standardisation, the examination of sons is performed by SRC, who is an endocrinologist and andrologist. For the few participants living interstate, the examination is performed by other endocrinologists with reproductive expertise.
- Auxology: weight and height are measured using standard equipment. BMI is calculated as weight in kilograms divided by height in meters squared (kg/m^2).
- Blood pressure: the mean is calculated of two resting seated measurements taken 5 minutes apart between 9 and 11 am, using a Welch Allyn ProBP 2000 automatic sphygmomanometer (Medshop Australia, Melbourne, Australia).

Serum hormone and metabolic measurement. Blood samples from sons are taken between 9 and 11 am after an overnight fast and processed

Table IV Summary of questionnaire data for ICSI study group and IVF control group.

Questionnaire data	Questionnaire source				
	ICSI mother	ICSI father	ICSI son	IVF/SC mother	IVF/SC son
Maternal health during pregnancy[a]	✓				
Maternal reproductive history	✓				
Obstetric and perinatal outcomes[b]	✓			✓	
Paternal health at conception[a]		✓			
Paternal reproductive history	✓	✓			
Maternal and paternal smoking and alcohol consumption	✓	✓		✓	
Location of residence[c]	✓	✓	✓	✓	✓
Occupation[d]	✓	✓	✓	✓	✓
Son growth and development	✓		✓	✓	✓
Son child special health care needs[e]	✓			✓	
Son hospitalisations	✓		✓	✓	✓
Son chronic health conditions[f]	✓		✓	✓	✓
Son pubertal development[g]	✓		✓	✓	✓
Son sexual orientation[l]	✓		✓	✓	✓
Son educational achievement	✓		✓	✓	✓
ICSI or IVF disclosure	✓		✓	✓	✓
Son quality of life[h]			✓		✓
Son psychosocial health[i]			✓		✓
Son attachment[j]			✓		✓
Son exercise behaviour[k]			✓		✓
Son relationship status[k]			✓		✓
Son reproductive history			✓		

[a]Chronic health conditions, hospitalisations during pregnancy (mother only), medications, BMI.
[b]Type of birth, obstetric complications, birth complications, singleton or multiple pregnancy, gestational age, birthweight, admission to special nursery, neonatal morbidities, congenital malformations.
[c]Residential postal code for each participant will be used to assign remoteness area codes to indicate if they reside in a metropolitan, regional or remote area in Australia (ABS, 2001).
[d]Occupations will be classified using the Australian and New Zealand Standard Classification of Occupations used by the Australian Bureau of Statistics (Pink and Bascand, 2009).
[e]Assessed using the Child Special Health Care Needs (CSHCN) Screener (Bethell *et al.*, 2002).
[f]Questions adapted from the Child Health Questionnaire (Waters *et al.*, 2000a,b).
[g]Assessed using the Adolescence Scale (AS-ICSM) (Gruzelier, 1999) .
[h]Measured using the WHOQol-Bref (WHOQoL Group, 1998).
[i]Assessed using the Kessler psychological distress scale, 'K10' (Kessler *et al.*, 2002).
[j]Assessed using the Parental Bonding Instruction (PBI) (Parker *et al.*, 1979).
[k]Assessed using questions from the Australian Temperament Project (AIHW, 2008).
[l]Assessed using questions from the Australian Study of Health and Relationships (Smith *et al.*, 2003).

by Australian Clinical Labs, Clayton, Victoria, Australia. Serum total testosterone, LH, FSH, sex hormone-binding globulin and insulin are measured using chemiluminescence methods on a Siemens Advia Centaur Immunoassay analyser (Siemens Australia). A complete lipid profile, glucose and highly sensitive C-reactive protein (hsCRP) are measured using spectrophotometric methods on a Siemens Advia Chemistry XPT analyser (Siemens Australia). hsCRP, a biomarker of inflammation, is an independent predictor of cardiovascular disease and has been incorporated into risk prediction models (Rutter *et al.*, 2004; Ridker *et al.*, 2007, 2008). The homeostasis model assessment-

estimated insulin resistance index will be calculated as a measure of insulin resistance, using the formula: [fasting glucose nmol/l*fasting insulin μU/ml]/22.5 (Matthews *et al.*, 1985).

Semen analysis. A semen sample is obtained by masturbation after 2–7 days of abstinence at the laboratory on the same day as the blood sample. The samples are analysed by Australian Clinical Labs according to the WHO manual for the examination and processing of human sperm (WHO, 2010a,b). This includes the assessment of ejaculate volume by weight, sperm density, total sperm count, total and progressive sperm motility, and percentage normal morphology. In the case of an abnormal result, a repeat semen analysis is offered at least 6 weeks after the first sample. Results are discussed with consenting sons, and two abnormal results prompt a consultation with a male fertility specialist, at no cost to the participant.

In sons with a sperm concentration <10 million/ml on two semen analyses, who give their additional consent, a karyotype is performed by a standard cytogenetics approach (Vincent *et al.*, 2002; Krausz and Riera-Escamilla, 2018). Y chromosome microdeletion screening is performed by PCR for those with a sperm concentration <5 million/ml on two semen analyses, according to best practice guidelines by the European Academy of Andrology and the European Molecular Genetics Quality Network (Krausz *et al.*, 2014).

Epigenetic (DNA methylation) analysis.

Two 4 ml EDTA blood samples are collected from all consenting sons. The samples are processed by the Murdoch Children's Research Institute (MCRI) Biobanking Facility, for storage of whole blood, plasma and buffy coat for viable peripheral blood mononuclear cells. All samples are frozen at −80°C within 2 h of collection. Epigenome-wide association studies will involve DNA methylation profiling of genomic DNA isolated from whole blood. We will use the Illumina Infinium Human Methylation EPIC Beadchip (EPIC array) (Illumina, the Netherlands). This is the platform of choice due to its relatively low cost, high genomic coverage, sensitivity and reproducibility. More than 850 000 CpG methylation values will be obtained, spanning 99% of all genes and regulatory regions implicated in complex disease aetiology. This platform was also used as part of the CHART study involving DNA methylation profiling of IVF-conceived adults, which forms one of the control groups (Novakovic *et al.*, 2019). It is the next generation from the 450 K arrays used by the Raine study for their blood samples of SC adults, which form another control group (Rauschert *et al.*, 2019).

Biospecimens for storage.

Ethics approval has been granted to ask sons for their consent to store biospecimens for future genetic, epigenetic and biochemical studies. Additional ethics approval regarding specific protocols will be sought at a later date.

Blood sample. We plan to perform whole genome sequencing (WGS) or whole exome sequencing (WES) on some of the DNA extracted from the whole blood of consenting sons, as described above.

Saliva sample. Saliva samples are collected from consenting sons. If a son has provided any biospecimen for storage (blood, saliva and/or semen), then his parents are also invited to provide a saliva sample. All samples are collected using Oragene (OG-500) tubes. Two 0.8 ml aliquots are stored at −80°C at the MCRI Biobanking Facility. We plan to perform WGS or WES on genomic DNA isolated from these samples.

Semen sample. Immediately following semen analysis, sperm and seminal fluid from consenting sons will be separated and stored in microfuge tubes at −80°C. Sperm samples will be aliquoted into 4 × 1 ml tubes and seminal fluid samples into 2 × 1.7 ml tubes for each individual. Samples will be transported to the MCRI Biobanking Facility for storage. Sperm samples will be used for future epigenetic and genetic studies, and seminal fluid for future biochemical analyses.

Data management and monitoring

Data will be stored in paper copy and electronic data file formats. Any paper forms, such as consent forms and hard copy questionnaires, will be stored securely. Information will be transcribed to a password protected REDCap (Research Electronic Data Capture) database hosted at the MCRI (Version 10.1.2, Vanderbilt University, TN, USA), where all participant data will be stored. Questionnaires completed online will automatically upload to REDCap. All participants on this database will be deidentified and issued with a unique study ID code. Regarding data from the clinical review, a letter and copy of all results will be posted to sons who have given consent. If a son's results are outside the normal range, they will be telephoned first to discuss them in detail by endocrinologist, SRC.

Confounders and modifiers

Data on important potential influences on the outcomes of interest are collected from the medical records of parents, and parent and son questionnaires. Depending on the aim, confounders and modifiers include: parental age and BMI; parity; household income; perception of financial situation; relationship status; parental occupation and education; maternal smoking and alcohol consumption during pregnancy; family structure; residential location; type of parental infertility; obstetric complications; mode of delivery; gestation at delivery or birthweight; source of sperm; sperm preparation; multiple birth; current age of son; son smoking status and alcohol consumption and son education and employment.

Addressing potential biases

Selection bias.

Using medical record data, it is possible to compare important characteristics between participating and non-participating ICSI parents, such as cause of infertility, indication for ICSI, paternal semen parameters and perinatal outcomes. Significant selection bias is unlikely between the ICSI study group, and both the ICSI and IVF control groups. All study groups have undergone IVF/ICSI, and therefore will have had similar experiences and we would expect to exhibit similar reporting styles. For non-participating ICSI sons, analysis of the maternal questionnaires will allow differences in chronic illness, development and special health care needs to be examined between the ICSI study group, and both the ICSI and IVF control groups. Despite this, it is impossible to exclude selection bias and results will need to be interpreted accordingly and multiple imputation analysis undertaken where appropriate. In terms of the SC control groups, derived from the CHART and Raine studies, selection bias in terms of fertility status is

also considered unlikely given their young age and the prospective study design of the Raine study.

Recall bias.

There is potential for recall bias given both concurrent and retrospective data are collected from ICSI parents. To minimise such bias, questions are arranged chronologically so that they are linked with objective events, such as pre-conception, pregnancy and birth, and so forth. It is possible that parents of ICSI- and IVF-conceived children recall aspects of their offspring's health and wellbeing more vividly than parents of SC children. However, they may recall positive outcomes as much as negative ones and a balance therefore will be achieved amongst participants.

Reporting bias.

A reporting bias may occur with some sensitive questions in the son's questionnaire, such as pubertal development and sexual identity. However, such bias is unlikely to differ between study groups. To minimise reporting bias, validated questions are used to assess such topics.

Sample size

ICSI cohort.

Of 1112 eligible ICSI parents (STF and OAZ fathers), 867 mothers and 823 fathers were traced and contacted. With current participation rates of 35% and 30% for mothers and fathers, respectively, we estimate a sample size of 303 mothers and 247 fathers completing the questionnaire for the ICSI study and ICSI control groups. With 70% of participating mothers (n = 212) allowing contact with their sons and a current participation rate of 60% for sons, we estimate a sample size of 127 ICSI sons participating in the questionnaire and clinical review components. We anticipate a ratio of 1:10 of participating sons born to fathers with OAZ (ICSI control group, n = 12) and sons born to fathers with STF (ICSI study group, n = 115). The 1:10 ratio of OAZ: STF ICSI groups will result in an estimated sample size of 28 mothers, 22 fathers and 275 mothers, 225 fathers for each group, respectively. This estimated sample size together with the sample size available for the other control groups are shown in Table I.

Aim 1: Health and development (questionnaire data).

For mother-reported number of son hospitalisations up to 18 years, one of the control groups will be mothers of SC sons (n = 428), recruited in the IYAS study (Halliday *et al.*, 2014). These SC control mothers reported a hospitalisation rate of 51% for their sons. With a sample size of 275 mothers reporting on their ICSI-conceived sons (ICSI study group), when compared with hospitalisations reported by the 428 SC control mothers, we would be able to detect an increase of hospitalisations from 51% to 58% in the ICSI study group, (alpha 0.05, power 80%, one sided test) (Stata version 16.0; StataCorp, Texas, USA).

For son-rated quality of life, as measured by the four Australian WHOQol-Bref domains, one of the control groups will be SC sons (n = 255), recruited in the IYAS study (Halliday *et al.*, 2014). The SC sons had a mean score (±SD) of 84.6 (±12) for the WHOQol-Bref physical domain. Using a two-sample comparison of the means in these two groups, a sample size of 255 SC sons and 115 ICSI-conceived sons (ICSI study group) will allow for the detection of a delta of 2.7, effect size = 0.23 (alpha 0.05, power 80%) (Stata version 16.0).

Aim 2: Reproductive health (clinical data).

We expect moderate-severe oligozoospermia in 5% of healthy young men and therefore a similar proportion in the Raine study SC sons (n = 365) (Hart *et al.*, 2015). A sample size of 365 Raine study SC sons and 115 ICSI-conceived sons born to fathers with STF (ICSI study group) participating in a semen analysis would allow us to detect an increase in oligozoospermia from 5% to 10% in the ICSI study group, reflected as a statistically significant difference at the 5% significance level with 80% power (one sided test). A sample size of 115 sons participating in the semen analysis in the ICSI study group (STF fathers) and 12 sons in the ICSI control group (OAZ fathers) will allow us to detect an increase in oligozoospermia from 5% to 27% in the ICSI study group, reflected as a statistically significant difference at the 5% significance level with 80% power (one sided test).

Aim 3: DNA methylation profiles (epigenetic data).

The sample size proposed (ICSI study group, n = 115; SC sons, n = 292) is larger than that used to identify robust associations for several other phenotypes/exposures, including identification of a long-term epigenetic 'signature' in 18-year old adults associated with prematurity (Cruickshank *et al.*, 2013), or exposure to maternal smoking in pregnancy (Novakovic *et al.*, 2014). The additional power this affords will allow us unparalleled capacity to identify even small ICSI-associated DNA methylation differences.

Statistical analyses

All analyses will be done with the latest version of Stata. The distributions of continuous exposures and outcomes will be examined for normality, and will be appropriately transformed if necessary. For each of the outcome measures, unadjusted differences between the ICSI study group and control group will be investigated. Chi square tests will be used to compare groups for categorical data and ANOVA will be used to compare means for continuous data. For each outcome, differences between the ICSI study group and control group will be investigated using multivariable linear and logistic regression for continuous and binary outcomes, respectively. Relevant potential confounders, effect modifiers or partial intermediates, reaching significance <0.1 in the univariate analysis, will be considered. The odds ratios will be estimated using both standard methods, as well as the method of marginal models fitted using Generalised Estimating Equations with information sandwich (robust) estimates of standard error, to check that the small number of sibling clusters does not affect the results. Results will be presented as adjusted odds ratios and 95% CIs.

Regarding epigenetic analysis, EPIC array DNA methylation data will be processed in the R computing environment using the missMethyl package (Phipson *et al.*, 2016). Normalisation will be performed using SWAN (Maksimovic *et al.*, 2012). EPIC probes known to cross-hybridise to other locations in the genome or overlapping with single nucleotide polymorphisms will be removed from the analysis. Methylation values will be transformed from beta (ranging from 0 to 1) to *M*-values, which have better statistical properties for analysis. To identify differentially methylated regions, we will first identify single CpG probe sites that show a difference in DNA methylation associated with ICSI. We will then scan the surrounding region for additional

probes that show the same methylation pattern (Peters *et al.*, 2015). This, in addition to setting a DNA methylation change cut-off of >5%, increases the confidence that the identified regions are true positives. A multivariate model will be used to account for technical effects and biological confounders, such as cell proportions in whole blood (Novakovic *et al.*, 2019).

Discussion

Given its relatively recent introduction, little is known about the health and reproductive potential of ICSI-conceived men whose fathers had STF. This study addresses the knowledge gap with a large and comprehensive review of the heath and fertility of young men conceived using ICSI from fathers with STF and their epigenetic patterning. The results of this study will improve clinical practice and couple counselling prior to ART, and encourage further research into male infertility and fertility preservation options.

There are many unique strengths of this study. These include: its comprehensive evaluation of health and fertility including both maternal- and son-reported health data and clinical data; epigenetic profiling of study populations to investigate underlying biological mechanisms for any differences in outcomes; the availability of health information on parents of ICSI sons including indication for ICSI and aetiology of male infertility; questionnaire, metabolic and epigenetic data on age-matched IVF- and SC controls who were recruited from a prior study using the same source population and same methodologies (Halliday *et al.*, 2014; Novakovic *et al.*, 2019); reproductive data on age-matched SC controls from a prospective birth cohort providing a valid population representative sample, which is ideal for comparing fertility measures and the prevalence of impaired semen quality (Hart *et al.*, 2015); a control group of OAZ-ICSI sons whose fathers had presumably normal spermatogenesis, which will permit isolation of the effects of paternal infertility and the ICSI technique; and the relatively large sample size compared with the only other similar study (Belva *et al.*, 2016, 2017).

Limitations include: current low recruitment rate, which is lower than in previous work with IVF-conceived adults, suggesting that male infertility may be a particularly sensitive subject for some couples to discuss with their offspring; invitation to sons with parental permission only; the requirement of a semen sample and testicular examination, which deters men's participation and hinders recruitment; potential selection, participation and reporting biases, as detailed previously and non-contemporaneous recruitment of IVF and SC controls.

This will be the largest cohort study to assess general health and reproductive outcomes, and epigenetic profiling of ICSI-conceived young men. In addition, this study will provide a valuable bioresource for further studies on the genetics of male infertility by providing genetic material from both parents and ICSI sons combined with accurate reproductive phenotyping of father and son pairs.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

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Authors' roles

S.R.C. was involved in the study design, ethics application, project management, data acquisition and drafted the manuscript. S.L. was involved in the study design, ethics application, statistical planning and drafted the manuscript. J.H. was involved in the study design, ethics application, project management and drafted the manuscript. J.K. was involved in the ethics application, project management, data acquisition and drafted the manuscript. M.K.O., D.J.A. and R.S. were involved in the study design and drafted the manuscript. J.M. and L.R. are the clinician signatures on the invitation letters, contributed to data acquisition and critically reviewed the manuscript. R.J.H. contributed to data acquisition and drafted the manuscript. R.I.M. conceived the idea for the study, contributed to the study design and ethics application, and drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

L.R. is a minority shareholder and the Group Medical Director for Monash IVF Group, and reports personal fees from Monash IVF group and Ferring Australia, honoraria from Ferring Australia, and travel fees from Merck Serono, MSD and Guerbet; R.J.H. is the Medical Director of Fertility Specialists of Western Australia and has equity in Western IVF; R.I.M. is a consultant for and a shareholder of Monash IVF Group and S.R.C. reports personal fees from Besins Healthcare and non-financial support from Merck outside of the submitted work. The remaining authors have no conflicts of interest to declare.

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