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Semen quality of young adult ICSI offspring: the first results

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STUDY QUESTION: What is the semen quality of young adult men who were conceived 18–22 years ago by ICSI for male infertility?

SUMMARY ANSWER: In this cohort of 54 young adult ICSI men, median sperm concentration, total sperm count and total motile sperm count were significantly lower than in spontaneously conceived peers.

WHAT IS KNOWN ALREADY: The oldest ICSI offspring cohort worldwide has recently reached adulthood. Hence, their reproductive health can now be investigated. Since these children were conceived by ICSI because of severe male-factor infertility, there is reasonable concern that male offspring have inherited the deficient spermatogenesis from their fathers. Previously normal pubertal development and adequate Sertoli and Leydig cell function have been described in pubertal ICSI boys; however, no information on their sperm quality is currently available.

STUDY DESIGN, SIZE, DURATION: This study was conducted at UZ Brussel between March 2013 and April 2016 and is part of a large follow-up project focussing on reproductive and metabolic health of young adults, between 18 and 22 years and conceived after ICSI with ejaculated sperm. Results of both a physical examination and semen analysis were compared between young ICSI men being part of a longitudinally followed cohort and spontaneously conceived controls who were recruited cross-sectionally.

PARTICIPANTS/MATERIALS, SETTING, METHOD: Results of a single semen sample in 54 young adult ICSI men and 57 spontaneously conceived men are reported. All young adults were individually assessed, and the results of their physical examination were completed by questionnaires. Data were analysed by multiple linear and logistic regression, adjusted for covariates. In addition, semen parameters of the ICSI fathers dating back from their ICSI treatment application were analysed for correlations.

MAIN RESULTS AND THE ROLE OF CHANCE: Young ICSI adults had a lower median sperm concentration (17.7 million/ml), lower median total sperm count (31.9 million) and lower median total motile sperm count (12.7 million) in comparison to spontaneously conceived peers (37.0 million/ml; 86.8 million; 38.6 million, respectively). The median percentage progressive and total motility, median percentage normal morphology and median semen volume were not significantly different between these groups. After adjustment for confounders (age, BMI, genital malformations, time from ejaculation to analysis, abstinence period), the statistically significant differences between ICSI men and spontaneously conceived peers remained: an almost doubled sperm concentration in spontaneously conceived peers in comparison to ICSI men (ratio 1.9, 95% CI 1.1–3.2) and a two-fold lower total sperm count (ratio 2.3, 95% CI 1.3–4.1) and total motile count (ratio 2.1, 95% CI 1.2–3.6) in ICSI men compared to controls were found. Furthermore, compared to men born after spontaneous conception, ICSI men were nearly three times more likely to have sperm concentrations below the WHO reference value of 15 million/ml (adjusted odds ratio (AOR) 2.7; 95% CI 1.1–6.7) and four times more likely to have total sperm counts below 39 million (AOR 4.3; 95% CI 1.7–11.3). In this small group of 54 father–son pairs, a weak negative correlation between total sperm count in fathers and their sons was found.

LIMITATIONS, REASONS FOR CAUTION: The main limitation is the small study population. Also, the results of this study where ICSI was performed with ejaculated sperm and for male-factor infertility cannot be generalized to all ICSI offspring because the indications for ICSI have nowadays been extended and ICSI is also being performed with non-ejaculated sperm and reported differences may thus either decrease or increase.

WIDER IMPLICATIONS OF THE FINDINGS: These first results in a small group of ICSI men indicate a lower semen quantity and quality in young adults born after ICSI for male infertility in their fathers.

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Key words: ICSI / offspring / sperm / reproduction / fertility

Introduction

In 1991, the introduction of ICSI into clinical practice was a real breakthrough for the treatment of male-factor infertility; men with impaired spermatogenesis could become the genetic father of their offspring which was not possible before (Van Steirteghem *et al.*, 1993). Nowadays, ICSI is a routinely performed ART, which has resulted in >2.5 million babies born worldwide (Van Steirteghem, 2012). Although ICSI was initially performed in couples with male-factor infertility, ICSI is now increasingly used even in the absence of abnormal semen parameters, which hinders conclusions on its role in male infertility per se.

Until now, the health of children conceived by IVF and ICSI has been described from infancy up to pubertal age but never beyond. Indeed, ART including ICSI has repeatedly been associated with an increased risk of adverse health outcomes in the perinatal period, including increased risk of low birth weight, prematurity and birth defects (Pinborg *et al*, 2013a, b). Although adverse cardiometabolic and vascular outcomes have been described in both IVF offspring (Ceelen *et al.*, 2007, 2008; Sakka *et al.*, 2010; Scherrer *et al.*, 2012; Valenzuela-Alcaraz *et al.*, 2013) and ICSI offspring (Belva *et al.*, 2007, 2011b), the impact of ART on the reproductive health of the offspring remains unknown. We previously described normal pubertal development (Belva *et al.*, 2012), normal levels of inhibin B, i.e. a marker of Sertoli cell function (Belva *et al.*, 2010) and normal levels of salivary testosterone, a marker of Leydig cell function in pubertal ICSI boys, as compared to spontaneously conceived peers (Belva *et al.*, 2011a). In a small group, both inhibin B and salivary testosterone levels were comparable in boys from fathers with severe oligoozoospermia to those in boys from fathers without severe oligozoospermia (Belva *et al.*, 2011a). But whether ICSI-conceived men born to fathers with impaired spermatogenesis are at risk of inheriting deficient spermatogenesis from their fathers could not be answered due to their young age.

Literature data regarding reproductive health of IVF and ICSI offspring is limited; only one study has described sperm parameters of young men whose mothers received 'fertility treatment' (Jensen *et al.*, 2007) and reported in a cohort of 47 men, a 46% lower sperm concentration and fewer motile and morphologically normal spermatozoa. Unfortunately, no information regarding the exact treatment mode was available. Therefore, it remains uncertain whether this finding is transferable to offspring who are conceived by ICSI mainly because of male-factor infertility.

Primary testicular dysfunction is the most frequent cause of male infertility, and although often unexplained, a genetic origin can be diagnosed in a subgroup (Massart *et al.*, 2012). Infertile men are known to have a higher prevalence of chromosomal abnormalities than fertile men (Ferlin *et al.*, 2007), and the transmission may result in affected offspring. Y-chromosomal microdeletions, particularly in the AZFc (azoospermia factor c) region, are the most frequently diagnosed genetic cause of impaired spermatogenesis with a prevalence of 6% in severely oligozoospermic men to 10% in azoospermic men (Oliva *et al.*, 1998; Vogt, 1998; Maurer and Simoni, 2000; Stouffs *et al.*, 2008). Transmission of Y-chromosomal deletions from father to son through ICSI has been reported (Kent-First *et al.*, 1996; Kamischke *et al.*, 1999; Page *et al.*, 1999). In view of the potential transgenerational transmission of male infertility, it remains to be elucidated whether the severity of the paternal spermatogenic failure (e.g. severe oligozoospermia) heralds impaired spermatogenesis in the offspring.

In this study, we compared semen parameters in the world oldest ICSI cohort, conceived by ICSI for mainly (severe) male-factor infertility, with spontaneously conceived peers. Furthermore, in order to investigate whether poor sperm quality in the fathers may be predictive for the sperm quality of their sons, we correlated the semen parameters of ICSI men with those of their fathers at the start of their ICSI treatment almost 20 years ago.

Materials and methods

Set-up and study groups

This study is part of a larger project investigating the cardiometabolic and reproductive profile of young ICSI adults, both female and male. Therefore, several examinations including physical examination, biometry, semen analysis and blood sampling were performed. Participants were offered the chance to deliver a second semen sample or blood sample in case of an abnormal result. In addition, participants were offered genetic testing (Yq deletions) in case of sperm concentrations <5 million/ml. Participants received written feedback regarding the results of their tests and were offered appropriate counselling, investigation and follow-up. All participants received an incentive by means of a gift voucher.

Young adults were eligible for inclusion if they were singleton, Caucasian and aged between 18 and 22 years in the study period between March 2013 and April 2016.

These adults, born between 1992 and 1996 after transfer of fresh embryos formed through ICSI using autologous, freshly ejaculated sperm, are part of a prospective follow-up cohort since their birth. The majority have been assessed earlier at one or more time points, i.e. 2 months, 1 year, 2 years, 5 years, 8 years and 14 years. All parents of eligible young ICSI adults in our database ($n = 423$) were sent a letter explaining the background and set-up of the study. Shortly after, these parents were contacted by phone in order to explore their own and their children's willingness to participate. After consent of the parents, the young adults were approached and invited to participate. The young adults were not approached directly, since from our study conducted at age 14 years, we learned that 20% of the parents did not disclose the mode of assisted conception to their children.

From the 215 eligible ICSI families with a male offspring, 149 could be reached. Eventually, 56 (37.5%) young ICSI men participated, but two were excluded since they were not able to produce a semen sample. Of the 93 refusing, 45 men declined participation themselves, while in 21 cases the parents decided that 'the family' was not interested in participating and thus their sons were not contacted. For 9 men, the mode of conception was not disclosed, and in 18 cases there was no objection to the study but an appointment for testing was cancelled and/or a suitable time for testing could not be arranged due to work or school. The reasons for declining for the 45 young adults were: 'I do not want to participate' ($n = 31$), 'I do not have the time to participate' (n=10), 'I do not want one/all examination(s) to be performed' (n=3), 'I am afraid to know my fertility potential' (n=1). Taking into account that the invitation to participate in this study reached only 119 young adults instead of 149, the actual participation rate is 47%. The educational level is known for 52 participants: 43 (83%) were attending university or non-university higher education.

A control group of spontaneously conceived peers aged between 18 and 22 years was recruited at college and university campuses. In order to balance groups, ICSI men were asked to invite a friend to serve as a participant in the control group. Recruitment was also performed by oral and written campaigns, resulting in 57 volunteers who were willing to participate, of whom 56 attended university or non-university higher education. Men who were only willing to take part in sub-examinations (physical examination and/or blood sampling) were not included in the study. Only young adults born after spontaneous conception without the use of any hormonal stimulation were eligible as controls.

Informed consent and Ethics Committee

Only participants who filled out the written informed consent after being informed were enroled. A separate informed consent was obtained for genetic testing. This study is part of a larger project on reproductive and metabolic health in young adults conceived by ICSI, which was approved by the Ethics Committee of the UZ Brussel.

Measurements

Physical examination

Weight and height were measured using standard equipment. BMI was calculated as weight in kilograms divided by the square of height in metres(kg/m^2).

Physical examinations were performed by urologists who were blinded to the mode of conception and to the participant's semen quality. Testicular volumes were assessed by a Prader orchidometer and by ultrasound (Behre *et al.*, 1989). Each testis was measured by estimating its maximal dimensions in three planes (width, length and height). The formula (length x width x height x 0.52) has been used to calculate the testicular volume based on ultrasound measurements (Lenz *et al.*, 1993; Bahk *et al.*, 2010). For analysis, the mean of the right and left testicular volume (based on the latter formula) was used. Genital examination (presence of varicocele, hydrocele, spermatocele, location of the testis in the scrotum, any other malformation) as well as penile length was measured.

Questionnaires

All participants were asked to fill out a questionnaire covering a large series of health parameters about themselves and their family, including information on lifestyle factors (sports, smoking habits, alcohol consumption and recreational drugs), chronic medication intake (at least 1 year), chronic illnesses (suffering at least 1 year), surgical interventions, any genital disorder (cryptorchidism, hydrocele, varicocele, inguinal hernia, other) and its treatment. In addition, educational level was asked as well as early life factors including *in utero* exposure to tobacco during pregnancy.

Before collection of the semen sample, staff members of the andrology laboratory recorded information on the abstinence time, the use of medication in the last 3 months and the occurrence of fever of >38 °C for at least 24 h in the past 3 months.

In case of missing or incomplete information, participants were re-contacted for further information.

Semen analysis

A semen sample was obtained by masturbation at the hospital. The participants had been instructed to abstain from ejaculation for at least 3 days prior to delivery of the sample. Semen analyses were performed blind to the mode of conception. The sample was analysed according to the most recent edition of the WHO manual for the examination and processing of human sperm (WHO, 2010). Ejaculate volume was measured by weight of the sample in a pre-weighed container. Sperm concentration was assessed by the use of the improved Neubauer haemocytometer, after dilution with a formalin solution. Total sperm count (semen volume x semen concentration) was reported. Sperm morphology was assessed after Shorr staining and according to strict criteria (Menkveld *et al.*, 1990; WHO, 2010). For sperm motility assessment, the percentage of progressive motility (A + B) and total motility (A + B + C) were calculated. Total motile count (semen volume x semen concentration x progressive motility/100) was also provided. For definitions of normal semen quality, the WHO reference values for human semen characteristics are adopted (Cooper *et al.*, 2010; WHO, 2010). The laboratory participates in an external quality control programme for semen assessment.

In the early 1990s, semen analysis of the fathers was performed according to the third edition of the WHO manual for human semen examination (WHO, 1992). Volume was assessed using graduated pipettes, and concentration and motility were evaluated in the Makler or improved Neubauer counting chamber. Regarding morphology, less strict criteria were applied in the early nineties.

In case of aberrant semen analysis results of the young adults, the delivery of a second semen sample was encouraged. However, only one ICSI male delivered a second semen sample, with results in line with those from the first sample. None of the spontaneously conceived men were willing to deliver a second semen sample.

Genetic analysis

In participants with a sperm concentration <5 million/ml, who gave their additional consent for genetic testing, Y chromosome microdeletion screening was performed in two multiplex PCR reactions, according to the recommendations described by Simoni *et al.* (2004) and Krausz *et al.* (2014). Six out of eight ICSI participants and one out of four of the controls in this situation gave their consent for genetic testing prior to the semen sampling.

Statistical analysis

Descriptive statistics were calculated on all parameters to determine the characteristics of the sample. In addition to mean and SD, also median and interquartile range (IQR) presenting the 25th–75th percentiles are reported, given the non-normal or near-normal distribution of several parameters. Sperm concentration, total count, total motile count and sperm morphology

were logarithmically transformed (log_{10}) to normalize their distribution. For ease of interpretability, mean values and 95% CIs were back-transformed for reporting. Volume, progressive motility and total motility were distributed close to normal and were analysed without transformation. Also, the distribution of testicular volume was close to normal and therefore not transformed for analysis. Chi-square test was used for categorical variables and Student's *t*-test (normalized) was used for continuous variables. Between-group differences (expressed as median) in sperm parameters were also tested with the non-parametric Mann–Whitney *U* test. Data analysis was performed using SPSS software version 23.

Multiple linear regression analysis was used to investigate differences in sperm parameters (concentration, total sperm count, total motile sperm count, morphology, progressive motility, total motility and volume) and multiple logistic regression was used to determine whether ICSI conception was associated with reduced semen quality: having a sperm concentration <15 million/ml, having a sperm concentration <5 million/ml, having a total sperm count <39 million, having <32% progressively motile sperm or having <4% morphologically normal sperm. Reference limits are adopted from the latest WHO guidelines (Cooper *et al.*, 2010).

Preliminary univariate regression analysis was performed in order to select covariates, known to affect sperm parameters and/or to be differently present among the two study groups, for inclusion in the final linear and logistic regression models. The following covariates were tested: age (continuous), time from ejaculation to analysis (continuous), abstinence period (≤2 days, 2–5 days, >5 days), genital malformation (yes/no according to combined information from questionnaire and physical examination), season of sampling (spring/summer versus fall/winter), BMI (<25, ≥25), smoking (yes/no), drugs (yes/no) and alcohol consumption (yes/no).

For the linear regression models, the covariates age, time from ejaculation to analysis, abstinence period and genital malformations were associated with at least one of the sperm parameters (see below). For the logistic regression models, the covariates age, abstinence period, genital malformation and BMI were associated with at least one of the outcomes (data not shown). For reasons of uniformity, the same covariates were used in the linear as well as in the logistic regression models. Therefore, the following covariates were used in all models: age, BMI, genital malformation, time from ejaculation to analysis and abstinence period.

Results are expressed as unstandardized regression coefficients (B) with a 95% CI. For log transformed outcome variables (concentration, total sperm count, total motile count and morphology), regression coefficients and 95% CIs were back-transformed and should be interpreted as a ratio.

The association between mean testicular volume and semen parameters in the ICSI men and controls was analysed with linear regression.

All semen parameters of the ICSI fathers, except volume, were logarithmically transformed (log_{10}) before correlation analysis. Volume was left untransformed.

The sample size was estimated from a power calculation was for the outcome total sperm count. With an aim of 55 participants in each group and an assumed overall mean (SD) total sperm count of 154 million (120) (Mendiola *et al.*, 2013), a reduction in total sperm count of ~30% could be detected with a power of 80% and a significance level alpha of 5% (one-sided test of log transformed total sperm count).

Results

Clinical characteristics

Characteristics of the participants are shown in Table I. ICSI men were slightly younger than their spontaneously conceived peers ($P = 0.003$). BMI was comparable between the two groups ($P = 0.17$). Self-reported alcohol consumption was different between the two groups, with more ICSI young adults reporting ever drinking alcohol ($P = 0.07$). The rate of genital malformations was comparable between the study groups ($P = 0.52$).

In both groups, three men had fever in the 2 months preceding the collection of the semen sample ($P = 0.95$) and 17 (31.5%) ICSI men and 15 (26.3%) controls had been taken medication ($P = 0.55$). Chronic medication use was recorded for 9 (16.7%) ICSI participants: 5 for allergies/asthma, 1 for attention-deficit hyperactivity disorder, 3 for acne using oral retinoids and for 13 men in the control group (22.8%): for allergies/asthma, for seizures, for depression and for attention-deficit hyperactivity disorder ($P = 0.512$).

There was no difference in mean testicular volume and glans type between the ICSI men and spontaneously conceived controls (Table I).

Regarding the ICSI men, ICSI was performed in 92.5% (50/54) of the cases because of male-factor infertility (48/54 only male infertility, 2/54 combined male and female infertility). Four couples suffered from idiopathic infertility.

Results of univariate linear regression analysis

Season of sampling, BMI, smoking, alcohol consumption and drug use were not associated with any of the semen parameters in our study.

Age was associated with total motile count ($P = 0.04$) and progressive motility ($P = 0.04$). Long abstinence time was negatively correlated with progressive motility ($P = 0.028$) and total motility ($P = 0.009$); short abstinence time was associated with volume ($P = 0.020$).

Although genital malformation was not associated with sperm concentration and total sperm count in the univariate analysis, the findings after stratification are noteworthy: in men having a genital malformation, a trend towards a four times lower sperm concentration (back-transformed mean: 7.3 versus 28.8 million/ml; $P = 0.08$) and total sperm count (back-transformed mean: 12.6 versus 50.1 million; $P = 0.08$) was observed in ICSI men versus controls. Even in the group of men without genital malformations, ICSI men had lower sperm concentrations and lower total sperm counts compared to controls (back-transformed mean: 16.5 versus 28.2 million/ml; $P = 0.04$ and back-transformed mean: 32.4 versus 70.9 million; $P = 0.01$).

Neither sperm concentration nor total sperm count were associated with mean testicular volume ($P = 0.17$; $P = 0.08$).

Comparison of semen parameters between ICSI men and controls

Time from ejaculation to analysis was well within 1 h and was comparable between the two groups ($P = 0.26$) (Table II). Abstinence time before sampling was mostly <2 days, both for ICSI as well as for control men. There was spilling during collection of the sample for three ICSI and four control men ($P = 0.75$).

ICSI men had lower median sperm concentrations (17.7 million/ml), lower median total sperm counts (31.9 million) and lower median total motile sperm (12.7 million) count in comparison to spontaneously conceived peers (37.0 million/ml, $P = 0.004$; 86.8 million, $P = 0.001$; 38.6 million, $P = 0.002$, respectively). (Table II). Median percentage progressive and total motility, median percentage normal morphology rate and median semen volume were not significantly different between the two groups ($P = 0.19$; 0.61; 0.29; 0.22,

Table I Characteristics of the two study populations.

	ICSI	SC	*P*-value
Clinical characteristics	*N* = 54	*N* = 57	
Birth weight (g)	3386.0 (592.3)	3492.4 (384.2)	0.28
Age (years)	19.4 (0.7)	20.0 (1.2)	0.003
Weight (kg)	75.2 (9.5)	72.2 (10.4)	0.12
Height (cm)	180.4 (6.7)	179.6 (6.3)	0.51
BMI (kg/m^2)	23.1 (2.7)	22.4 (2.9)	0.17
Smoking (yes/no)*	12 (24)	6 (11.5)	0.10
Drugs (yes/no)*	4 (8)	2 (3.8)	0.37
Alcohol (yes/no)*	42 (84)	33 (63.5)	0.02
Sports (hours/week)	4.5 (4.0)	5.9 (4.4)	0.07
Genital malformation (yes/no)*	8 (14.8)	6 (10.7)	0.52
Andrological examination	*N* = 47	*N* = 50	
Testicular volume right (US) (ml)	15.6 (6.5)	15.4 (4.9)	0.80
Testicular volume left (US) (ml)	16.4 (6.3)	15.2 (4.0)	0.28
Mean testicular volume (US) (ml)	16.0 (6.0)	15.3 (4.1)	0.45
Glans type*			
Helmet	24 (51.1)	34 (68.0)	0.32
Pointed	3 (6.4)	2 (4.0)	
Rounded cap	9 (19.1)	8 (16.0)	
Scoop	6 (12.8)	5 (10.0)	
Not determined	5 (10.3)	1 (2.0)	

ICSI = conceived by ICSI, SC = spontaneously conceived.
*Mean (SD) or number (%).

respectively). The type of defects (neck-midpiece, head and tail) was comparable among the ICSI offspring and the controls.

After adjustment for confounders (age, BMI, genital malformation, time from ejaculation to analysis, abstinence period), the statistically significant differences between ICSI men and spontaneously conceived peers remained: a doubled sperm concentration in spontaneously conceived peers in comparison to ICSI men (ratio: 1.9; 95% CI 1.1–3.2; $P = 0.02$) as well as a two-fold lower total sperm count (ratio: 2.3; 95% CI 1.3–4.1; $P = 0.005$) and total motile count (ratio: 2.1; 95% CI 1.2–3.6; $P = 0.007$) in ICSI men compared to controls (Table III).

Adjustment for confounders did not change the results of morphology, progressive motility, total motility and volume. For these parameters, differences between ICSI men and controls were not statistically significant.

Risk of reduced semen quality in ICSI offspring

Compared to men born after spontaneous conception, ICSI men were nearly three times more likely to have sperm concentrations <15 million/ml (AOR 2.7; 95% CI 1.1–6.7; $P = 0.035$) and four times more likely to have total sperm counts <39 million (AOR 4.3; 95% CI 1.7–11.3; $P = 0.002$) (Table IV). Also, the risk of having below-reference (<4% normal) sperm morphology rates was non-significantly higher in ICSI men (AOR 2.3; 95% CI 0.9–5.4; $P = 0.06$).

ICSI men were twice as likely to have extremely low-sperm concentration (<5 million/ml) and below-reference (<32%) progressive motile sperm, but this was not statistically significant (AOR 2.6; 95% CI 0.6–10.7 and AOR 2.3; 95% CI 0.7–7.9, respectively).

Six out of eight ICSI men with sperm concentration <5 million/ml were tested for Yq deletions but no abnormalities were found. From the four controls with sperm concentration <5 million/ml, one male was tested for Yq deletions and his result was normal.

Association of semen parameters of ICSI offspring with semen parameters of their fathers

More than 72% (39/54) of the fathers had sperm concentrations <15 million/ml, 26 out of 54 (48%) had sperm concentrations <5 million/ml and 38 (70%) had a total sperm count <39 million. Sperm characteristics of the ICSI fathers are provided in Table V.

Sperm concentration and total motile count in fathers were not correlated with corresponding values in their sons ($r = -0.2$; $P = 0.09$ and $r = -0.2$; $P = 0.07$). Also, percentage progressive and total motility in father–son pairs were not correlated ($r = 0.2$; $P = 0.14$ and $r = 0.0$; $P = 0.83$). Paternal total sperm count was weakly negatively correlated with total sperm count in the sons ($r = -0.3$; $P = 0.02$).

Table II Semen quality parameters of the men in the ICSI and control groups.

Semen parameters	ICSI (*N* = 54)	SC (*N* = 57)	*P*-value
Time from ejaculation to analysis (min)			
Mean (SD)	26.5 (17.5)	23.4 (11.2)	0.26
Abstinence category (days)			
≤2	28 (51.9)	37 (64.9)	0.13
2–5	14 (25.9)	15 (26.3)	
>5	12 (22.2)	5 (8.8)	
Concentration (million/ml)			
Mean (SD)	29.3 (35.0)	42.1 (32.0)	0.46
Back-transformed mean (95% CI)	14.6 (9.9–21.7)	28.1 (21.1–37.6)	0.008
Median (IQR)	17.7 (7.6–36.6)	37.0 (18.3–63.3)	0.004
Total count (million)			
Mean (SD)	70.1 (94.6)	117.5 (126.8)	0.03
Back-transformed mean (95% CI)	28.6 (18.2–45.1)	69.7 (50.8–95.7)	0.002
Median (IQR)	31.9 (11.7–90.3)	86.8 (43.8–149.7)	0.001
Progressive motility (A + B) (%)			
Mean (SD)	44.9 (18.2)	50.1 (13.4)	0.09
Median (IQR)	46.0 (30.2–59.0)	50.0 (39.0–60.0)	0.19
Total motile count (million)			
Mean (SD)	40.0 (64.2)	62.4 (70.5)	0.08
Back-transformed mean (95% CI)	15.4 (10.2–23.3)	36.5 (26.7–49.8)	0.001
Median (IQR)	12.7 (4.6–48.6)	38.6 (17.9–84.4)	0.002
Total motility (A + B + C) (%)			
Mean (SD)	53.6 (16.8)	56.4 (12.2)	0.32
Median (IQR)	55.0 (41.0–68.0)	56.0 (47.5–64.0)	0.61
Normal morphology (%)			
Mean (SD)	3.3 (2.4)	4.0 (3.2)	0.16
Back-transformed mean (95% CI)	3.6 (3.1–4.3)	4.0 (3.3–4.9)	0.43
Median (IQR)	3.0 (1.0–5.0)	4.0 (1.0–5.5)	0.29
Neck-midpiece defects, *n* (%)	26 (48.1)	30 (52.6)	0.64
Head defects, *n* (%)	40 (74.0)	45 (78.9)	0.55
Tail defects, *n* (%)	16 (29.6)	14 (24.5)	0.55
Volume (ml)			
Mean (SD)	2.5 (1.4)	2.8 (1.5)	0.25
Median (IQR)	2.2 (1.5–2.9)	2.6 (1.7–3.5)	0.22
Semen parameters according to WHO references			
Concentration < 15 million/ml, *n* (%)	23 (42.6)	12 (21.1)	0.01
Concentration < 5 million/ml, *n* (%)	8 (14.8)	4 (7.0)	0.19
Progressive motility <32%, *n* (%)	14 (26.0)	5 (8.8)	0.02
Normal morphology <4%, *n* (%)	34 (64.2)	26 (45.6)	0.05
Total count < 39 million, *n* (%)	28 (53.8)	13 (22.8)	0.001

IQR, interquartile range.

ICSI men were not more likely to have sperm concentrations <15 million/ml if their fathers had sperm concentration <15 million/ml (OR 0.4; 95% CI 0.1–1.2). Also, ICSI men were not more likely to have sperm concentrations <15 million/ml if their father had severe oligozoospermia (<5 million/ml) (OR 0.4; 95% CI 0.1–1.2). ICSI fathers with total sperm count <39 million were more likely to have sons with total sperm count >39 million (OR 0.1; 95% CI 0.0–0.4). Only 40% of the fathers with total sperm count <39 million had sons with total sperm count <39 million.

Table III Differences in sperm parameters between the ICSI and the control group: unadjusted and adjusted results from multiple linear regression analysis.

	Unadjusted			Adjusted for covariates		
	Ratio	**95% CI**	***P*-value**	**Ratio**	**95% CI**	***P*-value**
Concentration	1.9	1.2–3.1	0.008	1.9	1.1–3.2	0.017
Total count	2.4	1.4–4.2	0.001	2.3	1.3–4.1	0.005
Total motile count	2.3	1.4–3.9	0.001	2.1	1.2–3.6	0.007
Morphology	1.1	0.8–1.4	0.43	1.1	0.8–1.4	0.52
	Regression coefficient	**95% CI**	***P*-value**	**Regression coefficient**	**95% CI**	***P*-value**
Progressive motility	5.2	−0.8–11.1	0.09	1.9	−4.4–8.3	0.54
Total motility	2.8	−2.7–8.3	0.32	−0.5	−6.1–5.2	0.87
Volume	0.3	−0.2–0.9	0.25	0.2	−0.4–0.7	0.57

Adjustments made for age, BMI, genital malformation, time from ejaculation to analysis, abstinence period.

Table IV Risk of reduced semen quality in ICSI men versus controls: unadjusted and adjusted results from multiple logistic regression analysis.

	Unadjusted		Adjusted for covariates	
	OR (95% CI)	***P*-value**	**OR (95% CI)**	***P*-value**
Concentration <15 million/ml	2.8 (1.2–6.4)	0.016	2.7 (1.1–6.7)	0.035
Concentration <5 million/ml	2.3 (0.6–8.1)	0.19	2.6 (0.6–10.7)	0.20
Progressive motility <32%	3.6 (1.2–10.9)	0.02	2.3 (0.7–7.9)	0.15
Normal morphology <4%	2.1 (0.9–4.6)	0.05	2.3 (0.9–5.4)	0.06
Total count <39 million	3.9 (1.7–9.0)	0.001	4.3 (1.7–11.3)	0.002

Adjustments made for age, BMI, genital malformation, time from ejaculation to analysis, abstinence period.

Table V Semen characteristics of the ICSI fathers.

Semen characteristics	Mean (SD) *N* = 54	Median (IQR) *N* = 54
Concentration (million/ml)	11.6 (15.8)	5.7 (0.8–18.1)
Total sperm count (million)	37.1 (47.9)	16.4 (1.4–60.9)
Progressive motility (A + B) (%)	28.8 (25.1)	20.0 (9.7–50.0)
Total motile count (million)	12.1 (23.0)	2.6 (0.1–11.1)
Total motility (A + B + C) (%)	47.0 (24.6)	45.0 (31.7–64.5)
Normal morphology (%)	10.0 (10.9)	6.0 (2.0–15.2)
Volume (ml)	3.7 (1.9)	3.8 (2.3–5.0)

Discussion

This study investigated the reproductive health of the worldwide oldest cohort of ICSI offspring, having reached the age of 18–22 years. Compared to spontaneously conceived peers, young ICSI men showed lower sperm concentration, lower total sperm count as well as lower total motile sperm count. In terms of WHO (2010) reference values, young adult ICSI offspring were almost three times more likely to have sperm concentrations <15 million/ml and four times more likely to have total sperm count <39 million. ICSI men also had a non-statistically significant two-fold higher risk of having normal morphology rates <4%. In our rather small sample of 54 ICSI men, fathers with sperm concentrations <15 million/ml did not have offspring with sperm concentrations <15 million/ml.

Our findings are robust even after adjusting for covariates that are generally known to affect sperm parameters. In our data, no correlation between BMI, season of sampling, smoking, alcohol consumption and drug use could be found with any of the semen parameters. Also in the literature, BMI (MacDonald *et al.*, 2010), season of sampling (Francavilla *et al.*, 2007), alcohol consumption (Jensen *et al.*, 2014), drug (ab)use (Gundersen *et al.*, 2015) are not univocally correlated with semen parameters. Although smoking has been repeatedly negatively associated with conventional semen parameters (Sharma *et al.*, 2016), the effect of smoking might not be detectable in our rather young aged groups who only smoke occasionally. In our study, in contrast to a Japanese study (Sakamoto *et al.*, 2008), testicular volume was not correlated to semen parameters.

Comparing our outcomes to literature data is difficult since semen parameters of ICSI offspring are not described yet. Although a 46% lower sperm concentration and a 45% lower total sperm count were found in a study of 47 young adults born after maternal 'fertility treatment' in comparison with young adults born to mothers who did not receive any fertility treatment (Jensen *et al.*, 2007), our results in ICSI offspring show even more pronounced lower sperm concentrations

and lower total sperm counts in comparison to peers born without any fertility treatment, which might be explained by the different background of the parents (female- versus male-factor infertility).

Why this eldest cohort of ICSI-conceived men have both low-sperm quantity and quality is not clear? The unfavourable semen characteristics of the ICSI fathers are not predictive of the birth of a son facing deficient spermatogenesis, according to our rather small sample. However, this finding does not exclude the transgenerational inheritance of impaired spermatogenesis since the risk of low concentration and low total sperm count in ICSI offspring was found to be three to four times higher compared to a cross-sectionally recruited control group, even after ruling out Yq deletions. Also, copy number variants (Krausz *et al.*, 2012) and polymorphisms in other genes than those from the Y chromosome have been associated with male infertility (Aston *et al.*, 2010, Sato *et al.*, 2015). Unfortunately, the genetic origins of male-factor infertility remain largely unknown. Furthermore, epigenetic modifications, including aberrant DNA methylation in spermatozoa, have been associated with oligozoospermia, abnormal sperm morphology and decreased progressive motility (see supplementary table of Laurentino *et al.*, 2016). However, it remains to be elucidated if epimutations of spermatozoa can be transmitted to the offspring (Reik *et al.*, 2001). Further research should focus on several approaches to assess men's fertility potential: from exome and whole genome sequencing and epigenetics to novel seminal biomarkers. Since the cellular and molecular functioning of a spermatozoon remains poorly understood, which prevents insights into dysfunctional sperm cells, proteomics might reveal abnormal protein profiles associated with impaired spermatogenesis (Barratt, 2008). Importantly, given the results of this study, paired analysis of samples from fathers and sons is recommended. Alternatively, although birth weight as a proxy of foetal growth was comparable between the groups, suboptimal foetal growth patterns in ICSI offspring might be responsible for lower sperm quality, as has recently been shown in an unbiased cohort of Australian young men (Hart *et al.*, 2016).

To unravel the effect of the procedure, i.e. ICSI, versus the background risk, i.e. male infertility, on the reported findings in ICSI offspring is precarious. Whether the more invasive ICSI procedure is associated with adverse outcomes can be studied in male offspring conceived by conventional IVF (without ICSI) applied in couples with severe male-factor infertility, which is evidently very difficult. To link the male infertility factor with the reported findings, we should have included a cohort of male offspring conceived by ICSI for indications other than male infertility. However, in the early 1990s when ICSI was developed in our centre and patients were referred for treatment, the main indication for ICSI was male infertility. Outcomes in future generations of ICSI offspring may elucidate this hypothesis.

Considering that semen delivery in young adults is a serious challenge, our study had a rather high response rated since 37.5% (tested/reached) or 47% (tested/actually reached) of the ICSI-conceived men were willing to participate. The response rate in the control group could not be calculated since various ways of advertising were adopted. For both the ICSI and the control populations, the deposit of a semen sample turned out to be a major difficulty. Overall, acceptance rates of volunteers to provide a semen sample for research are between 13% and 19% and thus evidently do not represent the general population (Cooper *et al.*, 2010). One might speculate that the recruited control group in this study is more healthy than average given the apparently lower rates of tobacco use, drug use, alcohol consumption and the higher number of hours of sports per week, which may infer 'better outcomes' for semen parameters in the control group. When we compare the sperm concentration as observed in our control group with that described in the literature, the median sperm concentration of 37 million/ml in our control group was not significantly different from that in other unbiased cohorts: a cohort of 19-year-old Danish men who had a medical examination for military drafting (median 41 million/ml) (Andersen *et al.*, 2000) or the Western Pregnancy Cohort (Raine) consisting of 20- to 22-year-old men (median 45 million/ml) (Hart *et al.*, 2015) (Wilcoxon Signed Rank test: $P = 0.73$ and $P = 0.23$, respectively). Although population differences may exist, the median sperm concentration of the controls reported here are comparable to the literature data that confirm that our control group is comparable to volunteers in other countries. In addition, our group of spontaneously conceived men of the same age as the study participants had no prior knowledge of their own fertility status and thus this is unlikely to have influenced their motivation to participate. However, although participation bias in the ICSI group cannot be ruled out, this type of bias is unlikely too. One may hypothesize that parents who needed ICSI because of severely impaired semen quality would 'encourage' their sons to participate in order to discover whether their sons have compromised spermatogenesis too. Nearly one in three of the parents who refused participation disallowed us to contact their sons, either because of non-disclosure of the conception mode or because of 'the family is not interested', the latter pointing to a secretive attitude that is known to be more prominent in couples with male infertility than in those with female infertility (Hjelmstedt *et al.*, 1999). In any case, since the great majority of the non-participating ICSI families (88%, versus 89% in the participating families) had male-factor infertility as the indication for ICSI, we do not assume that the results observed in the group of participating ICSI men are biased towards a worse fertility status than in the non-participant groups.

An advantage of this study is that every individual was examined in one single centre. Indeed, data on weight and height but also information on genital malformations are retrieved from a physical examination in our centre and are thus more accurate than self-reported data as used in other outcome studies.

Strikingly, both in the ICSI and the control group, men with low-quality semen samples were invited to deliver a second sample, which occurred for only one ICSI men, and with comparable results in both samples. Although a large within-subject variability in semen parameters has been reported (Keel, 2006; Francavilla *et al.*, 2007), it has also been shown that within-subject coefficients of variation are lower in sperm donors compared with infertile men (Keel 2006). Other studies confirmed that in fertile men, a second semen analysis does not systematically differ from a first one (Stokes-Riner *et al.*, 2007; Zhu *et al.*, 2016). Nevertheless, our findings of semen parameters based on one semen sample should be interpreted accordingly.

In conclusion, the worldwide oldest ICSI-conceived adults showed significantly lower sperm concentration, lower total sperm count as well as lower total motile sperm count in comparison to a control group of spontaneously conceived peers. The risk of having sperm concentration and total sperm counts below the reference values was increased in ICSI offspring compared to controls. This finding should be interpreted in view of the background of the parents: ICSI was performed with ejaculated sperm because of impaired spermatogenesis. A generalization to all offspring born after ICSI, including ICSI for other indications than male infertility and ICSI in combination with non-

ejaculated sperm, can therefore not be made. Although we did not observe a clear correlation between the semen parameters of the ICSI men and their fathers, further genetic and epigenetic investigation of transgenerational passage of male infertility is highly mandatory.

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Authors' roles

This part of the project focussing on reproductive status was designed by F.B., M.B., G.V., H.T. and A.V.S. The protocol for physical assessments was designed by D.M. F.B. collected the data and analysed the data together with M.R.. All co-authors interpreted the data. F.B. wrote the paper, and it was finalized by all co-authors. All co-authors approved the definitive version of the manuscript.

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Conflict of interest

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