Testicular Function in a Birth Cohort of Young Men

Running title "Testicular function of Young Men"

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STUDY QUESTIONS: Using a novel approach to derive an unbiased population by investigating a birth cohort with a high on-going participation rate, what are the parameters and influences upon testicular function for a population not selected with regard to fertility?

SUMMARY ANSWER: While varicocele, cryptorchidism and obesity may impact on human testicular function, most common drug exposures and the presence of epididymal cysts appear to have no/minimal adverse impact. For each separate semen parameter 15-20% of men did not meet the WHO reference criteria for fertile men, and only 14.4% met all of the WHO reference range criteria.

WHAT IS KNOWN ALREADY: The majority of previous attempts to develop valid reference populations for spermatogenesis have relied on potentially biased sources such as recruits from infertility clinics, self-selected volunteer sperm donors for research or artificial insemination or once-fertile men who were seeking vasectomy. It is well known that studies requiring semen analysis have low recruitment rates, which consequently question their validity; however, there has been some concern that a surprisingly high proportion of young men may have semen variables that do not meet all the WHO reference range criteria for fertile men, with some studies reporting that up to one half of participants did not meet the reference range for fertile men. Reported median sperm concentrations ranged from 40-60 million sperm/ml

STUDY DESIGN, SIZE AND DURATION: The Western Australian Pregnancy Cohort (Raine) was established in 1989. At 20-22 years of age members of the cohort were contacted to attend for a general follow-up, with 753 participated of the 913 men contactable. Of these men 423 (56% of those participating in the 20-22 year cohort study, 46% of contactable men from the original cohort) participated in a testicular function study. Of the 423 men, 404 had testicular ultrasound, 365 provided at least one semen sample (287 provided a second) and 384 provided a blood sample.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Testicular ultrasound examinations were performed at King Edward Memorial Hospital, Subiaco, Perth, for testicular volume and presence of epididymal cysts and varicoceles. Semen samples were provided and analysed by standard semen assessment and a sperm chromatin structural assay (SCSA) at Fertility Specialists of Western Australia, Claremont, Perth. Serum blood samples were provided at the University of Western Australia, Crawley, Perth and were analysed for serum luteinising hormone (LH), follicular stimulating hormone (FSH), inhibin B, testosterone, dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), estradiol, estrone and the primary metabolites of DHT: 5α-androstane-3α,17β-diol (3α-diol) and 5-α androstane-3-β-17-beta-diol (3β-diol). Serum steroids were measured by liquid chromatography, mass spectrometry and LH, FSH and inhibin B by ELISA assays.

MAIN RESULTS AND THE ROLE OF CHANCE: Cryptorchidism was associated with significant reduction in testicular (p=0.047) and semen (p=0.027) volume, sperm concentration (p=0.007) and output (p=0.003). Varicocele was associated with smaller testis volume (p<0.001), lower sperm concentration (p=0.012) and total sperm output (p=0.030) and serum inhibin B (p=0.046). Smoking, alcohol intake, herniorrhaphy, an epididymal cyst, medication and illicit drugs were not associated with any significant semen variables, testicular volume or circulating reproductive hormones. BMI had significantly negative correlation with semen volume (r=-0.12, p=0.048), sperm output (r=-0.13, p=0.02), serum LH (r=-0.16, p=0.002), inhibin B (r=-0.16, p<0.001), testosterone (r=-0.23, p<0.001), DHT (r=-0.22, p<0.001) and positive correlation with 3αD (r=0.13, p=0.041) and DHEA (r=0.11, p=0.03). Second semen samples compared with the first semen samples in the 287 participants who provided two samples with no significant bias by Bland-Altman analysis.

Testis volume was significantly correlated positively with sperm concentration (r=0.25, p<0.001) and output (r=0.29, p<0.001) and inhibin B (r=0.42, p<0.001), and negatively with serum LH (r=-0.24, p<0.001) and FSH (r=-0.32, p<0.001). SCSA was inversely correlated with sperm motility (r=-0.20, p<0.001), and morphology (r=-0.16, p=0.005).

WHO semen reference criteria were all met by only 52 (14.4%) of men. Some criteria were not met at first analysis for semen volume (<1.5 mls, 14.8%), total sperm number (<39 million, 18.9%), sperm concentration (<15 million/ml, 17.5%), progressive motility (<32%, 14.4%), and morphologically normal sperm (<4%, 26.4%), while all five WHO criteria were not met for 4 participants (1.1%).

LIMITATIONS AND REASONS FOR CAUTION This was a large cohort study; however, potential for recruitment bias still exists. Men who did not participate in the testicular evaluation study (n=282) did not differ from those who did (n=423) with regard to age, weight, BMI, smoking or circulating reproductive hormones (LH, FSH, inhibin B, T, DHT, E_2, E1, DHEA, 3α-diol, 3β-diol), but were significantly shorter (178 vs 180 cm, p=0.008) and had lower alcohol consumption (p=0.019) than those who did participate.

WIDER IMPLICATIONS OF THE FINDINGS This study demonstrated the feasibility of establishing a birth cohort to provide a relatively unbiased insight into population-representative sperm output and function and investigating its determinants from common exposures. While varicocele, cryptorchidism and obesity may impact on human testicular function, most common drug exposures and the presence of epididymal cysts appear to have little adverse impact, and this study suggests that discrepancies from the WHO reference ranges are expected due to its derivation from non-population representative fertile populations.

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D.A.D No competing interests

RMcL is a shareholder in the Monash IVF Group

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Introduction

Both functions of the testis; spermatogenesis and steroidogenesis, require the development of valid population-based reference ranges. While identifying reference ranges for testosterone, the principal testicular androgen, is feasible if coupled with population-representative sampling. Spermatogenesis, however, is more difficult to calibrate as it require semen samples collected by masturbation, which is a deterrent to men's participation and hinders recruitment.

Most previous initiatives to identify valid reference ranges rely on studies of populations, such as infertility clinics, or non-infertile groups of men comprising sperm donors for research clinics, or laboratories, or for artificial insemination, or once-fertile men seeking vasectomy. However, each group has significant biases, notably low numbers of volunteers or biased participation rates, making them unsuitable to serve as valid reference populations. Studies of volunteer sperm donors typically feature very low participation rates (<20%), which may mask undefinable positive and negative participation biases with regard to the men's fertility. Since 1980 (World Health Organisation, 1980), the World Health Organization has published successive editions of its manual which standardized semen analysis and *inter alia* provided notional reference ranges based on expert group oracular statements until, in preparation for its 5th edition in 2010 World Health Organisation (2010), WHO's writing group undertook extensive evaluation to arrive at empirically-based reference range recommendations derived from populations of recently fertile men (Cooper, et al., 2010).

These uncertainties concerning valid population representative sampling have rendered it impossible to critically evaluate claims that sperm production in the general population may have been in decline. The present study has aimed to provide a novel approach to establishing a valid reference population which could be the basis for a comprehensive normative evaluation of human testicular function. In this study we profile a birth cohort of Australian men aged 20 years where the participation rates are relatively high and unbiased with regard to fertility. This study provides state-

of-the-art evaluations of testicular volume (by ultrasound) and a profile of circulating reproductive hormones (including steroid mass spectrometry profiling) together with detailed semen analysis findings.

Materials and Methods

The Raine study

The Western Australian Pregnancy Cohort (Raine) Study was formed from a pregnancy cohort study, in which 2,900 women were enrolled in a controlled trial by the 18th week of gestation from the antenatal booking clinics. Mothers were enrolled over a total of 30 months commencing in May 1989 and finishing in November 1991. The last children were born in April 1992. The 2868 children (including 1454 boys) born to 2804 mothers were retained to form the Western Australian Pregnancy Cohort (Raine) Study. The study aimed to investigate the role of perinatal events on subsequent childhood and adult health. The cohort is unique because detailed antenatal, postnatal and childhood measurements have been made. Cohort follow-up was undertaken at ages 1, 2, 3, 5, 8, 10, 14, 17, 20 and 22 years with the latest cohort including 1433 men still alive**,** making it one of the largest and most closely followed prospective cohorts of pregnancy, childhood and adolescence in the world. The current cohort of men aged 20-22 years included 913 men who were contactable of whom 753 participated in the 20-22 year follow-up study. Of these 753 men, 695 underwent physical examination, 608 completed medical questionnaires and 423 (56% of those participating in the 20-22 year cohort study, 46% of contactable men from the original cohort) participated in the testicular function study. Ethical approval was obtained from the University of Western Australia HREC, and all participants provided informed consent.

At 20 years of age members of the cohort were contacted to request they attend for follow-up. They were provided with information on the investigations which involved general health and a detailed eye assessment, dual energy X-ray absorptiometry measurement in one location, a testicular

ultrasound examination at another hospital and information on producing a semen sample at the IVF unit (Fertility Specialists of Western Australia). Members of the cohort were encouraged to take part in all the assessments, but were able to decline participation in some or all assessments if they desired. Of the 423 men, 404 had testicular ultrasound, 365 provided at least one semen sample (287 provided a second) and 384 provided a blood sample.

Semen analysis

Semen samples were provided at the IVF unit by masturbation, and the men were encouraged to have the recommended abstinence intervals (2-7 days) as per WHO semen manual guidelines (Cooper, et al., 2010). All volunteers were encouraged to perform a second semen sample regardless of the outcome of the first semen analysis. At the end of the study they were informed as to whether semen sample(s) was normal or abnormal and those with reduced semen parameters were offered appropriate counselling, investigation, follow-up and opportunity to cryostore semen if appropriate or required.

Standard semen assessment was performed according to the contemporaneous WHO semen manual (WHO, 1999) with results reported according to current WHO parameters (Cooper, et al., 2010) except that sperm concentration was determined by using a Makler chamber and semen volume was determined using a graduated, volumetric pipette, which differs from WHO recommendations. Sperm output was characterized as both sperm concentrations (million sperm per ml of ejaculate) and total sperm output (million per ejaculate). Total motile sperm (TMS) was derived from calculation of the seminal volume x concentration x motility (%A grade + %B grade). Sperm morphology was assessed according to the WHO strict criteria. Semen analyses were performed by experienced clinical scientists who undergo regular external and internal quality control assessment (EQASRM, Australia). For sperm concentration, morphology and motility, inter-laboratory and intra-laboratory results are plotted against the mean with strict warning (two

197 standard deviations from the mean) and action (three standard deviations from the mean) control
198 limits enforced, according to those recommended by the EQASRM program (Palacios, et al., 2012).
199
200 The sperm chromatin structural assay (SCSA) was performed as described (Evenson and Jost, 2000)
201 with slight modifications. In brief, the semen sample was diluted to a concentration of 5-10 × 10^6
202 spermatozoa/mL in a buffer solution (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA) diluted 10 x in
203 water at a pH of 7.5 and in a total volume of 500 µL. Samples were then snap frozen in liquid
204 nitrogen and stored for up to 14 days until analysis. External quality control samples were sourced
205 from the DNA fragmentation (by SCSA), quality assurance program (FertAid, NSW) and performed
206 prior to each sample batch. Assay controls were run in duplicate with an intra-assay average quality
207 control CV of 13.0%. Once thawed, samples were exposed to 30 seconds of treatment with acid
208 detergent comprised of 150 mM NaCl, 0.1% Triton X-100 at pH 1.2. After 30 sec, 6 µg/mL acridine
209 orange staining buffer was added to the sample for a further 2.5 min. Finally, a total of 5000 sperm
210 cells were analyzed by flow cytometry (FACSCallibur; Becton Dickinson). The DNA fragmentation
211 index (DFI) represents the percentage of sperm within the sample with fragmented or damaged DNA
212 The percentage of sperm with high DNA stainability (HDS) represent the percentage of sperm within
213 the sample with incomplete chromatin condensation.
214
215 *Hormone assays*
216 Serum inhibin B concentrations were measured in duplicate by Inhibin B Gen II ELISA from Beckman
217 Coulter Inc. (Brea, CA), which had a limit of detection of 2.6 pg/ml. Luteinising hormone (LH),
218 follicular stimulating hormone (FSH) levels were determined in duplicate using ELISA kits from IBL
219 International, Hamburg, Germany. The limit of detection of the LH assay was 0.4 IU/L (calibrated
220 against WHO IRP 80/552), while for FSH assay it was 0.2 IU/L (calibrated against NIBSC 92/510). The
221 intra-assay precision (CV) of the ELISAs ranged from 8-11% based on the mean values for low and
222 high value quality control samples from n=16-17 assays.

Serum steroids were measured by liquid chromatography, mass spectrometry as previously described (Harwood and Handelsman, 2009) with limits of quantitation for analytes as follows: testosterone (0.025 ng/ml), dihydrotestosterone (DHT, 0.1 ng/ml), dehydroepiandrosterone (DHEA, 0.05 ng/ml), estradiol (5 pg/ml) and estrone (5 pg/ml) and the primary metabolites of DHT: 5α-androstane-3α,17β-diol (3α-diol, 0.1 ng/ml) and 5-α androstane-3-β-17-beta-diol (3β-diol, 0.1 ng/ml). Reproducibility was <10% for each analyte in male serum.

Testicular Ultrasound

Ultrasound assessment of the scrotum was conducted by a single experienced operator with the men positioned supine and the scrotum supported by a rolled towel. All examinations were performed with light direct transducer contact using a 9-3 MHz linear array transducer (Philips IU22, Philips Healthcare). Each testis was measured by estimating its maximal dimensions in 3 planes, excluding the epididymis, using electronic calipers and testis volume was calculated using the formula for a prolate ellipsoid (length (L) x width (W) x height (H) x 0.52) (Lenz, et al., 1993, Sakamoto, et al., 2007). One value for L, W and H was recorded per examination. Testicular echogenicity and structures within the scrotum were also assessed including venous diameter measured in the supine position with Valsalva maneuver. Varicocele was defined as present when the maximal venous diameter was over 3mm, and increased with the Valvalva maneuver (Oyen, 2002, Pilatz, et al., 2011).

Statistical Analysis

Continuous data were summarized using means and standard deviations (SD) or medians and inter-quartile ranges (reported as Q1-Q3) when data followed a non-Gaussian distribution. Where data had a non-Gaussian distribution, power transformation was performed with cube-root transformation for sperm data (Handelsman, 2002) and other optimal power transforms according

to Box-Cox analysis (Box and Cox, 1964) using Shapiro-Wilks λ as an omnibus estimate of Gaussian distribution (Shapiro and Wilks, 1965) as required. Categorical data were summarized using frequency distributions. Paired t-tests (or Mann-Whitney tests for non-Gaussian data) for two groups or analysis of variance for more than two groups were used to compare subgroups of the cohort. Association between the transformed testicular and semen parameters were estimated using Pearson correlation coefficient. The reproducibility of semen analysis parameters between the first and second semen analysis was evaluated by Bland-Altman statistics for assessing agreement between two measurements of the same variable (Bland and Altman, 1986). Empirical curve fitting of semen variables on abstinence interval was performed using NCSS non-linear routines for fitting a curve rising to a maximum in a 3 parameter model of the form Y=a*(1-exp(-b(X-c))). Hypothesis tests were two-sided with p-values<0.05 considered statistically significant. SPSS (version 20.0, IBM SPSS) or NCSS (version 10, Kaysville, Utah, USA www.ncss.com) statistical software were used for data analysis.

Results

Description of study population

Among 423 cohort members who participated in the testicular function study, 365 (46.4%) underwent semen assessments with 287 (79% of those providing a first semen analysis, 68% of participants) providing a second semen sample. Testicular ultrasound examination was performed on 404 (51.4%) men with 346 having at least one semen sample and an ultrasound examination, 58 having just an ultrasound and 19 just a semen sample.

The anthropometric, testis volume, reproductive hormone and semen data from 423 men is provided in Table 1. Epididymal cysts were present in 125 men (32.8%, unilateral in 103 and equally common on left and right, 22 bilateral), varicocele was present in 100 men (24.9%, unilateral 76,

bilateral 24), a history of cryptorchidism in 9 (2.1%) and herniorraphy in 6 (1.5%); none reported a history of mumps orchitis.

Evaluation of participation bias

Men who did not participate in the testicular evaluation study (n=282) did not differ from those who did (n=423) in age, weight, BMI, smoking or circulating reproductive hormones (LH, FSH, inhibin B, T, DHT, E_2, E1, DHEA, 3α-diol, 3β-diol), but were significantly shorter (178 vs. 180 cm, p=0.008) and had lower alcohol consumption (p=0.019) than those who did participate.

Men who provided a second sample did not differ significantly from those who only provided a single semen sample in age, height, weight, BMI, alcohol intake, intake of medications, history of cryptorchidism, herniorraphy, or presence of a varicocele or an epididymal cyst, testis volume, semen variables or circulating reproductive hormones but were more likely to smoke (28.6% vs. 12.8%, p=0.004) and marginally more likely to use illicit drugs (p=0.048).

Evaluation of semen variables and their determinants

Azoospermia was present in 5 men's semen analysis, 4 on the first and 4 on the second semen sample, but only 3 men were azoospermic on both samples with the other two men having a low sperm output (<1 million) in the other sample. Those 3 men had low-normal testis volume (9.4-15.2 ml) with serum FSH high in one (14.3 IUL) while the other two had values (6.8, 7.0 IU/L) at the upper end of the reference range for young Australian men with normal semen analysis (8.4 IU/l) (Sikaris, et al., 2005). In addition sperm morphology could not be assessed in 11 men due to azoospermia or severe oligozoospermia (<5 million/ml). Abstinence interval of up to 4 days was a significant positive correlate of semen volume, but not sperm concentration or output. For abstinence intervals longer than 4 days abstinence intervals did not correlate with semen volume, sperm concentration or output.

Cryptorchidism was associated with significant reduction in testis (p=0.047) and semen (p=0.027) volume, sperm concentration (p=0.007) and output (p=0.003), but not other semen variables or circulating reproductive hormones (Table 2). Varicocele was associated with significantly lower body weight (p=0.019) and BMI (p=0.001), smaller testis volume (p<0.001), lower sperm concentration (p=0.012) and output (p=0.030) and serum inhibin B levels (p=0.046), but no other differences in height or other semen and reproductive hormone variables. Alcohol consumption was associated with reduced abstinence interval (p=0.029), but no other differences in testis volume, other semen variables or concentrations of circulating reproductive hormones. Smoking, herniorrhaphy, an epididymal cyst, medication (prescription or non-prescription) and illicit drugs (marijuana, others, both) were not associated with any significant differences in mean testis volume, any semen variable (semen volume, sperm concentration or output, motility) or circulating reproductive hormone levels (serum LH, FSH, inhibin B, testosterone, DHT, DHEA, estradiol, estrone, DHEA).

Testis volume was positively associated with markers of testicular function and BMI was negatively associated with markers of spermatogenesis. Correlations between semen parameters and between semen parameters and anthropometric and serum blood measurements are listed in tables 4 and 5.

Second semen samples compared with the first semen samples in the 287 participants who provided two samples exhibited no significant bias by Bland-Altman analysis for any semen variables (abstinence interval, semen volume, sperm concentration and output, TMS, sperm motility; data not shown). The mean coefficient of variation (natural scale) for sperm output was 47% (SD 33%, median 43%, 18% - 67%).

WHO semen reference criteria (Cooper, et al., 2010) were all met by only 52 (14.4%) men. In the remainder, some criteria were not met at the first semen analysis for semen volume (<1.5 ml, 14.8%), total sperm number (<39 million, 18.9%), sperm concentration (<15 million/ml, 17.5%), progressive motility (A + B grade motility <32%, 14.4%), and morphologically normal sperm (<4%, 26.4%) while all five WHO criteria were not met for 4 participants (1.1%).

Discussion

The main findings of this first birth cohort study of mature male reproductive function are consistent with previous reports identifying an impact of varicocele (Masson and Brannigan, 2014) and cryptorchidism (Thorup, et al., 2010) on spermatogenesis (but not on steroidogenesis), as well as the effects of obesity (MacDonald, et al., 2010, Sermondade, et al., 2013) on sperm output and reproductive hormones. On the other-hand cigarette smoking and alcohol and other drug consumption were not associated with a reduction in any markers of testicular function in this population of young men.

The present study of a birth cohort provides a novel approach to defining a reference population suitable for developing normative assessment for both major functions of the human testis. Using the Raine birth cohort, the present study provides a comprehensive description of spermatogenesis and steroidogenesis and their determinants using state-of-the-art methodologies. Many previous attempts to develop valid reference populations for spermatogenesis have had to rely mainly on sources such as infertility clinics, self-selected volunteer sperm donors for research or artificial insemination or once-fertile men who were seeking vasectomy, where recruitment is biased with regard to their fertility. It is well known that studies requiring semen analysis have low recruitment rates, raising questions as to their validity, and the potential for selection bias is often acknowledged by the authors (Handelsman, 1997, Muller, et al., 2004). The present study reduces the influence of employing biased sources by evaluating a pregnancy cohort which originated from recruitment of

unselected mothers during their pregnancy 20 years previously. Another approach to recruit an unbiased male population has been to study military conscripts undergoing compulsory medical examination in Nordic and Baltic countries, namely Denmark (Andersen, et al., 2000), Sweden (Richthoff, et al., 2002), Denmark, Norway, Estonia and Finland (Jorgensen, et al., 2002), Latvia (Tsarev, et al., 2005) and Germany (Paasch, et al., 2008). However, participation rates in those studies were very low (13-19%) or unstated (Paasch, et al., 2008) allowing for significant influence of volunteer participation bias. In addition, none provided comprehensive and integrated analysis of testicular function comparable with the present study. Other studies of volunteers mostly suffer from unknown participation bias as their source population for recruitment is usually unknown. An interesting exception was a Japanese study which adapted a modified capture-recapture method to enumerate an unknown population (Iwamoto, et al., 2013a) to determine that recruitment of University students in four cities was still only 16.6% with prominent between-site variability.

The present study still has some residual potential participation bias as we only recruited 56% of the men actively participating in the 20-22 year follow-up study, and there were differences in height and alcohol consumption between studied and non-studied men. Nevertheless, this represents a significant achievement, as most previous studies of men unselected for their fertility had recruitment rates mostly <20% (Cooper, et al., 2010), Iwamoto, et al., 2013a, Swan, et al., 2003). The Raine cohort was developed by enrolment of their pregnant mothers in 1990-1991 for a randomized study of ultrasound monitoring of fetal growth and the cohort has been followed closely since with high cohort retention (www.rainestudy.org.au). Hence original recruitment of males into the birth cohort was unbiased with regard to their fertility as adults, although those choosing to contribute semen samples within the follow-up study were self-selected.

This study has sufficient power to evaluate the impact on testicular function of many relatively common medical, demographic, anthropometric, environmental and physical exposures. For the

376 common exposures such as smoking, alcohol as well as illicit, over-the-counter or prescribed drugs
377 or the presence of epididymal cysts, their minimal or no impact on testicular function cannot be
378 realistically ascribed to participation bias or lack of study power. It has been proposed that there
379 may be other environmental factors that may impair sperm production or function and fertility, and
380 this cohort may allow for critical evaluation of the impact of postulated reproductive hazards such as
381 pollutants (Guven, et al., 2008), plastics (Liu, et al., 2012), pesticides (Hossain, et al., 2010),
382 sedentary activity (Gaskins, et al., 2013), cycling (Gebreegziabher, et al., 2004) and exposure to
383 breast milk (Laustsen, et al., 2011).
384
385 Our study confirms previous reports that a relatively high proportion of young men may have semen
386 variables that do not meet all the WHO reference range criteria (Lemcke, et al., 1997, Andersen, et
387 al., 2000, Richthoff, et al., 2002, Iwamoto, et al., 2013a, Jorgensen, et al., 2012) yet each of these
388 populations have relatively similar median sperm concentrations and sperm output to our findings.
389 The most probable reasons for this discrepancy compared to WHO recommended reference range
390 criteria is based on the origins of the WHO reference range which was derived by assembling and
391 pooling studies based on recently fertile men; however, they represent an elite rather than a
392 population-representative population. For example, characteristic of economically developed
393 countries, in 2014 Australia had 6.2 million men aged 18-55 years and 308,000 births, so that recent
394 fathers represent only ~5% of men of reproductive age. As male fertility is proportional to sperm
395 concentration up to a plateau at about 40 million spermatozoa per ml (Bonde, et al., 1998, Slama, et
396 al., 2002), selecting a reference population based on recently fertile men must inevitably produce
397 reference ranges that have superior sperm concentration and related variables to those of an
398 unselected group of men. This expectation is confirmed in the WHO reference range study (Cooper,
399 et al., 2010) as well as in Japan (Iwamoto et al. 2013a,Iwamoto et al. 2013b). Furthermore, the
400 construction of the WHO reference ranges was also primarily guided by the historical focus of semen
401 analysis on evaluation of male (in)fertility leading to a choice in setting the lower limit as a one-sided

95% confidence limit (Cooper, et al., 2010). This choice is consistent with the fact that neither enlarged testes (Meschede, et al., 1995) nor polyzoospermia (Chan, et al., 1986, Tournaye, et al., 1997) have any function significance for fertility. Nevertheless that choice also produces an elevated lower limit than two-sided confidence limits, which might be more appropriate for a reference range unselected for recent fertility, such as may be required for population or toxicological studies of men with undefined fertility. Previous studies which empaneled a eugonadal reference population from healthy volunteers, who had to have normal reproductive function verified by history, physical examination and laboratory tests, have also observed the need to exclude up to 15% of apparently healthy volunteers (Sikaris, et al., 2005).

Given the difficulty of obtaining semen samples in population-based studies an important practical finding from this study is that the first semen sample is unbiased with respect to a second sample. The validity of this interpretation is supported by the fact that men who provided two rather than a single semen sample did not differ significantly indicating no systematic bias in agreement to provide multiple semen samples. This finding is consistent with a previous report from a smaller, multi-centre study (Stokes-Riner, et al., 2007) and may simplify the task of studying populations whereby a single sample may be sufficient to evaluate study cohort for exposure to potential reproductive toxin(s). On the other hand, while a single sample is unbiased, it is subject to considerable biological variability; this creates large sample size requirements for studies aiming to establish effects on sperm output. Similarly, this high within-person variability is the basis for the longstanding recommendation that for individual fertility assessment, multiple semen samples are desirable.

The large and reasonably representative study population of the Raine birth cohort also provides uniquely valuable normative data on circulating reproductive hormones for young men, although it should be noted that the participants in this study were on average 2 cm shorter and consumed less alcohol than the non-participants, which may reflect residual bias in the men whose reproductive

function was studied. Furthermore while assessing sperm concentration with the Makler chamber and the seminal volume by a pipette may differ from WHO recommendations, these measurements potentially form a source of systematic and not a random error. Hence, although the absolute values may differ slightly according to the technique used it will have minimal or no impact on correlations of sperm variables with other factors. Furthermore as only nine men had cryptorchidism this may explain an lack of association with serum gonadotrophins.

We conclude that the Raine birth cohort provides novel, valid insights into human testicular function. The findings are consistent with other attempts to approximate population representative sampling but with a relatively higher participation rate. This study provided findings which confirm the effects of previously known adverse factors for male reproductive function such as varicocele, cryptorchidism and obesity, whereas other putatively hazardous exposures such as smoking, drug use (illicit, over-the-counter, prescription) or epididymal cysts had negligible effects on spermatogenesis or steroidogenesis. Further studies of this cohort will provide opportunity to evaluate the impact of other putative environmental toxins on this relatively unbiased young male population.

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Authors' roles

RJH conceived and established the study, and was primarily responsible for the manuscript

DAD performed statistical analysis and assisted with manuscript preparation

RIM assisted with planning of the study and assisted with manuscript preparation

MLW performed the semen analysis and assisted with manuscript preparation

JAK assisted with interpretation of the data and manuscript preparation

J.E. Dickinson analysed the testicular ultrasounds and assisted with manuscript preparation

NES assisted with planning of the study and with manuscript preparation

RJN assisted with planning of the study and with manuscript preparation

DJH assisted with planning of the study, performed data analysis and androgen assays, and was instrumental in manuscript preparation

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Conflict of Interest

R.H. is the Medical Director of Fertility Specialists of Western Australia and a shareholder in Western IVF. He has received educational sponsorship from MSD, Merck-Serono and Ferring Pharmaceuticals.

D.A.D No competing interests

RMcL is a shareholder in the Monash IVF Group

M.L.W No competing interests

JAK No competing interest

JED No competing interest

RJN is a shareholder in FertilitySA

480 DJH No competing interest

481

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References

Andersen AG, Jensen TK, Carlsen E, Jorgensen N, Andersson AM, Krarup T, Keiding N, Skakkebaek NE. High frequency of sub-optimal semen quality in an unselected population of young men. *Hum Reprod* 2000;15: 366-372.

Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;i: 307-310.

Bonde JP, Ernst E, Jensen TK, Hjollund NH, Kolstad H, Henriksen TB, Scheike T, Giwercman A, Olsen J, Skakkebaek NE. Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. *Lancet* 1998;352: 1172-1177.

Box GEP, Cox DR. An analysis of transformations. *Journal of the Royal Statistical Society B* 1964;26: 211-246.

Chan SY, Tang LC, Tang GW, Ho PC, Wang C. Spermatozoal fertilizing capacity in polyzoospermia: a preliminary study. *Andrologia* 1986;18: 208-213.

Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT *et al.* World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 2010;16: 231-245.

Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci* 2000;22: 169-189.

Gaskins AJ, Mendiola J, Afeiche M, Jorgensen N, Swan SH, Chavarro JE. Physical activity and television watching in relation to semen quality in young men. *Br J Sports Med* 2013.

Gebreegziabher Y, Marcos E, McKinon W, Rogers G. Sperm characteristics of endurance trained cyclists. *Int J Sports Med* 2004;25: 247-251.

Guven A, Kayikci A, Cam K, Arbak P, Balbay O, Cam M. Alterations in semen parameters of toll collectors working at motorways: does diesel exposure induce detrimental effects on semen? *Andrologia* 2008;40: 346-351.

Handelsman DJ. Sperm output of healthy men in Australia: magnitude of bias due to self-selected volunteers. *Hum Reprod* 1997;12: 2701-2705.

Handelsman DJ. Optimal power transformations for analysis of sperm concentration and other semen variables. *J Androl* 2002;23: 629-634.

Harwood DT, Handelsman DJ. Development and validation of a sensitive liquid chromatography-tandem mass spectrometry assay to simultaneously measure androgens and estrogens in serum without derivatization. *Clin Chim Acta* 2009;409: 78-84.

Hossain F, Ali O, D'Souza UJ, Naing DK. Effects of pesticide use on semen quality among farmers in rural areas of Sabah, Malaysia. *J Occup Health* 2010;52: 353-360.

Iwamoto T, Nozawa S, Mieno MN, Yamakawa K, Baba K, Yoshiike M, Namiki M, Koh E, Kanaya J, Okuyama A *et al.* Semen quality of 1559 young men from four cities in Japan: a cross-sectional population-based study. *BMJ Open* 2013;3.

Iwamoto T, Nozawa S, Yoshiike M, Namiki M, Koh E, Kanaya J, Okuyama A, Matsumiya K, Tsujimura A, Komatsu K *et al.* Semen quality of fertile Japanese men: a cross-sectional population-based study of 792 men. *BMJ Open* 2013;3.

Jorgensen N, Carlsen E, Nermoen I, Punab M, Suominen J, Andersen AG, Andersson AM, Haugen TB, Horte A, Jensen TK *et al.* East-West gradient in semen quality in the Nordic-Baltic area: a study of men from the general population in Denmark, Norway, Estonia and Finland. *Hum Reprod* 2002;17: 2199-2208.

Jorgensen N, Joensen UN, Jensen TK, Jensen MB, Almstrup K, Olesen IA, Juul A, Andersson AM, Carlsen E, Petersen JH *et al.* Human semen quality in the new millennium: a prospective cross-sectional population-based study of 4867 men. *BMJ Open* 2012;2.

Laustsen JM, Jensen MS, Thulstrup AM, Gabel P, Toft G, Bonde JP, Olsen J, Ramlau-Hansen CH. Does breastfeeding influence future sperm quality and reproductive hormones? *Int J Androl* 2011;34: 165-172.

534 Lemcke B, Behre HM, Nieschlag E. Frequently subnormal semen profiles of normal volunteers
535 recruited over 17 years. *Int J Androl* 1997;20: 144-152.
536 Lenz S, Giwercman A, Elsborg A, Cohr KH, Jelnes JE, Carlsen E, Skakkebaek NE. Ultrasonic testicular
537 texture and size in 444 men from the general population: correlation to semen quality. *Eur Urol*
538 1993;24: 231-238.
539 Liu L, Bao H, Liu F, Zhang J, Shen H. Phthalates exposure of Chinese reproductive age couples and its
540 effect on male semen quality, a primary study. *Environ Int* 2012;42: 78-83.
541 MacDonald AA, Herbison GP, Showell M, Farquhar CM. The impact of body mass index on semen
542 parameters and reproductive hormones in human males: a systematic review with meta-analysis.
543 *Hum Reprod Update* 2010;16: 293-311.
544 Masson P, Brannigan RE. The varicocele. *Urol Clin North Am* 2014;41: 129-144.
545 Meschede D, Behre HM, Nieschlag E. Endocrine and spermatological characteristics of 135 patients
546 with bilateral megalotestis. *Andrologia* 1995;27: 207-212.
547 Muller A, De La Rochebrochard E, Labbe-Decleves C, Jouannet P, Bujan L, Mieusset R, Le Lannou D,
548 Guerin JF, Benchaib M, Slama R *et al.* Selection bias in semen studies due to self-selection of
549 volunteers. *Hum Reprod* 2004;19: 2838-2844.
550 Oyen RH. Scrotal ultrasound. *Eur Radiol* 2002;12: 19-34.
551 Paasch U, Salzbrunn A, Glander HJ, Plambeck K, Salzbrunn H, Grunewald S, Stucke J, Vierula M,
552 Skakkebaek NE, Jorgensen N. Semen quality in sub-fertile range for a significant proportion of young
553 men from the general German population: a co-ordinated, controlled study of 791 men from
554 Hamburg and Leipzig. *Int J Androl* 2008;31: 93-102.
555 Palacios ER, Clavero A, Gonzalvo MC, Rosales A, Mozas J, Martinez L, Ramirez JP, Bjorndahl L,
556 Morancho-Zaragoza J, Fernandez-Pardo E *et al.* Acceptable variability in external quality assessment
557 programmes for basic semen analysis. *Hum Reprod* 2012;27: 314-322.

558 Pilatz A, Altinkilic B, Kohler E, Marconi M, Weidner W. Color Doppler ultrasound imaging in
559 varicoceles: is the venous diameter sufficient for predicting clinical and subclinical varicocele? *World*
560 *J Urol* 2011;29: 645-650.
561 Richthoff J, Rylander L, Hagmar L, Malm J, Giwercman A. Higher sperm counts in Southern Sweden
562 compared with Denmark. *Hum Reprod* 2002;17: 2468-2473.
563 Sakamoto H, Saito K, Oohta M, Inoue K, Ogawa Y, Yoshida H. Testicular volume measurement:
564 comparison of ultrasonography, orchidometry, and water displacement. *Urology* 2007;69: 152-157.
565 Sermondade N, Faure C, Fezeu L, Shayeb AG, Bonde JP, Jensen TK, Van Wely M, Cao J, Martini AC,
566 Eskandar M *et al.* BMI in relation to sperm count: an updated systematic review and collaborative
567 meta-analysis. *Hum Reprod Update* 2013;19: 221-231.
568 Shapiro SS, Wilks MB. An analysis of variance test for normality (complete samples). *Biometrika*
569 1965;52: 591-611.
570 Sikaris K, McLachlan RI, Kazlauskas R, de Kretser D, Holden CA, Handelsman DJ. Reproductive
571 hormone reference intervals for healthy fertile young men: evaluation of automated platform
572 assays. *J Clin Endocrinol Metab* 2005;90: 5928-5936.
573 Slama R, Eustache F, Ducot B, Jensen TK, Jorgensen N, Horte A, Irvine S, Suominen J, Andersen AG,
574 Auger J *et al.* Time to pregnancy and semen parameters: a cross-sectional study among fertile
575 couples from four European cities. *Hum Reprod* 2002;17: 503-515.
576 Stokes-Riner A, Thurston SW, Brazil C, Guzick D, Liu F, Overstreet JW, Wang C, Sparks A, Redmon JB,
577 Swan SH. One semen sample or 2? Insights from a study of fertile men. *J Androl* 2007;28: 638-643.
578 Swan SH, Brazil C, Drobnis EZ, Liu F, Kruse RL, Hatch M, Redmon JB, Wang C, Overstreet JW.
579 Geographic differences in semen quality of fertile U.S. males. *Environ Health Perspect* 2003;111:
580 414-420.
581 Thorup J, McLachlan R, Cortes D, Nation TR, Balic A, Southwell BR, Hutson JM. What is new in
582 cryptorchidism and hypospadias--a critical review on the testicular dysgenesis hypothesis. *J Pediatr*
583 *Surg* 2010;45: 2074-2086.

584 Tournaye H, Staessen C, Camus M, Verheyen G, Devroey P, Van Steirteghem A. No evidence for a
585 decreased fertilizing potential after in-vitro fertilization using spermatozoa from polyzoospermic
586 men. *Hum Reprod* 1997;12: 2183-2185.
587 Tsarev I, Gagonin V, Giwercman A, Erenpreiss J. Sperm concentration in Latvian military conscripts as
588 compared with other countries in the Nordic-Baltic area. *Int J Androl* 2005;28: 208-214.
589 WHO. Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus
590 Interaction. In Organisation WH (ed). 1999. Cambridge University Press, New York.
591 World Health Organisation. *WHO Laboratory Manual For The Examination of Human Semen and*
592 *Sperm-Cervical Mucus Interaction*. 1st edn, 1980. Press Concern, Singapore.
593 World Health Organisation. *WHO Laboratory Manual For The Examination and Processing of Human*
594 *Semen*. 5th edn, 2010. WHO, Geneva.

595

596 Table 1

597 Demographic variables of the men undergoing testicular assessment and the results of testicular
598 ultrasound examination, semen analysis and hormone measurement. Steroid concentrations in SI
599 units are provided in italics.

600

601 Table 2

602 The influence of demographic factors upon testicular volume, sperm output and concentration

603

604 Table 3

605 The influence of demographic factors upon serum hormone concentrations

606

607 Table 4.

608 Pearson correlation coefficients between semen sample parameters and anthropometric and serum
609 blood measurements. All bivariate statistically significant correlation remained statistically
610 significant with the adjustment for abstinence duration.

611

612 Table 5.

613 Bivariate correlation coefficients between semen parameters. All bivariate statistically significant
614 correlation remained statistically significant with the adjustment for abstinence duration.

615

616

Table 1

	Variable	N	Mean (SD)	Median (Q_1, Q_3)	Min-Max	($P_{2.5}$, $P_{97.5}$)
General	Age (yr)	423	20 (0.5)	20 (19.7, 20.3)	19.3-22.1	(19.4, 21.4)
	Height (cm)	410	179.6 (7.1)	180 (175, 184.8)	162-199	(165, 193.7)
	Weight (kg)	410	78.1 (14.3)	76.6 (68.4, 86.1)	52.2-137.5	(54.7, 112.4)
	BMI (kg/m2)	410	24.2 (3.9)	23.4 (21.4, 26.2)	16.7-39.3	(18.7, 35.2)
Testis Volume	Right (ml)	403	16.1 (4.1)	15.9 (13.2, 18.5)	3.2-30.5	(9, 25.1)
	Left (ml)	403	14.4 (3.9)	14.1 (11.8, 16.5)	4.8-30.8	(7.9, 23.8)
	Mean (ml)	402	15.2 (3.6)	14.9 (12.6, 17.2)	7.6-28.4	(9.1, 23.8)
Semen	Abstinence (days)	361	3.3 (12.6)	2 (2, 3)	0-240	(0, 7)
	Volume (ml)	365	3 (1.5)	2.8 (1.9, 3.8)	0.1-11	(0.8, 6.7)
	Sperm concentration (M/ml)	365	52.2 (40.1)	45 (21.8, 71.5)	0-220	(0.5, 154.3)
	Total sperm output (M)	365	153.1 (144.5)	112.2 (50.4, 205.6)	0-927.5	(0.7, 550.9)
	Motility (a+b, %)	361	53.8 (18.6)	58 (43, 66)	1-88	(9.1, 83)
	Morphology (N, %)	354	5.4 (3)	5 (3, 7)	0-18	(1, 12.1)
	SCSA %	358	4.1 (3.6)	3.1 (1.8, 5.2)	0.2-30	(0.7, 14.2)
	TMS [TMS]	365	90.9 (95)	63.6 (25.1, 125.8)	0-649.3	(0.1, 391.0)
Serum Hormones	LH (IU/l)	384	10.9 (3.6)	10.5 (8.3, 13)	2.3-28.4	(5.1, 18.7)
	FSH (IU/l)	384	4.9 (3.3)	4.4 (3, 6.2)	0.6-39.5	(1.3, 12)
	Inhibin B (pg/ml)	384	219 (73.3)	215 (166.4, 264.2)	4.5-431.2	(89.6, 382.2)
	Testosterone (ng/ml)	382	4.78 (1.52)	4.68 (3.69, 5.79)	1.07-9.75	(2.14, 8.06)
	(nmol/L)		*16.6 (5.3)*	*16.3 (12.8, 20.1)*	*3.7–33.9*	*(7.4, 28.0)*
	DHT (ng/ml)	377	0.35 (0.15)	0.34 (0.24, 0.45)	0.06-0.92	(0.11, 0.67)
	(nmol/L)		*1.21 (0.52)*	*1.17 (0.83, 1.55)*	*0.21-3.17*	*(0.38, 2.31)*
	3α-diol (ng/ml)	348	0.16 (0.09)	0.14 (0.09, 0.2)	0.05-0.58	(0.05, 0.39)
	(nmol/L)		*0.55 (0.31)*	*0.48 (0.31, 0.69)*	*0.17-2.00*	*(0.17, 1.34)*
	3β-diol (ng/ml)	262	0.15 (0.12)	0.12 (0.09, 0.17)	0.05-0.98	(0.05, 0.45)
	(nmol/L)		*0.52 (0.41)*	*0.41 (0.03, 0.69)*	*0.17-3.37*	*(0.17, 1.55)*
	Estradiol (pg/ml)	382	41.8 (13.6)	40.1 (34.2, 50.1)	9.4-88.6	(15.4, 74.4)
	(pmol/L)		*153.4 (48.8)*	*147.2 (125.5,183.9)*	*34.2-325.3*	*(56.5,273.1)*
	Estrone (pg/ml)	382	22.8 (8)	21.8 (17, 26.7)	6.7-58.4	(10.7, 42.1)
	(pmol/L)		*84.3 (29.6)*	*80.6 (62.9, 98.8)*	*24.8-216.0*	*(39.6,155.7)*
	DHEA (ng/ml)	382	7.1 (3.4)	6.5 (4.6, 8.7)	1.2-18.8	(2.3, 15.4)
	(nmol/L)		*24.6 (11.8)*	*22.5 (15.9,30.2)*	*4.2-65.2*	*(8.0, 53.4)*

Table 2

			Mean Testis Volume (ml)	Sperm Output (million/ejaculate)	Sperm concentration (million/ml)
Smoking	No	Med (Q1,Q3)	14.6 (12.5, 17)	113.4 (48.5, 220.7)	44 (19.1, 72.5)
		Min-Max	8.1-28.4	0-810	0-220
		N	263	245	245
	Yes	Med (Q1,Q3)	15.3 (13.1, 16.7)	97 (50.7, 175.4)	39.5 (21.5, 58)
		Min-Max	7.6-25	0-927.5	0-175
		N	45	46	46
Alcohol	Nil	Med (Q1,Q3)	14.4 (11.9, 16.5)	68 (31, 150.5)	40 (12, 60)
		Min-Max	8.6-25	0-533.6	0-138
		N	42	39	39
	Moderate	Med (Q1,Q3)	14.6 (12.3, 17.1)	116.6 (48.3, 224.8)	45.5 (18, 75.5)
		Min-Max	8.1-26.1	0-720	0-210
		N	159	152	152
	Binge	Med (Q1,Q3)	15.4 (13, 17)	118.4 (56, 199.8)	44 (27, 66)
		Min-Max	7.6-28.4	0-927.5	0-220
		N	106	99	99
Illicit Drugs	Nil	Med (Q1,Q3)	14.6 (12.5, 17.2)	113.5 (50.5, 213.7)	45 (22.1, 73.5)
		Min-Max	7.6-28.4	0-810	0-220
		N	259	272	272
	Marijuana Only	Med (Q1,Q3)	15.3 (13.2, 16.8)	113 (53.5, 200.3)	45 (21.1, 75)
		Min-Max	9.4-21.2	0-720	0-150
		N	45	50	50
	Other Only	Med (Q1,Q3)	15.5 (12.6, 18.2)	68 (24, 130.2)	31 (13.6, 53)
		Min-Max	8.1-27.8	0.4-217.6	0.1-136
		N	19	19	19
	Marijuana & Other	Med (Q1,Q3)	16.9 (15.6, 19.5)	156.6 (52.1, 215.8)	49.5 (16.4, 74.5)
		Min-Max	10.1-23.4	6-927.5	3.5-175
		N	20	22	22
Medication	Nil	Med (Q1,Q3)	14.8 (12.6, 17.1)	114 (55.4, 211.9)	44 (23, 74)
		Min-Max	7.6-28.4	0-927.5	0-220
		N	260	273	273
	Non-Prescription Only	Med (Q1,Q3)	14.4 (12.1, 16.7)	90 (46.3, 198)	43 (18, 59)
		Min-Max	9-26.1	0.7-345.8	0.2-121
		N	33	35	35
	Prescription Only	Med (Q1,Q3)	15.5 (13.2, 18.6)	109.8 (43.7, 180.5)	51.5 (25.4, 73.5)
		Min-Max	10.3-27.8	0-639.2	0-160
		N	46	48	48
	Non-Prescription & Prescription	Med (Q1,Q3)	17.4 (12.5, 18.3)	38.5 (32.4, 80.9)	16 (12.4, 18)
		Min-Max	11-18.4	10.6-194.4	11-108
		N	4	7	7
Cryptorchidism	No	Med (Q1,Q3)	15.2 (12.7, 17.3)	115.1 (53.6, 205.1)	47 (23, 73.3)
		Min-Max	7.6-28.4	0-927.5	0-220
		N	371	318	318
	Yes	Med (Q1,Q3)	13.1 (11.6, 15.6)	39.7 (19.7, 51.4)	17.8 (12.9, 32.3)
		Min-Max	8.6-16.8	0.8-83.6	1.2-38
		N	9	8	8
Herniorrhaphy	No	Med (Q1,Q3)	15 (12.6, 17.2)	112.1 (51.1, 202.2)	45 (22.6, 71)
		Min-Max	7.6-28.4	0-927.5	0-220
		N	383	328	328
	Yes	Med (Q1,Q3)	14.5 (11.2, 20.1)	37 (14.3, 197.5)	18.5 (8.6, 63.5)
		Min-Max	9.7-21.7	13.5-369.8	7.3-86
		N	6	6	6
Epididymal Cyst	Nil	Med (Q1,Q3)	15.2 (12.8, 17.6)	101.2 (50.4, 185.3)	41 (21.8, 66)
		Min-Max	7.6-28.4	0-810	0-210
		N	256	221	221
	Left Only	Med (Q1,Q3)	14.4 (13.1, 16.2)	143 (43.9, 291.4)	51 (15, 88.4)
		Min-Max	9-21	0-927.5	0-220
		N	52	40	40
	Right Only	Med (Q1,Q3)	13.9 (12, 17)	115.2 (47.9, 218.6)	47 (20, 74)
		Min-Max	8.1-23.2	0.4-551.8	0.1-200
		N	51	48	48
	Bilateral	Med (Q1,Q3)	14.6 (12.6, 17.8)	184 (129.2, 283.8)	50 (43, 68)
		Min-Max	9.8-21.9	33.8-526.4	16.1-124
		N	22	19	19
Varicoceles	Nil	Med (Q1,Q3)	15.5 (13.2, 17.5)	121 (54.8, 213.8)	47 (26, 75)
		Min-Max	8.6-28.4	0-810	0-210
		N	302	277	277

Unilateral	Med (Q1,Q3)	14.2 (11.1, 16.3)	79.2 (30.3, 219.9)	32.5 (10.5, 62.8)
<3mm	Min-Max	8.1-23.1	0-927.5	0-175
	N	36	32	32
Unilateral	Med (Q1,Q3)	13.7 (11.4, 15.9)	71.5 (27, 148.4)	27.5 (9.8, 50)
>3mm	Min-Max	7.6-21.2	0-280	0-88
	N	40	34	34
Bilateral	Med (Q1,Q3)	14.2 (11.6, 17.9)	132 (78.4, 330)	53 (31, 120)
<3mm	Min-Max	9.9-21	22.5-360	22.5-220
	N	13	11	11
Bilateral	Med (Q1,Q3)	14.1 (13.1, 17.8)	69 (50.4, 82.8)	23 (11, 51)
<3mm, >3mm	Min-Max	7.9-18	34.1-160.6	9.8-73
	N	7	7	7
Bilateral	Med (Q1,Q3)	11.8 (10.1, 13.4)	140.5 (87.5, 448.5)	51.5 (26.8, 85.3)
>3mm	Min-Max	9.7-13.9	70-551	20-95
	N	4	4	4

Table 3

			Serum T (ng/ml)	Serum DHT (ng/ml)	Serum Estradiol (pg/ml)	FSH (IU/L)	LH (IU/L)	Inhibin B (pg/ml)
Smoking	No	Med (Q1,Q3)	4.75 (3.73, 5.85)	0.34 (0.25, 0.44)	40.8 (34.9, 50.4)	4.6 (3.0, 6.5)	10.4 (8.5, 12.9)	214.9 (166.4, 268.2)
		Min-Max	1.28-9.75	0.06-0.92	9.4-88.6	0.6-16.7	4.3-22.9	4.5-431.2
		N	251	249	251	252	252	252
	Yes	Med (Q1,Q3)	4.42 (3.4, 5.53)	0.32 (0.27, 0.45)	41.5 (33.3, 53)	4.6 (3.0, 6.5)	12.0 (8.3, 14.0)	209.4 (156.2, 243.1)
		Min-Max	1.61-7.17	0.12-0.64	16.3-79.3	0.6-16.7	2.3-28.4	5.2-380.9
		N	45	43	45	45	45	45
Alcohol	Nil	Med (Q1,Q3)	4.39 (3.2, 5.75)	0.34 (0.21, 0.42)	40.8 (34.1, 46.7)	4.8 (3.3, 5.9)	10.5 (8.1, 13.5)	229.7 (176.9, 290.5)
		Min-Max	1.67-8.79	0.11-0.57	17.7-58.9	1.2-14.3	5.5-17.4	4.5-416.2
		N	39	38	39	39	39	39
	Moderate	Med (Q1,Q3)	4.56 (3.66, 5.81)	0.33 (0.22, 0.44)	40.2 (35, 51.5)	4.7 (2.9, 6.7)	10.4 (8.5, 12.9)	210.1 (163.1, 263.3)
		Min-Max	2.16-9.62	0.07-0.92	9.4-88.6	0.6-16.7	4.4-22.6	73.8-431.2
		N	154	154	154	154	154	154
	Binge	Med (Q1,Q3)	4.88 (3.96, 5.88)	0.38 (0.28, 0.45)	42.2 (34.4, 52.5)	4.2 (2.9, 6.2)	10.5 (8.7, 13.3)	213.4 (165.1, 261.3)
		Min-Max	1.28-9.75	0.06-0.85	15.2-79.3	0.7-39.5	4.3-28.4	5.2-408.8
		N	101	98	101	101	102	102
Illicit Drugs	Nil	Med (Q1,Q3)	4.71 (3.69, 5.91)	0.34 (0.25, 0.44)	39.8 (34.6, 50)	4.4 (2.8, 6.2)	10.5 (8.6, 13.1)	215.2 (165.5, 265.5)
		Min-Max	1.07-9.75	0.06-0.92	9.4-88.6	0.6-39.5	2.3-28.4	4.5-426.9
		N	250	248	250	251	251	251
	Marijuana Only	Med (Q1,Q3)	5.08 (3.75, 5.79)	0.36 (0.23, 0.46)	39.9 (31.1, 53.5)	3.9 (2.9, 6.2)	10.0 (7.7, 13.5)	221.1 (180.1, 264.8)
		Min-Max	2.23-8.42	0.08-0.7	15.9-75.6	1.4-26.9	6.1-18.9	46.5-431.2
		N	44	43	44	44	44	44
	Other Only	Med (Q1,Q3)	4.37 (3.75, 5.45)	0.33 (0.22, 0.44)	42.8 (36.4, 55.7)	4.3 (2.4, 7.0)	10.0 (7.7, 13.5)	221.1 (180.1, 264.8)
		Min-Max	2.56-7.72	0.16-0.64	11.6-77.6	1.2-9.0	6.1-18.9	46.5-431.2
		N	17	16	17	17	17	17
	Marijuana & Other	Med (Q1,Q3)	5.13 (4.34, 5.43)	0.39 (0.3, 0.47)	39.6 (28.7, 49.1)	4.9 (3.1, 6.4)	10.7 (7.6, 13.5)	217.2 (181.5, 304.8)
		Min-Max	2.61-9.08	0.12-0.64	12.2-66.1	1.6-15.9	5.2-17.1	101.7-345.9
		N	17	17	17	18	18	18
Medication	Nil	Med (Q1,Q3)	4.78 (3.69, 5.87)	0.34 (0.24, 0.45)	39.2 (33.6, 50.1)	4.4 (3.0, 6.4)	10.6 (8.5, 13.2)	218.1 (167.7, 267.3)
		Min-Max	1.07-9.62	0.06-0.92	9.4-83.7	0.6-39.5	3.3-28.4	4.5-431.2
		N	244	241	244	246	246	246
	Non-Prescription Only	Med (Q1,Q3)	4.42 (3.89, 5.63)	0.33 (0.28, 0.43)	42.9 (35.2, 49.6)	3.9 (2.8, 6.4)	9.6 (7.5, 13.3)	174.2 (139.4, 225.2)
		Min-Max	2.61-9.75	0.15-0.8	14.9-88.6	1.2-8.7	2.3-19.1	4.5-431.2
		N	33	33	33	33	33	33
	Prescription Only	Med (Q1,Q3)	4.7 (3.85, 5.86)	0.38 (0.25, 0.44)	40.9 (34.6, 52)	3.8 (2.4, 5.6)	10.0 (7.9, 12.0)	229.3 (194.7, 268.6)
		Min-Max	1.61-8.42	0.08-0.7	24.2-65	0.6-26.9	4.6-17.4	46.5-357.2
		N	45	44	45	45	45	45
	Non-Prescription & Prescription	Med (Q1,Q3)	5.91 (3.5, 7.51)	0.42 (0.25, 0.52)	43.5 (38.9, 55.7)	5.1 (3.6, 6.7)	12.5 (11.6, 15.6)	248.3 (176.8, 300.5)
		Min-Max	3.07-9.08	0.18-0.56	29.8-57.6	3.3-10.6	10.5-19.3	146.4-338.7
		N	6	6	6	6	6	6
Cryptorchidism	No	Med (Q1,Q3)	4.67 (3.68, 5.76)	0.34 (0.24, 0.44)	40 (34.3, 50)	4.3 (2.8, 6.2)	10.5 (8.3, 13.1)	217.6 (167.9, 267.3)
		Min-Max	1.07-9.62	0.06-0.92	9.4-88.6	0.6-39.5	2.3-28.4	146.4-338.7
		N	338	333	338	340	340	340
	Yes	Med (Q1,Q3)	4.48 (3.01, 7.03)	0.36 (0.23, 0.51)	40.1 (29.5, 51.5)	5.1 (3.2, 7.2)	10.4 (9.0, 13.5)	147.3 (128.3, 263.6)
		Min-Max	2.3-9.08	0.12-0.85	23.9-59.2	2.0-16.7	6.0-14.4	113.2-338.7
		N	8	8	8	8	8	8
Herniorrhaphy	No	Med (Q1,Q3)	4.64 (3.68, 5.74)	0.33 (0.24, 0.43)	40 (34.3, 50)	4.3 (2.8, 6.2)	10.6 (8.3, 13.1)	216.9 (167.5, 267.3)
		Min-Max	1.07-9.62	0.06-0.92	9.4-88.6	0.6-39.5	2.3-28.4	4.5-431.2
		N	350	345	350	351	351	351
	Yes	Med (Q1,Q3)	4.4 (3.12, 5.56)	0.27 (0.14, 0.7)	46.4 (36.6, 56.9)	3.6 (0.7, 7.3)	9.1 (5.7, 13.4)	212.2 (165.8, 311.2)
		Min-Max	2.87-5.76	0.09-0.85	34.6-59.2	0.6-39.5	2.3-28.4	4.5-431.2
		N	4	4	4	5	5	5
Epididymal Cyst	Nil	Med (Q1,Q3)	4.65 (3.71, 5.77)	0.33 (0.23, 0.43)	41.3 (34.7, 50.7)	4.2 (2.9, 5.7)	10.4 (8.2, 13.1)	217.2 (176.7, 267.7)
		Min-Max	1.07-9.75	0.06-0.92	10.2-88.6	0.6-39.5	2.3-28.4	5.2-431.2
		N	231	228	231	232	232	232
	Left Only	Med (Q1,Q3)	4.3 (3.39, 5.62)	0.36 (0.27, 0.47)	37.3 (34.2, 45.1)	4.0 (2.7, 6.6)	10.9 (8.4, 12.3)	211.5 (159.2, 267.3)
		Min-Max	2.11-7.19	0.1-0.69	14.2-79.6	1.1-11.6	4.2-18.0	4.5-426.9
		N	47	47	47	47	47	47
	Right Only	Med (Q1,Q3)	5.02 (3.87, 6.23)	0.37 (0.27, 0.43)	40 (32.3, 55.3)	5.1 (3.3, 6.4)	10.6 (9.1, 14.9)	214.2 (159.5, 283.0)
		Min-Max	1.61-9.62	0.11-0.6	9.4-83.7	0.6-11.9	5.5-22.9	92.7-416.2
		N	48	47	48	49	49	49
	Bilateral	Med (Q1,Q3)	4.17 (3.22, 5.32)	0.36 (0.21, 0.44)	36.4 (29.9, 41.9)	5.2 (2.3, 7.5)	10.0 (8.2, 13.5)	238.0 (157.3, 271.8)
		Min-Max	2.48-6.77	0.12-0.6	20.3-51.7	1.2-14.9	5.5-15.1	64.1-389.3
		N	19	18	19	19	19	19
Varicoceles	Nil	Med (Q1,Q3)	4.69 (3.69, 5.8)	0.34 (0.24, 0.45)	40.4 (34.3, 51.3)	4.2 (3.0, 5.8)	10.6 (8.5, 13.1)	215.9 (172.8, 268.0)
		Min-Max	1.07-9.75	0.07-0.8	9.4-88.6	0.6-15.9	3.3-22.9	4.5-431.2
		N	296	291	296	297	297	297
	Unilateral <3mm	Med (Q1,Q3)	4.15 (3.19, 5.1)	0.29 (0.19, 0.41)	38.6 (31.5, 48.2)	4.1 (2.1, 5.6)	9.5 (7.3, 12.2)	211.5 (158.8, 249.9)
		Min-Max	1.28-7.15	0.06-0.6	23.1-63.7	1.2-16.7	4.3-18.0	70.4-410.3

	N	33	33	33	33	33	33
Unilateral	Med (Q1,Q3)	4.97 (4.24, 5.88)	0.34 (0.26, 0.45)	44.1 (32, 52)	5.7 (3.6, 7.4)	11.5 (8.5, 15.2)	193.4 (140.4, 234.5)
>3mm	Min-Max	2.11-8.42	0.14-0.85	12.1-75.6	1.4-39.5	4.2-28.4	5.2-368.4
	N	35	35	35	36	36	36
Bilateral	Med (Q1,Q3)	5.18 (3.88, 5.37)	0.39 (0.28, 0.48)	35.5 (28.6, 39)	5.1 (3.3, 7.0)	8.6 (7.2, 10.2)	259.4 (169.2, 296.8)
<3mm	Min-Max	2.51-8.02	0.17-0.63	17.1-74.4	2.2-7.9	5.5-12.3	155.0-362.6
	N	9	9	9	9	9	9
Bilateral	Med (Q1,Q3)	6.04 (3.65, 6.1)	0.49 (0.33, 0.65)	34.7 (32.2, 37.7)	5.1 (3.1, 9.7)	12.5 (9.0, 16.5)	218.2 (166.6, 301.1)
<3mm, >3mm	Min-Max	2.53-6.15	0.26-0.67	31-39.2	3.0-11.6	7.1-18.8	144.5-319.4
	N	5	5	5	5	5	5
Bilateral	Med (Q1,Q3)	4.84 (2.68, 7.09)	0.35 (0.15, 0.79)	47.6 (44.4, 49.7)	4.6 (1.0, 8.0)	8.8 (3.7, 10.2)	185.9 (152.8, 208.5)
>3mm	Min-Max	2.61-7.19	0.09-0.92	43.6-50	0.7-8.1	2.3-10.4	145.6-212.2
	N	4	4	4	4	4	4

Table 4.

			Sperm					
	BMI	Testis Volume	Volume	Output	Concentration	SCSA	Morphology	Motility
Testis Volume	-	-	0.14*	0.29**	0.25**	0.03	0.14*	0.03
BMI	-	0.06	-0.12*	-0.13*	-0.06	0.02	0.04	-0.01
Height	0.06	0.28**	0.11*	-0.07	-0.14*	-0.01	-0.02	-0.03
LH	-0.16*	-0.24**	-0.01	-0.13*	-0.13*	0.05	-0.05	-0.07
FSH	-0.07	-0.32**	-0.01	-0.15*	-0.18**	-0.01	0.05	-0.04
Inhibin B	-0.16*	0.42**	0.04	0.21**	0.22**	0.02	-0.01	-0.01
T	-0.23**	0.05	0.05	0.08	0.06	0.10	0.08	0.01
DHT	-0.22**	0.06	0.07	0.08	0.05	0.12*	0.04	0.04
Estradiol	0.05	-0.11	0.06	0.00	-0.06	0.04	-0.09	-0.01
DHEA	0.11*	0.03	-0.01	-0.07	-0.05	0.01	-0.03	-0.02
3α-diol	0.13*	-0.01	0.03	0.04	0.03	0.14	-0.08	0.06
3β-diol	-0.04	0.04	-0.01	0.07	0.08	0.09	-0.07	0.07

** p<0.001; * p<0.05;

Table 5.

	Output	Concentration	SCSA	Morphology	Motility
Volume	0.51**	-0.01	0.06	0.06	0.20**
Output		0.74**	0.06	0.17**	0.30**
Concentration			0.07	0.23**	0.36**
SCSA				-0.16*	-0.20**
Morphology					0.25**

** p<0.001; * p<0.05;