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Correspondence:
Niels Jørgensen, Department of Growth and Reproduction, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen Ø, Denmark.
E-mail: niels.joergensen@rh.regionh.dk

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Possible involvement of the glucocorticoid receptor (NR3C1) and selected *NR3C1* gene variants in regulation of human testicular function

[1]L. Nordkap, [1]K. Almstrup, [1]J. E. Nielsen, [1]A. K. Bang, [1]L. Priskorn, [1]M. Krause, [1]S. A. Holmboe, [1]S. B. Winge, [1]D. L. Egeberg Palme, [1]N. Mørup, [1,2]J. H. Petersen, [1]A. Juul, [1]N. E. Skakkebæk, [1]E. Rajpert-De Meyts and [1]N. Jørgensen

[1]Department of Growth and Reproduction and International Center for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health (EDMaRC), Rigshospitalet, University of Copenhagen, Copenhagen Ø, Denmark, and [2]Department of Biostatistics, University of Copenhagen, Copenhagen, Denmark

SUMMARY

Perceived stress has been associated with decreased semen quality but the mechanisms have not been elucidated. It is not known whether cortisol, the major stress hormone in humans, can act directly via receptors in the testis, and whether variants in the gene encoding the glucocorticoid receptor (*NR3C1*) can possibly modulate the effect. To address these questions, we investigated the expression of the glucocorticoid receptor in human testicular tissue, including adult and fetal samples (n = 20) by immunohistochemical staining, and in silico analysis of publicly available datasets. In the adult testis NR3C1 protein was detected in peritubular cells, a subset of Leydig cells, Sertoli cells (weak), and spermatogonia, but not in spermatids. The NR3C1 expression pattern in fetal testis samples differed by a notably stronger reaction in Sertoli cells, lack of staining in gonocytes but the presence in a subset of prospermatogonia, and the almost absent reaction in nascent peritubular cells. In parallel, we explored the association between adult testicular function and three single nucleotide *NR3C1* polymorphisms (*Bcl*I [rs41423247], 9β [rs6198], and *Tth111*I [rs10052957]) affecting glucocorticoid sensitivity. Testicular function was determined by semen analysis and reproductive hormone profiling in 893 men from the general population. The *NR3C1* SNP *Bcl*I was associated with semen quality in an over-dominant manner with heterozygotes having better semen parameters compared to both homozygote constellations, and with sperm motility showing the strongest association. This association was supported by a higher inhibin B and inhibin B/FSH ratio, as well as a lower FSH in *Bcl*I heterozygotes. The SNPs 9β and *Tth111*I were not associated with semen parameters. Although the clinical impact of the findings is limited, the results substantiate a suggested link between stress and testicular function. Hence this investigation should be regarded as a discovery study generating hypotheses for future studies.

INTRODUCTION

Several lines of evidence suggest an adverse effect of stress on testicular function (Whirledge & Cidlowski, 2010; Li *et al.*, 2011; Nordkap *et al.*, 2016). The mechanisms are yet unknown, but stress is assumed to increase the glucocorticoid (GC) production from the adrenal glands, and high GC exposure in patients with Cushing's disease has been associated with impaired testicular function (Gabrilove *et al.*, 1974; Tsigos & Chrousos, 2002). Whether the effects of adrenal GCs on the testicular function in such patients are due to direct testicular effects or indirect effects via a hypothalamic-pituitary inhibition remains unclear.

Data from rodents suggest that stress directly affects the testis, as a stress-induced increase in corticosterone secretion resulted in apoptosis of Leydig cells, spermatogonia, and primary spermatocytes as well as decreased testosterone levels (Yazawa *et al.*, 1999; Gao *et al.*, 2002; Chen *et al.*, 2012; Silva *et al.*, 2014). On

the other hand, disruption of GC production due to adrenalectomy led to damage of seminiferous tubules and ineffective spermiation (Silva *et al.*, 2014). Thus, optimal testicular function may potentially require GC levels within a certain optimal range, which opens the possibility that fine-tuning of the receptor expression is required. Due to the limited information available in humans, we decided to explore the expression of the glucocorticoid receptor, encoded by *NR3C1* (nuclear receptor subfamily 3 group C member 1), in the human testis, as well as a possible modulating effect of the common *NR3C1* gene variants on testicular function. One study has recently reported expression of NR3C1 in the human testis (Chihara *et al.*, 2016), but did not specify the cellular localization. Thus, in the present study, we used immunohistochemistry to identify in which cell types the receptor was present and to investigate possible differences of the expression pattern during testicular development.

NR3C1 transcripts are found in many tissues, but several different isoforms and splice variants of *NR3C1* exist. Hence, single nucleotide polymorphisms (SNPs) in or around *NR3C1* may affect NR3C1 differently depending on the isoform or splice variant (Koper *et al.*, 2014). Several of these SNPs have been associated with inter-individual differences in GC sensitivity in vivo and in vitro (Manenschijn *et al.*, 2009; Quax *et al.*, 2013). In this study, we have explored three of the best described *NR3C1* SNPs: *Bcl*I (rs41423247), 9β (rs6198), and *Tth111*I (rs10052957). *Bcl*I is located in the intron between exon 2 and 3 of the full-length *NR3C1* and is believed to be involved in alternative splicing of NR3C1 (van Rossum *et al.*, 2003; Koper *et al.*, 2014). The *Bcl*I polymorphism has been associated with increased GC sensitivity and clinical outcomes in G-allele carriers including increased BMI, total body fat mass, abdominal obesity, blood pressure, fasting glucose, and insulin levels (Buemann *et al.*, 1997; Rosmond *et al.*, 2000; van Rossum *et al.*, 2003; Rosmond & Holm, 2008; Szappanos *et al.*, 2009; Geelen *et al.*, 2013; Koper *et al.*, 2014). The 9β SNP is located in the 3′UTR of *NR3C1* in the exon 9β splice variant and has been suggested to be associated with a less stable transcript, and thereby reduced expression of the 9β variant and increased GC resistance (Derijk *et al.*, 2001; Quax *et al.*, 2013; Koper *et al.*, 2014). Carriage of the 9β G-allele has been associated with reduced central adiposity (Syed *et al.*, 2006). *Tth111*I is located upstream of exon 1 in a putative promoter or enhancer region, and carriage of the T-allele has been associated with increased cortisol levels (Rosmond *et al.*, 2000). To investigate whether the three *NR3C1* variants were associated with testicular function, we used a well-characterized cohort of 893 healthy young men from the general population.

MATERIALS AND METHODS

Testicular tissue samples

The tissue material used in this study included formalin-fixed and paraffin-embedded samples from 20 individuals; 15 from adult patients and five from aborted fetuses. The 15 adult testis samples were prepared after orchiectomy performed in patients treated for testicular cancer. The tissue specimens selected for the studies were excised from morphologically normal tissue removed together with the tumors. Twelve samples contained normal testicular parenchyma with complete ongoing spermatogenesis, including late spermatids, and three samples were from dysgenetic areas, where some tubules contained morphologically immature Sertoli cells. Care was taken to exclude areas with germ cell neoplasia in situ by morphological evaluation supported by immunohistochemical staining for markers of neoplasia (Rajpert-De Meyts *et al.*, 2015).

Five fetal testis samples were obtained from fetuses from elective abortions or miscarriages at gestational week (GW) 9, GW 10, GW 14, GW 15, and GW 20. The specimens, all with normal morphology, were collected in the course of previous studies (Rajpert-De Meyts *et al.*, 2004; Jorgensen *et al.*, 2015) and stored in the tissue archive of the Department of Growth & Reproduction, Rigshospitalet.

Immunohistochemical staining

Immunohistochemical staining (IHC) for the glucocorticoid receptor protein was performed according to a standard indirect peroxidase method. Briefly, the specimens were cut at 4–6 μm, deparaffinized, and rehydrated. Antigen retrieval was performed by microwaving the sections for 15 min in a citrate buffer (10 mM, pH = 6.0), and the sections were incubated in 0.5% H_2O_2 to inhibit the activity of endogenous peroxidase, followed by incubation with goat serum to quench unspecific staining. Subsequently, the mouse-anti-human GCR primary antibody (cat. nr. sc-393232, Santa Cruz Biotechnologies, Heidelberg, Germany) was applied in a dilution 1 : 100 and the sections were incubated overnight at 4 °C. In addition, a mouse monoclonal antibody against human OCT4 (cat. nr. sc-5279, Santa Cruz Biotech., Heidelberg, Germany) was used in fetal testis samples to detect the presence of gonocytes, which retain a high expression of pluripotency factors, in contrast to pre-spermatogonia, that are OCT4 negative (Rajpert-De Meyts *et al.*, 2004). The following day, the sections were incubated with biotinylated goat anti-mouse IgG (Zymed Histostain kit, San Francisco, CA, USA), followed by a peroxidase-conjugated streptavidin complex (Zymed Histostain kit). Visualization of the reaction was performed using amino ethyl carbazole (a red color), and the sections were counterstained with Mayer's hematoxylin (a blue color). The sections were washed in Tris-buffered saline (TBS) buffer between each step, except after the pre-incubation with goat serum and application of the primary antibody. For negative controls, serial sections from each tissue sample were processed with the primary antibody replaced by TBS. A nuclear staining of endothelial cells and smooth muscle cells in blood vessels was considered an internal positive control, because these cells are known to express NR3C1 in other organs (Sato *et al.*, 1995; Balkaya *et al.*, 2011). The antibody was shown to recognize NR3C1 in human and rodent tissues, and its specificity has been tested by the manufacturer and previous research (Sasse *et al.*, 2015).

The stained sections were first investigated manually on a light microscope (Nikon Microphot-FXA) and then scanned using a NanoZoomer 2.0 HT (Hamamatsu Photonics, Herrsching am Ammersee, Germany). The analysis of staining was performed using the software NDPVIEW version 1.2.36 (Hamamatsu Photonics). The intensity of staining was scored according to an arbitrary semi-quantitative scale; +++ (strong staining); ++ (moderate staining); + (weak staining); +/− (few cells stained); and - (no staining). The cell types were identified by location (e.g. inside seminiferous tubules or in the interstitial compartment) as well as by morphological features.

In silico analysis of NR3C1 expression in public datasets

We evaluated previously published data on the expression of the GC receptor in the human testis, both at the protein and the transcriptional level. The cellular distribution of the NR3C1 protein in the adult testis tissue obtained by immunohistochemistry was examined in the Human Protein Atlas (www.proteinatlas.org), which also reports the presence of transcripts (Uhlen *et al.*, 2015). In addition, the presence of the NR3C1 transcripts was examined in two public gene expression databases; in the GTEx Portal (www.gtexportal.org) and in the In silico Transcriptomics (IST) Online (http://ist.medisapiens.com). We have also examined a relative expression of *NR3C1* in adult and fetal Leydig cells, by interrogating data from our previous micro-array study of the global transcriptome of Leydig cells (Lottrup *et al.*, 2017).

Subjects for the population study and their andrological characterization

Young men from the general population (median age 20 years) filled in a detailed questionnaire on general and reproductive health and lifestyle factors, underwent a physical examination including a scrotal ultrasound, delivered a semen sample for analysis, and had a blood sample drawn for assessment of reproductive hormones and isolation of DNA. Recruitment and examinations were identical with the set-up described in details previously (Nordkap *et al.*, 2016), but the study subjects were not overlapping. Briefly, men were offered participation in the study when they attended a compulsory physical examination at around 18–20 years of age to declare them fit for military service or not. All Danish men, except those suffering from severe chronic disease, are called upon to present themselves at this examination.

Initially, 935 men were included, but 42 men were subsequently excluded due to the following reasons: inconclusive genotyping results probably due to insufficient DNA quality (n = 18), azoospermia (n = 5), missing data on sperm concentration and total sperm count (n = 4), ejaculatory duct obstruction (n = 4), diagnosis of testicular cancer during the study (n = 1), previous treatment of testicular cancer (n = 1), only one testis in the scrotum (n = 2), or active abuse of anabolic steroids (n = 7). Thus, in total, 893 men were included during the years 2012 to 2014 in the final analyses.

Semen analysis

All men provided a semen sample by masturbation in a room close to the EAA-certified semen laboratory. The men had been instructed to abstain from ejaculation for at least 48 h prior to participation, and the exact duration of ejaculation abstinence was recorded. The sample was analyzed for semen volume, sperm concentration, motility, and morphology according to the WHO criteria as described elsewhere (WHO, 2010; Jorgensen *et al.*, 2012). Briefly, semen volume was assessed by weighing. For sperm motility assessment, 10 μL of well-mixed semen was placed on a glass slide kept at 37 °C, and immediately examined on the heated stage of a microscope at 37 °C at ×400 magnification. The spermatozoa were classified as progressively motile, non-progressively motile, or immotile. For the assessment of the sperm concentration, the samples were diluted in a solution of 0.6 mol/L NaHCO3 and 0.4% (v/v) formaldehyde in distilled water, and subsequently assessed using a Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Only spermatozoa with tails were counted. For evaluation of sperm morphology, smears were Papanicolaou stained, and assessed by two experienced technicians according to 'stricter criteria' (Menkveld *et al.*, 1990).

Blood sampling and hormone analysis

On the day of attendance, all participants had a blood sample drawn for assessment of reproductive hormone levels and isolation of DNA. Blood samples were drawn between 8:30 and 13:00 with the exact time recorded (median 10:40). Inhibin B was measured by a specific two-sided enzyme immunometric assay (Inhibin B gen II, Beckman Coulter Ltd., High Wycombe, UK). Assessment of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) was performed by time-resolved immunofluorometric assays (Delfia Wallac, Turku, Finland). Testosterone and sex hormone-binding globulin (SHBG) were assessed by a time-resolved immunofluorometric assay (Delfia) for men included in 2012 and 2013 and by a chemiluminescence immunoassay (Access2, Beckman Coulter Ltd., High Wycombe, UK) for men included in 2014, thus, the results were subjected to assay-specific correction factors to make them comparable. Intra-assay and inter-assay coefficients of variation were below 10% for all hormones. Ratios between relevant hormones were calculated.

Isolation of DNA and *NR3C1* genotyping

Genomic DNA was isolated from samples of EDTA-preserved peripheral blood using SEV AS1010 DNA purification kits on Maxwell 16-MDx instrument (Promega, Madison, WI, USA) and quantified on a NanoDrop ND-1000 spectrophotometer (Saveen Werner, Limhamn, Sweden). Three different SNPs in or around the gene encoding the glucocorticoid receptor (*NR3C1* at chromosome 5) were analyzed using KASP™ competitive allele-specific PCR (LGC Genomics, Hoddesdon, UK). A master mix containing a low amount of ROX fluorophore was used for internal normalization and quantitative assessment of the competitive PCR was performed on a Stratagene Mx3000P qPCR instrument (Agilent Technologies, Santa Clara, CA, USA). DNA was omitted in the reaction mixture of the negative controls which in all cases showed no amplification. A standard touchdown PCR program was used (as advised by LGC Genomics), and all assays separated samples into clear genotype clusters. A total of 18 samples could not be assigned to a genotype cluster and were excluded. Primers for analysis of *Bcl*I (rs41423247) (chromosome 5, forward strand, position 143399010, GRCh38.p7) were designed by LGC Genomics against the following sequence: CTCTYAAAGAGATT[S]ATSAGCAGACATAACTT. Many previous studies did not consider what strand they analyzed, which may cause confusion when the polymorphism is common and is between a G and a C. For *Bcl*I, we consider the homozygotic wild type (non-carriers) as CC, which is the most common homozygotic constellation and the minor allele homozygotes as GG. Primers for analysis of *Tth111*I (rs10052957) (chromosome 5, forward strand position 143407136, GRCh38.p7) were designed by LGC Genomics against the following sequence: TATTCAGACTCA[R]TCAAKGCAAGGACCTGAT. Primers for analysis of 9β (rs6198) (chromosome 5, forward strand position 143278056, GRCh38.p7) were designed by LGC Genomics against the following sequence: ACAGCAAATTTAAA[Y]GAAAAAAWAAAAGT.

Genotyping was performed at Department of Growth and Reproduction, Rigshospitalet, Copenhagen.

Statistics

*Bcl*I genotype groups were compared with regard to lifestyle and health factors by chi-squared test (categorical variables) and Kruskal–Wallis test (continuous variables) to identify potential confounders. The initial evaluation indicated that *Bcl*I was more strongly associated with semen quality than *Tth111*I and 9β; therefore, the basic characteristics of the study population are shown stratified according to *Bcl*I genotypes.

The Hardy–Weinberg equilibriums were tested using the chi-squared test by comparing the expected and actual allelic frequencies. Allele distributions were consistent with the Hardy–Weinberg equilibrium (Hardy–Weinberg equilibrium $p = 0.6$ (*Bcl*I), $p = 0.1$ (*Tth111*I), and $p = 0.9$ (9β).

Distributions of reproductive parameters according to genotypes were assessed by two genetic models; the commonly used additive model and the rarely used over-dominant model (Elston *et al.*, 2012; Hedrick, 2012). Application of the over-dominant model was justified by rodent studies indicating that too little or too much GC may not be beneficial. With this in mind, homozygote constellations could be unfavorable compared to heterozygotes.

Multiple linear regression analyses were used to test differences in reproductive outcomes between genotypes of *Bcl*I, *Tth111*I, and 9β. For linear regression analysis, sperm concentration, total sperm count, semen volume, total motile count, and total morphologically normal count were normalized by a cubic root transformation before analysis to accommodate skewed distribution of residuals and variance heteroscedasticity. Percentage of morphologically normal spermatozoa was close to normally distributed and entered in the model untransformed. The percentage of motile spermatozoa and reproductive hormones were transformed by use of natural logarithm. Analyses were initially performed unadjusted followed by analyses taking into account covariates known to be associated with semen parameters or reproductive hormones. Using backwards elimination, covariates were excluded stepwise if they did not change the effect estimates more than 10%. Semen volume, sperm concentration, total sperm count, and total morphologically normal counts were adjusted for ejaculation abstinence period, daily cigarette smoking (yes/no), and varicocoele stage 2 or 3 detected during the physical examination. Percentage of motile spermatozoa was adjusted for ejaculation abstinence period, time from ejaculation until motility analysis, BMI, varicocoele, and self-reported scrotal injury (trauma i.e. kick or stroke to the scrotum). Percentage of morphologically normal spermatozoa was not adjusted for any covariates. Reproductive hormones were adjusted for age, BMI, daily cigarette smoking, maternal smoking during pregnancy, and time of day for blood sampling.

Our study was dimensioned to find a standardized difference of 0.19 (group difference/standard deviation) with a power of 81% when testing on a 5% significance level [431 participants *Bcl*I heterozygotes (CG) and 462 homozygotes (GG and CC)]. Our study was thus reasonably powered to find clinically relevant differences.

Statistical analyses were conducted using IBM SPSS STATISTICS version 22 (IBM, Armonk, NY, USA). p-values <0.05 were considered statistically significant. We did not correct for multiple testing.

Ethical approval

Approval of the study was obtained from the local science ethics committee (H-KF-289428) and men were only included after informed written consent. The tissue samples were collected and stored in the tissue archive after obtaining consent from the adult patients or women donating the aborted tissue. Use of stored tissue samples for immunohistochemistry was approved in separate applications (KF 01-186/01 and H-1-2012-007).

RESULTS

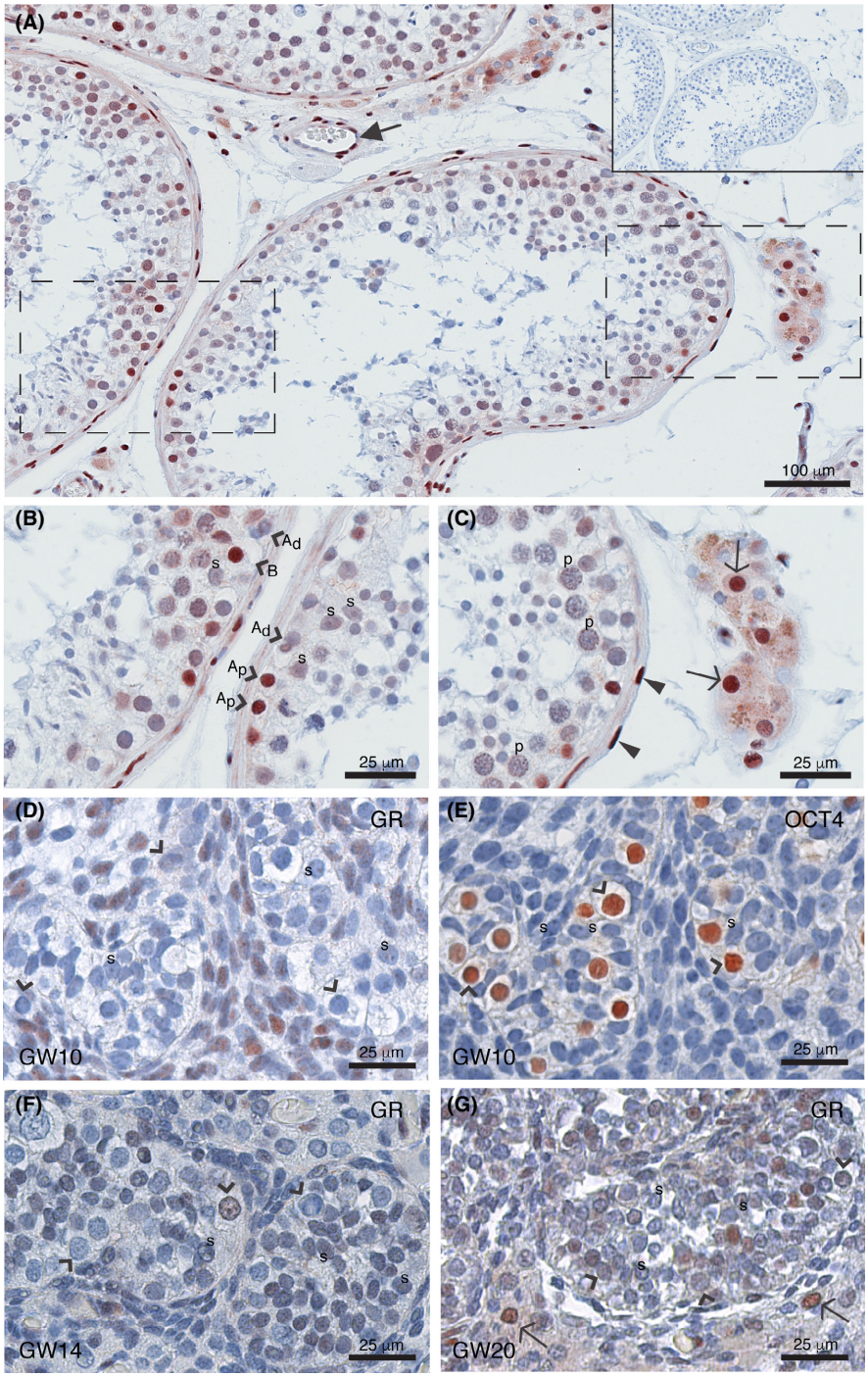
Expression of NR3C1 in the human testis

The general pattern of the glucocorticoid receptor protein expression studied by immunohistochemistry was similar and reproducible in all investigated tissue samples. However, differences were observed regarding the expression in different cell types during development (described in details below). In all cell types, NR3C1 protein was localized within the nucleus, with Leydig cells as the exception, where some weak cytoplasmic staining was also visible. Representative images are presented in Fig. 1.

In the normal adult testes, NR3C1 was detected in all peritubular myoid cells (intensity score +++/ ++), in the majority of Leydig cells (score +++/++), with a subset of Leydig cells and other interstitial cells negative, in endothelial and myoid cells of blood vessels (+++/ ++ with a few negative cells), and in a subset of germ cells (Fig. 1A–C). Regarding germ cells, the strongest staining was observed in a subset of spermatogonia (ranging from +++ to +), with much weaker reaction in pachytene and late spermatocytes (+ to +\−). No expression of NR3C1 was detected in spermatids. The analysis of spermatogonial expression suggests that spermatogonia A dark was NR3C1 negative, while the expression in spermatogonia A pale was very variable, ranging from +++ to negative. In addition to the subset of A pale cells, the strongest expression was observed in a subpopulation of spermatogonia with the largest round nuclei, likely spermatogonia B, possibly also some preleptotene primary spermatocytes (Fig. 1B). The expression in Sertoli cells was generally weak (+ to negative). A similar pattern was observed in the available images in the Human Protein Atlas, where the immunohistochemical staining was performed in testis samples from three adult individuals, using two different antibodies. The above findings are also supported by the gene expression data at the RNA level. In all three examined public datasets, the expression of NR3C1 was present in the testis, but at a low level when compared to other tissue types.

The pattern of expression of the glucocorticoid receptor was different in the fetal testis, and some changes were also observed in various cell lineages during early fetal maturation (Fig. 1D–G). In the youngest specimens (9–10 GW), the NR3C1 protein was hardly detected within the forming seminiferous cords—with only very week reaction in a subset of Sertoli cells appearing at GW 10. The strongest reaction at this early stage of development was observed in mesenchymal cells located under the testis capsule and in the subset of interstitial cells in between the cords. In older specimens, there was progressively stronger expression of NR3C1 in the Sertoli cells, which though retained a heterogeneous pattern of expression. Fetal Leydig cells displayed a weak staining, much lower than in the control adult samples. A

Figure 1 Immunohistochemical expression of the glucocorticoid receptor (GR) in the human testis. The microphotographs show representative patterns in the adult testis (A–C) and in the fetal testes (D–G) at different developmental ages between 10 and 20 gestational weeks (GW). (A) An overview image showing NR3C1 positivity (red staining) in nuclei of different cell types within the seminiferous tubules and in the interstitial compartment, including endothelial cells and smooth muscle cells (arrow) in the wall of a blood vessel. The insert in the right upper corner shows a negative control processed without the primary antibody. Two areas marked with dashed lines are shown in close-ups below. (B) A high-power image of seminiferous tubules. A heterogeneous pattern of expression in germ cells was observed. Expression ranged from very strong staining in a subset of spermatogonia, especially type B and A pale (A_p) to lack of reaction in another subset of spermatogonia, including A dark (A_d) as well as in both, round and late spermatids. Pachytene spermatocytes showed a weak reaction. Note also a relatively weak reaction in Sertoli cell nuclei (s). (C) A higher magnification image of a part of a seminiferous tubule displaying the same pattern as in image (B), and a group of Leydig cells (arrows), with strongly positive nuclei and faint reaction in the cytoplasm, which was not observed in other cell types. Note a strong staining in peritubular myoid cells (arrowheads) and nearly negative pachytene spermatocytes (p). (D) GR staining in the fetal testis at GW10—the reaction in mainly confined to the interstitial compartment in a subset of cells with immature mesenchymal cell appearance. Note lack of reaction in fetal gonocytes (arrowheads) and in fetal Sertoli cells (s). At this age, all germ cells are gonocytes identified by the strong expression of pluripotency factor OCT4 (cells with red nuclei marked by arrowheads) shown in (E), in a serial section from the same specimen. (F) At GW14, the GR reaction appears in a subset of Sertoli cells (s) and in a small subset of germ cells (marked with arrowheads) but gonocytes remain GR negative. (G) At GW20, there is a further increase in GR staining in Sertoli cells (S) and in a growing proportion of pre-spermatogonia (arrowheads) as well as in fetal Leydig cells (arrows), but with a marked heterogeneity. Nascent peritubular cells are largely GR negative. [Colour figure can be viewed at wileyonlinelibrary.com].



moderately lower level of *NR3C1* transcripts in fetal vs. adult Leydig cells was also observed in a previous transcriptome study (Lottrup *et al.*, 2017). Interestingly, nascent peritubular cells were NR3C1 negative. With regard to fetal germ cells, gonocytes (distinguished by OCT4 positivity) were NR3C1 negative, while pre-spermatogonia displayed a heterogeneous pattern, ranging from negative to ++.

To investigate whether immature Sertoli cells retain a high expression of NR3C1 regardless of the individual's age, we examined three different specimens from adult patients with testicular cancer and tubules containing visibly undifferentiated Sertoli cells, which are one of the hallmarks of testicular dysgenesis syndrome (Skakkebaek *et al.*, 2001). Interestingly, the immature Sertoli cells in the adults were largely NR3C1 negative, with only trace of reaction in one specimen.

NR3C1 SNPs; population study of association between genotypes and testicular function

Minor allele frequencies and genotype distribution for *Bcl*I, *Tth111*I, and 9β are shown in Table S1.

BclI (rs41423247)

The basic characteristics of the entire group of 893 men as well as characteristics of the men stratified according to *Bcl*I genotypes are shown in Table 1. *Bcl*I non-carriers (CC) more frequently reported an experience of scrotal injury ($p = 0.04$). Apart from this, there were no significant differences in health or lifestyle characteristics among the different *Bcl*I genotypes. Of the 143 men that reported use of medicine within the last 3 months prior to participation in the study, the majority reported occasional use of over-the-counter painkillers or medications to treat acne and allergy, but 13 men (1.5%) reported intake of morphine, antidepressants, or medication as part of ADHD-treatment, and 39 men (4.4%) reported intake of GC as inhalation or dermal application. There were no differences in frequencies of men reporting use of medicine between *Bcl*I genotypes.

Table 2 shows the semen parameters and reproductive hormone levels stratified according to *Bcl*I genotypes. The associations between *Bcl*I and testicular function were found to best fit with the over-dominant model. None of the parameters differed significantly in an additive model. In the over-dominant model, sperm motility was the semen parameter most strongly associated with *Bcl*I. The fully adjusted estimates of percentages of motile spermatozoa were 59% for heterozygotes (CG) and 53% for homozygotes (CC *and* GG) ($p < 0.001$). Sperm concentration, total sperm count, and percentage of morphologically normal spermatozoa also tended to be higher in heterozygotes but this did not reach statistical significance. Inhibin B tended to be higher and FSH lower in heterozygote *Bcl*I carriers and consequently the inhibin B/FSH ratio was significantly higher among *Bcl*I heterozygotes ($p = 0.048$) (Table 2). No associations were detected between *Bcl*I and testosterone, LH, or SHBG.

Comparing *Bcl*I heterozygotes (CG) with either non-carriers (CC) or homozygotes (GG), the effect size estimates indicated that men with the CC genotype had the poorest semen quality except for percentage of morphologically normal spermatozoa (data not shown). However, a direct comparison of the CC and the GG genotypes did not reveal any significant differences in levels of reproductive hormones or semen parameters (data not shown).

Tth111I (rs10052957) and 9β (rs6198)

*Tth111*I was not significantly associated with any semen parameters. Crude results indicated, however, that *Tth111*I minor allelic homozygotes (TT) may have better testicular function (Table S2). Also, *Tth111*I homozygotes (TT) had a significantly larger testis volume. The 9β polymorphism was not associated with any reproductive parameters (Table S3).

Combined effects of BclI, Tth111I, and 9β

Even though *Tth111*l and 9β were not associated with semen quality on their own, we tested if they modified the association

Table 1 Basic description of the study participants, in the total population and stratified according to *NR3C1 Bcl*I (rs41423247) genotypes

	No.[a]	Total group ($n = 893$)	*Bcl*I genotypes			*p*-value[b]
			CC, $n = 333$ (37.3%)	CG, $n = 431$ (48.3%)	GG, $n = 129$ (14.5%)	
Age (years)	892	20 (18; 23)	20 (18; 23)	20 (18;23)	20 (18;22)	0.64
Weight (kg)	892	74.7 (58.9; 93.1)	74.9 (57.3; 93.4)	74.2 (59.4; 92.9)	75.9 (60.8; 95.3)	0.22
Height (m)	892	1.82 (1.72; 1.93)	1.82 (1.71; 1.93)	1.83 (1.72; 1.93)	1.83 (1.74; 1.95)	0.24
BMI (kg/m^2)	892	22.4 (18.2; 27.6)	22.6 (18.1; 28.2)	22.3 (18.2; 26.8)	22.6 (18.8; 28.3)	0.32
Ejaculation abstinence (h)	891	61 (37; 153)	62 (37; 155)	61 (36; 155)	62 (37; 131)	0.87
Time between ejaculation and motility analysis (min)	875	30 (11; 75)	30 (11; 72)	30 (12; 75)	35 (13; 76)	0.04
Time of day, blood sampling (AM) (hh:min)	893	10:40 (9:11; 12:16)	10:38 (9:10; 12:14)	10:42 (9:15; 12:19)	10:40 (9:02; 12:19)	0.79
Varicocoele, grade 2 or 3	878	11	9	11	13	0.57
STD[c], epididymitis, or orchitis previously	893	7	7	6	7	0.87
Born with cryptorchidism	893	4	3	4	4	0.63
Testicular torsion	893	0.6	0.8	0.9	0	0.22
Previous scrotal injury	893	9	12	8	5	0.04
Fever >38C° (>24 h) within the last 3 months	893	8	7	8	7	0.14
Smoking cigarettes daily	893	26	27	28	19	0.13
Smoking hash/pot/marihuana daily	892	3	2	3	5	0.33
Mother smoked during pregnancy	893	15	13	16	16	0.71
Alcohol >14 units recent week	893	21	24	23	19	0.60
Use of medicine >3 consecutive days (last 3 months)	893	16	13	17	20	0.08

Values are medians (5th–95th percentiles) or percentages. [a]No. refers to number of men for which information was available. [b]Kruskal–Wallis test used for continuous variables and chi-square test for categorical variables. [c]Sexually transmitted diseases (chlamydia, gonorrhea, or syphilis).

Table 2 Semen parameters, reproductive hormones, and testis size according to *NR3C1 Bcl*I (rs41423247) genotypes

Semen parameters	No.[a]	Total group	*Bcl*I genotypes			Additive model[c]	Over-dominant model[d]
		n = 893	CC (*n* = 333)	CG (*n* = 431)	GG (*n* = 129)	*p*-value	*p*-value
Semen volume (mL)	889	3.3 (1.3; 6.2)	3.4 (1.4; 5.9)	3.2 (1.3; 6.4)	3.4 (1.1; 6.2)	0.75	0.65
Sperm concentration (mill/mL)	893	43 (4; 145)	39 (2; 158)	45 (6; 148)	46 (3; 138)	0.22	0.063
Total sperm count (mill)	889	135 (11; 464)	130 (5; 531)	140 (19; 435)	132 (6; 604)	0.26	0.15
Motile spermatozoa (%)	889	67 (31; 87)	66 (25; 86)	68 (37; 87)	65 (23; 88)	0.08	<0.0001
Total motile count (mill)	885	87 (5; 334)	79 (2; 351)	96 (11; 328)	92 (1; 366)	0.22	0.047[e]
Morphologically normal forms (%)	888	6.5 (0.5; 12.3)	5.5 (0.5; 16.0)	7.0 (1.0; 15.5)	6.0 (0.5; 14.5)	0.79	0.11
Morphologically normal forms in total (mill)	884	8.3 (0.2; 50.1)	7.0 (0.05; 49.0)	9.0 (0.4; 50.5)	8.5 (0.1; 63.2)	0.26	0.025[e]
Levels of reproductive hormones							
Testosterone (nmol/L)	887	19.9 (12.0; 31.7)	20.0 (12.1; 30.9)	20.1 (12.0; 32.0)	19.3 (12.5; 32.6)	0.75	0.77
LH (U/L)	887	3.5 (1.7; 6.8)	3.6 (1.7; 7.2)	3.5 (1.7; 6.7)	3.3 (1.6; 6.6)	0.36	0.73
FSH (U/L)	887	2.8 (1.0; 7.0)	2.7 (1.1; 7.2)	2.7 (0.9; 6.7)	3.0 (1.1; 7.1)	0.75	0.11
SHBG (nmol/L)	886	32 (17; 57)	31 (17;56)	32 (17;59)	32 (17; 50)	0.32	0.38
Inhibin B (pg/mL)	886	169 (81; 283)	168 (67; 267)	172 (87; 290)	164 (77; 284)	0.35	0.056
Inhibin B/FSH	885	66 (15; 227)	62 (10; 211)	66 (17; 238)	53 (13; 234)	0.83	0.048
Testis size							
Testis size, palpation (mL)[b]	875	20 (13; 28)	20 (12; 27)	21 (13; 28)	20 (12; 29)	0.57	0.13
Testis size, ultrasound (mL)[b]	880	13.4 (8.0; 20.4)	13.1 (7.9; 19.6)	13.3 (8.0; 20.5)	12.9 (7.8; 21.0)	0.63	0.13

Values are medians (5th–95th percentiles). [a]No. refers to number of men for which information was available. [b]Mean of both testicles. [c]Adjusted *p*-values obtained from multivariate linear regression analysis (additive model). [d]Adjusted *p*-values obtained from multivariate linear regression analyses (over-dominant model). Heterozygote carriers (CG) used as reference group. [e]*p*-values from unadjusted over-dominant models were a little higher for total motile count ($p = 0.051$), morphologically normal forms in total ($p = 0.057$), and a little lower for Inhibin B ($p = 0.041$) and Inhibin B/FSH ($p = 0.036$). Estimates (not shown) did not change.

between *Bcl*I and semen quality. Within each *Bcl*I genotype, men were stratified based on whether they were *Tth111*I T-allele carriers. We did not find any significant modifying effect of *Tth111*I on semen parameters for *Bcl*I-heterozygote (CG) men. *Bcl*I non-carriers (CC) tended to have better semen parameters and higher inhibin B/FSH ratio if they were *Tth111*I T-allele carriers (significant for sperm motility, $p = 0.039$, data not shown). Homozygote *Bcl*I men (GG) tended to have better semen parameters if they were *not Tth111*I T-allele carriers. No tendency was observed for reproductive hormones.

Stratifying men within each *Bcl*I genotype according to the presence of the 9β G-allele did not reveal any modifying effect on semen quality. No interactions between *Tth111*I and 9β were found for any outcome variables.

DISCUSSION

In this study, we described the cellular localization of the glucocorticoid receptor in the human adult and fetal testis and provided evidence of an association between semen quality and a genetic polymorphism *Bcl*I (rs41423247) in the *NR3C1* gene.

The immunohistochemical investigations indicated that GC might affect spermatogenesis directly, because the receptor was detected in germ cells, but possibly also indirectly via somatic cells. The presence of GR in peritubular cells, which was previously reported only in rodents (Levy *et al.*, 1989; Hazra *et al.*, 2014), is interesting because these cells not only insure the shape and contractility of the tubules but also secrete paracrine factors that regulate the spermatogonial niche (Flenkenthaler *et al.*, 2014; Winge *et al.*, 2015). We observed lack of the receptor expression in the developing fetal peritubular cells, indicating that glucocorticoids may be needed for peritubular cell function to support post-pubertal spermatogenesis. The strong reaction of the NR3C1 protein in adult Leydig cells is consistent with the previously suggested link between GC and testicular steroid hormone production (Norman & Smith, 1992; Vermeulen, 1993). However, the heterogeneity of expression along with a lower level of expression of the GC receptor in fetal Leydig cells, which are also producing large amounts of androgens, suggests a possible involvement of GC in another function.

We demonstrated in this study for the first time, that the expression of the GC receptor in germ cells depends on their differentiation and maturation stage. NR3C1 was completely absent in fetal gonocytes and appeared first in a subset of fetal pre-spermatogonia. The variable immunohistochemical reaction in adult spermatogonia, especially the strong reaction in spermatogonia type B, as well as the lack of immunohistochemical staining in haploid germ cells is intriguing. Further functional investigations are required to specify whether the heterogeneous expression of the receptor in mitotic spermatogonia is linked to the cell cycle stage or if GC are involved in the regulation of the onset of meiosis.

The antibody that we used was a monoclonal antibody raised against amino acids 121–420 of NR3C1, which is a part of the receptor included in most known splice variants. Our results therefore do not reveal whether any specific isoforms are present in the testis but rather give an overall picture of NR3C1 expression. In a previous study, NR3C1 was also shown to be expressed in the human testis by immunohistochemistry using a different antibody, but the authors did not describe in which cell types the protein was present (Chihara *et al.*, 2016). The authors concluded that the NR3C1 expression was stronger in tissues with non-obstructive azoospermia, but they did not take into account cellularity and a greater number of Leydig and peritubular cells in samples without ongoing spermatogenesis.

Our results are partly in agreement with results from rodent studies where NR3C1 have been detected in Leydig cells as well as other types of interstitial cells, Sertoli cells, and primary spermatocytes (Levy *et al.*, 1989; Schultz *et al.*, 1993; Hazra *et al.*, 2014). The observed minor discrepancies in germ cell expression pattern might be due to different methodology or genuine

species differences. Expression of the GC receptor in the murine Sertoli cells was reported high during development but down-regulated in the adult testis (Hazra *et al.*, 2014), and in our study, we have for the first time confirmed this development pattern in humans. Conditional knock-out of the GC in Sertoli cells caused adverse morphological changes including reduced numbers of Sertoli —and germ cells along with decreased levels of gonadotropins, indicating a function of glucocorticoid in testis development (Hazra *et al.*, 2014).

The presence of the receptor in the testis is consistent with a modulation of spermatogenesis by some of the variants of the *NR3C1* gene. One previous study reported an association between a *NR3C1* SNP (rs852977) and non-obstructive azoospermia (Chihara *et al.*, 2016), but we have not included this particular SNP in our study. Our results indicate that men with the *Bcl*I heterozygote genotype (CG) have better testicular function than men with either the CC-allele (the less sensitive genotype) or the GG genotype (the sensitive genotype), although the differences between groups from a clinical perspective was rather small. However, in terms of pathophysiology, it is unknown whether a sensitive receptor or a less sensitive receptor is beneficial for testis function. Carriage of the G-allele of the *Bcl*I *NR3C1* SNP has previously been associated with *increased* glucocorticoid sensitivity but also with a *decreased* expression of GR-α (the active receptor) (van Rossum *et al.*, 2003; Sinclair *et al.*, 2012) which is, the splice variant predominantly expressed in the testis (Ensembl transcript: ENST00000394464) [http://www.gtexportal.org/home/gene/NR3C1 (The Genotype-Tissue Expression (GTEx) pilot analysis: Multitissue gene regulation in humans BY THE GTEX CONSORTIUM, Science 08 May 2015: 648–660 last accessed 27.06.2017)]; Russcher *et al.*, 2007. This is opposed to C-carriers that have decreased glucocorticoid sensitivity and higher GR-α expression. Our data indicate that balancing these two situations, with a GC genotype, seems to be the most beneficial combination for testicular function. It may therefore be speculated that intermediate glucocorticoid levels, not too high and not too low, may be beneficial, which is in line with the conclusion from the most extended and thoroughly conducted review (Whirledge & Cidlowski, 2010). This is also in line with the 'over-dominant' genetic model that best described the association between *Bcl*I and testicular function. This model, often synonymously termed heterozygote advantage, states that individuals heterozygous at a locus have a greater fitness than both homozygote genotypes (Hedrick, 2012). However, only few examples of heterozygote advantages have been proposed (Witchel *et al.*, 1997). The heterozygote advantage that we observed might be explained by a potentially important positive influence on male fertility favoring selection toward the heterozygote genotype. This selection might also be influenced by the pleiotropic nature of GC; on one hand, corticosteroids are fundamentally necessary due to potent anti-inflammatory effects and involvement in the stress response, on the other hand, corticosteroids can potentially be harmful due to adverse metabolic effects. GC-sensitive phenotypes may have a health advantage due to anti-inflammatory properties but a health disadvantage due to adverse effects of GC, and vice versa for GC-resistant phenotypes. Indeed, carriage of the *Bcl*I G-allele has been associated with both adverse metabolic outcomes (like increased BMI, total body fat mass, abdominal obesity, blood pressure, fasting glucose, and insulin) but also a *reduced* susceptibility to develop autoimmune diseases (Rosmond & Holm, 2008; Geelen *et al.*, 2013; Koper *et al.*, 2014).

The strengths of our study are that we combined an immunohistochemical NR3C1 localization study with a population study of the receptor's genetic variants and detected results that are mutually supporting each other. Furthermore, the results from the population study relied on a large population of healthy men unselected regarding fertility status or testicular function, and with thorough information on lifestyle and reproductive health making it possible to control for relevant covariates. Minor allele frequencies of the investigated SNPs in our population matched closely those reported the 1000 Genomes project in European populations; 38% (*Bcl*I), 32% (*Tth111*I), and 17% (9β), respectively [1000genomes.org (www.ebi.ac.uk/arrayexpress. last accessed 27.06.2017); (www.internationalgenome.org/category/ensembl. last accessed 27.06.2017)].

Our study also has limitations. The tissue expression study was performed using only one approach and one antibody, and although we found supporting data at the transcript level in available databases, an independent validation at the mRNA level would be preferable. But that would require either in situ hybridization, which is difficult in paraffin-embedded tissues, or microdissection of different cell types and subpopulations. Men in the population study did not have the blood samples drawn at exactly the same time which may not be optimal due to the diurnal rhythm of testosterone and Inhibin B (Carlsen *et al.*, 1999). However, there was no difference in time of day for blood sampling between genotype groups and we also included time of day for blood sampling in the models adjusting for a possible bias. We assessed only three *NR3C1* SNPs even though other interesting variants, for example rs852977, rs6195, and rs6189/rs6190, have been described. However, the three polymorphisms were selected because they were well studied and previously have been associated with a variety of health outcomes suggesting widespread potential effects. In addition, they were relatively common in contrast to other described SNPs. We investigated several different associations but chose not to adjust for multiple comparisons. Although such adjustment would protect against type I error, it would deflate the type 2 error. Furthermore, the various semen parameters and reproductive hormones are highly interrelated making multiple comparison correction less straightforward. As can be seen in Table 2, the *p*-value for percentage of motile spermatozoa obtained in the over-dominant model is highly significant and would remain statistically significant even if one were to correct the *p*-value with a factor of 150. We therefore feel confident that the reported associations are relevant.

CONCLUSION

The human testis is a target for glucocorticoids as evidenced by the presence of the GC receptor in several cell types. The developmental expression pattern suggests a role for GCs in regulation of spermatogenesis. The *Bcl*I variant (rs41423247) of the *NR3C1* gene may play a functional role in regulating the GC exposure in the testis and may be important for testicular function, especially sperm motility. This association was substantiated by a higher Inhibin B and Inhibin B/FSH ratio, and lower FSH in *Bcl*I heterozygotes. Although the clinical relevance of the findings is limited, the results substantiate a previously suggested link between stress and testicular function and should be

regarded as a discovery study generating hypotheses for future studies.

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DISCLOSURES

The authors have nothing to disclose.

AUTHORS' CONTRIBUTIONS

Substantial contributions to conception and design performed by LN, KA, JEN, AKB, LP, ERM, AJ, NES, and NJ. Data acquisition performed by LN, KA, JEN, AKB, LP, MK, SAH, SBW, DLEP, and NM. Data analysis performed by LN, KA, JEN, ERM, and NJ. Statistical evaluation performed by JHP. Data interpretation performed by all authors. Drafting the manuscript and critical discussion performed by LN, KA, ERM, NES, and NJ. Revising manuscript critically for important intellectual content performed by all authors. Final approval of the manuscript performed by all authors.

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[Correction added on October 26, 2017, after online publication: The name Rajpert-De Meyts E has been corrected throughout the references.]

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Distribution of *NR3C1 Bcl*I (rs41423247), *Tth111*I (rs10052957), and 9β (rs6198) genotypes in Danish men stratified according to *Bcl*I genotypes.

Table S2 Semen parameters and reproductive hormones according to *Tth111*I (rs10052957) genotypes.

Table S3 Semen parametres and reproductive hormones according to 9β (rs6198) genotypes.