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ORIGINAL ARTICLE ***Reproductive endocrinology***

Gonadotropin suppression in men leads to a reduction in claudin-11 at the Sertoli cell tight junction

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STUDY QUESTION: Are Sertoli cell tight junctions (TJs) disrupted in men undergoing hormonal contraception?

SUMMARY ANSWER: Localization of the key Sertoli cell TJ protein, claudin-11, was markedly disrupted by 8 weeks of gonadotropin suppression, the degree of which was related to the extent of adluminal germ cell suppression.

WHAT IS KNOWN ALREADY: Sertoli cell TJs are vital components of the blood–testis barrier (BTB) that sequester developing adluminal meiotic germ cells and spermatids from the vascular compartment. Claudin-11 knockout mice are infertile; additionally claudin-11 is spatially disrupted in chronically gonadotropin-suppressed rats coincident with a loss of BTB function, and claudin-11 is disorganized in various human testicular disorders. These data support the Sertoli cell TJ as a potential site of hormonal contraceptive action.

STUDY DESIGN, SIZE, DURATION: BTB proteins were assessed by immunohistochemistry ($n = 16$ samples) and mRNA ($n = 18$ samples) expression levels in available archived testis tissue from a previous study of 22 men who had undergone 8 weeks of gonadotropin suppression and for whom meiotic and post-meiotic germ cell numbers were available. The gonadotropin suppression regimens were (i) testosterone enanthate (TE) plus the GnRH antagonist, acyline (A); (ii) TE + the progestin, levonorgestrel, (LNG); (iii) TE + LNG + A or (iv) TE + LNG + the 5α-reductase inhibitor, dutasteride (D). A control group consisted of seven additional men, with three archived samples available for this study.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Immunohistochemical localization of claudin-11 (TJ) and other junctional type markers [ZO-1 (cytoplasmic plaque), β-catenin (adherens junction), connexin-43 (gap junction), vinculin (ectoplasmic specialization) and β-actin (cytoskeleton)] and quantitative PCR was conducted using matched frozen testis tissue.

MAIN RESULTS AND THE ROLE OF CHANCE: Claudin-11 formed a continuous staining pattern at the BTB in control men. Regardless of gonadotropin suppression treatment, claudin-11 localization was markedly disrupted and was broadly associated with the extent of meiotic/post-meiotic germ cell suppression; claudin-11 staining was (i) punctate (i.e. 'spotty' appearance) at the basal aspect of tubules when the average numbers of adluminal germ cells were $<15\%$ of control, (ii) presented as short fragments with cytoplasmic extensions when numbers were 15–25% of control or (iii) remained continuous when numbers were $>40\%$ of control. Changes in localization of connexin-43 and vinculin were also observed (smaller effects than for claudin-11) but ZO-1, β-catenin and β-actin did not differ, compared with control.

LIMITATIONS, REASONS FOR CAUTION: Claudin-11 was the only Sertoli cell TJ protein investigated, but it is considered to be the most pivotal of constituent proteins given its known implication in infertility and BTB function. We were limited to testis samples which had been gonadotropin-suppressed for 8 weeks, shorter than the 74-day spermatogenic wave, which may account for the heterogeneity in claudin-11 and germ cell response observed among the men. Longer suppression (12–24 weeks) is known to suppress germ cells further and claudin-11 disruption may be more uniform, although we could not access such samples.

WIDER IMPLICATIONS OF THE FINDINGS: These findings are important for our understanding of the sites of action of male hormonal contraception, because they suggest that BTB function could be ablated following long-term hormone suppression treatment.

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Introduction

The blood–testis barrier (BTB) is a dynamic junctional complex between Sertoli cells which functions to segregate meiotic and post-meiotic germ cells into an immune-privileged adluminal compartment separate from other testicular cells and the vasculature in the basal compartment (Setchell, 2008). Loss or impairment of BTB function, whether by targeted knock-down (Gow *et al.*, 1999; Mazaud-Guittot *et al.*, 2010) or suppression of key protein components (Tarulli *et al.*, 2006, 2008; McCabe *et al.*, 2010; Haverfield *et al.*, 2014) in rodent models, or by poorly understood mechanisms in men (Cavicchia *et al.*, 1996; Alves *et al.*, 2014), causes major apoptotic loss of adluminal germ cells and in some cases infiltration of immune cells into the epithelium (Mital *et al.*, 2011; Silva *et al.*, 2014; Zhao *et al.*, 2014; Michel *et al.*, 2015), underscoring the essential role of the BTB to male fertility.

Various inter-Sertoli junctional types contribute to the BTB, including ectoplasmic specializations, gap and adherens junctions, with involvement of actin cytoskeleton and cytoplasmic plaques, but it is well established that the Sertoli cell tight junction (TJ) forms the major physical component of the BTB (for reviews see Pelletier, 2011; Li *et al.* 2015). TJs 'seal' adjacent Sertoli cell membranes via interactions between transmembrane proteins including occludin (Saitou *et al.*, 1997; Morita *et al.*, 1998), junctional adhesion molecule (JAM) family members (Glick *et al.*, 2004) and coxsackie and adenovirus receptor (Su *et al.*, 2012). Members of the claudin family, the tissue distribution of which is highly specific (Lapierre, 2000; González-Mariscal *et al.*, 2003), are also key players in the seminiferous epithelium (Morita *et al.*, 1999; Meng *et al.*, 2005; Hermo *et al.*, 2010; Pelletier, 2011; Chakraborty *et al.*, 2014; McMillan *et al.*, 2014). Claudin-11, which is also expressed in the oligodendrocytes of the central nervous system (Morita *et al.*, 1999), is arguably the most important testicular TJ protein as selective gene knock out in the murine testis results in the cessation of spermatogenesis and sterility (Gow *et al.*, 1999; Mazaud-Guittot *et al.*, 2010), and this phenotype is transgenically reversible (Wu *et al.*, 2012). However other claudins, including claudin-3 (Meng *et al.*, 2005; Smith and Braun, 2012) and claudin-5 (Morrow *et al.*, 2009), also appear to contribute to TJ function in the rodent. In infertile men with BTB failure, claudin-11 protein shifts location from TJs to the Sertoli cell cytoplasm (Fink *et al.*, 2009; Haverfield *et al.*, 2013), suggestive that the cellular mechanisms which direct TJ proteins to TJs (Su *et al.*, 2010) are faulty.

The formation of functional Sertoli cell TJs, as shown using electron-opaque or protein-based tracers such as lanthanum and horseradish peroxidase, occurs concomitantly with the onset of puberty and commencement of spermatogenesis, when there is an increase in circulating LH/testosterone and FSH (de Kretser and Burger, 1972; Russell and Peterson, 1985). In an immature rat Sertoli cell culture model, testosterone regulates claudin-11 mRNA expression and claudin-11 protein localization to the TJ where it contributes significantly to function, while FSH also regulates claudin-11 mRNA expression (Kaitu'u-Lino *et al.*, 2007; McCabe *et al.*, 2015). *In vivo*, gonadotropins and/or androgen exert several effects: (i) regulating the formation of functional TJs in a congenitally hypogonadal (*hpg*) mouse model (McCabe *et al.*, 2012), (ii) maintaining and restoring TJ function in GnRH antagonist-treated adult rats (McCabe *et al.*, 2010; Haverfield *et al.*, 2014) and (iii) involvement in both the loss and gain of TJ function in the seasonally breeding Djungarian hamster model (Tarulli *et al.*, 2006, 2008). In each case, the suppressed levels of circulating gonadotropins and spermatogenesis were associated with spatial changes in the localization of claudin-11 away from TJs, which became permeable to the small molecular weight tracer, biotin. Such phenotypes were at least partially reversible by short to mid-term gonadotropin administration (Tarulli *et al.*, 2006, 2008; McCabe *et al.*, 2010; Haverfield *et al.*, 2014). Thus, successful spermatogenesis is critically dependent upon a functional Sertoli cell TJ consisting of appropriately distributed transmembrane proteins such as claudin-11, and the presence of circulating gonadotropins.

In the adult human, claudin-11 is localized in a discrete filamentous pattern around the basal aspect of seminiferous tubules, comparable with animal models (Fink *et al.*, 2009; Nah *et al.*, 2011; Chiba *et al.*, 2012; Haverfield *et al.*, 2013), but becomes disorganized with punctate staining in Sertoli cell cytoplasm in testis sections from infertile men with non-obstructive azoospermia (Nah *et al.*, 2011; Chiba *et al.*, 2012; Haverfield *et al.*, 2013). Similarly, claudin-11 staining, together with other junctional proteins, including ZO-1 and ZO-2, becomes diffuse and cytoplasmic in testicular intraepithelial neoplasia in conjunction with a loss of BTB function (Fink *et al.*, 2006, 2009). Collectively these studies, supported by the animal models, establish claudin-11 localization as a de facto marker of BTB function in men. On this basis, Ilani *et al.* (2012) set out to determine whether the human BTB was affected by short-term gonadotropin suppression associated with male contraception, but found no change in the localization of several TJ proteins including claudin-11. Potential limitations of that study included the short duration of the suppression treatment (9 weeks, prior to BTB assessment) and the extent of spermatogenic suppression achieved by the combined testosterone and progestin (levonorgestrel; LNG) treatment regime (Ilani *et al.*, 2012).

Our laboratory has examined the effectiveness of various hormone suppression regimes on germ cell populations in men (Zhengwei *et al.*, 1998; McLachlan *et al.*, 2002; Matthiesson *et al.*, 2005a,b). In particular, a testosterone plus LNG treatment similar to Ilani *et al.* (2012), but with added GnRH antagonist (acyline) or 5α-reductase inhibitor (dutasteride) was found to provide a 25–40% greater suppression of spermatogenesis than testosterone and LNG alone (Matthiesson *et al.*, 2005a,b). With this in mind, we hypothesized that the expression and localization of claudin-11 at the BTB would be disrupted by male hormonal contraception. The aim of this study was to assess the potential for claudin-11 as a target of male hormonal contraception by analysing changes in the

localization of this protein and other BTB markers following gonadotropin suppression in testis biopsies from the Matthiesson *et al.* (2005a,b) studies.

Materials and Methods

Experimental design

As outlined in Matthiesson *et al.* (2005a), all men ($n = 29$) included in the study underwent medical interview with physical and biochemical investigations. Subjects had to fulfill each of six criteria: (i) aged 21–55 years, (ii) normal physical findings and testicular volumes, (iii) two normal semen analyses according to World Health Organization criteria, (iv) normal serum FSH and LH, (v) normal serum testosterone and (vi) normal liver and renal function and complete blood count. As a result of the inclusion criteria, there were no differences seen in mean age, testicular or ejaculate volume, sperm concentration or motility or serum gonadotropins. Mean BMI ranged from 25 to 27 kg/m^2. Men who were excluded from the study included those with a history of hypertension or significant cardiovascular, renal, hepatic, prostatic or testicular disease or infertility, as well as men taking significant prescribed medications and those involved in competitive sports, who were tested for androgen usage.

The Institutional Review Board at the University of Washington, WA, USA, approved all experimental protocols and subjects gave written informed consent before screening.

In brief, after an initial 2-week screening phase, all men were randomly assigned to a control group ($n = 7$) which proceeded straight to surgery or to an 8-week course of one of four gonadotropin suppressive treatments ($n = 5–6$/group) which all included 100 mg testosterone enanthate (TE; Delestestryl, Bristol-Myers, Squibb, Princeton, NJ, USA) injected i.m. weekly, in combination with one of the following: (i) 125 μg progestin levonorgestrel (LNG; Wyeth, Madison, NJ, USA), orally, daily ($n = 5$), (ii) LNG + 0.5 mg 5α-reductase inhibitor dutasteride (D; GlaxoSmithKline, Research Triangle Park, NC, USA), orally, daily ($n = 6$), (iii) 300 μg/kg GnRH antagonist acyline (A; Multiple Peptide Systems, San Diego, CA, USA), s.c, fortnightly ($n = 6$), or iv) LNG + A ($n = 5$) (Matthiesson *et al.*, 2005a,b).

Following the treatment phase and prior to a 4-week recovery phase, testicular biopsies were taken from each man in conjunction with a pre-planned vasectomy and either fixed in Bouin's fixative for 3 h and embedded in paraffin wax, or used for total RNA extraction. Available samples for this study for immunohistochemistry ($n = 2–5$/group) and RT–PCR ($n = 3–5$/group) are displayed in Supplementary data, Table SI. Note that not all samples available for immunohistochemistry were available for RT–PCR and vice versa. Data for quantification of germ cell numbers, intratesticular steroids and other endocrine and morphological end-points have already been published (Matthiesson *et al.*, 2005a,b).

Immunohistochemistry

Tissue sections were prepared and visualized by immunofluorescence as previously described (Haverfield *et al.*, 2013, 2014). Primary antibodies, rabbit anti-claudin-11 (Zymed #36–4500; 1.25 μg/ml, 1 h), rabbit anti-ZO-1 (Zymed #61–7300; 1.25 μg/ml, overnight), mouse anti-β-catenin (Transduction Laboratories #610154; 0.33 μg/ml, 1 h), mouse anti-connexin-43 (Sigma ascites fluid #C8093; 1:800 of stock, 1 h), mouse anti-vinculin (Sigma ascites fluid #V4505; 1:400 of stock, 1 h) and mouse anti-β-actin (MP Biomedicals ascites fluid #69100; 1:200 of stock, 1 h) were diluted in 10% normal goat serum (Chemicon International, Temecula, CA, USA) in phosphate-buffered saline and applied to the sections. Negative controls substituted rabbit/mouse immunoglobulin G at the same concentration as the primary antibody. The secondary antibody (30 min) was goat anti-rabbit/mouse Alexa-488 (Molecular Probes, Eugene, OR, USA) at 5 μg/ml (2.5 μg/ml for ZO-1). Experiments were repeated 2–3 times with all staining and microscopy-analysis within an experiment conducted at the same time. Multiple immunofluorescence images were captured for each section. For morphological analyses, one tissue section from each man was re-stained with haematoxylin and eosin (H&E). Images of each section in their entirety were captured for analysis.

RNA amplification and quantitative real-time PCR

Archived testis mRNA was obtained for some men from the Matthiesson *et al.* (2005a,b) studies for real-time PCR analysis (primer and RT–PCR conditions presented in Supplementary data, Table SII). All mRNA was initially amplified to maximize availability of DNA for RT–PCR (see Supplementary data, Methods for details).

Results

Localization of claudin-11 in control and gonadotropin suppressed human testes

All control men characteristically displayed a largely continuous 'scalloping' appearance of claudin-11 at the Sertoli cell TJs at the outer edges of the tubules (Fig. 1A). However, variable staining patterns were observed in that some displayed clear punctate staining, particularly #142, and the staining intensity of claudin-11 also varied within and across sections (Fig. 1A). The scarcity of tissue precluded quantification of these variations by western blot analysis.

After 8 weeks of gonadotropin suppression, claudin-11 localization fell into three distinct categories (Fig. 1B). In Category 1, staining appeared similar to controls with the peripheral 'scalloping' appearance. In Category 2, the neat scalloping pattern had changed into a fragmented phenotype with short vertical cytoplasmic extensions. In Category 3, there appeared to be a complete loss of the scalloping claudin-11 appearance, instead replaced with punctate structures, typically at the basal aspect of the tubule, but also scattered towards the centre (Fig. 1B). This category was the most common and appeared in 10 of the 13 gonadotropin suppressed men assessed (Table I). No other claudin-11 staining pattern was observed in six of these men. Qualitatively in the remaining four, one (#126) appeared to have more Category 3 than Category 2 staining, while the other 3 had more Category 1 than Category 3 (#117, 120, 132) (Table I). Differences in the extent of claudin-11 disruption were apparent within the treatment groups, but not between them (see below).

Comparison of germ cell numbers with claudin-11 localization

We then sought to determine if these changes in claudin-11 corresponded to alterations in adluminal germ cell numbers in each testis sample, using published data from Matthiesson *et al.* (2005b). The germ cell quantification data averaged across each treatment group appears in Supplementary data, Fig. S1 and representative images of H&E stained testis sections in Supplementary data, Fig. S2, which allows visualization of remaining adluminal germ cell types from men within each treatment group for comparison to each other and to control.

To summarize the findings of Matthiesson *et al.* (2005b), 8 weeks of gonadotropin suppression significantly reduced germ cell numbers across all treatment groups from type B spermatogonia onward, to

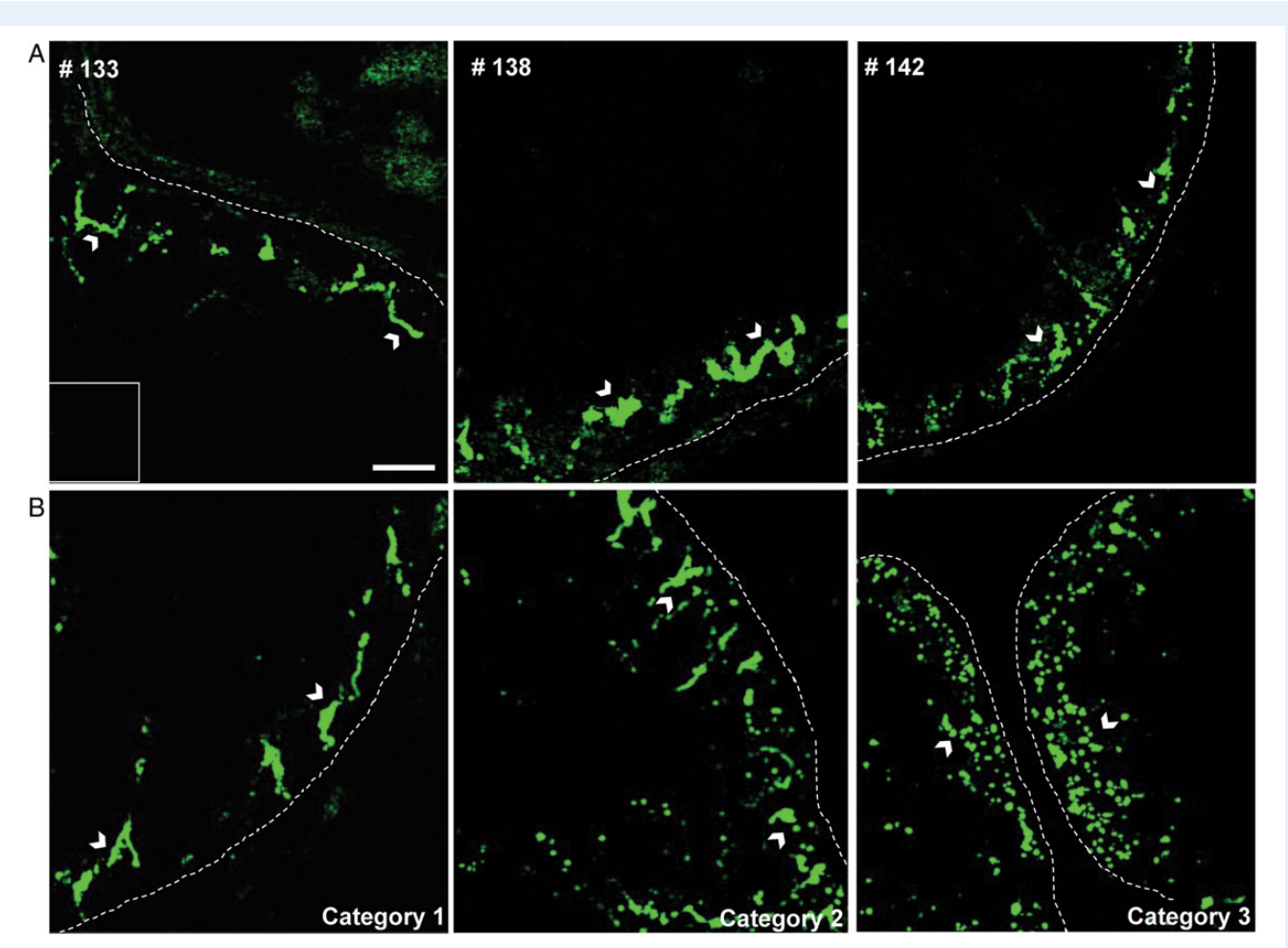


Figure 1 Localization of claudin-11 in control and hormone suppressed human testes. Bouin's fixed, paraffin embedded human testis biopsies were processed by immunohistochemistry and stained for the tight junction (TJ) protein, claudin-11 (green; arrowheads). (**A**) Biopsies from three control men, with subject identifiers shown by the # symbol identical to those used by Matthiesson *et al.* (2005a). The boundary of each tubule is denoted by white dashed lines. (**B**) Representative examples of the three distinct categories for claudin-11 staining observed across all gonadotropin suppression treatment groups; Category 1 = continuous staining, Category 2 = filamentous staining with cytoplasmic extensions and Category 3 = punctate staining. Scale bar = 17 μm. Inset = negative control.

an average of 22% of control (Table I and Supplementary data, Fig. S1), with no significant differences between treatment groups (Matthiesson *et al.*, 2005b). However, the extent of germ cell loss was heterogeneous and variations were apparent within each group, as shown in Fig. 2. For example, acyline was the only treatment that significantly ($P < 0.05$) suppressed type B spermatogonia and preleptotene spermatocytes in some men, whereas only LNG-containing treatments resulted in significant decreases in step 3–6 spermatids (Fig. 2). However, within each treatment group are clear examples where extensive suppression from B spermatogonia or preleptotene spermatocytes onwards was achieved (Fig. 2). The small peak at step 3–6 spermatids observed across all samples reflects the fact that the human spermatogenic wave is 74 days in length (Amann, 2008) and the 56-day suppression treatment was not long enough for lesions in the earlier germ cells to flow through. Indeed 12–24 weeks of suppression is known to profoundly lower these cell types (Zhengwei *et al.*, 1998; McLachlan *et al.*, 2002).

We then examined whether claudin-11 protein localization varied with the extent of germ cell suppression. Representative images for each treatment group are presented in Fig. 2, with all images displayed in Supplementary data, Fig. S3. Images in Supplementary data, Fig. S3 are arranged within treatment groups in order from maximal adluminal germ cell suppression to the lowest. This analysis revealed that the punctate claudin-11 staining pattern representative of Category 3 was mostly observed with maximal suppression of adluminal germ cells (defined as combined number of leptotene/zygotene spermatocytes to step 7–8 elongated spermatids) to <15% of control (see Table I and Fig. 2 and Supplementary data, Fig. S3), whereas the filamentous/cytoplasmic extension staining pattern was observed in samples with adluminal germ cells at 15–25% of control (#122, #131; Table I and Fig. 2 and Supplementary data, Fig. S3). The continuous claudin-11 staining pattern similar to control (Category 1) was mostly observed when adluminal germ cell numbers >40% of control were present (Table I and Fig. 2 and Supplementary data, Fig. S3).

Table I Comparison between claudin-11 staining, adluminal germ cell numbers and presence of tubule lumens in immunohistochemical sections from human testes.

Treatment	Patient number	Claudin-11 staining category	Percentage (%) adluminal germ cells compared with control*	Percentage (%) tubules with lumens	Percentage (%) tubules without lumens**	Number of tubules counted***
Control	133	1	100	60	21	214
Control	138	1	100	51	30	43
Control	142	1	100	58	38	52
TE + A	101	3	5.3	65	31	261
TE + A	102	3	14.4	1.5	96	135
TE + A	116	3	18.8	1	99	144
TE + A	122	2	24.9	31	63	232
TE + A	137	1	40.9	8	92	191
TE + LNG	109	3	2.6	51	49	86
TE + LNG	131	2	21.7	27	73	221
TE + LNG + A	117	1 and 3	11.8	45	55	51
TE + LNG + A	136	3	12.1	37	63	481
TE + LNG + A	126	3 and 2	15.5	18	82	163
TE + LNG + D	120	1 and 3	5.3	16	84	161
TE + LNG + D	107	3	12.9	34	60	129
TE + LNG + D	132	1 and 3	44.1	18	82	156

A, acyline; D, dutasteride; LNG, levonorgestrel; TE, testosterone enanthate.
*Samples are listed within their treatment groups in order of percentage adluminal germ cell suppression; defined as combined leptotene/zygotene spermatocytes to step 7–8 elongated spermatids.
**Parts of some immunohistochemical sections were too difficult to determine whether the tubules contained lumens or not; hence, there are percentages that do not equal 100% in every section. These particular areas were excluded from all analyses.
***All tubules within a tissue section were counted.

Only four men [#117 and #126 (TE + LNG + A), and #120 and #132 (TE + LNG + D)] presented mixed claudin-11 staining categories, which did not fit our observed trend. To illustrate this point, two representative images of claudin-11 staining in #117 (TE + LNG + A; Fig. 2) show tubules with continuous claudin-11 staining while others were punctate.

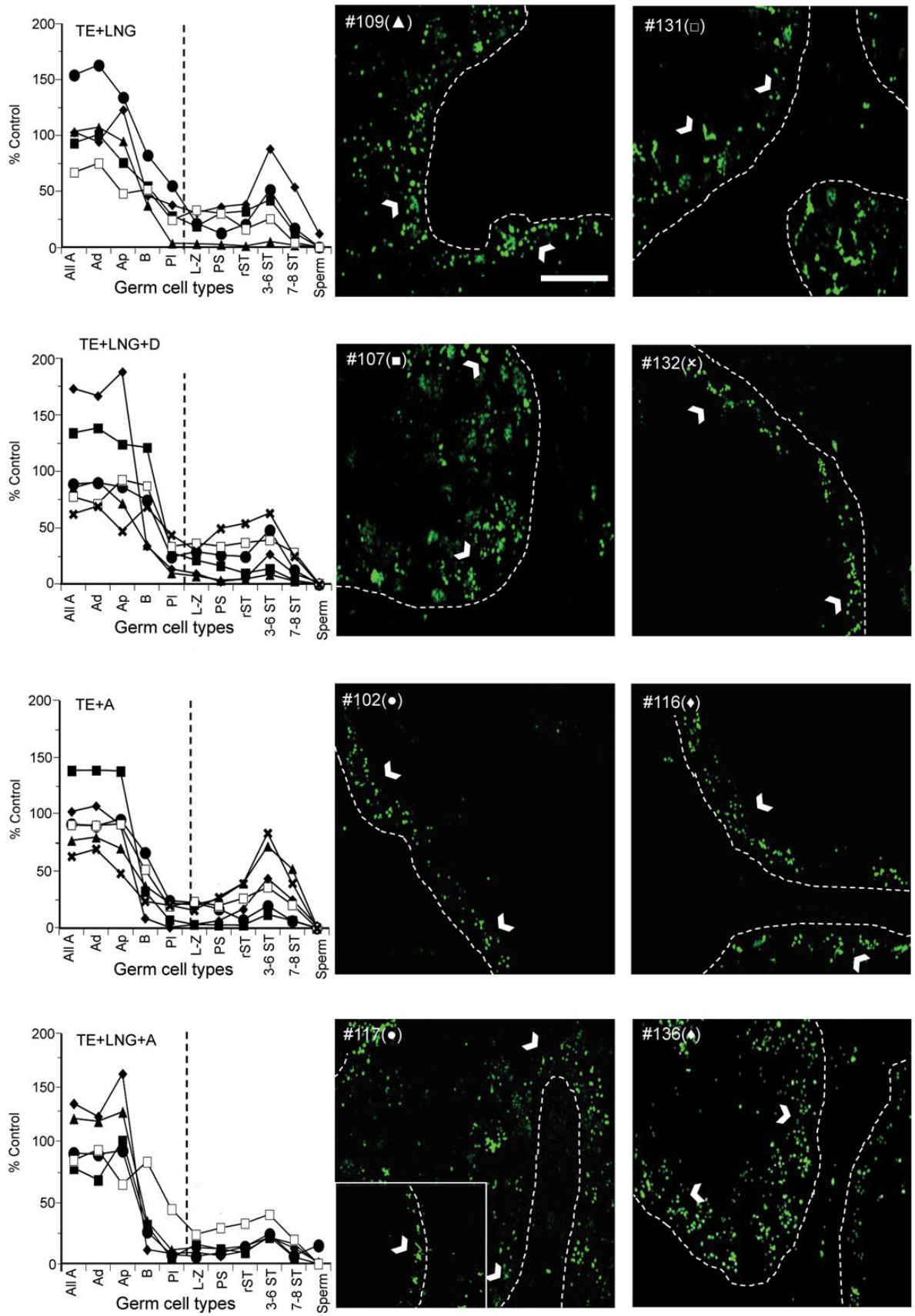
The effect of gonadotropin suppression on seminiferous tubule lumens

All tubules were assessed for the presence or absence of a lumen following H&E staining (Fig. 3 and Supplementary data, Fig. S4 and Table I), as the presence of a lumen is accepted as qualitative evidence of BTB functionality in various animal models (Pelletier, 1990, Setchell, 2008). Representative images from the TE + A group against a control image are presented in Fig. 3, with additional images again arranged in order of greatest adluminal germ cell suppression to lowest within treatment groups, presented in Supplementary data, Fig. S4. Overall, there was no association between the extent of claudin-11 dislocation or germ cell suppression and the percentage of tubules where lumens were present (Table I). For example, #101 and #102 (TE + A group) were designated Category 3 for their punctate claudin-11 staining and both had markedly suppressed adluminal germ cell numbers (~5–14% of control), but demonstrated widely variable tubule lumen positivity (65% for #101, #1.5% for #102, Table I and Fig. 3). Similarly, only 8% of tubules in #137 (TE + A) contained lumens, but claudin-11 staining was in Category 1, similar to controls.

The effect of gonadotropin suppression on other BTB junction protein markers in men

Given the effect of gonadotropin suppression upon claudin-11 of the TJ and its close proximity to other junctional types in the BTB, we next assessed whether gonadotropins could also regulate the localization of β-actin (cytoskeleton), β-catenin (adherens junction), vinculin (ectoplasmic specialization), connexin-43 (gap junction) and ZO-1 (underlying cytoplasmic plaque). Results were consistent across all controls and treated men and we present representative data from #109 (TE + LNG) in Fig. 4. This individual had punctate claudin-11 staining (Category 3) associated with marked suppression of adluminal germ cells to <3% of control (Table I).

Staining for all junction-related proteins was extensive in control and all treatment groups (Fig. 4). In controls, β-actin and β-catenin were localized around all germ cell types, while connexin-43 and ZO-1 were localized at the BTB towards the outer edges of the tubules. Vinculin staining was apparent at ectoplasmic specializations localized at the BTB, and also extending towards the apically located ectoplasmic specializations between Sertoli cells and round and elongating spermatids near tubule lumens (Fig. 4). Following gonadotropin suppression for 8 weeks, β-actin and β-catenin remained present around remaining germ cells, and ZO-1 was still localized in extensive belts at the outer edge of the tubules as in controls. Connexin-43 staining appeared reduced in intensity and not as continuous as in controls. Basal ectoplasmic specializations at the BTB appeared intact with no apparent reduction in vinculin staining; however, apical staining had become diffuse and cytoplasmic



in Sertoli cells, reflective of a loss of late germ cell types (Fig. 4). Overall, the effect of gonadotropin suppression appeared to be greater on claudin-11 localization than for any other protein assessed.

Effects of gonadotropin suppression on junctional mRNA expression levels in men

Gonadotropin suppression did not significantly alter the mRNA expression levels of any of the TJ proteins (claudin-11, claudin-3, occludin, JAM-A) or other BTB proteins (ZO-1, β-catenin, connexin-43, vinculin, β-actin) tested (Supplementary data, Fig. S5). Androgen regulated INSL-3 decreased 20-fold ($P < 0.001$) in each of the suppression treatments compared with control (Supplementary data, Fig. S5), consistent with a decrease of ~98% in intratesticular testosterone (Matthiesson *et al.*, 2005b).

Discussion

We provide evidence that claudin-11, the key protein of the human Sertoli cell TJ, is regulated by gonadotropins *in vivo*. Its organization was markedly altered into a punctate staining pattern in gonadotropin-suppressed men, compared with its continuous staining pattern in controls, and identifies a pathway relevant to the efficacy of long-term male hormonal contraception. We also observed more modest changes in the localization of gap junction protein, connexin-43, which provides communication channels between Sertoli cells and germ cells or other Sertoli cells, and for which regulation has been closely linked with TJ function at the BTB (Li *et al.*, 2009; Carette *et al.*, 2010; Gerber *et al.*, 2014, 2016; Weider *et al.*, 2011). The loss of apical ectoplasmic specialization structural integrity as shown by diffuse vinculin staining is consistent with the loss of later germ cell types including spermatids, and its known dependence on gonadotropins (O'Donnell *et al.*, 2000; Wong *et al.*, 2005; Sluka *et al.*, 2006). Finally, the relationship between TJ claudin-11 organization and the extent of suppression of meiotic and post-meiotic germ cells suggests a signalling mechanism between adluminal germ cells and the BTB that has yet to be characterized.

While several studies have observed disorganized claudin-11 staining in testes from infertile men (Nah *et al.*, 2011; Chiba *et al.*, 2012; Haverfield *et al.*, 2013), or in cancerous testis tissue with loss of BTB function (Fink *et al.*, 2006, 2009), the current study provides the first evidence that claudin-11 localization at the BTB in men is altered by chronic gonadotropin suppression. This result is supported by studies in rats where long-term suppression with the same GnRH-antagonist, acyline, as used herein, caused claudin-11 localization to become cytoplasmic and BTB function to be lost (McCabe *et al.*, 2010; Haverfield *et al.*, 2014). The same trend is observed when gonadotropins are naturally suppressed in a seasonal breeder model, the Djungarian hamster (Tarulli *et al.*, 2006, 2008) and, as in the rat model above, is concomitant with reduced germ cell numbers and absent spermatogenic activity. These models are consistent with the infertile phenotype of the murine claudin-11 knockout animal (Gow *et al.*, 1999; Mazaud-Guittot *et al.*, 2010). Restoration of testicular androgen levels by hCG (LH analogue) or testosterone treatment in rats (McCabe *et al.*, 2010; Haverfield *et al.*, 2014), or human FSH in Djungarian hamsters (Tarulli *et al.*, 2006, 2008), led to a restoration of claudin-11 localization and BTB function, and re-initiation of spermatogenesis, underscoring the hormonal dependence of the BTB.

In contrast, a recent study also investigated BTB protein expression and localization in men given hormonal contraception, and concluded that BTB integrity was not affected in terms of TJ proteins including claudin-11, claudin-3 and JAM A (Ilani *et al.*, 2012). There are several differences between the Ilani *et al.* (2012) study and our current data including the extent to which FSH and testicular androgen suppression was achieved, the extent of germ cell suppression, and the time course of suppression and point at which BTB function was assessed. The Ilani *et al.* (2012) study used a model reported by Wang *et al.* (2007), which in common with Matthiesson *et al.* (2005a), applied gonadotropin suppression regimes based on the administration of long-acting testosterone esters and the progestin LNG. Yet germ cell populations were more suppressed in Matthiesson *et al.* (2005a) [mean 19.4% pachytene spermatocytes plus round spermatids (Supplementary data, Fig. S1) compared with 37.5% in the Ilani *et al.* (2012) study] suggesting a more profound gonadotropin suppression at sampling time.

Within our different treatment groups, we found individuals wherein pachytene spermatocyte and round spermatid numbers ranged from 2.7 to 39.5% of control [Fig. 2 and Matthiesson *et al.* (2005b)], and we found a corresponding change in the extent of claudin-11 localization at the BTB. We, therefore, propose a link between the presence of meiotic and post-meiotic germ cells and the structure and function of the BTB in men, and suggest that the continued presence of these germ cell

Figure 2 Comparison of claudin-11 protein localization with extent of adluminal germ cell suppression. Data for adluminal germ cell numbers (i.e. all germ cell types on the right-hand side of dashed lines) in individual men treated with, TE + LNG, TE + LNG + D, TE + A, TE + LNG + A (see below) for 8 weeks ($n = 5–6$/group) were obtained from Matthiesson *et al.* (reprinted with permission from Matthiesson *et al.*, 2005a,b), and compared with claudin-11 localization (green; arrowheads) in testis biopsies obtained from the same men. Representative images from each treatment group are shown here, with additional images for other men arranged in order of adluminal germ cell suppression (greatest to least; Table I) within treatment groups, included in Supplementary data, Fig. S3. Subject numbers were allocated by Matthiesson *et al.* (2005a) and are listed with their corresponding graph symbol within each image, as well as below. Note that, two representative images have been provided for subject #117 (see insert). Germ cell types presented are: All type A spermatogonia (All A), type A dark spermatogonia (Ad), type A pale spermatogonia (Ap), type B spermatogonia (B), preleptotene spermatocytes (Pl), leptotene and zygotene spermatocytes (L–Z), pachytene spermatocytes (PS), round spermatids (rST), step 3–6 elongating spermatids (3–6 ST), step 7–8 elongating spermatids (7–8 ST) and sperm concentration in the ejaculate on the day of testicular biopsy. A, acyline; D, dutasteride; LNG, levonorgestrel; TE, testosterone enanthate. Edges of the seminiferous tubules have been indicated by white dashed lines. TE + LNG: #105; filled squares, #106; filled circles, #109; triangles, #123; open squares, #131; filled diamonds, TE + LNG + D: #107; filled squares, #108; filled circles, #120; triangles, #124; filled diamonds, #127; open squares, #132; X, TE + A: #101; filled squares, #102; filled circles, #114; triangles, #116; filled diamonds, #122; open squares, #137; X, TE + LNG + A: #104; filled squares, #117; filled circles, #126; triangles, #136; filled diamonds, #141; open squares, scale bar = 30 μm.

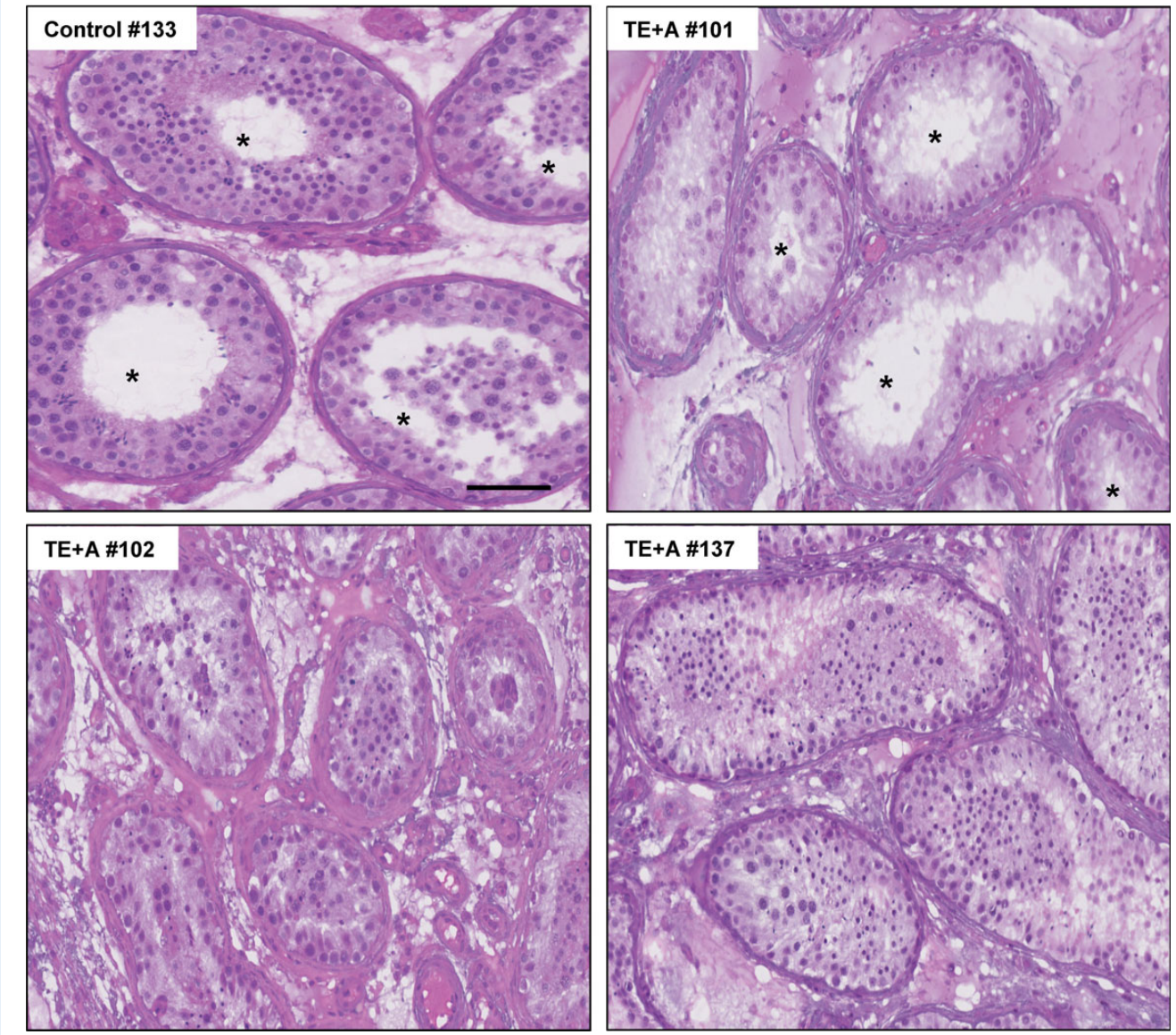


Figure 3 The presence/absence of seminiferous tubule lumens across treatment groups. Representative haematoxylin and eosin images of testis sections from men receiving control and gonadotropin-suppressing TE + A treatment for 8 weeks. Images are representative of typical tubule patterns observed across treatment groups. For images from all men analysed, with subject/patient number arranged in order of greatest adluminal germ cell suppression to lowest within the treatment groups (Table I), see Supplementary data, Fig. S4. Tubule lumens across sections are indicated with asterisks for reference. Scale bar = 100 μm.

types above a threshold of ~30% of control may have accounted for the Ilani *et al.* (2012) study failing to detect changes in claudin-11 organization. Indeed, we have previously demonstrated a positive role of meiotic and post-meiotic germ cells on BTB function in adult rats (Haverfield *et al.*, 2014). Unfortunately it was not possible to take multiple testis biopsies from the same man over the course of the study. The small residual peak in step 3–6 round spermatids in Matthiesson *et al.* (2005b) suggested a longer suppression was required and indeed we predict that in men suppressed for 12–24 weeks (beyond the 74-day-long spermatogenic wave) where profound decreases in these cells are observed (Zhengwei *et al.*, 1998; McLachlan *et al.*, 2002; Amann, 2008), claudin-11 disorganization would be more consistent.

The observed change in claudin-11 localization in men differed from rat models of gonadotropin suppression using acyline, where claudin-11 protein was localized predominantly in the Sertoli cell cytoplasm (McCabe *et al.*, 2010; Haverfield *et al.*, 2014). Besides the germ cell contribution mentioned above, a key difference could be the residual testicular androgen concentration, which was very low at ~7 nmol/l in rats after acyline treatment (Porter *et al.*, 2006), but 47–65 nmol/l in men after TE + LNG ± acyline (Matthiesson *et al.*, 2005b). These higher residual testicular androgen levels in men may be due to the near-normal serum testosterone concentration (18–19 nmol/l) maintained by the TE treatment, something the rats did not receive. Alternatively, LH-independent constitutive androgen secretion by the human Leydig

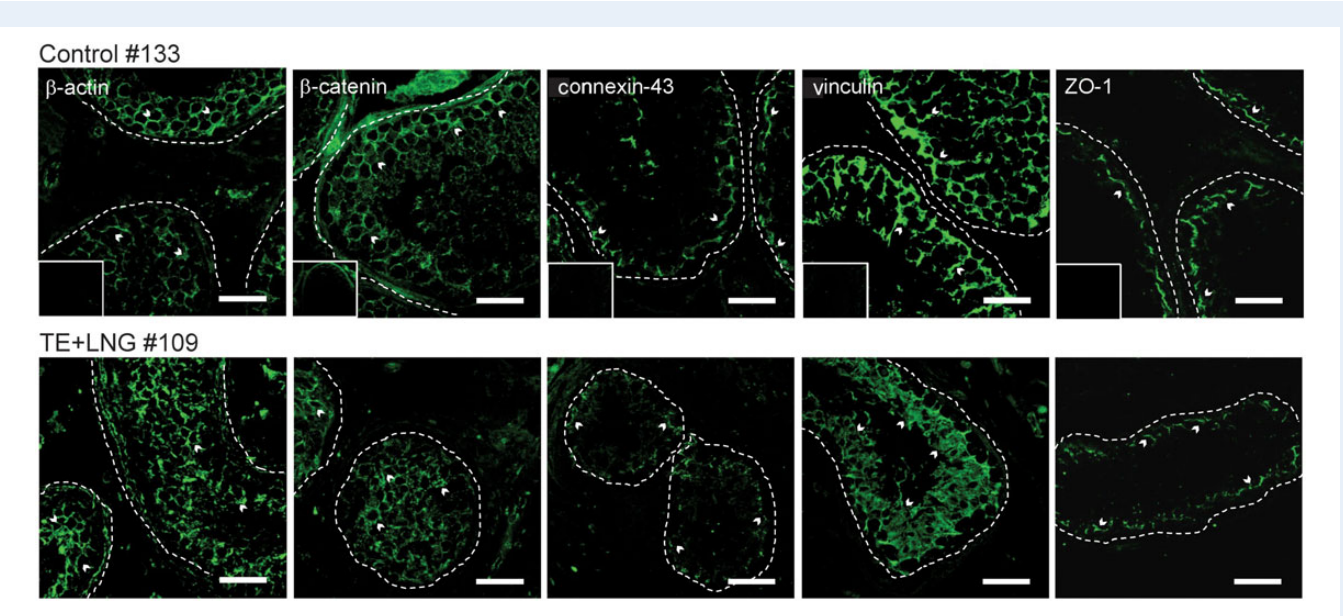


Figure 4 The effect of gonadotropin suppression on other junctional types at the human blood-testis barrier. Bouin's fixed, paraffin embedded human testis biopsies were processed by immunohistochemistry and stained for (i) cytoskeletal protein β-actin, (ii) adherens junction protein β-catenin, (iii) gap junction protein connexin-43, (iv) ectoplasmic specialization protein vinculin and (v) cytoplasmic plaque protein ZO-1 (all green; indicated by arrowheads). Biopsies came from control #133, and #109 from the TE + LNG (testosterone enanthate + levonorgestrel) treatment group. This individual demonstrated maximal disruption to claudin-11 localization and adluminal germ cells and is representative of other men from different treatment groups. Numbers were allocated by Matthiesson *et al.* (2005a). The boundary of each tubule is denoted by white dashed lines. Bar = 50 μm. Inset = negative control.

cells could have maintained high intratesticular levels (Haywood *et al.*, 2002, 2003; O'Shaughnessy *et al.*, 2009). It is well known that claudin-11 mRNA and protein expression in Sertoli cells is androgen dependent *in vitro* (Florin *et al.*, 2005; Kaitu'u-Lino *et al.*, 2007) and *in vivo* (de Gendt *et al.*, 2004; Meng *et al.*, 2005; McCabe *et al.*, 2010, 2012). Additionally, testosterone promotes the localization of various TJ proteins, including claudin-11, from the cytoplasm to Sertoli cell junctions (Kaitu'u-Lino *et al.*, 2007) via an endocytic recycling process proposed to allow breakdown and re-formation of TJs at the BTB (Yan *et al.*, 2008; Su *et al.*, 2010). BTB dynamics are also regulated by cytokines such as transforming growth factor-β3, which activates the protein degradation pathway (Su *et al.*, 2010); for a recent, comprehensive review about mammalian BTB regulation see Mruk and Cheng (2015). Collectively, these studies suggest that the claudin-11 endocytosis-recycling process was either compromised in the treated men due to residual testicular testosterone (~2% of controls, Matthiesson *et al.*, 2005b), or follows a different mechanism to that observed in rodents, and in low levels of testicular androgens is processed for protein degradation, perhaps consistent with its punctate appearance.

As mentioned earlier, various testicular pathologies are associated with altered localization of several BTB proteins (Fink *et al.*, 2006, 2009; Nah *et al.*, 2011; Chiba *et al.*, 2012; Haverfield *et al.*, 2013) and further analyses of some of these have been attempted *in vitro* (Ma *et al.*, 2013; Hai *et al.*, 2015) and *in vivo,* including one study directly showing a loss of BTB function in testicular carcinoma *in situ* (Fink *et al.*, 2006). However, it remains unknown whether BTB functionality was altered in the current study even when maximal claudin-11 disorganization was observed. Recent data show that the BTB can be selectively permeable to tracers of increasing molecular weight in rats following hormone suppression and replacement (Haverfield *et al.*, 2014), but similar tracer techniques have not been applied to men undergoing male hormonal contraception. As an alternative measure of BTB function, we looked for the presence of tubule lumens in treated men, but found no correlations with either the extent of germ cell suppression or claudin-11 disorganization. In comparative terms, an animal model that may emulate the impact of gonadotropin suppression and germ cell loss on BTB function is the seasonal breeding Djungarian hamster. Testicular regression in this model occurs naturally following exposure to short-day length which suppresses pituitary FSH, LH and testicular testosterone, leading to progressive loss of germ cell types (Bergmann, 1987; Lerchl *et al.*, 1993; Meachem *et al.*, 2005). The BTB becomes permeable to tracer at the point when only spermatogonia and a few primary spermatocytes remain (Bergmann, 1987), and TJ proteins including claudin-11 are disorganized (Tarulli *et al.*, 2006, 2008), which is similar to the germ cell phenotype and claudin-11 staining pattern of some of the men (e.g. #109, #120, #101, Fig. 2) in the current study, suggesting a loss of human Sertoli cell TJ function.

In conclusion, we show here that the organization of the key Sertoli cell TJ protein, claudin-11, at the BTB in men is markedly reduced following chronic gonadotropin suppression. Furthermore, the degree of claudin-11 change within individual patients was linked to the extent of germ cell loss from the seminiferous epithelium. These findings are important for our understanding of the sites of action of male hormonal contraception (McLachlan *et al.*, 2002), because they suggest that BTB function could be ablated following long-term hormone suppression treatment.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

This work was conducted in the Male Fertility Regulation laboratory led by P.G.S. P.G.S. supervised the project and with M.J.M., produced the study design and the production of this manuscript. M.J.M. carried out all of the techniques presented herein, including their optimization. M.E.D. provided guidance for the production of this manuscript and also provided experimental suggestions. G.A.T. provided expert guidance with respect to immunohistochemical techniques and assisted with the production of this manuscript. G.L.-L. conducted the H&E staining and captured microscopy images of testis sections for Fig. 3 and Supplementary data, Fig. S2. S.J.M., K.L.M. and R.I.M. kindly provided the testis sections and mRNA used in this study, which had been archived from a previous study led by R.I.M.

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Conflict of interest

None declared.

References

Alves MG, Dias TR, Silva BM, Oliveira PF. Metabolic cooperation in testis as a pharmacological target: from disease to contraception. *Curr Mol Pharmacol* 2014;**7**:83–93.

Amann RP. The cycle of the seminiferous epithelium in humans: a need to revisit? *J Androl* 2008;**29**:469–487.

Bergmann M. Photoperiod and testicular function in *Phodopus sungorus*. *Adv Anat Embryol Cell Biol* 1987;**105**:1–76.

Carette D, Weider K, Gilleron J, Giese S, Dompierre J, Bergmann M, Brehm R, Denizot JP, Segretain D, Pointis G. Major involvement of connexin 43 in seminiferous epithelial junction dynamics and male fertility. *Dev Biol* 2010;**346**:54–67.

Cavicchia JC, Sacerdote FL, Ortiz L. The human blood-testis barrier in impaired spermatogenesis. *Ultrastruct Pathol* 1996;**20**:211–218.

Chakraborty P, William Buaas F, Sharma M, Smith BE, Greenlee AR, Eacker SM, Braun RE. Androgen-dependent Sertoli cell tight junction remodeling is mediated by multiple tight junction components. *Mol Endocrinol* 2014;**28**:1055–1072.

Chen Z, Evans WH, Pflugfelder SC, Li DQ. Gap junction protein connexin 43 serves as a negative marker for a stem cell-containing population of human limbal epithelial cells. *Stem Cells* 2006;**24**:1265–1273.

Chiba K, Yamaguchi K, Ando M, Miyake H, Fujisawa M. Expression pattern of testicular claudin-11 in infertile men. *Urology* 2012;**80**:1161.e13–1161.e17.

de Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lécureuil C *et al.* A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc Natl Acad Sci USA* 2004;**101**:1327–1132.

de Kretser DM, Burger H. Ultrastructural studies of the human Sertoli cell in normal men and males with hypogonadotrophic hypogonadism before and after gonadotrophic treatment. In: Saxena GG, Beling HM (ed). *Gonadotrophins*. New York, USA: Wiley-Interscience, 1972, 640–656.

Fink C, Weigel R, Hembes T, Lauke-Wettwer H, Kliesch S, Bergmann M, Brehm RH. Altered expression of ZO-1 and ZO-2 in Sertoli cells and loss of blood-testis barrier integrity in testicular carcinoma in situ. *Neoplasia* 2006;**8**:1019–1027.

Fink C, Weigel R, Fink L, Wilhelm J, Kliesch S, Zeiler M, Bergmann M, Brehm R. Claudin-11 is over-expressed and dislocated from the blood-testis barrier in Sertoli cells associated with testicular intraepithelial neoplasia in men. *Histochem Cell Biol* 2009;**131**:755–764.

Florin A, Maire M, Bozec A, Hellani A, Chater S, Bars R, Chuzel F, Benahmed M. Androgens and postmeiotic germ cells regulate claudin-11 expression in rat Sertoli cell. *Endocrinology* 2005;**146**:1532–1540.

Gerber J, Weider K, Hambruch N, Brehm R. Loss of connexin43 (Cx43) in Sertoli cells leads to spatio-temporal alterations in occludin expression. *Histol Histopathol* 2014;**29**:935–948.

Gerber J, Heinrich J, Brehm R. Blood-testis barrier and Sertoli cell function: lessons from SCCx43 knockout mice. *Reproduction* 2016;**151**:R15–R27.

Ghassemifar MR, Sheth B, Papenbrock T, Leese HJ, Houghton FD, Fleming TP. Occludin TM4(-): an isoform of the tight junction protein present in primates lacking the fourth transmembrane domain. *J Cell Sci* 2002;**115**:3171–3180.

Glick G, Ebnet K, Aurrand-Lions M, Imhof BA, Adams RH. Spermatid differentiation requires the assembly of a cell polarity complex downstream of junctional adhesion molecule-C. *Nature* 2004;**431**:320–324.

González-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. *Prog Biophys Mol Biol* 2003;**81**:1–44.

Gow A, Southwood CM, Li JS, Pariali M, Riordan GP, Brodie SE, Danias J, Bronstein JM, Kachar B, Lazzarini RA. CNS myelin and Sertoli cell tight junction strands are absent in Osp/claudin-11 null mice. *Cell* 1999;**99**:649–659.

Hai Y, Sun M, Niu M, Yuan Q, Guo Y, Li Z, He Z. BMP4 promotes human Sertoli cell proliferation via Smad1/5 and ID2/3 pathway and its abnormality is associated with azoospermia. *Discov Med* 2015;**105**:311–325.

Haverfield JT, Meachem SJ, O'Bryan MK, McLachlan RI, Stanton PG. Claudin-11 and connexin-43 display altered spatial patterns of organization in men with primary seminiferous tubule failure compared with controls. *Fertil Steril* 2013;**100**:658–666.

Haverfield JT, Meachem SJ, Nicholls PK, Rainczuk KE, Simpson ER, Stanton PG. Differential permeability of the blood-testis barrier during reinitiation of spermatogenesis in adult male rats. *Endocrinology* 2014;**155**:1131–1144.

Haywood M, Tymchenko N, Spaliviero J, Koch A, Jimenez M, Gromoll J, Simoni M, Nordhoff V, Handelsman DJ, Allan CM. An activated human follicle-stimulating hormone (FSH) receptor stimulates FSH-like activity in gonadotropin-deficient transgenic mice. *Mol Endocrinol* 2002;**16**:2582–2591.

Haywood M, Spaliviero J, Jimenez M, King NJ, Handelsman DJ, Allan CM. Sertoli and germ cell development in hypogonadal (hpg) alone or in combination with testosterone. *Endocrinology* 2003;**144**:509–517.

Hermo L, Pelletier RM, Cyr DG, Smith CE. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 5: intercellular junctions and contacts between germ cells and Sertoli cells and their regulatory interactions, testicular cholesterol, and genes/proteins associated with more than one germ cell generation. *Microsc Res Tech* 2010;**73**:409–494.

Ilani N, Armanious N, Lue YH, Swerdloff RS, Baravarian S, Adler A, Tsang C, Jia Y, Cui YG, Wang XH *et al.* Integrity of the blood-testis barrier in healthy men after suppression of spermatogenesis with testosterone and levonorgestrel. *Hum Reprod* 2012;**27**:3403–3411.

Kaitu'u-Lino TJ, Sluka P, Foo CFH, Stanton PG. Claudin-11 expression and localisation is regulated by androgens in rat Sertoli cells *in vitro*. *Reproduction* 2007;**133**:1169–1179.

Lapierre LA. The molecular structure of the tight junction. *Adv Drug Deliv Rev* 2000;**41**:255–264.

Lerchl A, Sotiriadou S, Behre HM, Pierce J, Weinbauer GF, Kliesch S, Nieschlag E. Restorations of spermatogenesis by follicle-stimulating hormone despite low intratesticular testosterone in photoinhibited hypogonadotropic Djungarian hamsters (Phodopus sungorus). *Biol Reprod* 1993;**49**:1108–1116.

Li MW, Mruk DD, Lee WM, Cheng CY. Connexin 43 and plakophilin-2 as a protein complex that regulates blood-testis barrier dynamics. *Proc Natl Acad Sci USA* 2009;**106**:10213–10218.

Li N, Mruk DD, Cheng CY. Actin binding proteins in blood-testis barrier function. *Curr Opin Endocrinol Diabetes Obes* 2015;**22**:238–247.

Ma M, Yang S, Zhang Z, Li P, Gong Y, Liu L, Zhu Y, Tian R, Liu Y, Wang X *et al.* Sertoli cells from non-obstructive azoospermia and obstructive azoospermia patients show distinct morphology, Raman spectrum and biochemical phenotype. *Hum Reprod* 2013;**28**:1863–1873.

Matthiesson KL, Amory JK, Berger R, Ugoni A, McLachlan RI, Bremner WJ. Novel male hormonal contraceptive combinations: the hormonal and spermatogenic effects of testosterone and levonorgestrel combined with a 5alpha-reductase inhibitor or gonadotropin-releasing hormone antagonist. *J Clin Endocrinol Metab* 2005a;**90**:91–97.

Matthiesson KL, Stanton PG, O'Donnell L, Meachem SJ, Amory JK, Berger R, Bremner WJ, McLachlan RI. Effects of testosterone and levonorgestrel combined with a 5alpha-reductase inhibitor or gonadotropin-releasing hormone antagonist on spermatogenesis and intratesticular steroid levels in normal men. *J Clin Endocrinol Metab* 2005b;**90**:5647–5655.

Mazaud-Guittot S, Meugnier E, Presenti S, Wu X, Vidal H, Gow A, Le Magueresse-Battistoni B. Claudin-11 deficiency in mice results in loss of the Sertoli cell epithelial phenotype in the testis. *Biol Reprod* 2010; **82**:2012–2013.

McCabe MJ, Tarulli GA, Meachem SJ, Robertson DM, Smooker PM, Stanton PG. Gonadotrophins regulate rat testicular tight junctions *in vivo*. *Endocrinology* 2010;**151**:2911–2922.

McCabe MJ, Allan CM, Foo CF, Nicholls PK, McTavish KJ, Stanton PG. Androgen initiates Sertoli cell tight junction formation in the hypogonadal (hpg) mouse. *Biol Reprod* 2012;**87**:1–8.

McCabe MJ, Foo CF, Dinger ME, Smooker PM, Stanton PG. Claudin-11 and occludin are major contributors to Sertoli cell tight junction function, *in vitro*. *Asian J Androl* 2015; in press.

McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de Kretser DM, Pratis K, Robertson DM. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Prog Horm Res* 2002;**57**:149–179.

McMillan M, Andronicos N, Davey R, Stockwell S, Hinch G, Schmoelzl S. Claudin-8 expression in Sertoli cells and putative spermatogonial stem cells in bovine testis. *Reprod Fertil Dev* 2014;**26**:633–644.

Meachem SJ, Stanton PG, Schlatt S. Follicle-stimulating hormone regulates both Sertoli cell and spermatogonial populations in the adult photoinhibited Djungarian hamster testis. *Biol Reprod* 2005;**72**:1187–1193.

Meng J, Holdcraft RW, Shima JE, Griswold MD, Braun RE. Androgens regulate the permeability of the blood-testis barrier. *Proc Natl Acad Sci USA* 2005;**102**:16696–16700.

Michel V, Pilatz A, Hedger MP, Meinhardt A. Epididymitis: revelations at the convergence of clinical and basic sciences. *Asian J Androl* 2015; **17**:756–763.

Mital P, Hinton BT, Dufour JM. The blood-testis and blood-epididymis barriers are more than just their tight junctions. *Biol Reprod* 2011; **84**:851–858.

Morita K, Itoh M, Saitou M, Ando-Akatsuka Y, Furuse M, Yoneda K, Imamura S, Fujimoto K, Tsukita S. Subcellular distribution of tight junction-associated proteins (occludin, ZO-1, ZO-2) in rodent skin. *J Invest Dermatol* 1998;**110**:862–866.

Morita K, Sasaki H, Fujimoto K, Furuse M, Tsukita S. Claudin-11/OSP-based tight junctions of myelin sheaths in brain and Sertoli cells in testis. *J Cell Biol* 1999;**145**:579–588.

Morrow CM, Tyagi G, Simon L, Carnes K, Murphy KM, Cooke PS, Hofmann MC, Hess RA. Claudin 5 expression in mouse seminiferous epithelium is dependent upon the transcription factor ETS variant 5 and contributes to blood-testis barrier function. *Biol Reprod* 2009; **81**:871–879.

Mruk DD, Cheng CY. The mammalian blood-testis barrier: its biology and regulation. *Endocr Rev* 2015;**36**:564–591.

Nah WH, Lee JE, Park HJ, Park NC, Gye MC. Claudin-11 expression increased in spermatogenic defect in human testes. *Fertil Steril* 2011; **95**:385–388.

O'Donnell L, Stanton PG, Bartles JR, Robertson DM. Sertoli cell ectoplasmic specializations in the seminiferous epithelium of the testosterone-suppressed adult rat. *Biol Reprod* 2000;**63**:99–108.

O'Shaughnessy PJ, Morris ID, Huhtaniemi I, Baker PJ, Abel MH. Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: data from mutant and genetically modified mice. *Mol Cell Endocrinol* 2009;**306**:2–8.

Pelletier RM. A novel perspective: the occluding zonule encircles the apex of the Sertoli cell as observed in birds. *Am J Anat* 1990;**188**:87–108.

Pelletier RM. The blood-testis barrier: the junctional permeability, the proteins and the lipids. *Prog Histochem Cytochem* 2011;**46**:49–127.

Porter KL, Shetty G, Meistrich ML. Testicular edema is associated with spermatogonial arrest in irradiated rats. *Endocrinology* 2006;**147**:1297–1305.

Russell LD, Peterson RN. Sertoli cell junctions: morphological and functional correlates. *Int Rev Cytol* 1985;**94**:177–211.

Saitou M, Ando-Akatsuka Y, Itoh M, Furuse M, Inazawa J, Fujimoto K, Tsukita S. Mammalian occludin in epithelial cells: its expression and subcellular distribution. *Eur J Cell Biol* 1997;**73**:222–231.

Setchell BP. Blood-testis barrier, junctional and transport proteins and spermatogenesis. In: Cheng CY (ed). *Molecular Mechanisms in Spermatogenesis*. New York, USA: Landes Bioscience, Austin, TX/ Springer Science, 2008, 212–233.

Silva CA, Cocuzza M, Carvalho JF, Bonfá E. Diagnosis and classification of autoimmune orchitis. *Autoimm Rev.* 2014;**13**:431–434.

Sluka P, O'Donnell L, Bartles JR, Stanton PG. FSH regulates the formation of adherens junctions and ectoplasmic specialisations between rat Sertoli cells *in vitro* and *in vivo*. *J Endocrinol* 2006;**189**:381–395.

Smith BE, Braun RE. Germ cell migration across Sertoli cell tight junctions. *Science* 2012;**338**:798–802.

Su L, Mruk DD, Lee WM, Cheng CY. Differential effects of testosterone and TGF-β3 on endocytic vesicle-mediated protein trafficking events at the blood-testis barrier. *Exp Cell Res* 2010;**316**:2945–2960.

Su L, Mruk DD, Cheng CY. Regulation of the blood-testis barrier by coxsackievirus and adenovirus receptor. *Am J Physiol Cell Physiol* 2012; **303**:C843–C853.

Tarulli GA, Stanton PG, Lerchl A, Meachem SJ. Adult Sertoli cells are not terminally differentiated in the Djungarian hamster: effect of FSH on proliferation and junction protein organization. *Biol Reprod* 2006; **74**:798–806.

Tarulli GA, Meachem SJ, Schlatt S, Stanton PG. Regulation of testicular tight junctions by gonadotrophins in the adult Djungarian hamster *in vivo*. *Reproduction* 2008;**135**:867–877.

Wang C, Cui YG, Jia Y, Sinha Hikim A, Lue YH, Tong JS, Qian LX, Sha JH, Zhou ZM, Hull L *et al.* Transient scrotal hyperthermia and levonorgestrel enhance testosterone-induced spermatogenesis suppression in men through increased germ cell apoptosis. *J Clin Endocrinol Metab* 2007; **92**:3292–3304.

Weider K, Bergmann M, Brehm R. Connexin 43: its regulatory role in testicular junction dynamics and spermatogenesis. *Histol Histopathol* 2011;**26**:1343–1352.

Wong CH, Xia W, Lee NP, Mruk DD, Lee WM, Cheng CY. Regulation of ectoplasmic specialization dynamics in the seminiferous epithelium by focal adhesion-associated proteins in testosterone-suppressed rat testes. *Endocrinology* 2005;**146**:1192–1204.

Wu X, Peppi M, Vengalil MJ, Maheras KJ, Southwood CM, Bradley M, Gow A. Transgene-mediated rescue of spermatogenesis in *Cldn11*-null mice. *Biol Reprod* 2012;**86**:1–11.

Yan HHN, Mruk DD, Lee WM, Cheng CY. Blood-testis barrier dynamics are regulated by testosterone and cytokines *via* their differential effects on the kinetics of protein endocytosis and recycling in Sertoli cells. *FASEB J* 2008;**22**:1945–1959.

Zhao S, Zhu W, Xue S, Han D. Testicular defense systems: immune privilege and innate immunity. *Cell Mol Immunol* 2014;**11**:428–437.

Zhengwei Y, Wreford NG, Royce P, de Kretser DM, McLachlan RI. Stereological evaluation of human spermatogenesis after suppression by testosterone treatment: heterogeneous pattern of spermatogenic impairment. *J Clin Endocrinol Metab* 1998;**83**:1284–1291.