Dynamic GnRH and hCG testing: establishment of new diagnostic reference levels

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Abstract

Objective: Gonadotropin-releasing hormone (GnRH) and human chorionic gonadotropin (hCG) stimulation tests may be used to evaluate the pituitary and testicular capacity. Our aim was to evaluate changes in follicular-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone after GnRH and hCG stimulation in healthy men and assess the impact of six single nucleotide polymorphisms on the responses.

Design: GnRH and hCG stimulation tests were performed on 77 healthy men, 18–40 years (reference group) at a specialized andrology referral center at a university hospital. The potential influence of the tests was illustrated by results from 45 patients suspected of disordered hypothalamic–pituitary–gonadal axis.

Methods: Baseline, stimulated, relative and absolute changes in serum FSH and LH were determined by ultrasensitive TRIFMA, and testosterone was determined by LC–MS/MS.

Results: For the reference group, LH and FSH increased almost 400% and 40% during GnRH testing, stimulated levels varied from 4.4 to 58.8 U/L and 0.2 to 11.8 U/L and FSH decreased in nine men. Testosterone increased approximately 110% (range: 18.7–67.6 nmol/L) during hCG testing. None of the polymorphisms had any major impact on the test results. Results from GnRH and hCG tests in patients compared with the reference group showed that the stimulated level and absolute increase in LH showed superior identification of patients compared with the relative increase, and the absolute change in testosterone was superior in identifying men with Leydig cell insufficiency, compared with the relative increase.

Conclusions: We provide novel reference ranges for GnRH and hCG test in healthy men, which allows future diagnostic evaluation of hypothalamic–pituitary–gonadal disorders in men.

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Introduction

The hypothalamic–pituitary–gonadal (HPG) axis regulates the development and maintenance of the male reproductive system. Secretion of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary gland is mainly stimulated by the hypothalamic gonadotropin-releasing

hormone (GnRH). Simplified, FSH and LH stimulate spermatogenesis and testosterone (T) production in the testicles respectively.

Decreased testicular function can be due to a primary testicular dysfunction or a dysfunction of the pituitary gland or hypothalamus. Dynamic testing of the HPG

axis may facilitate the diagnostic process of testosterone deficiency. The capacity of the pituitary gland for gonadotropin secretion can be assessed by the GnRH test, whereas the capacity of the Leydig cells in the testicles to produce T can be assessed by the human chorionic gonadotropin (hCG) test (1, 2, 3, 4, 5, 6, 7, 8).

The single nucleotide polymorphism (SNP) *FSHB* -211G>T (rs10835638) in the promoter of the gene coding for the FSH B-subunit leads to a reduced FSH production (9, 10, 11). In addition, both sensitivity and transcriptional activity of the FSH receptor (*FSHR*) have been linked to the polymorphisms *FSHR* 2039A>G(rs6166) and the promoter polymorphism *FSHR* -29G>A (rs1394205) and all three SNPs have been associated to male reproduction (11, 12, 13). Genetic variants in the LH receptor (*LHCGR*) might alter the receptor function, and rs2293275 has been associated with spermatogenic damage (14) and rs7371084 and rs4953617 have been associated with testicular germ cell cancer (15). To which degree these polymorphisms affect the GnRH and hCG test responses, have, to our knowledge, not been described. To provide updated reference levels for the GnRH and hCG tests and examine the possible confounding by genetic effect of six gonadotropin-related SNPs, we examined 77 healthy Danish men. The potential usefulness of these reference levels is illustrated by results from 45 patients suspected of disordered HPG axis who had previously been tested.

Subjects and methods

Healthy participants (reference group)

Eighty healthy Danish men participating in an ongoing study of testicular function (16) were invited to take part in the hormone tests within a period of three weeks from the primary study. Inclusion criteria were no medical history of chronic diseases, testicular surgery or trauma. During the primary study, the men completed a questionnaire regarding medical history and lifestyle factors, delivered a semen sample and underwent a physical examination (body weight and height, assessment of testicular volume and ultrasound scan of the testicles). All examinations, tests and laboratory assessments were performed at Dept. of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark between 2012 and 2014. All men received 500 DKK (~68 Euro) for their participation. Seventy-seven men completed the GnRH and hCG tests. However, five did not show up for the final blood sampling for the hCG tests, leaving results for 72 men on the hCG test.

The study has been approved by the 'Ethical board of the Capital region' (permit number H-2-2012-092).

Hormone testing

All men had a GnRH test performed directly followed by the initiation of an hCG test. All GnRH tests were started between 08:00 and 12:00h. Prior to the tests, a baseline blood sample was drawn, which counted as the baseline blood sample for both tests.

GnRH stimulation test

An intravenous injection of 100µg GnRH (Relefact) was given, and a blood sample was collected after 30 min.

Table 1 Basic description of 77 healthy men.

Characteristics	Values
Basic description, median (min; max)	
Age (years)	27.0 (18; 40)
Height (cm)	183 (165; 197)
Weight (kg)	77 (58; 113)
Body mass index (kg/m ²)	22.9 (17.3; 30.9)
Mean of testis size (mL) ^a	14.0 (7.7; 26.0)
Total sperm count (million)	221 (7; 827)
Alcohol intake (units/week) ^b	7.5 (0; 60)
Smoking, % (n)	
Daily	11.7 (9)
Occasionally	28.6 (22)
Proven fatherhood	53.2 (41)
Genotypes, % (n)	
rs10835638 (<i>FSHB</i> -211G>T)	
GG	68.8 (53)
GT	26.0 (20)
TT	5.2 (4)
rs1394205 (<i>FSHR</i> -29G>A)	
GG	55.8 (43)
GA	36.4 (28)
AA	7.8 (6)
rs6166 (<i>FSHR</i> 2039A>G)	
AA	23.4 (18)
AG	46.8 (36)
GG	29.9 (23)
rs2293275 (<i>LHCGR</i> S312N)	
CC	37.3 (28)
CT	46.7 (35)
TT	16.0 (12)
rs7371084 (<i>LHCGR</i>)	
TT	64.0 (48)
TC	36.0 (27)
CC	0 (0)
rs4953617 (<i>LHCGR</i>)	
TT	85.3 (64)
TC	14.7 (11)
CC	0 (0)

^aMeasured by ultrasound and calculated as mean value of left and right testicle; ^b1 unit=12g alcohol. Alcohol intake is the sum of intake of beer, wine and strong alcohol the recent week prior to participation in study.

hCG

An injection of 5000 IU hCG (Pregnyl) was given in the gluteal muscle after the final blood sample was taken for the GnRH test. The follow-up blood sample was taken 72 h later.

All men also had an adrenocorticotrophic hormone (ACTH) test performed simultaneously with the GnRH test for another study. Results of this test will not be described here.

Patient group

We retrospectively identified five patient groups with a total of 45 men from our out-patient clinic. They underwent a GnRH test and/or an hCG test performed the same way as the healthy participants, as part of their clinical work-up of suspected testosterone deficiency between 1996 and 2015. Fourteen patients had been unilaterally orchiectomized due to testicular germ cell cancer and had received irradiation against germ cell neoplasia *in situ* (GCNIS) in the remaining testis. Eleven were suspected of hypogonadotropic hypogonadism on various backgrounds; *pituitary abnormalities* (five tumors

and one empty sella), *normal pituitary morphology* (MRI validated) but absence of puberty (two with Kallmann syndrome and three with isolated hypogonadotropic hypogonadism (IHH)). Nine were suspected of *medically induced* testosterone deficiency due to treatment with methadone, morphine, gabapentin or baclofen. Eleven patients were *obese men* (BMI >30) with a low-normal baseline total testosterone where it was concluded from the clinical work-up that they did not have testosterone deficiency, and therefore, were not offered testosterone treatment.

Hormone and semen analysis

The blood samples obtained from the healthy participants were allowed to clot, thereafter centrifuged, and serum

Table 2 Serum hormone levels in the reference group of healthy men undergoing GnRH and hCG test.

		Median (2.5; 97.5 percentile)
GnRH test (n=77)		
FSH	Baseline (IU/L)	2.6 (0.7; 7.2)
	Stimulated (IU/L)	3.7 (0.5; 10.5)
	Absolute increase (IU/L)	0.9 (–1.4; 4.7)
	Relative increase (%)	40 (–52; 128)
LH	Baseline (IU/L)	3.6 (1.8; 8.4)
	Stimulated (IU/L)	17.8 (7.4; 54.2)
	Absolute increase (IU/L)	14.1 (3.3; 48.6)
	Relative increase (%)	392 (69; 1068)
hCG test (n=72)		
Testosterone	Baseline (nmol/L)	19.1 (9.0; 34.5)
	Stimulated (nmol/L)	40.9 (22.8; 67.5)
	Absolute increase (nmol/L)	20.7 (7.5; 46.4)
	Relative increase (%)	111 (35; 347)
Estradiol	Baseline (pmol/L)	64 (17; 121)
	Stimulated (pmol/L)	166 (102; 265)
	Absolute increase (pmol/L)	99 (17; 214)
	Relative increase (%)	150 (14; 760)
Free testosterone	Baseline (pmol/L)	426 (239; 712)
	Stimulated (pmol/L)	1079 (554; 1791)
	Absolute increase (pmol/L)	606 (203; 1444)
	Relative increase (%)	138 (39; 477)

Figure 1

(A, B, C and D) Hormone levels at baseline and after GnRH and hCG-test in the reference group of healthy men (n=77/72). Results stratified and colored according to baseline quartiles (Blue: 1st quartile, Green: 2nd quartile, Light brown: 3rd quartile, Purple: 4th quartile). (A) LH levels at baseline and 30 min. after GnRH stimulation. (B) FSH levels at baseline and 30 min. after GnRH stimulation. (C) Testosterone levels at baseline and 72 h after hCG stimulation. (D) Calculated free testosterone levels at baseline and 72 h after hCG stimulation.

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was frozen at -20°C until analysis. Hormone analyses were performed when all subjects had completed their participation. Patients had their hormone analysis done at the time of the visit in the clinic. Serum levels of FSH and LH were determined using a time-resolved immunofluorometric assay (TRIFMA) (Delfia, Wallac, Turku, Finland). Sex hormone-binding globulin (SHBG) was determined by time-resolved chemiluminescent immunoassay (Access, Beckman Coulter). Estradiol levels were determined using radioimmunoassay (Biotech-IGG, Pantex). Inter- and intra-assay coefficients of variation (CVs) for measurements of the hormones were FSH (3% and 2%), LH (2% and 3%), SHBG (5% and 4%) and estradiol (15% and 8%) respectively. Serum testosterone of the healthy volunteers was analyzed by a TurboFlow-LC-MS/MS method (17). The CVs of the low and high level quality control samples were 10%

and 5.7% respectively in the detection range of 0.071–36 nmol/L. Limit of quantification (LOQ) was 0.10 nmol/L. Serum testosterone of the patients had been analyzed by radioimmunoassay/RIA (Coat-a-Count, Siemens) with a detection limit of 0.23 nmol/L, intra-assay CV was 17% and an inter-assay CV was 13%. For T, the conversion factor to LC-MS/MS from RIA was 1.24 for T (RIA) >5 nmol/L and 1.91 for T (RIA) <5 nmol/L. Patients T-values were corrected according to these factors. Free testosterone was calculated (cFT) according to Vermeulen *et al.* (18) assuming a fixed albumin value of 43.8 g/L.

All participants produced semen samples by masturbation, and the samples were assessed as previously described (16). Semen volume was assessed by weighing, assuming a density of 1 g/mL. For the assessment of the sperm concentration, the samples were diluted in a

Table 3 FSH and LH levels before and after GnRH stimulation stratified according to the 6 different polymorphisms in the reference group of healthy men.

		Median (min; max)			P-value (ANOVA)		
		GG (n=53)	GT (n=20)	TT (n=4)	Additive	Dominant	Recessive
rs10835638 (FSHB -211G > T)	FSH						
	Baseline (IU/L)	3.0 (0.4; 7.9)	2.3 (0.7; 5.4)	2.9 (1.1; 3.7)	0.228	0.154	0.863
	Stimulated (IU/L)	4.0 (0.2; 10.4)	2.8 (1.1; 11.8)	3.4 (1.4; 5.8)	0.491	0.447	0.835
	Absolute increase (IU/L)	1.0 (–1.6; 4.6)	0.6 (0.1; 6.6)	0.9 (–0.5; 2.07)	0.918	0.751	0.694
	Relative increase (%)	45 (–54; 109)	36 (3; 152)	44 (–14; 57)	0.625	0.452	0.730
rs1394205 (FSHR -29G > A)	FSH						
	Baseline (IU/L)	2.6 (0.4; 7.9)	3.3 (1.1; 7.2)	2.2 (0.9; 6.6)	0.454	0.213	0.604
	Stimulated (IU/L)	3.3 (0.2; 7.4)	4.4 (0.6; 11.8)	3.2 (1.5; 10.1)	0.164	0.066	0.933
	Absolute increase (IU/L)	0.9 (–1.6; 2.7)	1.4 (–0.6; 6.6)	1.0 (0.1; 3.5)	0.027	0.010	0.626
	Relative increase (%)	39 (–54; 109)	46 (–52; 152)	56 (10; 80)	0.077	0.052	0.055
rs6166 (FSHR 2039A > G)	FSH						
	Baseline (IU/L)	2.2 (0.4; 6.6)	3.0 (0.9; 7.9)	2.6 (0.9; 6.0)	0.309	0.124	0.842
	Stimulated (IU/L)	3.2 (0.2; 10.1)	4.0 (0.6; 11.8)	4.0 (1.2; 9.9)	0.238	0.200	0.493
	Absolute increase (IU/L)	0.8 (–1.2; 3.5)	1.0 (–1.5; 6.6)	1.0 (–0.5; 4.6)	0.450	0.525	0.542
	Relative increase (%)	46 (–54; 93)	37 (–52; 152)	54 (–14; 109)	0.569	0.784	0.373
rs2293275 (S312N)	LH						
	Baseline (IU/L)	3.9 (2.1; 8.9)	3.3 (1.9; 8.4)	3.6 (1.6; 5.5)	0.455	0.534	0.540
	Stimulated (IU/L)	18.8 (8.2; 54.0)	17.8 (7.6; 58.8)	14.8 (4.4; 31.7)	0.276	0.498	0.249
	Absolute increase (IU/L)	15.9 (3.8; 48.5)	14.1 (3.4; 50.4)	11.0 (1.8; 26.9)	0.394	0.561	0.398
	Relative increase (%)	420 (60; 1122)	375 (70; 1065)	368 (71; 869)	0.392	0.414	0.569
rs7371084	LH						
	Baseline (IU/L)	3.8 (1.6; 7.3)	3.2 (1.9; 8.9)			0.614	
	Stimulated (IU/L)	17.8 (4.4; 43.7)	18.2 (8.2; 58.9)			0.412	
	Absolute increase (IU/L)	13.9 (1.8; 36.7)	15.3 (3.4; 50.4)			0.358	
	Relative increase (%)	364 (71; 1065)	501 (60; 1122)			0.082	
rs4953617	LH						
	Baseline (IU/L)	3.4 (1.6; 8.9)	4.2 (2.3; 7.1)			0.458	
	Stimulated (IU/L)	17.7 (4.4; 58.8)	24.7 (9.2; 44.0)			0.337	
	Absolute increase (IU/L)	13.9 (1.8; 50.4)	19.5 (5.1; 38.1)			0.297	
	Relative increase (%)	400 (60; 1122)	392 (122; 869)			0.888	

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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). Total sperm count was calculated as semen volume × sperm concentration.

Genotyping

Genotyping was performed as described before (19). In short, EDTA-preserved peripheral blood was used for isolation of genomic DNA using the Promega Maxwell 16 DNA purification kits (Promega Biotech AB) and quantified on a NanoDrop ND-1000 spectrophotometer (Saveen Werner, Limhamn, Sweden). KASP SNP genotyping assays (LGC Genomics, Hoddesdon, UK) were used to determine the genotypes by competitive PCR. KASP genotyping assays were designed by LGC Genomics toward the

following sequences: *FSHB* -211G>T (rs10835638), TATCAAATTTAATT[G/T]TACAAAATCATCAT; *FSHR* -29G>A (rs1394205), TCTCTGCAAATGCAG[A/G]AAGAAA TCAGGTGG; *FSHR*2039A>G (rs6166), ATGTAAGTGGAA CCA[C/T]TGGTGACTCTGGGA. Genotyping assays for the three *LHCGR* variants were designed by LGC Genomics toward the following sequences: rs2293275, ACAGTGTTTTTRTTR[Y]TCACTKYCCTTACTGT; rs7371084, ACACTGTGGC[Y]TAGRTYKGTAAAGTA; rs4953617, TCCT GGCCAAG[Y]AGATTCTGCCCC.

A master mix containing a low concentration of the ROX fluorophore was used for internal normalization and as negative controls DNA was omitted in the reaction mixture and in all cases showed no amplification. All assays separated samples into clear genotype clusters.

All 77 healthy men were genotyped for the *FSHB* and the two *FSHR* variants but only 75 men were genotyped for the *LHCGR* variants.

Table 4 Testosterone and calculated free testosterone levels before and after hCG stimulation stratified according to the 3 *LHCGR* polymorphisms in the reference group of healthy men.

					P-value (ANOVA)		
		Median (min; max)			Additive	Dominant	Recessive
rs2293275 (S312N)		CC (n=27)	CT (n=32)	TT (n=11)			
Testosterone	Baseline (nmol/L)	20.3 (7.1; 33.6)	18.6 (11.2; 30.3)	15.5 (9.4; 38.9)	0.488	0.458	0.722
	Stimulated (nmol/L)	42.7 (29.1; 67.5)	40.0 (18.7; 58.2)	39.0 (23.7; 67.6)	0.691	0.085	0.280
	Absolute increase (nmol/L)	20.6 (7.8; 48.2)	20.2 (6.2; 35.9)	21.1 (14.3; 39.3)	0.948	0.423	0.365
Free testosterone	Relative increase (%)	102 (37; 412)	107 (28; 297)	130 (74; 253)	0.657	0.798	0.187
	Baseline (pmol/L)	485 (171; 774)	416 (260; 663)	395 (258; 609)	0.338	0.435	0.445
	Stimulated (pmol/L)	1135 (701; 1907)	1018 (495; 1521)	1057 (705; 1444)	0.188	0.114	0.676
	Absolute increase (pmol/L)	636 (213; 1513)	586 (202; 1136)	639 (425; 1031)	0.961	0.691	0.531
	Relative increase (%)	133 (39; 596)	135 (38; 437)	152 (109; 397)	0.886	0.795	0.253
rs7371084		TT (n=46)	TC (n=24)	CC (n=0)			
Testosterone	Baseline (nmol/L)	18.2 (7.1; 38.9)	20.0 (9.4; 33.6)			0.488	
	Stimulated (nmol/L)	39.3 (18.7; 67.6)	42.8 (23.7; 61.5)			0.285	
	Absolute increase (nmol/L)	20.8 (6.2; 48.2)	20.3 (7.8; 39.0)			0.824	
Free testosterone	Relative increase (%)	112 (49; 412)	108 (28; 333)			0.711	
	Baseline (pmol/L)	415 (171; 699)	472 (302; 774)			0.385	
	Stimulated (pmol/L)	1026 (495; 1767)	1138 (701; 1907)			0.270	
	Absolute increase (pmol/L)	593 (201; 1513)	642 (213; 1351)			0.984	
	Relative increase (%)	141 (56; 596)	138 (38; 401)			0.851	
rs4953617		TT (n=61)	TC (n=9)	CC (n=0)			
Testosterone	Baseline (nmol/L)	18.9 (7.1; 38.9)	21.3 (12.7; 30.3)			0.416	
	Stimulated (nmol/L)	40.9 (18.7; 67.6)	50.0 (31.4; 58.1)			0.232	
	Absolute increase (nmol/L)	20.6 (6.2; 48.2)	21.0 (14.8; 39.3)			0.282	
Free testosterone	Relative increase (%)	111 (28; 412)	110 (62; 253)			0.666	
	Baseline (pmol/L)	423 (171; 774)	485 (260; 626)			0.986	
	Stimulated (pmol/L)	1027 (495; 1907)	1140 (872; 1457)			0.365	
	Absolute increase (pmol/L)	602 (201;1513)	731 (449; 1031)			0.363	
	Relative increase (%)	139 (38;596)	137 (83; 397)			0.376	

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Statistical analysis

For descriptive statistics, medians and 2.5–97.5th percentiles or frequencies were calculated for the healthy men used as reference group. Correlations between serum FSH and LH were tested by Spearman's rank-order correlation. Responses to the hormone stimulations were calculated as absolute increase (stimulated-baseline) or relative increase ((stimulated-baseline)/baseline). Paired samples *t*-test was used to compare baseline and stimulated hormonal values. Multivariate linear regression analysis was used to detect the differences in the hormone levels between the different genotype groups (tested as additive, dominant and recessive models). Hormone values were transformed by natural logarithm to obtain variance stability and normalize data in the analysis. Covariates

evaluated in the statistical model included factors possibly associated with levels of reproductive hormones (blood sampling hour, age, smoking status, BMI and alcohol consumption) and were included if significant. $P < 0.05$ was considered statistically significant. The 2.5th percentiles of the stimulated levels and increases (absolute and relative) from the healthy reference group were used as cut-off values when comparing with the results from the patients' hormone stimulation tests. SPSS, version 22 for Windows was used for all statistical analyses.

Results

Table 1 describes the group of healthy men, including genotypes and distribution of alleles. Three men had

Table 5 Testosterone and calculated free testosterone levels before and after hCG stimulation stratified according to the 3 FSH related polymorphisms, in the reference group of healthy men.

		Median (min; max)			P-value (ANOVA)		
		GG (n=50)	GT (n=18)	TT (n=4)	Additive	Dominant	Recessive
<i>rs10835638 (FSHB -211G > T)</i>	Testosterone						
	Baseline (nmol/L)	19.1 (7.1; 33.6)	19.4 (12.7; 38.9)	16.3 (12.5; 20.2)	0.949	0.615	0.244
	Stimulated (nmol/L)	41.3 (23.7; 67.5)	40.7 (25.4; 67.6)	34.3 (18.7; 52.2)	0.162	0.499	0.026
	Absolute increase (nmol/L)	20.6 (7.8; 48.2)	20.7 (9.2; 39.3)	18.0 (6.2; 31.8)	0.308	0.611	0.113
	Relative increase (%)	113 (28; 412)	103 (49; 253)	106 (49; 158)	0.386	0.410	0.578
	Free testosterone						
<i>rs1394205 (FSHR -29G > A)</i>	Baseline (pmol/L)	432 (171; 774)	430 (258; 609)	369 (294; 423)	0.405	0.743	0.143
	Stimulated (pmol/L)	1097 (701; 1907)	992 (651; 1445)	852 (495; 1189)	0.023	0.099	0.013
	Absolute increase (pmol/L)	620 (212; 1513)	582 (307; 1031)	482 (201; 766)	0.097	0.283	0.037
	Relative increase (%)	142 (38; 596)	138 (63; 397)	125 (56; 203)	0.267	0.363	0.316
	Testosterone						
	Baseline (nmol/L)	18.3 (9.4; 38.9)	19.8 (7.1; 33.5)	22.5 (15.5; 31.0)	0.404	0.900	0.077
<i>rs6166 (FSHR 2039A > G)</i>	Stimulated (nmol/L)	40.3 (18.7; 67.6)	39.0 (24.8; 64.0)	46.5 (40.9; 58.2)	0.260	0.573	0.105
	Absolute increase (nmol/L)	20.0 (6.2; 48.2)	23.0 (7.8; 35.9)	22.9 (16.7; 39.3)	0.360	0.493	0.364
	Relative increase (%)	110 (28; 412)	122 (37; 322)	106 (62; 253)	0.899	0.602	0.519
	Free testosterone						
	Baseline (pmol/L)	423 (254; 773)	429 (171; 699)	467 (260; 666)	0.652	0.278	0.366
	Stimulated (pmol/L)	1046 (567; 1907)	1121 (495; 1523)	1183 (938; 1319)	0.915	0.819	0.509
<i>rs1394205 (FSHR -29G > A)</i>	Absolute increase (pmol/L)	602 (203; 1513)	636 (201; 1136)	620 (485; 1031)	0.699	0.813	0.631
	Relative increase (%)	137 (38; 596)	152 (39; 437)	146 (80; 397)	0.660	0.456	0.755
	Testosterone						
	Baseline (nmol/L)	15.7 (7.1; 33.6)	19.8 (11.2; 38.9)	18.6 (11.7; 33.5)	0.334	0.088	0.989
	Stimulated (nmol/L)	41.3 (23.7; 67.5)	40.6 (30.2; 67.6)	40.7 (18.7; 64.0)	0.064	0.146	0.115
	Absolute increase (nmol/L)	23.0 (14.3; 48.2)	20.4 (11.3; 34.7)	20.4 (6.2; 39.0)	0.021	0.053	0.065
<i>rs1394205 (FSHR -29G > A)</i>	Relative increase (%)	139 (53; 412)	112 (49; 214)	105 (28; 333)	0.007	0.003	0.111
	Free testosterone						
	Baseline (pmol/L)	382 (171; 774)	433 (280; 666)	391 (284; 644)	0.450	0.138	0.884
	Stimulated (pmol/L)	1160 (764; 1906)	1055 (701; 1521)	1075 (495; 1688)	0.039	0.098	0.084
	Absolute increase (pmol/L)	625 (288; 1513)	606 (336; 1027)	614 (201; 1351)	0.015	0.043	0.052
	Relative increase (%)	157 (50; 596)	135 (63; 273)	135 (38; 401)	0.007	0.006	0.090

used medicine (antihistamine; $n=2$, dermally applied glucocorticoid; $n=1$) at some point during the last 3 months prior to participation. Use of medicine was not significantly associated with any of the hormone levels (data not shown). Among the smokers, 14 men used cannabis or other recreational drugs; for all less than once a month and the use was not associated to any hormone levels (data not shown). Thus, adjusting for men who used the drugs or analyzing data excluding these men did not change the results.

Of the 77 men, 41 men were proven fathers and 36 had unproven fertility status. Total sperm count ranged from 7 to 827 million, with only two men with less than 39 million (7 and 38 million). None of the participants were homozygous minor allele (TT) carriers of *LHCGR* variants rs7371084 and rs4953617.

GnRH test

The results of the GnRH and hCG tests in the healthy men are shown in Table 2 and colored according to baseline quartiles in Fig. 1. Tables 3, 4 and 5 show the results stratified according to the polymorphisms. The stimulated gonadotropin levels showed a large variation (Fig. 1A and B), with stimulated FSH and LH values in the ranges 0.2–11.8 IU/L and 4.4–58.8 IU/L respectively. For FSH, the stimulated level decreased in nine men. The median (min–max) testis size of these men were 14 mL (12–22 mL), and total sperm counts 202 million (45–614 million). LH increased in all 77 men. Stimulated values correlated with baseline values for both FSH (r_s : 0.872, $P<0.01$) and LH (r_s : 0.292, $P<0.01$). The relative increase of the LH level (392%) was much higher than the increase of FSH (40%), but overall, the serum levels of the two gonadotropins correlated positively (baseline r_s : 0.415, stimulated r_s : 0.606, absolute increase r_s : 0.665 and relative increase r_s : 0.826, all $P<0.01$) (Fig. 2). Age of the men was negatively correlated with the stimulated level (r_s : -0.35 , $P=0.002$) and absolute increase in LH (r_s : -0.31 , $P=0.005$), but not with the change in any other hormones. Smoking status or BMI had no influence on the hormonal increase after GnRH stimulation ($P>0.05$).

FSH levels were associated to the *FSHR* -29G>A genotypes; A-allele carriers had higher absolute increase in FSH: median 0.9 IU/L in the GG group, 1.4 IU/L in the GA group and 1.0 IU/L in the AA group (additive model, $P=0.027$ and dominant model, $P=0.010$), but no difference in baseline levels. No other SNPs were associated with the LH or FSH response (Table 3). The decrease in

FSH levels after GnRH stimulation in the nine men was not associated to any genotype.

hCG test

Results of the hCG tests are shown in Table 2. Figure 1C and D illustrate the increases in T and cFT (both $P<0.01$) stratified into quartiles based on baseline hormone level. The absolute increase in stimulated T and cFT ranged from 18.7 to 67.6 nmol/L and 495 to 1907 pmol/L respectively. The median relative increase in T was 111% and in cFT 138%. Estradiol also increased ($P<0.01$), whereas SHBG did not change ($P=0.27$) (data not shown). Stimulated levels correlated with baseline values for T (r : 0.475, $P<0.01$) and cFT (r : 0.299, $P=0.011$). Stimulated levels of estradiol were positively correlated with the increase in T (r : 0.524, $P<0.01$) and cFT (r : 0.565, $P<0.01$). Age and BMI were negatively associated with baseline levels of T ($P=0.003$ and $P=0.004$) and cFT ($P=0.012$ and $P=0.001$), but not associated with stimulated levels ($P>0.05$). Smoking status was not associated to the T or cFT response ($P>0.05$).

None of the 3 *LHCGR* polymorphisms showed any significant effect on the stimulated T levels following

Figure 2

Correlation between the absolute change in FSH and LH 30 min after GnRH stimulation in the reference group of 77 healthy men.

an hCG test (Table 4). The *FSHB* -211G>T and the *FSHR* 2039A>G genotypes were associated to the testosterone response after hCG stimulation (Table 5). The T-allele (*FSHB* -211G>T) carriers had lower stimulated testosterone as well as lower stimulated and absolute cFT increase ($P<0.05$). For the *FSHR* 2039A>G genotypes, the G-allele carriers had lower absolute and relative testosterone as well as cFT increase ($P<0.05$).

Patient groups

Figure 3A and B show the LH and T levels before and after GnRH and hCG stimulation in the healthy men and the 45 patients (Table 6: medians and range). The 2.5th percentiles from the healthy reference group (Table 2) were used as cut-off, and we classified the patients' responses to the two tests on whether or not they reached the 2.5th

percentile of the LH levels (stimulated: 7.4 IU/L, absolute increase: 3.3 IU/L and relative increase: 69%) and T levels (stimulated: 23 nmol/L, absolute increase: 7.5 nmol/L and relative increase: 35%).

GnRH test

All patients, except one with IHH, had a relative LH increase above the 2.5th percentile of the healthy participants. Evaluating the absolute increases showed that the two Kallmann patients and two patients with pituitary abnormalities did not reach the cut-off. However, assessing the stimulated level showed that four of the six patients with pituitary abnormalities and one in the group with suspicion of medically induced testosterone deficiency did not reach the cut-off level. All the obese patients and the GCNIS-irradiated patients

Figure 3

(A and B) LH and testosterone levels before and after GnRH/hCG stimulation in the reference group of healthy men ($n=77/72$), and in 5 groups of patients suspected of testosterone sufficiency. Patients with pituitary abnormalities ($n=6/4$), Patients with Kallman syndrome/IHH ($n=5/5$), Patients suspected of medically induced testosterone deficiency ($n=9/8$), Obese patients (BMI >30) ($n=11/8$), Patients who had been unilaterally orchiectomized due to testicular germ cell cancer and received irradiation against germ cell neoplasia *in situ* (GCNIS) in the remaining testis ($n=14$). Marked line indicates 2.5th percentile for the reference group of healthy men.

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Table 6 Hormon values at baseline and after hormone stimulation stratified according to 5 groups of patients suspected of testosterone sufficiency.

		Patient categories				
		1. Pituitary abnormalities	2. Kallmann syndrome/IHH	3. On medication	4. Obese	5. GCNIS irradiated
		n=6	n=5	n=9	n=11	n=14
GnRH test	FSH					
	Baseline (IU/L)	2.4 (1.3; 4.9)	0.8 (0.1; 1.2)	2.9 (1.5; 13.9)	3.8 (1.2; 24.4)	40.6 (23.9; 59.9)
	Stimulated (IU/L)	3.3 (1.6; 7.2)	2.0 (0.4; 2.4)	4.0 (2.0; 16.3)	6.8 (1.8; 36.1)	61.1 (28.9; 92.9)
	Absolute increase (IU/L)	1.0 (0.4; 2.4)	0.9 (−0.1; 1.4)	1.5 (0.3; 3.6)	2.2 (0.6; 11.7)	19.1 (−2.0; 45.7)
	Relative increase (%)	43 (28; 57)	101 (−9; 465)	41 (7; 143)	48 (33; 122)	43 (−4; 100)
LH	Baseline (IU/L)	1.4 (0.8; 2.52)	0.5 (0.0; 0.8)	2.0 (0.4; 6.0)	2.8 (1.6; 7.5)	14.9 (7.0; 32.0)
	Stimulated (IU/L)	6.5 (2.8; 12.6)	5.4 (0.1; 13.7)	11.0 (6.4; 23.4)	16.9 (9.5; 50.5)	71.2 (37.5; 123.0)
	Absolute increase (IU/L)	5.3 (2.0; 11.1)	4.7 (0.0; 12.9)	9.0 (5.0; 21.1)	14.8 (7.9; 43.1)	60.4 (23.5; 108.6)
	Relative increase (%)	374 (164; 757)	1097 (20; 1679)	576 (161; 1726)	496 (287; 950)	345 (96; 904)
		n=4	n=5	n=8	n=8	n=14
hCG test	Testosterone					
	Baseline (nmol/L)	5.3 (0.4; 6.2)	2.6 (1.2; 4.4)	7.8 (0.9; 10.4)	7.4 (6.8; 11.5)	8.6 (5.8; 15.8)
	Stimulated (nmol/L)	22.9 (17.9; 27.5)	6.4 (2.5; 13.5)	21.9 (18.3; 32.2)	17.9 (12.7; 53.7)	12.9 (6.5; 21.7)
	Absolute increase (nmol/L)	19.6 (13.1; 21.7)	2.1 (1.0; 12.4)	12.8 (9.8; 25.9)	10.7 (5.6; 42.2)	3.6 (0.7; 8.9)
	Relative increase (%)	523 (374; 6855)	128 (49; 1687)	189 (109; 4596)	122 (78; 366)	30 (11; 122)

Values are medians (min;max). Group 1: patients with pituitary abnormalities, group 2: patients with kallman syndrome/IHH, group 3: patients suspected of medically induced testosterone deficiency, group 4: obese patients (BMI >30), group 5: patients who had been unilaterally orchiectomized due to testicular germ cell cancer and received irradiation against germ cell neoplasia *in situ* (GCNIS) in the remaining testis.

had an LH response higher than the cut-off of both the stimulated and the absolute increase.

hCG test

Of the 39 patients who had hCG tests performed, only 8 men had a stimulated T level above the 2.5th percentile cut-off. For the GCNIS-irradiated patients, all had stimulated levels and absolute increases lower than the cut-off levels in accordance with their clinical symptoms (except one), whereas the relative increase in T levels only detected eight of these men as having an insufficient response. For three of the men with normal pituitary morphology (one with Kallmann syndrome and two with IHH) and four out of the eight obese men, the absolute increase in T was below the cut-off. All men suspected of medically induced testosterone deficiency and those with pituitary abnormalities had an absolute T increase higher than the cut-off.

Discussion

In this study, we examined the responses to GnRH and hCG stimulation in healthy Danish men and established normal reference ranges. For both tests, we detected large variations in the stimulated hormone levels, but polymorphisms in genes involved in gonadotropin

signaling (*FSHB* -211G>T, *FSHR* -29G>A and *FSHR* 2039A>G and LHCGR (rs2293275, rs7371084 and rs4953617)) did not seem to contribute significantly to this variation.

When comparing the results obtained from the reference group with those of 45 patients suspected of disorders of the HPG axes, it became clear that evaluation of the GnRH test cannot rely on assessing the relative increase in LH as almost all the patients with pituitary failure had a relative increase larger than the 2.5th percentile of the reference group. Using the absolute increase alone was not always sufficient either. Thus, we recommend that a normal GnRH response should rely on a stimulated LH level ≥ 7.4 IU/L combined with an absolute increase of ≥ 3.3 IU/L. Four of the six patients with pituitary abnormalities had an impaired LH response according to the suggested criteria. The two patients with sufficient responses both had pituitary prolactinomas, and their lower baseline LH may be caused by reduced endogenous GnRH secretion rather than a failure of the gonadotrophic cells. Of the five patients with normal pituitary morphology, the two with Kallmann's syndrome responded normally to the GnRH test, and therefore, most likely have a hypothalamic insufficiency rather than a pituitary. In the group with suspicion of medically induced testosterone deficiency, all except one had normal GnRH test responses, in accordance with the inhibitory effect of the drugs primarily on the hypothalamic function (20).

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Evaluating the hCG tests of the patients, only 8 patients actually exceeded the 2.5th percentile of the stimulated T levels of the healthy participants. Although the relative increase in T level could be used to identify men with severe testosterone deficiency, a relative increase can be difficult to use in a clinical setting as very low baseline values can show a markedly relative increase without reaching normal absolute increase or stimulated level. We suggest that a normal adult response to an hCG test should be an absolute increase in $T \geq 7.5$ nmol/L. This also implies that men with secondary testosterone deficiency can increase in T, but not as much as healthy men, and still be considered to have a normal Leydig cell capacity.

The group of obese men may be challenging from a diagnostic point of view. In our study, they all had normal LH responses to GnRH tests indicating that the low baseline LH level detected in this group is not due to a pituitary insufficiency. Among the obese men, four had an abnormal absolute T change after the hCG stimulation, indicating a reduced Leydig cell capacity, which is in accordance with other studies (21, 22). However, this reduced capacity does not exclude that their baseline T level is sufficient, and in our opinion, it is evident that the clinical decision on whether they need testosterone substitution should not only be based on total T but also on free T and symptoms associated with testosterone deficiency. However, if men suspected of testosterone deficiency have large testicles, good semen quality and high serum level of inhibin B, this may additionally be arguments against the presence of testosterone deficiency (23). We found a negative association between BMI and baseline total and free T, but found no association between the increase in T and BMI in the group of healthy men, probably due to the narrow range in BMI.

Our main objective for this study was to describe the hormone changes for GnRH and hCG test in healthy men and provide novel reference ranges. We included the 45 patients suspected of testosterone deficiency to illustrate the potential use of the cut-off values, not to validate them. It would have been preferable to be able to calculate the diagnostic accuracy for our suggested cut-off values, but our patient group was too small and heterogeneous. In the clinical work-up of patients, their hormone levels are sometimes in a 'gray zone' in contrast to other patients who show clearly abnormal or normal levels. For example, the baseline LH is often very low in patients with hypogonadotropic hypogonadism, and the diagnosis might be obvious and without need for hormone stimulation test, whereas the testosterone

levels in obese men are low, and at the same time, they may have low LH. Thus, in such situations, the stimulation tests may help to distinguish between men having a hypogonadotropic hypogonadism and those having an adequately low testosterone level as an adaptation to low SHBG caused by the obesity.

We cannot provide any information of why nine men had a decrease in FSH instead of an increase after the GnRH stimulation. As mentioned in the results, the FSH levels correlated with the LH levels, and the decrease was not associated with their testis size, semen quality or genotypes. However, it is well known that the FSH response is much smaller than the LH response after GnRH stimulation (3, 8, 24).

Both tests revealed large variations in the stimulated hormone levels among the healthy men. We anticipated that especially the promoter polymorphism *FSHB* -211G>T could have been an important modulator of the response to GnRH stimulation due to its strong association to the FSH secretion because other studies have shown that minor allele carriers have lower FSH levels, inhibin B levels and smaller testis volumes (9, 10, 11). The T-allele carriers of our participants had a tendency for a lower baseline and a lower stimulated FSH level, however, within the range observed for non-carriers. This polymorphism has also been suggested to be adversely associated with androgen production (9, 11). In accordance, the T-allele carriers had slightly lower T and cFT levels after hCG stimulation. The promoter polymorphism *FSHR* -29G>A A-allele leads to a reduced *FSHR* gene expression and is associated to a higher baseline FSH (13, 24, 25). We also detected a slightly, but significantly, larger increase in FSH level upon GnRH stimulation in the A-allele carriers. For the *FSHR* 2039A>G polymorphism, which is believed to reduce the *FSHR* response to FSH (26), our detection of a lower T response and tendency toward higher levels of stimulated FSH in the G-allele carriers is compatible with previous publications (27, 28). Even though we detected significant associations between the FSH-related SNPs and the hormonal responses, the differences were small. None of the tested LHCGR polymorphisms modulated the hCG response. The *rs2293275* is located in exon 10 near a glycosylation site, which has been hypothesized to reduce the receptor activity (14, 27, 29). Although associations between the LHCGR polymorphism and impaired spermatogenesis and under-masculinization have been detected (14, 27, 30), it has also been shown that deletion of exon 10 only affects the sensitivity to LH and not hCG, which could be an explanation for

the lack of an association (31). For two of the LHCGR polymorphisms, none were homozygous carriers of the minor allele, and in the FSH-related genotype groups, some of the minor allele groups were small. Men carrying more than one minor allele might be more affected. Testing of a sufficiently large population could probably reveal significant differences in hormone responses between genotypes, and there may be other SNPs that alone or in combination could have a larger effect on the stimulation tests. The investigated SNPs are known to affect steady state levels of hormones, but the effect of genetic variants may be different when the system is stimulated. Based on our results, we recommend that interpretation of both tests can be done without accounting for the six investigated gonadotropin-related polymorphisms.

The strength of our setup was that all 77 participants were healthy without any diseases affecting the testicular function, the first such setup study found in literature. Though previous studies have published results on both GnRH and hCG tests, those studies have included administration of different doses of GnRH/hCG and often with only a few or without healthy controls (2, 5, 32, 33, 34, 35, 36). We used the LC-MS/MS method for testosterone analysis in this group of healthy men. LC-MS/MS-based tests have a higher specificity than immunoassays and are currently believed to be the golden standard in testosterone measurements.

Our study also has some limitations. The hormone levels of the patients in the retrospective part of this study were analyzed by immunoassays as LC-MS/MS was not yet available. Previous studies have shown a good agreement between the LC-MS/MS method and immunoassays, though in the low concentration range, the correlation was lower (17). However, we corrected for the difference between the two methods before comparisons. In the setup of our study, all men had an ACTH test performed simultaneously with the GnRH test for another study. In the routine clinical setting, these tests are often done simultaneously because suspicion of pituitary deficiency should lead to the validation of the other pituitary axes. The knowledge whether the tests influence each other's response is, sparse, but studies have shown that combined pituitary testing does not lead to differences in the hormone responses compared to separate tests (37, 38, 39). Results from rodents indicate that ACTH can stimulate T production in the neonatal Leydig cells through the ACTH receptor, but apparently without any effect post-pubertally (40).

Also, Leydig cells may have glucocorticoid receptors, and high levels of glucocorticoids have been shown to decrease testosterone production (41).

Even though the age range in our reference group was narrow, we detected a negative association of age on the LH response. This is in accordance with Harman *et al.* who showed that even though baseline gonadotropins increase slightly with age, there is a lower incremental response to GnRH test (42).

This is the first study to evaluate the dynamic changes in FSH, LH and T after GnRH and hCG stimulation respectively, in a large cohort of healthy men. Hereby, we provide new reference data, which allow future evaluation of men suspected of HPG disorders.

Declaration of interest

The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. None of the authors have any competing interests to declare.

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Author contribution statement

Substantial contributions to conception and design: A K B, N J, N L and A J. Data acquisition: A K B, L N, L P and K A. Data analysis: A K B. Data interpretation: All authors. Drafting the manuscript: A K B and N J. Revising manuscript critically for important intellectual content: All authors. Final approval of the manuscript: All authors.

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Clinical Study	A K Bang and others	Reference levels on GnRH and hCG tests	176:4	391
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