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Insulin-Like Factor 3 Serum Levels in 135 Normal Men and 85 Men with Testicular Disorders: Relationship to the Luteinizing Hormone-Testosterone Axis

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Insulin-like factor 3 (INSL3) serum levels were measured in 135 andrologically well-characterized normal men and 85 patients with testicular disorders to investigate how the hormone, which is a major secretory product of human Leydig cells, is related to testosterone (T), LH, and semen quality. INSL3 was measured by using a newly developed fluorescence immunoassay.

Median (2.5–97.5 percentiles) INSL3 serum levels were as follows: normal men (n = 135), 0.99 (0.55–1.73) ng/ml; infertile men (n = 23), 1.11 (0.60–2.07) ng/ml; anorchid men (n = 21), nondetectable (ND); patients with 47, XXY, Klinefelter syndrome (n = 21), 0.12 (ND–0.78) ng/ml; men with hypogonadotropic hypogonadism and T substitution (n = 11), ND; and men with hypogonadotropic hypogonadism and human chorionic gonadotropin (hCG) treatment (n = 5), 0.36 (0.13–0.73) ng/ml. Before testicular biopsy, two infertile men had blood samples drawn directly from vena spermatica. Here, the serum INSL3 levels were 15-fold higher than in serum from peripheral blood samples (13.84 and 14.00 ng/ml, respectively). In two unilaterally orchiectomized former testis cancer patients, who underwent hCG stimulation test, INSL3 serum levels were unchanged 72 and 96 h after hCG stimulation. In conclusion, we provide a normal range for INSL3 serum levels in adult men and show that the majority, if not all, circulating INSL3 derives from the testes. Furthermore, our data strongly indicate that INSL3 secretion is dependent on the differentiating effect of LH on Leydig cells but independent of the steroidogenic LH-mediated action. Thus, even though T and INSL3 are both dependent on LH, these two Leydig cell hormones are regulated differently. **(*J Clin Endocrinol Metab* 90: 3410–3418, 2005)**

INSULIN-LIKE FACTOR 3 (INSL3) is a novel member of the insulin-like hormone superfamily. In the male, the *INSL3* gene and its product are expressed in testicular Leydig cells in all mammalian species investigated, including humans (1–4).

INSL3 produced by prenatal Leydig cells is essential for the transabdominal part of testis descent via stimulation of outgrowth and differentiation of the gubernaculum, as shown in rodents (5, 6). Numerous human mutation analyses have sought to elucidate the role of INSL3 and its receptor, LGR8 (7–9), in human cryptorchidism (10). Despite this, clear evidence is still lacking for an involvement of INSL3 in human testicular descent (11, 12). INSL3 is also abundantly expressed in adult Leydig cells (4); however, the effect of INSL3 in adult life is not clear. A recent finding of LGR8 expression in germ cells and the suppression of apoptosis through binding of INSL3 to LGR8 suggest a paracrine role of INSL3 in germ cell survival (13). The *LGR8* gene is also expressed in human pituitary gland, thyroid, and uterus, among other tissues (14, 15). Even though the expression of LGR8 has not been investigated at the peptide level, the wide distribution of *LGR8* gene expression in combination with a high concentration of INSL3 in postpubertal male serum (15–17) strongly suggest additional, yet unrecognized, endocrine effects of INSL3.

To enable further investigations of INSL3 in humans, we have developed a time-resolved fluorescence immunoassay (TR-FIA) for determination of INSL3 in human serum. Using this assay, we have measured the level of serum INSL3 in 135 young men from the general Danish population to establish a reference interval for this hormone and to relate it to other reproductive hormones as well as to semen parameters. Moreover, serum samples from a number of men with different testis-related pathologies, including infertile men and patients with hypogonadotropic hypogonadism and 47, XXY, Klinefelter syndrome, have also been measured. Taken together, our data enable a thorough evaluation of the regulation of INSL3 in human Leydig cells.

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Abbreviations: CV, Coefficient(s) of variation; DL, detection limit; DTPA, diethylenetriaminepentaacetic acid dianhydride; hCG, human chorionic gonadotropin; hINSL3, human INSL3; HSA, human serum albumin; INSL3, insulin-like factor 3; MES, 2-morpholinoethansulfonic acid; ND, nondetectable; T, testosterone; TR-FIA, time-resolved fluorescence immunoassay.

Patients and Methods

Study populations

Normal men. Since 1996, every year, young men from the general Danish population have been voluntarily enrolled in studies on semen quality in connection with a compulsory medical examination for military service. In Denmark, all young men, except those suffering from chronic severe disease (<15%), are required to attend a compulsory medical

examination before they may be considered for military service. Therefore, such men may be considered representative of the general population of young men. In addition to semen samples, the participants had a blood sample taken for analysis of reproductive hormones. See (18) for further details. For the present study, serum samples from a random subgroup (chosen via a random draw of identification numbers in our SPSS database) of 135 of the participants enrolled from 1998–2002 have been analyzed for INSL3. Of the 135, only 98 (73%) had sperm concentration more than 20×10^6/ml, which is the lower reference concentration according to World Health Organization (WHO) (19). In accordance with these numbers, investigations of semen quality in young Danish men have shown that a little more than 20% have sperm concentration below the WHO reference concentration of 20×10^6/ml. Characteristics of all 135 men and the subgroup of 98 men are given in Table 1.

Patients. From our andrology clinic, the following patients were included in the study. Infertile men included 23 men referred to our clinic due to fertility problems and subsequently diagnosed with severe oligozoospermia (sperm concentration $< 5 \times 10^6$/ml) or azoospermia without indications of obstruction and with no history of testicular cancer. Three of these had a history of cryptorchidism. Characteristics of these infertile men are also given in Table 1. Anorchid men included 21 men who have been bilaterally orchiectomized due to testis cancer or torsion or have no testes due to testis aplasia. All these received testosterone (T) replacement therapy in the form of intramuscular injections every other week (Testoviron Depot, 135 mg). Klinefelter syndrome included 21 men diagnosed with 47, XXY, Klinefelter syndrome. Thirteen of these received T injections (Testoviron Depot, 135 mg every 2nd week), three received oral T replacement (T undecanoate, 80 + 40 mg every day), three were not in need of treatment, and two were included at the time of diagnosis, before start of treatment. Eight of 21 patients with 47, XXY had their semen quality examined, and they all had severe oligo- or azoospermia. Hypogonadotropic hypogonadism included 16 male patients with hypogonadotropic hypogonadism. Eleven of these received T treatment (Testoviron Depot, 135 mg every 2nd wk), and five received human chorionic gonadotropin (hCG) treatment. hCG stimulation included two former testis cancer patients, unilaterally orchiectomized, who went through an hCG stimulation test and had blood samples taken at the time of hCG injection as well as 72 and 96 h after stimulation. Characteristics of the male patient groups are given in Table 2. Vena spermatica included two patients who had blood samples taken from a peripheral vein and from vena spermatica before testicular biopsy. The one was infertile due to retrograde ejaculation after a total colectomy, and the other had severe oligozoospermia for unknown reasons. Both had normal testicular histology and complete spermatogenesis as evaluated by histological examination of testis biopsies. Characteristics of these two patients are given in Table 3.

For all included patients, blood samples were taken as a part of their treatment and were analyzed for INSL3 with the written consent of the patient as well as the authorization of the local ethical committee. For all included patients, analyses of T, LH, FSH, and inhibin B were available.

Development of an immunoassay for human INSL3 (hINSL3)

To determine INSL3 concentrations directly in human serum, we developed a semicompetitive TR-FIA, based on synthetic hINSL3 of high purity, an hINSL3-specific rat antiserum, an hINSL3 tracer labeled with chelated Eu^{3+} ions, and a secondary goat antirat γ-globulin antibody immobilized on the surface of microtiter wells.

Reagents and buffers used. Chemically synthesized mature hINSL3 consisting of A and B chain and controlled for the correctness of structure and for the purity of protein (20) was purchased from the laboratory of Erika Büllesbach and Chris Schwabe (Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC). Human serum albumin (HSA) was from Sigma-Aldrich (St. Louis, MO), as well as *N*-ethyl-*N*′-[3-dimethylaminopropyl]carbodiimide hydrochloride, 1-hydroxy-2,5-dioxo-3-pyrrolidinesulfonic acid monosodium salt hydrate, and diethylenetriaminepentaacetic acid dianhydride (DTPA). Assay buffer was 0.2 M Tris/HCl, 1.8% NaCl, 2 mM EDTA, 2% BSA, 1% crude egg white from fresh eggs, 0.01% bovine γ-globulin, 0.04% Tween 20, and 0.2% NaN_3 (pH 7.3) at 22 C. Tracer buffer was 0.1 M Tris/HCl, 0.1% NaN_3, 0.9% NaCl, 0.01% Tween 20, 0.1% BSA (stabilizer, Wallac, Turku, Finland), and 0.0005% bromophenol blue sodium salt (pH 7.5). Washing solution is 0.02% Tween 20 and 0.5% NaCl. Bovine INSL3 was recombinantly produced in the yeast *Pichia pastoris* and consisted of bovine A and B chains with the C chain being replaced by the tripeptide AAK (M. Wald, S. Hartung, and R. Ivell, unpublished data). Human insulin and human IGF-II were from Sigma-Aldrich, and porcine relaxin (>85% purity, resulting in a single Coomassie-stainable band in PAGE) was a gift from O. D. Sherwood (Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL).

Production of antiserum. Rats were immunized with pure synthetic hINSL3 in combination with hINSL3 molecules coupled to HSA. For the coupling, 1 mg HSA in 50 μl 0.5 M sodium phosphate (pH 7.4) was slowly added to 0.5 mg (83 nmol) synthetic hINSL3 in 200 μl water. The resulting protein solution was transferred to a microtube containing 838

TABLE 1. Characteristics of men from the general population and infertile men

	Men from the general population, median (2.5–97.5 percentiles)			Infertile men, median (2.5–97.5 percentiles)	P^a	
	All	Sperm conc. $>20 \times 10^6$/ml	Sperm conc. $<20 \times 10^6$/ml	Sperm conc. $<5 \times 10^6$/ml	a	b
n	135	98	37	23		
Age (yr)	19 (18–25)	19 (18–25)	19 (18–26)	30 (23–51)	**<0.001**	0.439
BMI (kg/m^2)	22.4 (17.9–31.0)	22.4 (18.0–31.0)	22.4 (16.4–32.1)	25.3 (19.4–36.3)	**0.005**	0.598
Sperm conc. ($\times 10^6$/ml)	42 (0.6–198.5)	58 (20.5–208.3)	12 (0–19)	0.7 (0–3.9)	**<0.001**	**<0.000**
Semen volume (ml)	3.0 (1.3–7.6)	2.8 (1.3–7.6)	3.4 (1.1–8.7)	3.9 (0.4–8.2)	0.097	**0.027**
Motile sperm (%)	68 (36–84)	69 (42–86)	62 (10–76)	26 (3–60)	**<0.001**	**0.004**
Normal morphology (%)	7 (1–19)[b]	8 (1–20)[b]	4 (0–16)	13 (3–29)[b]		**<0.000**
INSL3 (ng/ml)	0.99 (0.55–1.73)	1.00 (0.55–1.88)	0.94 (0.41–1.70)	1.11 (0.60–2.07)	0.086	0.436
Testosterone (nmol/liter)	23.8 (12.1–39.3)	23.9 (14.9–39.2)	23.0 (11.4–46.0)	14.7 (6.6–35.2)	**<0.001**	0.459
LH (IU/liter)	3.85 (1.72–8.57)	3.54 (1.73–7.67)	4.79 (1.61–10.4)	4.23 (1.38–11)	**0.040**	**0.001**
FSH (IU/liter)	3.32 (0.96–10.64)	3.05 (1.02–10.32)	3.94 (0.64–14.58)	10.1 (3.2–21.4)	**<0.001**	0.066
Inhibin B (pg/ml)	209 (79–395)	219 (80–395)	195 (44–484)	84 (ND–173)	**<0.001**	0.097

[a] Mann-Whitney *U* test performed between (a) the 98 men from the general population with sperm concentration (conc.) greater than 20×10^6/ml and the 23 infertile men and (b) between men from the general population with sperm conc. greater than and less than 20×10^6/ml. *P* values at or below 0.05 are indicated in *bold*.

[b] Morphology is evaluated by different criteria in men from the general population and in infertile men. Therefore, no statistics for the 98 men from the general population with sperm conc. greater than 20×10^6/ml and the 23 infertile men has been made for this variable. ND, Nondetectable.

TABLE 2. Characteristics of different patient groups, median (2.5–97.5 percentiles)

	Anorchism	Hypogonadotropic hypogonadism		Klinefelter Syndrome			
	T (injections)	T (injections)	hCG treatment	T (injections)	T (oral adm.)	No treatment	New diagnosis
n	21	11	5	13	3	3	2
Age (yr)	44 (18–72)	29 (19–57)	26.3 (22.8–45)	31 (22–44)	23 (20–26)	30 (21–32)	31.5 and 42.9
BMI (kg/m^2)	24.2 (19.9–27.2)	22.5 (21.6–24.0)	21.8 (19.7–24.6)	25.4 (18.7–30.4)	20.9 (17.6–27.2)	24 (20–25)	29.2 and 24.1
INSL3 (ng/ml)	ND (ND–0.06)[a]	ND (ND–0.06)[a]	0.36 (0.13–0.73)	0.06 (ND–0.62)[a]	0.70 (0.12–0.78)	0.86 (0.83–0.95)	0.07 and 0.31
T (nmol/liter)	17.6 (5.4–31.4)[a]	10.1 (ND–18.8)[a]	17.4 (12.6–38.9)	14.9 (4.4–37.5)[a]	8.0 (4.4–11.7)	15 (12.8–17.3)	ND and 9.3
LH (IU/liter)	2.95 (ND–26.7)[a]	ND (ND–0.3)[a]	ND (ND–0.1)	2.4 (ND–15.6)[a]	19.3 (15.5–19.4)	11 (9.2–12.0)	4.7 and 12.8
FSH (IU/liter)	18.0 (0.4–74.6)[a]	0.2 (ND–0.6)[a]	0.2 (0.1–2.9)	3.45 (0.24–33.5)[a]	39.9 (23.7–58.4)	29.8 (22.0–31.9)	16 and 34.9
Inh B (pg/ml)	ND[a]	ND (ND–44)[a]	82 (30–118)	ND[a]	ND	ND	ND

[a] Blood samples taken at different time-points after T injection. ND, Nondetectable; Inh B, inhibin B; adm., administration.

μg *N*-ethyl-*N*′-[3-dimethylaminopropyl]carbodiimide hydrochloride and 950 μg 1-hydroxy-2,5-dioxo-3-pyrrolidinesulfonic acid monosodium salt hydrate, and the mixture was stirred for 90 min at 20 C. The reaction was stopped by the addition of 640 μg imidazole in 20 μl water, followed by an incubation for 20 min at 20 C under stirring. After centrifugation at 10,000 rpm for 1 min, the pellet was saved, and the supernatant was subjected to gel permeation chromatography using a trisacryl GF05M column (1 × 8.5 cm, cutoff at molecular weight 3000; Pall, East Hills, NY) and 0.9% NaCl as eluent. Cross-linked and unmodified INSL3 passed the column with the void volume (detection at 280 nm). The protein peak fraction (1.4 ml) was collected manually and combined with the protein pellet from the above centrifugation step. After resuspension of the pellet, volume was adjusted to 2 ml with 0.9% NaCl, and the mixture was shock-frozen in 200-μl aliquots, each containing approximately 150 μg total protein.

Four male Wistar rats were injected sc with 600 μl of a fine emulsion of 1 vol complete Freund's adjuvant (Invitrogen, Carlsbad, CA) and 1 vol 0.76% NaCl containing 70 μg hINSL3-HSA conjugate and 23 μg free synthetic hINSL3, followed by four boost injections in monthly intervals with a similar emulsion made with incomplete Freund's adjuvant (Invitrogen). The rats were kept in the central animal breeding facility of the University Hospital Hamburg-Eppendorf, and injections and serum recovery were carried out by expert staff.

Tracer synthesis. Tracer was synthesized by coupling DTPA-chelated Eu^{3+} to the three available amino groups of the A and B chains of synthetic hINSL3. In detail, 25 μg freeze-dried hINSL3 (4.17 nmol) were dissolved in 27 μl 0.26 M $NaHCO_3$ and added to 225 μg (630 nmol) DTPA anhydride freshly dissolved in 10 μl *N*,*N*-dimethylformamide. After 2 min moderate stirring and 5 min incubation at 20 C without stirring, the reaction mixture was brought to pH 6.0 by slowly adding 20 μl 0.5 M 2-morpholinoethansulfonic acid (MES). Then, 1.26 μmol $EuCl_3$ (2-fold excess to DTPA) in 15 μl 40 mM MES (pH 6.0) was successively added in 3-μl portions, followed by moderate vortexing after each addition. After incubation for 30 min at 4 C under mild agitation, the reaction mixture was diluted with 20 μl elution buffer [50 mM Tris/HCl (pH 7.55), 0.9% NaCl, and 0.1% NaN_3] and 5 μl 1 M $NaHCO_3$, centrifuged (10,000 × *g*, 1 min), and purified by gel permeation chromatography without delay (column, Superdex Peptide, 10 × 300 mm, Amersham Biosciences Inc., Piscataway, NJ; flow, 1 ml/min with elution buffer as above; fraction size, 1 ml). The peak fraction of the Eu^{3+}-labeled hINSL3 was localized by counting 0.05 and 2.0 μl of each fraction after mixing with 200 μl enhancement solution (Wallac, see below). Fractions 10–13 containing the Eu^{3+}-labeled hINSL3 were pooled, brought to a final volume of 45 ml with tracer buffer containing and additional 0.5% BSA (stabilizer, Wallac), and lyophilized in 500-μl aliquots.

Immunoassay procedure. Incubations were performed in microtiter strips (NUNC GmbH & Co. KG, Wiesbaden, Germany) coated with 1 μg/well goat antirat IgG antibody (Sigma Chemie, Deisenhofen, Germany), being stored frozen at −20 C. Before use, the strips were equilibrated to room temperature and washed once with 300 μl/well washing solution. One hundred microliters serum sample (or 100 μl standard, see below) were mixed (5 min) with 100 μl rat anti-hINSL3 antiserum diluted 1:70,000 in assay buffer and incubated 18–20 h at 4 C in a dark and humid chamber to allow binding of hINSL3 to the rat antibody, which in turn binds to the goat antirat antibody attached to the well surface. Standards consisted of 100 μl blank serum (pooled serum samples from postmenopausal women, won by informed consent of probands at the University Hospital Hamburg-Eppendorf and University Hospital of Copenhagen) spiked with defined concentrations of synthetic hINSL3 (0.05–3.2 ng/ml). This first incubation was terminated by washing once with washing solution, thereby removing the serum matrix. Then, 200 μl ice-cold tracer buffer containing approximately 500,000 cps hINSL3-Eu^{3+} tracer was added, followed by an incubation for 90 min at 4 C in a dark and humid chamber. During this incubation, the tracer occupies the remaining free binding sites on the rat anti-hINSL3 antibodies. Finally, the wells were washed three times, filled with 200 μl enhancement solution (Wallac), and incubated for 1 h at room temperature on a plate shaker. This released the incorporated Eu^{3+} ions, which in turn formed new highly fluorescent stable complexes with compounds in the enhancement so-

TABLE 3. Characteristics of infertile men who had blood samples drawn from vena spermatica

Characteristics	Patient 1	Patient 2
Age (yr)	29	28
BMI (kg/m^2)	25	26
Sperm conc. (×10^6/ml)	5	0
Semen volume (ml)	5.2	0
Clinical information	None specific	Retrograde ejaculation
Testis size (orchidometry)	20/20	15/15
Testicular histology	Complete spermatogenesis	Complete spermatogenesis

	Blood sample location			
	Periphery	V. spermatica	Periphery	V. spermatica
INSL3 (ng/ml)	0.98	14.00	0.90	13.84
T (nmol/liter)	15.4	2195	29.9	1655
LH (IU/liter)	3.65	3.25	1.03	0.98
FSH (IU/liter)	5.75	5.56	1.01	0.95
Inhibin B (pg/ml)	161	401	206	1067

lution. Time-resolved fluorescence was measured in a Wallac Victor (2) 1420 multilabel counter (PerkinElmer Life and Analytical Sciences, Boston, MA) by pulsing samples 1000/sec with excitation light of 340 nm and detecting emitted light at 615 nm, with a delay of 400 μsec after each flash to minimize nonspecific background fluorescence. All samples were measured in duplicates.

Blood samples and additional hormone analyses

Blood samples were drawn from an antecubital vein and centrifuged after clotting. Serum was stored at −20 C until analysis. Storage time before analysis ranged from 0–6 yr. In normal men, T was determined using a time-resolved fluoroimmunoassay (DELFIA, Wallac) with a detection limit (DL) of 0.23 nmol/liter and intra- and interassay coefficients of variation (CV) less than 6%. In patients, T was measured using a solid-phase RIA (Coat-a-count; DPC, Los Angeles, CA) with a DL of 0.23 nmol/liter and intra- and interassay CV of 9.8 and 9.9%, respectively. LH and FSH were measured by time-resolved immunofluorometric assays (DELFIA) with DL of 0.05 and 0.06 IU/liter, respectively, and intra- and interassay CV less than 8% in the full range. Inhibin B was measured by use of a specific two-sided enzyme immunometric assay (Oxford Bio-Innovation Ltd., Oxford, UK). The sensitivity of this assay was 20 pg/ml, and the intra- and interassay CV were less than 12 and 17%, respectively. All samples were measured in duplicate.

Semen concentration and semen volume

The semen samples were obtained by masturbation and ejaculated into a clean collection tube in the privacy of a room adjacent to the laboratory. The semen samples were maintained at 37 C until analysis. The analysis of semen samples was performed according to WHO guidelines (21) but was further specified after a study of interlaboratory variation (22). Ejaculate volume was estimated by weighing the collection tube. Phase-contrast microscopy (positive phase-contrast optics) was used for the examination of fresh semen. For assessment of the sperm concentration, the samples were diluted in a solution of 0.6 mol/liter $NaHCO_3$ and 0.4% (vol/vol) formaldehyde in distilled water. The sperm concentration was assessed using a hemocytometer (Bürker-Türk, Friedrichsdorf, Germany). Only sperm with tails were counted. For the assessment of sperm motility, 10 μl well-mixed semen was placed on a clean glass slide (which had been kept at 37 C) and covered with a 22- × 22-mm coverslip. The preparation was placed on the heating stage of a microscope (37 C) and immediately examined at ×400 magnification. The sperm were classified as either motile (WHO motility classes A + B + C) or immotile (WHO motility class D) to report the percentage of motile sperm. The motility assessment was performed in duplicate, and the average value was calculated for both samples. Finally, smears for morphology assessment were made. After fixation and Papanicolaou staining, all smears from the 135 men from the general population were sent to Turku, Finland, for a centralized morphology assessment according to strict criteria (23). Sperm morphology of the infertile patients was assessed according to WHO (24).

Biopsy, catheterization, and blood sampling

Bilateral testicular biopsies were taken as an outpatient procedure. The preparation and examination of the biopsies was as described previously (25). The tissues were stained with hematoxylin and eosin, and histology was evaluated by light microscopy. Catheterization of vena spermatica was performed before the testicular biopsy. The catheterization was done under fluoroscopic guidance, and the catheter was introduced as far as possible into the left spermatic vein. The venous anatomy was evaluated by angiography to rule out dilution of spermatic venous blood from various tributes. A peripheral serum sample was taken initially from the right femoral vein, which was the position where the catheter was introduced.

Statistics

Descriptive statistics are given as median values and 2.5 and 97.5 percentiles. Spearman's correlation coefficients were used to evaluate the correlations between hormones, sperm concentrations, sperm volume, and sperm motility in the men from the general population as well as in the infertile men. Mann-Whitney *U* test was used to compare hormone levels between infertile men and normospermic men from the general population as well as hormone levels in men from the general population with a sperm concentration less than and more than 20×10^6/ml. SPSS for Windows version 10.1 (SPSS Inc., Chicago, IL) was used for all calculations and statistical analyses.

Results

hINSL3 assay characteristics

Specificity. TR-FIA with serial dilutions of hINSL3 and related peptides belonging to the insulin/relaxin family of hormones were performed. As can be seen in Fig. 1A, only hINSL3 itself is able to effectively occupy binding sites of the antibodies present in the antiserum, resulting in the less binding of labeled hINSL3 tracer the more unlabeled specific antigen was added first. A weak occupation (less than 3% cross-reactivity at EC_{70}; an EC_{50} was not reached in the concentration range used) is observable with the bovine homolog. No binding could be observed with human insulin, porcine relaxin, and human IGF-II within the used amounts, all of which exceed physiological concentrations in serum.

Sensitivity. To minimize matrix effect, increase sensitivity, and avoid further purification steps, hINSL3 was measured directly in human serum, using pools of sera from postmenopausal women (presumed to be devoid of INSL3) to be spiked with standard. The reason to choose sera from postmenopausal women as zero matrix is that although no human tissue, besides the testis, appears to express the *INSL3* gene in appreciable amounts, the functional ovary has been identified as a potential low-level source of the hormone (26). A typical standard curve is shown in Fig. 1B, showing that the assay covers a reliable detection range between 0.05 and 3.2 ng hINSL3/ml serum. In the Copenhagen laboratory, in which all samples were measured, the DL of the assay, as calculated by the concentration corresponding to the cps of blank serum minus 2 SDs, was confirmed to be 0.05 ng/ml.

Accuracy. Individual sera from male and female humans were spiked with defined serial amounts of synthetic hINSL3 (from 0.075–1.2 ng/ml serum). Recovery was 79.2–136.0% of the added standard in female (n = 4) and 81.6–110.4% in male (n = 5) sera, which is a reasonable accuracy and allows quite reliable measurements of actual serum samples.

Precision. Intra- and interassay variations were measured in the Hamburg laboratory by spiking serum samples of postmenopausal women with synthetic hINSL3 in amounts within the detection range (0.05–3.2 ng/ml) and assaying these samples in replicates (n = 6) either in parallel or in separate runs. The CV varied between 24.7% in the lower, to 2.9% in the mid, detection range of the assay within the same run and between 7.7% and 1.6% in separate runs, respectively. In the Copenhagen laboratory, the intraassay variation, as evaluated by 10 parallel measurements of serum from one male, was 11.3%. The interassay CV was evaluated by 38 separate measurements (four replicates in each run) of serum from one male (middetection range) and by 25 separate measurements (four replicates in each run) of a mixture of male and female serum (low detection range). In both, the interassay CV was 10%.

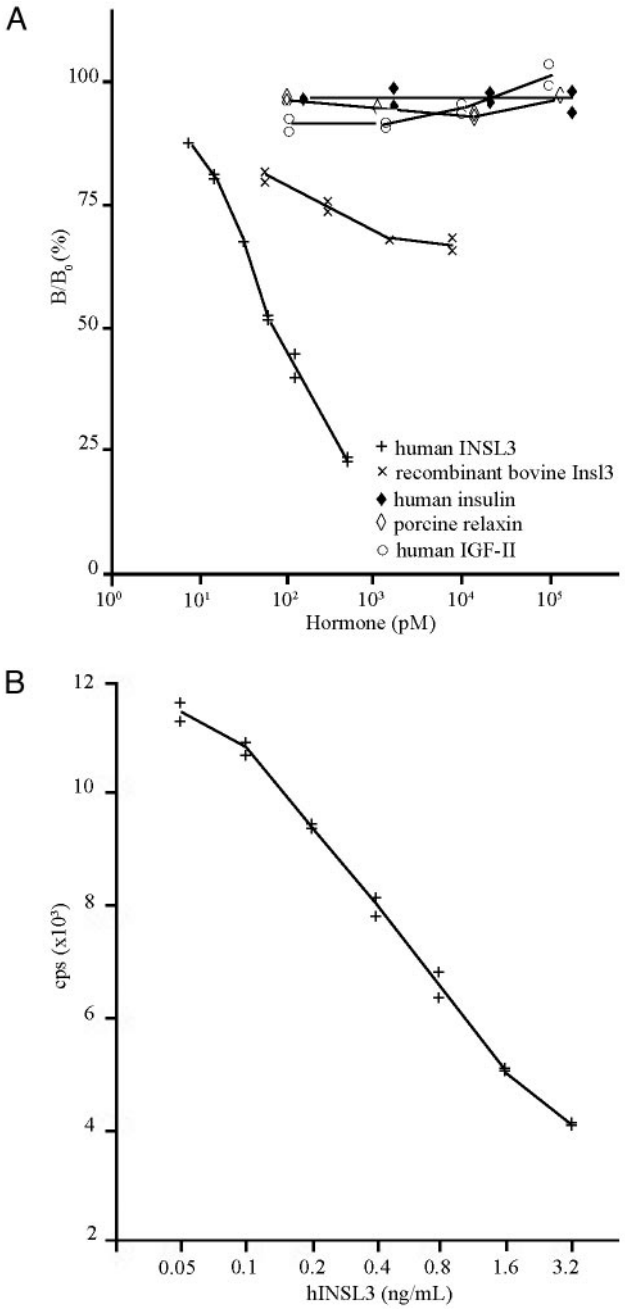

FIG. 1. A, Specificity of the hINSL3 TR-FIA. hINSL3 and several related peptide hormones were tested for binding to the antiserum in duplicate reactions. Only bovine Insl3, the closest molecular relative among the peptides tested, showed a weak cross-reaction. B, Standard curve for the assay. Standards consisted of pooled serum from postmenopausal women spiked with defined amounts of synthetic hINSL3, giving a concentration range of 0.05–3.2 ng/ml.

Influence of freezing and thawing. The effect of repeated cycles of freezing and thawing was evaluated in the Hamburg laboratory by spiking buffer and serum samples with hINSL3, aliquoting the spiked samples and measuring the samples (in the same assay) after zero to four cycles of 1 h freezing (−20 C) followed by thawing at room temperature. There was no effect of repeated freezing and thawing on the measured concentration of hINSL3.

INSL3 levels in young men from the general population and in infertile men

Median (2.5–97.5 percentiles) levels of INSL3 in all men from the general population and men from the general population with normospermia and infertile men are given in Table 1, and individual levels of INSL3 are shown in Fig. 2. The median (2.5–97.5 percentiles) serum INSL3 level in all 135 men from the general population was 0.99 (0.55–1.73) ng/ml. The INSL3 levels did not change if only men with normospermia were selected. As a group, the infertile men with severe oligo- or azoospermia did not differ significantly in INSL3 levels from the normospermic subgroup of men from the general population, although a tendency toward higher INSL3 serum levels in infertile men was observed ($P = 0.086$). T was significantly lower ($P < 0.001$) and LH significantly higher ($P = 0.040$) in the infertile men compared with men with normospermia.

Correlation among INSL3, reproductive hormones, and sperm parameters

In young men from the general population, the subgroup of these with normospermia, as well as in the 23 infertile men, correlation analyses were performed for INSL3 and T, LH, FSH, inhibin B, and the sperm parameters concentration, volume, motility, and morphology. A significant positive correlation was found between INSL3 and T in men from the general population ($r = 0.289$, $P = 0.001$) as well as in the normospermic subgroup ($r = 0.285$, $P = 0.004$). A similar correlation was not found in the infertile men ($P = 0.642$). None of the other parameters correlated significantly to INSL3 in any of these groups (data not shown), although a tendency toward a positive correlation was found between INSL3 and sperm morphology ($P = 0.089$) in men from the general population.

Hormone levels in different male patient groups

Median (2.5–97.5 percentiles) levels of INSL3 in the different patient groups are given in Table 2, and individual levels of INSL3 are given in Fig. 2.

Anorchid men. In the 21 anorchid men, INSL3 serum levels were in the range of less than 0.05–0.06 ng/ml, at or below the DL of the assay.

Klinefelter syndrome. Of the 13 patients with Klinefelter syndrome receiving T injections, one patient presented with an INSL3 serum concentration within the lower normal range, six were below the normal range, and six even below the DL of the assay. Two of the three Klinefelter patients receiving oral T replacement had INSL3 serum levels within the normal range, and one had an INSL3 serum level below the normal range. The three Klinefelter patients with the highest INSL3 levels were those who were not in need of T substitution. The two Klinefelter patients diagnosed at the time of INSL3 measurement showed clearly reduced T and INSL3 levels as well as increased LH.

Hypogonadotropic hypogonadism. All 16 patients with hypogonadotropic hypogonadism exhibited levels below the normal range; the 11 of those receiving T injections all had

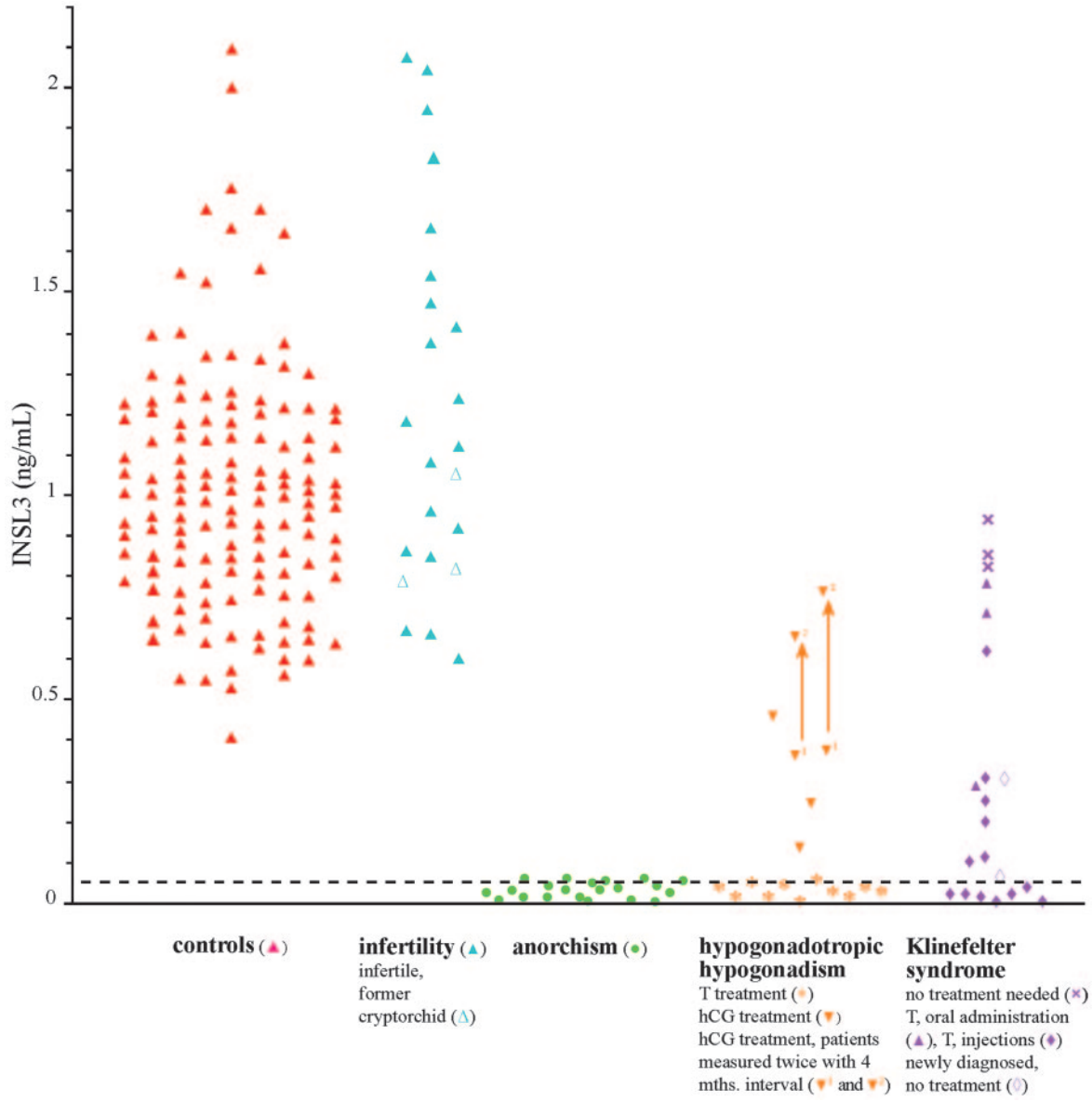

FIG. 2. Individual INSL3 serum levels measured in 135 men from the general population (controls) and 81 patients with testicular disorders. *Arrows* indicate the increase in INSL3 levels over time in two patients with hypogonadotropic hypogonadism receiving hCG treatment and measured with 4-month interval. *Black dotted line*, DL of the hINSL3 assay.

nondetectable (ND) INSL3 serum levels, whereas the five patients receiving stimulating hCG treatment showed clearly detectable INSL3. One patient measured after 2 and 5.5 months of hCG stimulation (1000 IU two times a week) showed an increase in INSL3 serum concentration over time from 0.38 to 0.73 ng/ml, and another patient measured after 8 and 12 months of hCG treatment (1500 IU two times a week) showed an increase in INSL3 from 0.36 to 0.68 ng/ml.

hCG stimulation. The two patients who had an hCG stimulation test showed strong increases in T after 72 and 96 h, whereas INSL3 serum levels stayed the same at all timepoints (Fig. 3).

Testicular biopsies and blood samples from vena spermatica

In the two infertile men investigated, the histological examinations of the bilateral testis biopsies showed normal conditions and complete spermatogenesis. Left vena spermatica serum INSL3 levels in the two men were 14.00 and 13.84 ng/ml, respectively, meaning approximately 15 times higher compared with peripheral INSL3 levels in the same persons. Characteristics are given in Table 3.

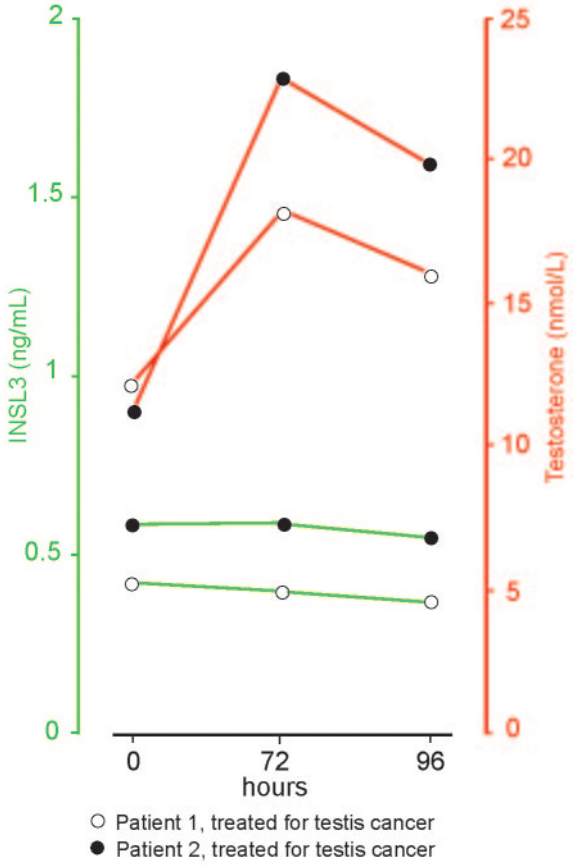

FIG. 3. INSL3 and T serum levels measured at the time of hCG stimulation and 72 and 96 h after stimulation.

Discussion

A comparison between INSL3 serum levels in our andrologically well-characterized control group of 135 men from the general population and the unmeasurable INSL3 serum levels in our group of 21 anorchid men established that the production of circulating INSL3 is specific for the testis. A testicular origin of INSL3 is further demonstrated by the fact that the concentration of INSL3, in accordance with the concentration of other testicular hormones (T and inhibin B) was very high in blood samples drawn directly from vena spermatica. These *in vivo* findings strongly supports previous *in vitro* cDNA analyses, suggesting that INSL3 expression is specifically confined to the testis in the human male (2) and are furthermore in line with recently published INSL3 serum analyses (15). The possibility, however, still exists that tissues other than the testis express paracrine-acting INSL3, which is not secreted to the blood or, if so, in amounts too limited to be detectable in our assay. Within the human testis, the source of secreted INSL3 has previously been shown to be the Leydig cells (27). All individuals studied were young adults with mature Leydig cells (except those with hypogonadotropic hypogonadism), although the average patient was 10 yr older than the controls.

Our data from male patients with hypogonadotropic hypogonadism clearly showed that INSL3 production and secretion was dependent on LH. This was illustrated by the ND INSL3 levels of patients receiving T replacement therapy and the clearly detectable INSL3 serum levels in those receiving hCG-stimulating therapy. The fundamental role of LH was further emphasized by the increase in INSL3 serum levels in the two patients measured at different timepoints after onset of long-term hCG treatment. In contrast, the unaltered INSL3 after short-term hCG stimulation opposed an acute regulatory effect of LH on INSL3. Hence, we observed, on the one hand, a clear increase in INSL3 in response to repeated injections with hCG, capable of inducing Leydig cell differentiation and, on the other hand, unchanged INSL3 production 3–4 d after a single dose of hCG, which in itself is unlikely to influence Leydig cell differentiation but indeed stimulates steroidogenesis. In all, the findings are in accordance with a constitutive production and secretion of INSL3, maintained by the long-term trophic effect of LH on Leydig cell structure and function and independent of the acute steroidogenic effect of LH. Our human data support previous findings in rodents, where Insl3 is up-regulated during puberty and hypogonadal mice respond with increasing Insl3 production only after several days of Leydig cell-differentiating hCG treatment (28, 29).

In line with the lack of an acute stimulatory effect of LH on INSL3 synthesis, we did not find any correlation between LH and INSL3 in our 135 normal men. In contrast, Foresta *et al.* (15) found a positive correlation between LH and INSL3 in 40 normal men. The reason for these discrepant results is not immediately clear.

Interestingly, hormone analyses from our infertile men exemplified a situation in which T serum levels, but not INSL3 serum levels, were affected, compared with our group of normospermic men from the general population. The discrepancy between supranormal LH levels, reduced T levels, and normal INSL3 levels in infertile men pointed to a disturbed steroidogenesis of the Leydig cells but not to a general impairment of Leydig cell function or differentiation status because INSL3 production seemed to be normal. Similar differences in T and LH levels between fertile and idiopathic infertile men have previously been published by our group (30). In that article, we hypothesized that the observed reduced T levels in infertile men might reflect an impaired paracrine communication between the seminiferous epithelium and the Leydig cells. If so, this impaired cross-talk did not seem to influence INSL3 production. An apparent irrelevance of INSL3 for fertility evaluation was further supported by the finding of similar INSL3 serum levels in combination with significantly increased LH levels and higher FSH levels in the subgroup of men from the general population with a sperm concentration less than 20×10^6/ml. In contrast, Foresta *et al.* (15) reported decreased INSL3 in the copresence of normal LH and T serum levels in infertile men with severe hypospermatogenesis. The discrepancy between data and following interpretations in the two studies may very well be the occasion of heterogeneity in the studied infertile men because infertile populations hold a wide spectrum of identified as well as unidentified pathologies. However, our finding of normal INSL3 serum levels in infertile men is in agreement with our observed lack of correlation between INSL3 and semen parameters found in the present study and previous findings of normal INSL3 expression in

Leydig cells of men with severe oligo- or azoospermia as evaluated by immunostaining (4).

Hyperplastic Leydig cells have previously been shown to have a reduced INSL3 expression, in accordance with a less differentiated state of the cells (31). Men with Klinefelter syndrome are known to have histologically abnormal Leydig cells with big Leydig cell clusters, often described as hyperplastic (32, 33), which might partly explain the generally reduced INSL3 serum levels measured in this patient group. The copresence of low T levels and low INSL3 levels were in accordance with a generally impaired (or less mature) state of the Leydig cells and, thereby, clearly distinguishable from the Leydig cell function of our infertile men, in which only steroidogenesis was affected. From a clinical aspect, it is interesting that the subgroup of 47, XXY patients not receiving therapy presented with INSL3 levels that clearly correlated to the functional state of their Leydig cells, as evaluated by T and LH measurements. However, it was difficult to evaluate the Leydig cell function of the large subgroup of Klinefelter patients receiving T injections by the traditional serum markers T and LH because these were strongly influenced by the treatment. In these situations as well as in evaluation of untreated 47, XXY patients, INSL3 measurements may prove to be clinically useful by providing a direct estimate of the current basal Leydig cell function, irrespective of any treatment.

It was evident from our data that a number of different patient groups had strongly reduced or ND INSL3 serum levels. Many of these patients were treated with T injections, which may have further suppressed any potential capability of producing INSL3 via suppression of LH, as indicated by the low LH median value in the subgroup of patients with Klinefelter syndrome receiving T injections. The recent finding that binding of INSL3 to germ cells serve to protect these from undergoing apoptosis (13) raises the question of the impact of reduced INSL3 levels on fertility. Fertility was, for various reasons, already strongly impaired in the described patient groups, which camouflaged the potential effect and perhaps also minimized the importance, if any, of reduced INSL3 levels. The question still remains, however, of whether the INSL3 produced by the Leydig cells has an effect on other organs and tissues, as suggested by the substantial serum levels and the widely distributed expression of its receptor *LGR8*. The well-documented efficacy of T replacement therapy in bilaterally castrated patients suggests that the eventual endocrine effects of INSL3 may be subtle. However, it remains to be seen whether some andrological patients, besides having symptoms of T insufficiency, in addition could be affected by reduced levels of INSL3.

The presented data are all based on a newly developed TR-FIA specific for hINSL3. The recently published paper by Foresta *et al.* (15) was based on the use of a commercially available INSL3 RIA kit, which to our knowledge is the only commercially available INSL3 kit to date. Apparently, the commercial assay measure somewhat lower concentrations compared with our assay, a plausible explanation for which may be differences in standardizations.

In conclusion, we have established that in the human male, the majority, if not all, circulating INSL3 is produced in the Leydig cells. The production of INSL3 is dependent on the long-term, trophic effect of LH and independent of the acute steroidogenic LH effect; INSL3 and T are thereby regulated differently. Patients with testicular disorders often have reduced or absent INSL3 serum levels, emphasizing the need for increased knowledge of the role of INSL3 in adult men.

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