Serum insulin-like factor 3 is highly correlated with intratesticular testosterone in normal men with acute, experimental gonadotropin deficiency stimulated with low-dose human chorionic gonadotropin: a randomized, controlled trial

Mara Y. Roth, M.D.,[a] Kat Lin, M.D.,[b] Katrine Bay, M.Sc., Ph.D.,[c] John K. Amory, M.D., M.P.H.,[a] Bradley D. Anawalt, M.D.,[a] Alvin M. Matsumoto, M.D.,[a,d] Brett T. Marck, B.S.,[d] William J. Bremner, M.D., Ph.D.,[a] and Stephanie T. Page, M.D., Ph.D.[a]

[a] Center for Research in Reproduction and Contraception, Department of Medicine; and [b] Department of Obstetrics and Gynecology, University of Washington, Seattle, Washington; [c] University Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark; and [d] Geriatric Research, Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle, Washington

Objective: To study the potential role for using serum biomarkers, including insulin-like factor 3 (INSL3), 17α-hydroxyprogesterone, antimüllerian hormone, and inhibin B, as correlates of intratesticular T (IT-T) concentrations in men.
Design: Prospective, randomized, controlled trial.
Setting: University-based medical center.
Patient(s): Thirty-seven healthy men aged 18–50 years.
Intervention(s): All men received the GnRH antagonist acyline, plus very low doses of hCG (0 IU, 15 IU, 60 IU, or 125 IU) SC every other day or 7.5 g T gel daily (75 mg delivered). The IT-T concentrations obtained by percutaneous testicular aspiration with simultaneous serum protein and steroid concentrations were measured at baseline and after 10 days of treatment.
Main Outcome Measure(s): Intratesticular and serum hormone and gonadotropin concentrations.
Result(s): After 10 days of gonadotropin suppression, serum INSL3 decreased by more than 90% and correlated highly with IT-T concentrations. In contrast, serum inhibin B, antimüllerian hormone, and 17α-hydroxyprogesterone did not correlate with IT-T. Serum INSL3 increased with the dose of hCG administered and returned to baseline after treatment.
Conclusion(s): Serum INSL3 correlates highly with IT-T and serum T concentrations during acute gonadotropin suppression in men. Human chorionic gonadotropin stimulates dose-dependent increases in INSL3 and IT-T in healthy men and might be a useful biomarker of IT-T concentration in some clinical settings.
Clinical Trial Registration Number: NCT# 00839319. (Fertil Steril® 2013;99:132–9. ©2013 by American Society for Reproductive Medicine.)
Key Words: Androgens, INSL3, male infertility, gonadotropins, intratesticular testosterone

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/rothmy-insl3-intratesticular-testosterone-gonadotropins/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Received February 9, 2012; revised September 5, 2012; accepted September 6, 2012; published online October 3, 2012.
M.Y.R. has nothing to disclose. K.L. has nothing to disclose. K.B. has nothing to disclose. J.K.A. receives research funds as a site in a phase III trial from Clarus Pharmaceuticals (unrelated to this work). B.D.A. has nothing to disclose. A.M.M. has nothing to disclose. B.T.M. has nothing to disclose. W.J.B. has nothing to disclose. S.T.P. has nothing to disclose.
The Eunice Kennedy Shriver National Institute of Child Health and Human Development supported this work through cooperative agreement U54 HD-42454 as part of the Cooperative Contraceptive Research Centers Program. M.Y.R. is supported, in part, by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (grant K12 HD053984). A.M.M. is supported by the US Department of Veterans Affairs.
Reprint requests: Mara Y. Roth, M.D., Department of Medicine, University of Washington, 1959 NE Pacific Street, Box 357138, Seattle, Washington 98195 (E-mail: mylang@u.washington.edu).

Fertility and Sterility® Vol. 99, No. 1, January 2013 0015-0282/$36.00
Copyright ©2013 American Society for Reproductive Medicine, Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.fertnstert.2012.09.009

Male factor infertility accounts for 20% of infertile couples, and in as many as 60% of these cases the underlying cause of their infertility is unknown (1). Androgens play a vital role in spermatogenesis. Testosterone, secreted by testicular Leydig cells upon stimulation by LH, is present in very high concentrations within the testes, 100–1,000-fold higher than the concentrations found in the circulation (2–7). Infertile men with low gonadotropins, and in some cases men with idiopathic infertility, can be treated with exogenous gonadotropins in an effort to initiate or optimize spermatogenesis. Human chorionic gonadotropin, which stimulates Leydig cells similarly to LH, is used to increase T production in the testes before the addition of FSH. In men with hypogonadotropic hypogonadism, the dose of hCG is often adjusted to normalize serum T, yet the intratesticular T (IT-T) concentration is one of the keys to inducing spermatogenesis. In some men, normal serum T concentrations can be achieved by administering hCG without normalizing IT-T concentrations or stimulating spermatogenesis (3, 8, 9), raising the possibility that serum T may not be an accurate marker of IT-T. In addition, given that gonadotropin therapy to induce spermatogenesis often requires several years to achieve conception (10), identifying serum markers to potentially predict response to therapy would benefit infertility patients. Unfortunately, measurement of intratesticular androgens is invasive, requiring either percutaneous testicular aspiration or surgical testicular biopsy, so IT-T concentrations are not often evaluated in infertile men. A serum biomarker of IT-T might be useful in the diagnosis, prognosis, and treatment monitoring of some men with infertility.

Insulin-like factor 3 (INSL3), antimüllerian hormone (AMH), inhibin B (INHB), and the T precursor 17α-hydroxyprogesterone (17-OHP) are candidate substances, quantifiable in serum, and have the potential to serve as serum correlates of IT-T. Similar to T, INSL3 is a protein produced by Leydig cells and is known to play a key role in testicular descent during fetal development (11, 12). Expression of INSL3 increases during pubertal development (13) and is abundant in serum of adult men (14). Although the role of INSL3 in adults remains unclear, some studies suggest that serum concentrations of INSL3 are lower in men with infertility, even those with normal serum T concentrations (15). Production of INSL3 is clearly regulated by LH, although it has yet to be fully determined whether this regulation is direct or via IT-T (13, 16). Although T concentrations often vary widely in a given individual over the course of a day (17), INSL3 seems to be constitutively expressed in normal adult men and declines with age (18). Therefore, serum INSL3 has the potential to reflect steady-state IT-T levels without the confounding pulsatility of serum LH and T (19, 20).

Antimüllerian hormone and INHB are glycoproteins secreted by Sertoli cells. Antimüllerian hormone plays a key role in male embryos to trigger müllerian duct regression (21). Although AMH is present in high concentrations in adult men, its functional role is unclear (22). The glycoprotein INHB, also secreted by Sertoli cells, correlates strongly with sperm count, concentration, and testicular volume in adult men (23). Inhibin B largely functions to regulate the release of FSH (24–26). Both INHB and AMH have been reported to be lower in men with nonobstructive azoospermia (27), although attempts to use INHB and AMH as predictors for the success of infertility treatment in azoospermic men have not been successful to date (28, 29).

In addition to these testicular products, previous studies have suggested that serum concentrations of the T precursor 17-OHP reflect IT-T when normal concentrations of serum T are maintained (30). Approximately 70% of circulating 17-OHP is thought to be of testicular origin, and similar to T, production of 17-OHP is stimulated by LH and hCG (31, 32). Whether serum 17-OHP concentrations are a good biomarker for IT-T when IT-T concentrations are very low has not been investigated to date.

To investigate the relationship between serum markers of testicular function and IT-T, we evaluated serum INSL3, AMH, INHB, and 17-OHP in healthy men undergoing experimental gonadotropin suppression (3). In conjunction with the administration of a potent GnRH antagonist that effectively suppresses gonadotropins for 2 weeks, acyline (33), subjects were randomized to receive low doses of hCG to create a continuum of IT-T concentrations across the experimental groups (3). On the basis of their regulation by LH, we hypothesized that serum 17-OHP and INSL3 would be highly correlated with IT-T in the setting of low-dose LH (hCG) stimulation, whereas AMH and INHB concentrations, which are stimulated primarily by FSH, would not change.

MATERIALS AND METHODS

Subjects

The study design has been reported previously (3). In brief, we enrolled 40 healthy men, aged 18–50 years, with normal serum gonadotropins, serum T concentrations, and normal results on seminal fluid analyses. Men were excluded if they were in poor general health or had abnormal blood test results, active skin conditions that would prevent the use of T gel, active alcohol or drug abuse, history of testicular or scrotal surgery, infertility, chronic pain syndrome, use of steroids, T, or medications that might affect androgen metabolism, including ketoconazole, glucocorticoids, known bleeding disorder, or if they were using anticoagulant medications such as aspirin or warfarin. Thirty-seven men completed all study procedures. A blood sample for the assessment of serum hormones and proteins and a unilateral testicular fine-needle aspiration were performed on day 1 (2, 34). We then administered acyline (NeoMPS), a GnRH antagonist, 300 μg/kg SC to all subjects. On the same day, subjects then received their first dose of hCG (Pregnyl; Organon) or 1% T gel (Androgel; Solvay) based on treatment group randomization (groups 1–5): placebo hCG (normal saline), 15 IU hCG SC, 60 IU hCG SC, 125 IU hCG SC, or 1% T gel 7.5 g daily (resulting in 75 mg absorbed daily). Administration of hCG/placebo continued every other day for five doses in groups 1–4. Group 5 applied the T gel to the skin daily for 10 days. A blood sample was collected on day 7 to assess for effective suppression of gonadotropins as indicated by a serum LH concentration $<$1.2 IU/L. On day 10 subjects underwent a second testicular fine-needle

aspiration of the contralateral testis, and a blood sample for protein and hormone quantification was collected. A follow-up visit on day 40 was performed to ensure the testicular examination, serum, and semen parameters had all returned to normal. The University of Washington institutional review board approved the study, and all subjects provided written, informed consent before study procedures. The study was registered in advance on clinicaltrials.gov as NCT #00839319.

Measurements

Testicular fluid samples were immediately placed on ice, centrifuged at 300 × *g* to remove any aspirated cells; the supernatant fluid was decanted and stored at −70°C. Serum was stored at −20°C. Testicular fluid and serum samples were assayed simultaneously for T by liquid chromatography–tandem mass spectrometry on a Waters Aquity ultra performance liquid chromatography (UPLC) coupled with a Micromass Premiere-XE tandem quadrupole mass spectrometer (Waters) as previously reported (3). Serum LH, FSH, and hCG concentrations were quantified by immunofluorometric assay as reported previously (3).

Serum samples were shipped to Copenhagen, Denmark, on dry ice for INSL3 analysis and were in transit for <48 hours. Serum concentrations of INSL3 were measured using a time-resolved fluorescence immunoassay with a detection limit of 0.05 ng/mL and inter- and intra-assay coefficients of variation of 11.3% and 8.0%, respectively. Development and evaluation of this assay has been described in detail previously (20).

Serum INHB and AMH were both measured by enzyme-linked immunosorbent assay (Kamaya Biomedical). The lower limit of detection for INHB was 31 pg/mL, and the inter- and intra-assay coefficients of variation were 7% and 8.5%, respectively. The lower limit of detection for the AMH assay was 0.15 ng/mL, and the interassay coefficient of variation was 8%. Serum 17-OHP was quantified using a radioimmunoassay (Siemens Healthcare Diagnostics) with a lower limit of detection of 0.1 ng/mL. The inter- and intra-assay coefficients of variation were 11% and 6.7%, respectively, for low pools, and 8.5% and 3.5% for high pools. All samples for all subjects were batched and measured in a single assay.

Statistical Analysis

The primary endpoint of the study was the differences in intratesticular T concentration between groups. Eight subjects per group conferred an 80% power to detect a difference of >8 ng/mL, with a variance of 20% between the groups at an α of 0.05 when adjusted for multiple comparisons. Because of nonnormality, the data are expressed as medians and 25th and 75th percentiles. Six subjects (one each in groups 1 and 2 and two each in groups 3 and 4) had evidence of incomplete gonadotropin suppression, with serum LH values >1.2 IU/L on day 7. These subjects were excluded from further analysis because their serum and intratesticular hormones were confounded by normal serum concentrations of LH (3). Therefore, analysis of baseline and end-of-treatment hormone concentrations was performed on the 31 subjects who suppressed serum LH below the lower limit of the normal range by day 7. Comparisons of hormone concentrations between groups were performed in a nonparametric fashion using Kruskal-Wallis analysis of variance with a Wilcoxon rank-sum post hoc test. Comparisons between baseline and end-of-treatment hormone concentrations within a group were performed using a nonparametric Wilcoxon sign-rank (paired) test. Correlations between serum and intratesticular hormones concentrations were performed using the nonparametric Spearman technique and graphed using best linear fit lines. No corrections were made for multiple comparisons. All statistical analyses were performed using STATA version 10.0 (StataCorp). For all comparisons, an α of <0.05 was considered significant.

RESULTS

Baseline Hormone Concentrations

A complete description of the subjects screened and enrolled in this study, including the complete inclusion and exclusion criteria, has been previously reported (3). There were no serious adverse events during this study, and the testicular aspiration procedure was well tolerated. Testicular aspirate volumes were similar at baseline and after 10 days of treatment with hCG or T, with a median volume of 10 μL.

The subject's baseline characteristics and serum hormones are reported in Table 1. Serum gonadotropins and androgens as well as intratesticular hormone data have been previously reported (3, 35). There were no statistically significant differences in any of the measurements between the treatment groups at baseline. Baseline hormone concentrations did not correlate with age, race, or body mass index (data not shown).

Baseline INSL3 was normally distributed for the 31 subjects. Neither baseline IT-T nor serum T correlated significantly with baseline INSL3 ($r = 0.31$, $P=.09$ and $r = 0.19$, $P=.3$, respectively), but serum LH did correlate significantly with INSL3 at baseline (Fig. 1). Reanalysis of the data after removal of outliers in the data did not change the results. As presented previously, serum T also did not correlate with IT-T at baseline (3). Inhibin B did not correlate with serum T, FSH, or LH but had a negative correlation with IT-T at baseline ($r = -0.4$, $P<.05$). Neither AMH nor 17-OHP correlated significantly with serum T, IT-T, FSH, or LH at baseline (data not shown).

Posttreatment Hormone Concentrations

Serum INSL3 decreased by >90% compared with baseline with the administration of the GnRH antagonist acyline by itself and in combination with T gel and was not significantly different after treatment between these two groups (Fig. 2, Table 1). As reported previously, serum LH decreased by nearly 95% from baseline (median [25th, 75th percentiles] of 3.4 [2.6, 4.9] IU/L to 0.19 [0.1, 0.5] IU/L), and serum FSH decreased by 89% from baseline (median of 2.4 [1.5, 3.0] IU/L to 0.27 [0.2, 0.4] IU/L), with acyline administration alone ($P<.001$ for both comparisons) (3). Insulin-like factor 3 decreased by 80% from baseline ($P<.05$) in the group receiving 15 IU hCG but did not differ significantly from posttreatment concentrations of the acyline-alone group or acyline plus T

TABLE 1

Baseline characteristics and baseline and posttreatment serum hormones of 31 participants by treatment group.

Characteristic	0 IU hCG (n = 6)	15 IU hCG (n = 7)	60 IU hCG (n = 5)	125 IU hCG (n = 5)	T gel (n = 8)	All subjects (n = 31)
Age (y)	21 (20, 26)	25 (20, 29)	22 (20, 24)	22 (21, 26)	22 (20, 24)	22 (20, 26)
BMI (kg/m^2)	24.8 (23.6, 26.3)	24.1 (23.2, 26.7)	24.9 (21.2, 26.3)	25.8 (22.9, 26)	23.7 (21.1, 25.4)	24.1 (22.9, 26.3)
Baseline hormones						
Serum T (ng/mL)	3.7 (3.2, 4.8)	4.3 (3.3, 6.0)	4.1 (3.6, 4.3)	4.8 (4.1, 5.3)	4.3 (3.9, 5.2)	4.2 (3.4, 4.9)
Serum INSL3 (ng/mL)	0.9 (0.7, 1.1)	1.0 (0.7, 1.5)	1.1 (1.0, 1.2)	0.9 (0.8, 1.3)	1.1 (1.1, 1.3)	1.1 (0.8, 1.2)
Serum Inhibin-B (pg/mL)	86.5 (78, 124)	109 (96, 165)	136 (135, 180)	117 (104, 142)	123 (88, 147)	114 (100, 151)
Serum AMH (ng/mL)	5.8 (2.1, 9.1)	2.5 (0.6, 28)	2.7 (2.4, 3.9)	1.0 (0.8, 1.6)	2.5 (0.6, 12)	2.5 (0.9, 6.8)
Serum 17-OHP (ng/mL)	4.7 (3.8, 7.8)	4.9 (4.2, 6.5)	5.9 (5.1, 7.6)	4.3 (4, 4.5)	4.6 (3.7, 5.4)	4.9 (3.9, 6.5)
Intratesticular T (ng/mL)	991 (717, 1098)	694 (486, 967)	521 (501, 690)	1,002 (659, 1,132)	839 (437, 970)	717 (501, 1,002)
Posttreatment hormones						
Serum T (ng/mL)	0.13 (0.11, 0.19)	0.3 (0.11, 0.34)	0.86 (0.31, 1.01)	2.36 (1.79, 3.44)	5.08 (4.11, 6.97)	
Serum INSL3 (ng/mL)	0.07 (0.06, 0.17)	0.18 (0.14, 0.26)	0.29 (0.28, 0.44)	0.84 (0.64, 1.04)	0.1 (0.06, 0.17)	
Serum INHB (pg/mL)	96 (81, 106)	114 (103, 157)	147 (134, 175)	138 (98, 139)	138 (93, 172)	
Serum AMH (ng/mL)	6.4 (2, 24)	4.2 (1.1, 33)	3.6 (2.8, 4)	2.0 (0.9, 2.1)	5.9 (2.2, 16.7)	
Serum 17-OHP (ng/mL)	3.4 (1.8, 4.3)	2.4 (1.6, 4.3)	2.8 (2.8, 5.6)	3.2 (2.7, 5.3)	2.3 (1.4, 3.2)	
Intratesticular T (ng/mL)	21.6 (11.4, 63.8)	38.9 (22.6, 73.8)	91.2 (39.8, 702)	282.3 (256.0, 357.5)	20.9 (9.7, 75.5)	

Note: Values are median (25th, 75th percentiles).

Roth. INSL3 and intratesticular T. Fertil Steril 2013.

gel group, both of which also decreased significantly from baseline ($P<.05$ compared with baseline for acyline alone and acyline plus T gel). Insulin-like factor 3 decreased by 70% from baseline ($P<.05$) in the group receiving 60 IU hCG and was significantly different from all other treatment groups ($P<.01$). The INSL3 concentration in the group receiving the highest hCG dose, 125 IU, did not change significantly from baseline ($P=.22$), but the posttreatment INSL3 concentration in the 125 IU group differed significantly from all other treatment groups ($P<.01$).

Suppression of INSL3 production with acyline, then stimulation of INSL3 with low doses of hCG, established a dose–response relationship between hCG dose and serum INSL3 concentrations (Fig. 2). In addition, after 10 days of hCG treatment, serum INSL3 correlated highly with IT-T, serum T, and serum hCG (Fig. 3A–3C). After treatment with hCG, serum INSL3 and IT-T correlated strongly ($r = 0.79$, $P<.001$) in a pattern similar to the significant correlation of serum T and IT-T after suppression with a GnRH analog ($r = 0.86$, $P<.001$ [3]).

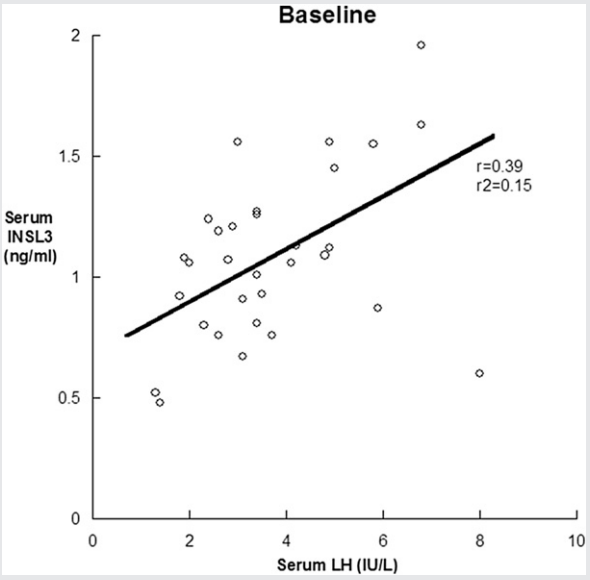
Serum INHB, AMH, and 17-OHP concentrations did not change significantly from baseline after gonadotropin suppression followed by 10 days of hCG or T therapy (Table 1). As compared with baseline, posttreatment serum INHB concentrations did not correlate significantly with IT-T ($r=-0.3$, $P=.07$). In addition, neither AMH nor 17-OHP concentrations correlated with IT-T after treatment ($r = -0.2$, $P=.2$ and $r = 0.2$, $P=.2$, respectively). A negative correlation between serum INHB and FSH approached significance after 10 days of hCG treatment ($r = -0.4$, $P=.07$), but serum AMH did not correlate with FSH after treatment ($r = 0.2$, $P=.4$).

DISCUSSION

In this placebo-controlled, randomized clinical trial we have shown that serum INSL3 concentrations in normal men decrease dramatically with acute gonadotropin suppression and increase in a dose–response relationship with low-dose hCG stimulation, correlating highly with IT-T and serum T concentrations. In contrast, serum INHB, AMH, and 17-OHP did not significantly change with acute gonadotropin suppression or hCG stimulation and did not correlate with IT-T. Our results expand upon previous observations by Bay et al. examining the effect of high-dose gonadotropin stimulation on INSL3 concentrations (19, 20). The prior data illustrate that INSL3 seems to be maximally produced at baseline in normal men and does not increase with gonadotropin stimulation (20). In infertile men with hypogonadotropic hypogonadism, high hCG doses are capable of stimulating otherwise suppressed INSL3 production (19). Here, use of gonadotropin suppression with a GnRH antagonist, acyline, coupled with very-low-dose hCG stimulation (to mimic LH stimulation), demonstrates a dose–response relationship between hCG/LH stimulation and INSL3 concentrations in a low-gonadotropin environment.

Because serum INSL3 correlates highly with IT-T concentrations, serum INSL3 might be useful as a serum biomarker for the effect of hCG therapy and LH-like stimulation of

FIGURE 1

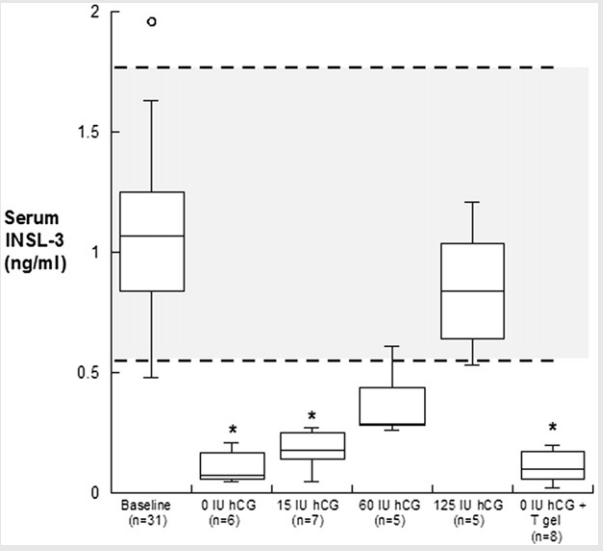


Baseline correlation between serum INSL3 and serum LH for all 31 subjects. The line shown represents the best linear fit.

Roth. INSL3 and intratesticular T. Fertil Steril 2013.

Leydig cells. Studies evaluating INSL3 concentrations in hypogonadotropic hypogonadal men treated with hCG vs. T therapy have suggested that INSL3 production and secretion is regulated by LH rather than IT-T (20). Our data cannot help to distinguish whether LH or IT-T stimulates INSL3 production, because both correlate similarly with INSL3 in this study.

FIGURE 2



Box plot of serum INSL3 concentration in gonadotropin-suppressed subjects at baseline (n = 31) and on day 10 by treatment group (n = 6 for the 0-IU hCG group, n = 7 for the 15-IU hCG group, and n = 5 for the 60-IU hCG group and 125-IU hCG group). *$P<.05$ compared with baseline, 60-IU hCG group, and 125-IU hCG group. *Dotted lines* and *gray shading* represent the normal range for serum INSL3.

Roth. INSL3 and intratesticular T. Fertil Steril 2013.

During hCG therapy in normal men with experimentally induced hypogonadotropic hypogonadism, serum T also correlates highly with IT-T (3), but men may achieve a normal serum T concentration while still having an IT-T concentration below the normal range. In addition, men receiving exogenous T therapy may have a normal serum T concentration but significantly suppressed IT-T, suggesting that perhaps an alternative serum biomarker for IT-T could be useful. Because serum INSL3 is a protein, it may be a more stable marker of IT-T than serum T in infertile men being treated with hCG who are intermittently compliant with their injections. In addition, a marker of IT-T in men receiving exogenous T may be useful in understanding suppression of spermatogenesis while using a male hormonal contraceptive regimen.

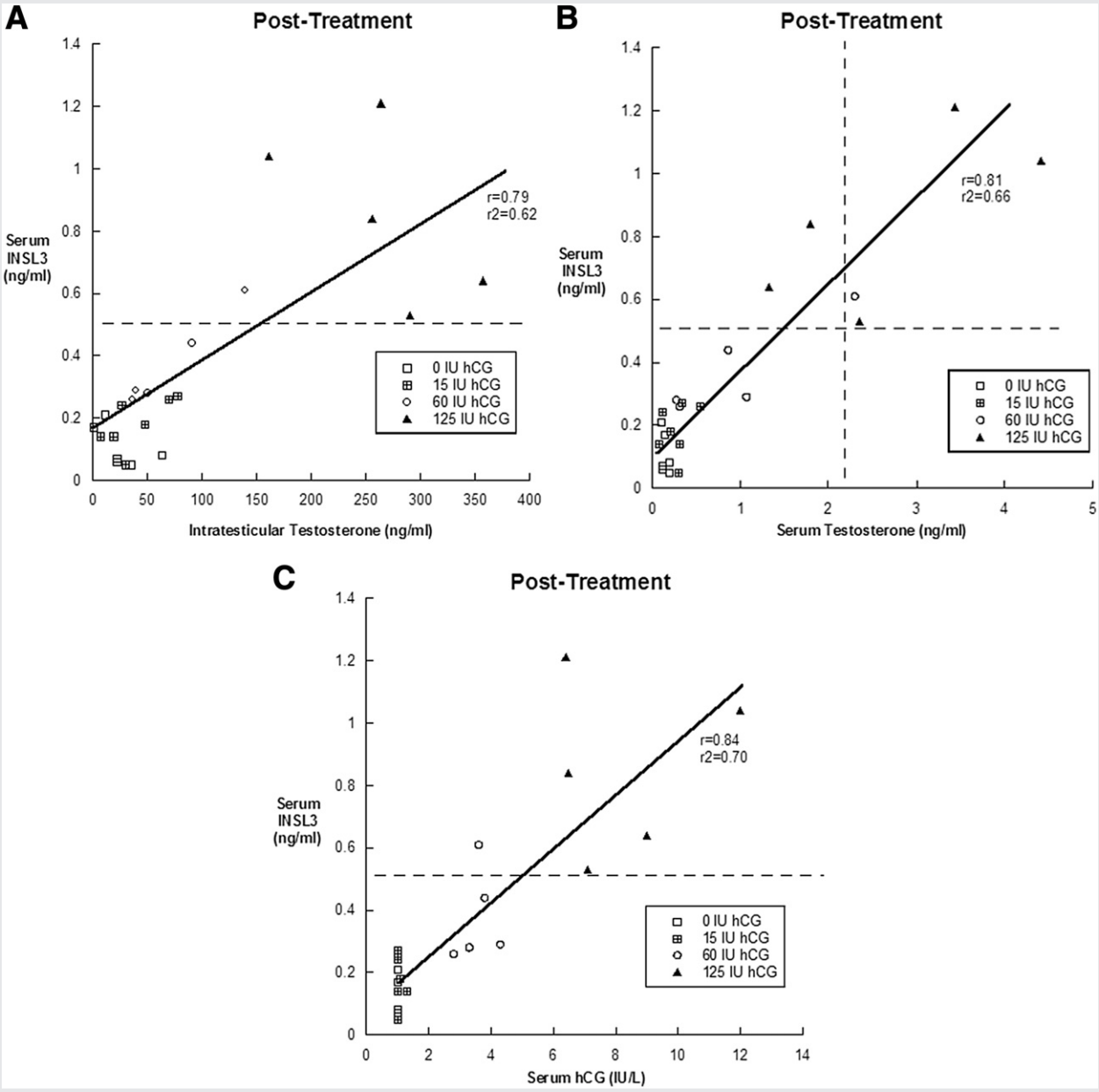
Inhibin B did not change significantly with acute gonadotropin suppression or hCG stimulation. Inhibin B has been identified as a product of Sertoli cells and as a clinical marker of spermatogenesis (24, 26–29). In the normal male, FSH receptors are found only on Sertoli cells (36), and FSH stimulates INHB production. Inhibin B inhibits FSH secretion from pituitary gonadotropes via negative feedback (26). On the basis of prior data in male hormonal contraceptive trials, we anticipated that serum INHB concentrations would significantly decrease as FSH levels fell in all treatment groups and that serum INHB production would not be stimulated by hCG stimulation (37). The lack of INHB suppression in our study is likely related to the brief duration of gonadotropin suppression and less suppression of FSH (89%) as compared with LH (95%) (3).

In contrast to INHB, AMH is primarily produced by prepubertal Sertoli cells in response to FSH stimulation (38). Antimüllerian hormone subsequently declines with rising LH stimulation and seems to be suppressed by high intratesticular androgen concentrations (39). Although AMH concentrations might be expected to rise in settings of low IT-T, the lack of FSH stimulation in our experimental model may explain the lack of rise in AMH concentrations. Alternatively, the AMH results were highly variable between subjects, raising doubt about the reliability of the assay used for these measurements.

In addition, our study did not show a correlation between 17-OHP, a T precursor, and IT-T in the setting of acute gonadotropin suppression or in response to low-dose hCG stimulation. A previous study done by Amory et al. using T enanthate for gonadotropin suppression and treating with higher doses of hCG suggested that 17-OHP may serve as a useful correlate for IT-T in men receiving gonadotropin therapy for infertility (30). However, when comparing the lowest hCG dose in that study, 125 IU every other day, with the group receiving gonadotropin suppression alone, the 17-OHP concentration was the same. Therefore, it seems possible that serum 17-OHP correlates with IT-T in the presence of normal or near-normal hCG stimulation but not with very-low-dose hCG stimulation.

One limitation of this was study is our limited sample size. Correlations of several of the hormones with one another approached but did not attain statistical significance in our

FIGURE 3



(A–C) Correlations between posttreatment serum INSL3 for all 23 subjects receiving hCG therapy intratesticular T (A), serum T (B), and serum hCG (C). *Dotted lines* represent the lower limit of the normal range. *Lines* shown represent the best linear fit.

Roth. INSL3 and intratesticular T. Fertil Steril 2013.

study. Because the study was powered to determine the differences in intratesticular T between dose groups of hCG, it may have lacked the necessary power to identify all significant associations between the various hormones. Future, larger studies designed with adequate power to examine these relationships in men with infertility will be needed to clarify the relative utility of these serum markers for IT-T during hCG therapy. A second possible limitation is the use of normal men in this study, not infertile men. Future studies investigating the best serum biomarker for IT-T during hCG therapy should be conducted in infertile men, because the ability to extrapolate the findings discussed here may not reflect the associations observed in infertile men.

In summary, we demonstrated an acute decrease in serum INSL3 concentrations in response to gonadotropin suppression and a dose–response relationship between INSL3 and IT-T concentrations using low-dose hCG stimulation in normal men. We have also shown that serum INHB, AMH, and 17-OHP do not correlate significantly with IT-T. This work may have relevance in the use of serum INSL3 as a potential marker for IT-T concentrations. Although IT-T concentrations vary significantly among fertile men and reflect LH pulsatility

(2), both the minimal and the "ideal" concentration of IT-T for optimal spermatogenesis remain unknown. However, given that spermatogenesis depends upon extremely high concentrations of IT-T and that serum T concentrations do not accurately reflect IT-T concentrations in some settings (for example, in men receiving therapeutic T replacement), the use of INSL3 as an alternative serum marker for IT-T concentrations might allow for more accurate monitoring of hCG therapy in infertile men. Whether INSL3 is a superior marker to serum T as a reflection of Leydig cell function during therapy for infertility will require a future, larger study in infertile men.

Acknowledgments: The authors thank Ms. Iris Nielsen, Ms. Marilyn Busher, Ms. Dorothy McGuiness, and Ms. Connie Pete for their assistance with this study, as well as the study volunteers, without whom this research would not be possible.

REFERENCES

1. Bhasin S. Approach to the infertile man. J Clin Endocrinol Metab 2007;92:1995–2004.
2. Roth MY, Lin K, Amory JK, Matsumoto AM, Anawalt BD, Snyder CN, et al. Serum LH correlates highly with intratesticular steroid levels in normal men. J Androl 2010;31:138–45.
3. Roth MY, Page ST, Lin K, Anawalt BD, Matsumoto AM, Snyder CN, et al. Dose-dependent increase in intratesticular testosterone by very low-dose human chorionic gonadotropin in normal men with experimental gonadotropin deficiency. J Clin Endocrinol Metab 2010;95:3806–13.
4. Zhao M, Baker SD, Yan X, Zhao Y, Wright WW, Zirkin BR, et al. Simultaneous determination of steroid composition of human testicular fluid using liquid chromatography tandem mass spectrometry. Steroids 2004;69:721–6.
5. Matthiesson KL, Stanton PG, O'Donnell L, Meachem SJ, Amory JK, Berger R, et al. 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 2005;90:5647–55.
6. McLachlan RI, O'Donnell L, Stanton PG, Balourdos G, Frydenberg M, de Kretser DM, et al. Effects of testosterone plus medroxyprogesterone acetate on semen quality, reproductive hormones, and germ cell populations in normal young men. J Clin Endocrinol Metab 2002;87:546–56.
7. Coviello AD, Matsumoto AM, Bremner WJ, Herbst KL, Amory JK, Anawalt BD, et al. Low-dose human chorionic gonadotropin maintains intratesticular testosterone in normal men with testosterone-induced gonadotropin suppression. J Clin Endocrinol Metab 2005;90:2595–602.
8. Coviello AD, Bremner WJ, Matsumoto AM, Herbst KL, Amory JK, Anawalt BD, et al. Intratesticular testosterone concentrations comparable with serum levels are not sufficient to maintain normal sperm production in men receiving a hormonal contraceptive regimen. J Androl 2004;25:931–8.
9. Page ST, Kalhorn TF, Bremner WJ, Anawalt BD, Matsumoto AM, Amory JK. Intratesticular androgens and spermatogenesis during severe gonadotropin suppression induced by male hormonal contraceptive treatment. J Androl 2007;28:734–41.
10. Liu PY, Baker HWG, Jayadev V, Zacharin M, Conway AJ, Handelsman DJ. Induction of spermatogenesis and fertility during gonadotropin treatment of gonadotropin-deficient infertile men: predictors of fertility outcome. J Clin Endocrinol Metab 2009;94:801–8.
11. Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, et al. Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. Mol Endocrinol 1999;13:681–91.
12. Nef S, Parada LF. Cryptorchidism in mice mutant for Insl3. Nat Genet 1999;22:295–9.
13. Wikstrom AM, Bay K, Hero M, Andersson AM, Dunkel L. Serum insulin-like factor 3 levels during puberty in healthy boys and boys with Klinefelter syndrome. J Clin Endocrinol Metab 2006;91:4705–8.
14. Ferlin A, Garolla A, Rigon F, Rasi Caldogno L, Lenzi A, Foresta C. Changes in serum insulin-like factor 3 during normal male puberty. J Clin Endocrinol Metab 2006;91:3426–31.
15. Foresta C, Bettella A, Vinanzi C, Dabrilli P, Meriggiola MC, Garolla A, et al. A novel circulating hormone of testis origin in humans. J Clin Endocrinol Metab 2004;89:5952–8.
16. Bay K, Andersson AM. Human testicular insulin-like factor 3: in relation to development, reproductive hormones and andrological disorders. Int J Androl 2011;34:97–109.
17. Tenover JS, Matsumoto AM, Clifton DK, Bremner WJ. Age-related alterations in the circadian rhythms of pulsatile luteinizing hormone and testosterone secretion in healthy men. J Gerontol 1988;43:M163–9.
18. Anand-Ivell R, Wohlgemuth J, Haren MT, Hope PJ, Hatzinikolas G, Wittert G, et al. Peripheral INSL3 concentrations decline with age in a large population of Australian men. Int J Androl 2006;29:618–26.
19. Bay K, Matthiesson KL, McLachlan RI, Andersson AM. The effects of gonadotropin suppression and selective replacement on insulin-like factor 3 secretion in normal adult men. J Clin Endocrinol Metab 2006;91:1108–11.
20. Bay K, Hartung S, Ivell R, Schumacher M, Jurgensen D, Jorgensen N, et al. Insulin-like factor 3 serum levels in 135 normal men and 85 men with testicular disorders: relationship to the luteinizing hormone-testosterone axis. J Clin Endocrinol Metab 2005;90:3410–8.
21. Teixeira J, Maheswaran S, Donahoe PK. Mullerian inhibiting substance: an instructive developmental hormone with diagnostic and possible therapeutic applications. Endocr Rev 2001;22:657–74.
22. Pietilainen KH, Kaprio J, Vaaralahti K, Rissanen A, Raivio T. Circulating antimullerian hormone levels in adult men are under a strong genetic influence. J Clin Endocrinol Metab 2012;97:E161–4.
23. Meachem SJ, Nieschlag E, Simoni M. Inhibin B in male reproduction: pathophysiology and clinical relevance. Eur J Endocrinol 2001;145:561–71.
24. Illingworth PJ, Groome NP, Byrd W, Rainey WE, McNeilly AS, Mather JP, et al. Inhibin-B: a likely candidate for the physiologically important form of inhibin in men. J Clin Endocrinol Metab 1996;81:1321–5.
25. O'Connor AE, De Kretser DM. Inhibins in normal male physiology. Semin Reprod Med 2004;22:177–85.
26. Anawalt BD, Bebb RA, Matsumoto AM, Groome NP, Illingworth PJ, McNeilly AS, et al. Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. J Clin Endocrinol Metab 1996;81:3341–5.
27. Muttukrishna S, Yussoff H, Naidu M, Barua J, Arambage K, Suharjono H, et al. Serum anti-Mullerian hormone and inhibin B in disorders of spermatogenesis. Fertil Steril 2007;88:516–8.
28. Goulis DG, Tsametis C, Iliadou PK, Polychronou P, Kantartzi PD, Tarlatzis BC, et al. Serum inhibin B and anti-Mullerian hormone are not superior to follicle-stimulating hormone as predictors of the presence of sperm in testicular fine-needle aspiration in men with azoospermia. Fertil Steril 2009;91:1279–84.
29. Toulis KA, Iliadou PK, Venetis CA, Tsametis C, Tarlatzis BC, Papadimas I, et al. Inhibin B and anti-Mullerian hormone as markers of persistent spermatogenesis in men with non-obstructive azoospermia: a meta-analysis of diagnostic accuracy studies. Hum Reprod Update 2010;16:713–24.
30. Amory JK, Coviello AD, Page ST, Anawalt BD, Matsumoto AM, Bremner WJ. Serum 17-hydroxyprogesterone strongly correlates with intratesticular testosterone in gonadotropin-suppressed normal men receiving various dosages of human chorionic gonadotropin. Fertil Steril 2008;89:380–6.
31. Stege R, Eriksson A, Henriksson P, Carlstrom K. Orchidectomy or oestrogen treatment in prostatic cancer: effects on serum levels of adrenal androgens and related steroids. Int J Androl 1987;10:581–7.
32. Carlstrom K, Stege R. Adrenocortical function in prostatic cancer patients: effects of orchidectomy or different modes of estrogen treatment on basal

steroid levels and on the response to exogenous adrenocorticotropic hormone. Urol Int 1990;45:160–3.
33. Herbst KL, Coviello AD, Page S, Amory JK, Anawalt BD, Bremner WJ. A single dose of the potent gonadotropin-releasing hormone antagonist acyline suppresses gonadotropins and testosterone for 2 weeks in healthy young men. J Clin Endocrinol Metab 2004;89:5959–65.
34. Jarow JP, Chen H, Rosner TW, Trentacoste S, Zirkin BR. Assessment of the androgen environment within the human testis: minimally invasive method to obtain intratesticular fluid. J Androl 2001;22:640–5.
35. Roth MY, Page ST, Lin K, Anawalt BD, Matsumoto AM, Marck B, et al. The effect of gonadotropin withdrawal and stimulation with human chorionic gonadotropin on intratesticular androstenedione and DHEA in normal men. J Clin Endocrinol Metab 2011;96:1175–81.
36. Asatiani K, Gromoll J, Eckardstein SV, Zitzmann M, Nieschlag E, Simoni M. Distribution and function of FSH receptor genetic variants in normal men. Andrologia 2002;34:172–6.
37. Anderson RA, Wallace EM, Groome NP, Bellis AJ, Wu FC. Physiological relationships between inhibin B, follicle stimulating hormone secretion and spermatogenesis in normal men and response to gonadotrophin suppression by exogenous testosterone. Hum Reprod 1997;12:746–51.
38. Rey R. Endocrine, paracrine and cellular regulation of postnatal anti-mullerian hormone secretion by sertoli cells. Trends Endocrinol Metab 1998;9:271–6.
39. Young J, Chanson P, Salenave S, Noel M, Brailly S, O'Flaherty M, et al. Testicular anti-mullerian hormone secretion is stimulated by recombinant human FSH in patients with congenital hypogonadotropic hypogonadism. J Clin Endocrinol Metab 2005;90:724–8.