Serum Insulin-like Factor 3, Testosterone and Luteinizing Hormone in Experimental and Therapeutic Testicular Suppression

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Abstract

Background:

Serum INSL3 is a Leydig cell biomarker, but little is known about the circulating concentration of INSL3 during hypothalamus-pituitary-testicular suppression.

Aim:

To study the concomitant changes in serum concentrations of INSL3, testosterone and LH during experimental and therapeutic testicular suppression.

Methods:

We included serum samples from three different cohorts comprising subjects before and after testicular suppression; 1) Six healthy young men who were treated with androgens (Sustanon®, Aspen Pharma, Dublin, Ireland), 2) Ten transgender girls (male sex assigned at birth) who were treated with three-monthly GnRH agonist injections (Leuprorelinacetat, Abacus Medicine, Copenhagen, Denmark) and 3) Fifty-five patients with prostate cancer who were randomized to surgical castration (bilateral subcapsular orchiectomy) or treatment with GnRH agonist (Triptorelin, Ipsen Pharma, Kista, Sweden). Serum INSL3 and testosterone concentrations were quantified in stored serum samples using validated LC-MS/MS methodologies, and LH was measured by an ultrasensitive immunoassay.

Results:

The circulating concentrations of INSL3, testosterone and LH decreased during experimental testicular suppression in healthy young men by Sustanon injections and subsequently returned to baseline levels after release of suppression. All three hormones decreased during therapeutic hormonal hypothalamus-pituitary-testicular suppression in transgender girls and in prostate cancer patients.

Conclusion:

INSL3 resembles testosterone as a sensitive marker of testicular suppression and reflects Leydig cell function, also during exposure to exogenous testosterone. Serum INSL3 measurements may complement testosterone as a Leydig cell marker in male reproductive disorders, during therapeutic testicular suppression as well as in surveillance of illicit use of androgens.

Introduction

The peptide hormone Insulin-like factor 3 (INSL3) is produced by the testicular Leydig cells and by the theca cells of the ovaries (1). INSL3 is important for testicular descent in prenatal life and targets the receptor Relaxin Family Peptide Receptor 2 (RXFP2). Mutations in the *INSL3* or *RXFP2* genes result in impaired testicular descent during embryogenesis in rodents and are rare causes of cryptorchidism in humans (2, 3). Little is known about its postnatal function, but INSL3 may be involved in regulation of male germ cell survival (4), bone metabolism (5) and muscle growth (6). INSL3 is of clinical interest since the circulating level of INSL3 reflects Leydig cell maturity and function (8) and INSL3 is commonly reduced in men with hypogonadism (9, 10).

It has been proposed that INSL3 may serve as a complementary diagnostic marker to testosterone – the other major secretory product of the Leydig cells and an established biochemical marker in the clinic. Testosterone is not only expressed by the testicular Leydig cells, but also originates from the adrenal glands and this complicates the diagnostic interpretation of serum testosterone as a biochemical marker of hypogonadism. In addition, interpretation of testosterone levels is further complicated by its association with body composition and lifestyle, which may influence sex hormone binding globulin (SHBG) and thereby the biologically active free fraction of testosterone. By contrast, INSL3 is expressed only by the Leydig cells and the relationship between INSL3 and lifestyle factors appear to be less pronounced. Thus, INSL3 is not associated with BMI, whereas higher BMI is associated with lower total testosterone (11). Smoking is associated with lower INSL3 (11, 12), but with higher testosterone. In addition, although the two Leydig cell hormones INSL3 and testosterone are both regulated by the pituitary luteinizing hormone (LH), they appear to differ in their response to changes in LH (11). Here we investigate concomitant changes in circulating INSL3, testosterone and LH before and after experimental hormonal testicular suppression by androgens in healthy young men, and in patients undergoing therapeutic testicular suppression by gonadotropin-releasing hormone agonists (GnRHa) or orchiectomy.

Methods and materials

Subjects and ethical considerations

This paper is based on blood samples collected in conjunction with three studies (Table 1):

Four healthy adult men (mean age: 23.5 years (SD=1.9)) were subjected to experimental testicular suppression by intramuscular Sustanon injections (250 mg, Aspen Pharma, Dublin, Ireland), as previously described (13). Intramuscular Sustanon was injected on day 1 and 21, and serum testosterone, LH and INSL3 were measured at baseline (day 0) and on day 1, 3, 5, 10, 14, 21, 22, 24, 26, 31, 35 and 42. Sustanon is a mixture of four different testosterone esters; i.e. 30 mg testosterone propionate, 60 mg testosterone isocaproate, 60 mg testosterone phenylpropionate and 100 mg testosterone decanoate which all contribute to the quantification of serum testosterone by our LC-MS/MS method. The participants were fully informed, both orally and in writing, of the experimental procedures and of potential risks and discomforts associated with participation, before signing a written consent. The study was approved by the local ethics committee of Copenhagen, Denmark (H-17011319), and performed in accordance with the Declaration of Helsinki of 1964 and its later amendments.

Ten transgender girls (mean age: 15.9 (1.7) years) were treated with three-monthly GnRH agonist at day 1, 30 and 90, respectively. Testosterone, LH and INSL3 were measured in serum collected immediately prior to each treatment (day 1) and after 30 and 90 days. The treatment included two injections of GnRH agonist given as leuproreline acetate (Abacus Medicine, Copenhagen, Denmark) 11.25 mg with an interval of 30 and 90 days, respectively. The GnRH analogue treatment was part of the routine gender-affirming hormone treatment (GAHT) of transgender girls . All serum samples were collected in connection with routine blood samples. Written informed consent were obtained either by the adolescent person itself (above 18 years of age), the parents (less than 15 years of age) or the parents and the young person (between 15 and 18 years of age). The

study was approved by the Danish Data Protection Agency (P-2019-230) and the ethics committee (H-18050607).

Patients with prostate cancer (n=55) were randomized to either subcapsular orchiectomy (SO) (n=26, mean age: 72(8.8) years) or GnRH agonist injection (n=29, mean age: 71(5.8) years) given as triptorelin 22.5 mg intramuscular (IPSEN, Kista, Sweden). Participants allocated to the GnRH arm were treated with the anti-androgen bicalutamide 50 mg daily for 30 days upon first injection (as previously described (14). Serum testosterone, LH and INSL3 were measured on day 1 immediately prior to treatment, and 90 and 180 days after treatment. The trial was conducted in accordance with the Declaration of Helsinki and the legal regulations in Denmark. Permission was obtained from the Danish Medicines Agency (EudraCT 2013-002553-29; registered on www.clinicaltrialsregister.eu) and the Capital Regional Committee on Health Research Ethics in Denmark (H-2-2013-107). All patients gave oral and written consent prior to inclusion.

Hormone analysis

All blood samples were drawn from an antecubital vein, clotted and centrifugated, and serum was stored at -20 or -80°C until analysis. In all participants of this study, serum INSL3 was determined by LC-MS/MS as previously described (15). The limit of detection (LOD) and quantification were 0.03 and 0.15 µg/l, respectively, and the intraassay variation was < 10%. Serum testosterone was measured in the group of Sustanon treated healthy men and in the group of transgender girls by LC-MS/MS as previously described (13). The quantification limit was 0.1 nM and the intra-assay variation was < 5%. In the Sustanon-treated men and the transgender girls, serum LH was determined by using the time-resolved immunofluorometric assay (Delfia; PerkinElmer, Boston, MA, USA, RRID: AB278338) and the limit of detection was 0.05 U/l and the inter-assay variation was < 2%. In the prostate cancer patients, serum testosterone was measured by LC-MS/MS and the limit of quantification

was 8.6 ng/dl and the inter-assay variation was < 7% and LH was measured by immunoassay (ADVIA Centaur, Siemens, Germany, RRID: AB2905665) and the limit of detection was 0.3 IU/l and the intra-assay variation was < 3%, as previously described (14).

Statistical methods

Means, standard deviations and p-values (t-test for means) were calculated in Excel (Microsoft, 2016).

Results

Testicular suppression by androgens in healthy men

Serum testosterone increased immediately after each treatment with Sustanon from a baseline mean of 20.9 (3.4) nM to peak mean levels of 64.9(21.0) and 81.4 (22.9) nM on day 1 and 21, respectively (Figure 1A). Serum testosterone declined on day 3 and reached nadir levels below the respective baseline levels in all four men after 10-14 days. Hereafter testosterone increased slightly and reached baseline levels. Serum LH decreased after each Sustanon injection from a baseline mean of 4.7 (1.6) U/l and reached nadir values of 0.9(0.3) and 0.8(0.2) U/l after 3 -10 days, respectively. Hereafter serum LH increased and reached baseline levels approximately 14-21 days after each Sustanon injection (Figure 1B). Like LH, serum INSL3 decreased after each Sustanon injection from a baseline mean of 1.1(0.2) µg/L and reached nadir values of 0.1(0.1) µg/L 5-10 days later (Figure 1C) and returned to baseline levels approximately 21-22 days after each injection. The nadir levels of LH and INSL3 were reached 7.9(3.0) and 9.4(1.8) days after Sustanon injection, respectively (t-test for means, p = 0.23). Return to baseline levels for LH and INSL3 occurred 19.3(3.2) and 21.1(0.4) days after Sustanon injection, respectively (p = 0.13). Thus, the changes in INSL3 were slightly delayed as compared to LH, but this was not statistically significant.

Testicular suppression by GnRH agonist in Transgender girls

Serum testosterone decreased from a baseline mean of 13.5 (6.5) nM at day 1 to 0.9(0.6) and 3.3 (6.3) nM after 30 and 90 days, respectively (Figure 2A). Mean serum LH decreased from a mean baseline of 3.1 (1.4) U/l to 0.7 (0.5) and 1.1 (1.4) U/l after 30 and 90 days, respectively (Figure 2B). Mean serum INSL3 decreased from a baseline mean of 0.8 (0.4) μg/L to 0.1 (0.1) and 0.3 (0.3) μg/L after 30 and 90 days, respectively (Figure 2C).

Testicular suppression by GnRH agonist in prostate cancer patients

In the subcapsular orchiectomy (SO) treated group, mean serum testosterone decreased from 16(5.7) nM to 0.6(0.2) and 0.5(0.2) nM after 90 and 180 days, respectively (Figure 3A). Serum LH increased from a baseline mean of 5.5(3.3) U/l to mean levels of 31.2(12.9) and 32.6(13.8) U/l after 90 and 180 days, respectively (Figure 3B). Serum INSL3 decreased from a baseline mean of 0.44(0.28) μg/L to below the limit of detection (LOD: 0.03 μg/L) in all samples collected 90 and 180 days after treatment (Figure 3A). In 28 of the 29 patients treated with Triptorelin, serum testosterone decreased from a baseline mean of 13.1(6.4) nM to mean levels of 0.4(0.1) and 0.4(0.2) nM after 90 and 180 days, respectively (Figure 3D). Serum LH decreased from a baseline mean of 6.7(7.4) to a mean of 0.3(0.07) U/l and 0.3 (0.07) U/l after 90 and 180 days, respectively (Figure 3E). In 22 of the patients treated with triptorelin (D-F), mean serum INSL3 decreased from 0.47(0.34) μg/L to below the LOD at both 90 and 180 days after treatment, whereas six patients treated with triptorelin differed slightly in that serum INSL3 remained above the LOD (0.03 to 0.09 μg/L) in serum collected 90 and 180 days after treatment (Figure 3D). Finally, of the 29 patients treated with Triptorelin there was one significant outlier. In this patient both testosterone, LH and INSL3 decreased from baseline and until 90 days after treatment, after which the

levels of all three hormones increased again in serum collected at 180 days after treatment (Figure 3D-F, dark green line).

Discussion

We investigated the dynamics of serum INSL3, LH and testosterone in experimental and therapeutic testicular suppression. The measured concentrations of serum testosterone increased immediately after Sustanon treatment, as expected. This was followed by an immediate decrease in the level of circulating testosterone likely due to rapid clearance of testosterone from the circulation in parallel with a suppression of endogenous testosterone secretion due to negative feedback of the Hypothalamic-Pituitary-Gonadal (HPG) axis. The induced negative feedback may also explain why serum testosterone decreased to levels even below the respective baseline levels in each of the four men, after which it gradually returned to baseline levels. The testosterone level in men following testosterone injections is composed of exogenous as well as endogenous testosterone and must be interpreted with caution. Following an intramuscular depot injection, the testosterone esters are slowly released into the blood stream where the esters are hydrolyzed by esterase enzymes to give active testosterone. In the present study “non-stabilizing tubes” were used for collection of serum and this may cause the testosterone esters to further hydrolyze (16). Serum LH and INSL3 were decreased immediately after Sustanon injections suggesting an immediate induction of negative feedback of the HPG axis after exposure to exogenous testosterone. In this context, serum INSL3 complements testosterone measurements and reflects the immediate reduction in Leydig cell function that is masked by the high levels of exogenous testosterone. As such, INSL3 is a potential complementary biomarker for detection of exogenous testosterone administration within anti-doping. Three previous studies have shown that serum INSL3 decrease significantly during experimental gonadotropin suppression in healthy men (17, 18, 19) and

testosterone appeared to recover better than INSL3 after long-term suppression (17). In the present study there was no evidence that short term testicular suppression has a lasting impact on the circulating levels of INSL3, testosterone or LH. However, a recent study found that long term testicular suppression by illicit use of AAS resulted in reduced serum INSL3 for many years after AAS cessation in men, independently of testosterone, suggesting persistently impaired Leydig cell function (20).

In the transgender girls treated with GnRH analogue on day 1 and 30, all three hormones were strongly suppressed on day 30 and 90 and serum testosterone and INSL3 appeared equally sensitive as markers of testicular suppression. The mean baseline INSL3 was 0.8 µg/L in the ten transgender girls (mean age = 16.1 years), as compared to a mean of 1.0 µg/L in the four adult healthy men subjected to Sustanon injections (mean age = 23.5 years). This can be explained by the normal increase in serum INSL3 during pubertal development where the maximum level is reach at approximately 20 years of age (11). In the transgender girls INSL3 decreased approximately two-fold after treatment (mean=0.3 µg/L, after 90 days), compared to a ten-fold decrease in INSL3 in the group of healthy adult men treated with Sustanon. Although based on a small cohort of subjects, these results indicate that complete testicular suppression may be more challenging in adolescents during pubertal development, as compared to adult men.

In the third group of 55 older Danish men with prostate cancer the average baseline INSL3 was 0.45 µg/L, as compared to 1.0 ug/L in the group of four young healthy men. Cohort studies (11, 12, 21) have now established that older age is associated with a continuing and relatively pronounced decrease in circulating INSL3, as compared to a relatively minor decrease in testosterone. This supports a gradual impairment of Leydig cell function as men age and it supports that the compensatory increase in LH commonly observed in older men is sufficient to maintain testosterone, but not INSL3 levels. Thus, the relatively low baseline serum INSL3 level observed in the older patients is considered physiological for older men and likely not associated with prostate cancer. In the group of older prostate cancer patients that were treated with orchiectomy there was a

substantial increase in serum LH, caused by positive feedback of the HPG axis due to depletion of circulating testosterone, as expected. Despite high circulating levels of LH, serum INSL3 decreased to undetectable levels supporting that in men the testes are the primary, and possibly only, source of circulating INSL3. Serum testosterone also decreased dramatically following orchiectomy but remained present in detectable levels likely due to a persistent production of testosterone in the adrenal glands. In this context, serum INSL3 may complement testosterone in that it reflects the Leydig cell function only. In the group of prostate cancer patients treated with Triptorelin, all three hormones decreased except for one patient, a “fast metabolizer”, where all three hormones increased again at day 180. The fact that INSL3 remained above the LOD after treatment with Triptorelin in some, but not all, patients may suggest varying interindividual sensitivity to GnRH suppression. Also, 5 out of 6 Triptorelin treated patients with measureable levels of INSL3 had LH < LOD. Thus, INSL3 may not be entirely dependent on LH. Nonetheless, it is well-established that Triptorelin is effective in suppressing testosterone in prostate cancer patients (14).

A limitation of the study is the few samplings in the study groups 2 and 3 (Table 1). A more frequent sampling could possibly reveal differences in the dynamics of INSL3 and testosterone not visible in the present study. A second limitation of our study is the small number of participants in two of the three sample groups (four and ten individuals, respectively, Table 1). Accordingly, there is a need to replicate these results in a larger number of participants. A third limitation of our study is that the experiments were not originally designed with the purpose of studying INSL3 as the primary end point after testicular suppression. To conclude, serum INSL3 and testosterone appear to be equally sensitive and similar markers of testicular suppression. Serum INSL3 measurements may complement testosterone as a Leydig cell marker in male reproductive disorders, during therapeutic testicular suppression, and in anti-doping and illicit use of AAS.

Data Availability

All data generated or analyzed during this study are included in this article or in the data repositories listed in References.

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Tables

Table 1

Group	Treatment	Participants (n)	Gender	Age (years)	BMI (kg/m2)
1: Healthy volunteers	Experimental androgen injections (sustanon)	4	M	23.5 (1.9)	27.2 (4.4)
2: Transgender girlss	Androgen deprivation therapy by GnRH agonist (leuproreline acetate)	10	MtF	15.9 (1.7)	21.6 (4.4)
3: Prostate cancer patients	Androgen deprivation therapy by GnRH agonist (triptorelin)	29	M	75 (5.8)	27.6 (3.5)
4: Prostate cancer patients	Androgen deprivation therapy by subcapsular orchiectomy	28	M	72 (8.8)	26.9 (4.8)

Mean (SD); BMI, body mass index; MtF, Male to Female transgender individuals

Figure legends

Figure 1

Four adult healthy men (mean age: 23.5(1.9) years) were treated with Sustanon on day 1 and 21. Serum testosterone (A), LH (B) and INSL3 (C) were measured at baseline (day 0) and at day 1, 3, 5, 10, 14, 21, 22, 24, 26, 31, 35 and 42. The vertical black dotted lines indicate the time of treatment (day 1 & 21). The normal reference range for healthy men aged 20-25 years is indicated by the horizontal black (mean) and grey lines (normal limits).

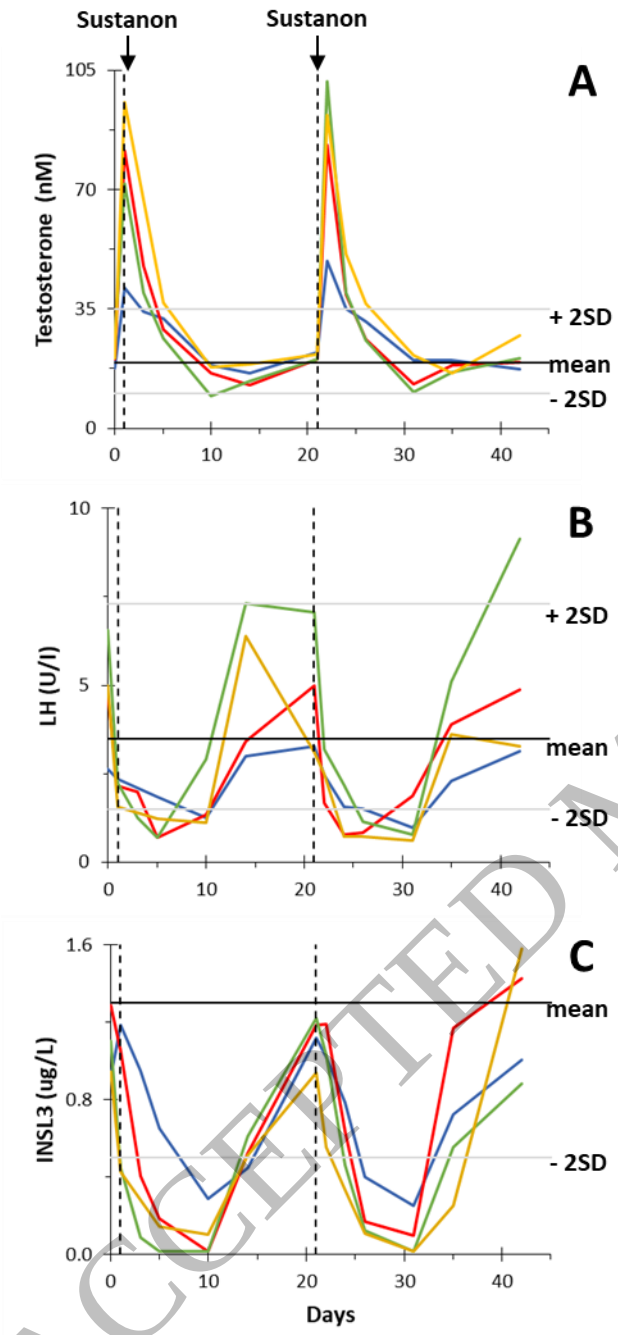
Figure 2

Ten transgender girls (male sex assigned at birth) (mean age: 16.1(1.6) years) were treated with GnRH agonist on day 1 and 30. Serum testosterone (A), LH (B) and INSL3 (C) were measured on day 1, 30 and 90. The black dotted lines indicate the time of treatment (day 1 & 30). The normal reference range for healthy cis male adolescents aged 15-18 years is indicated by the horizontal black (mean) and grey lines (normal limits).

Figure 3

A total of 55 prostate cancer patients (mean age: 71.5(6.2) years) were treated with subcapsular orchiectomy (n=26) (A, B, C) or Triptorelin (n=29) (D, E, F) on day 1. Serum testosterone (A&D), LH (B&E) and INSL3 (C&F) were measured on day 1, 90 and 180. The vertical black dotted lines indicate the time of treatment (day 1). The normal reference range for healthy men aged 65-76 years is indicated by the horizontal black (mean) and grey lines (normal limits).

Figure 1



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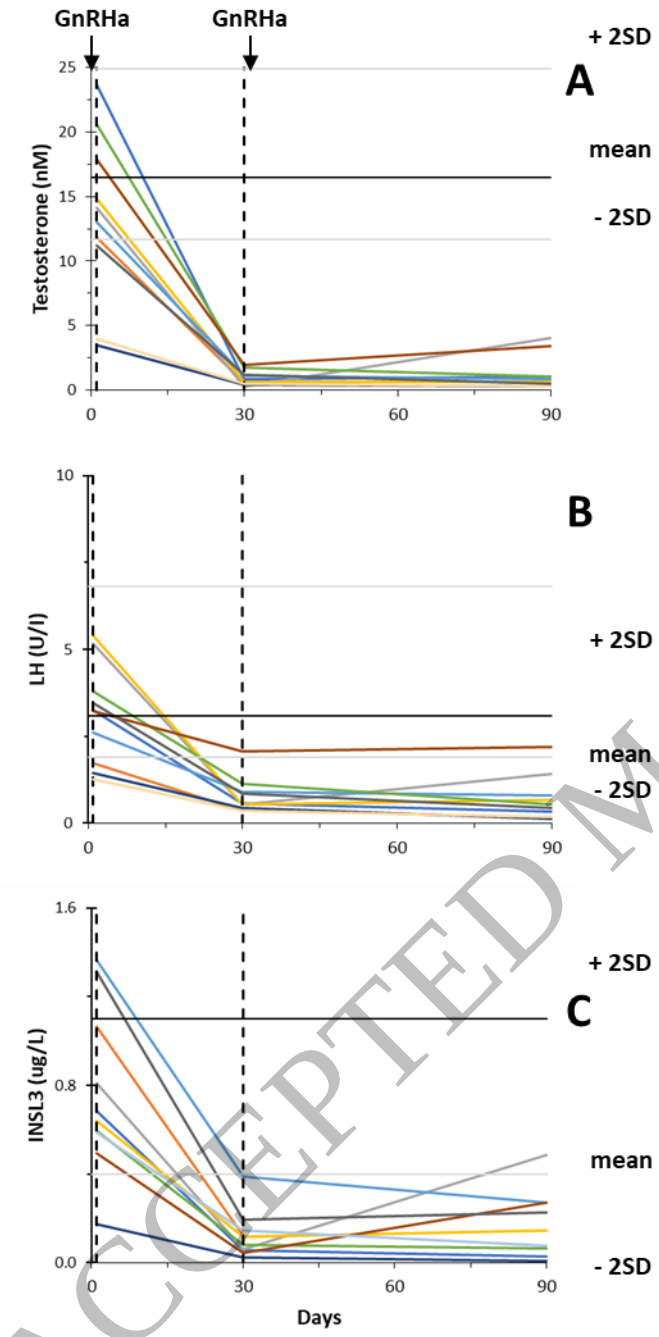
2 **Figure 2**



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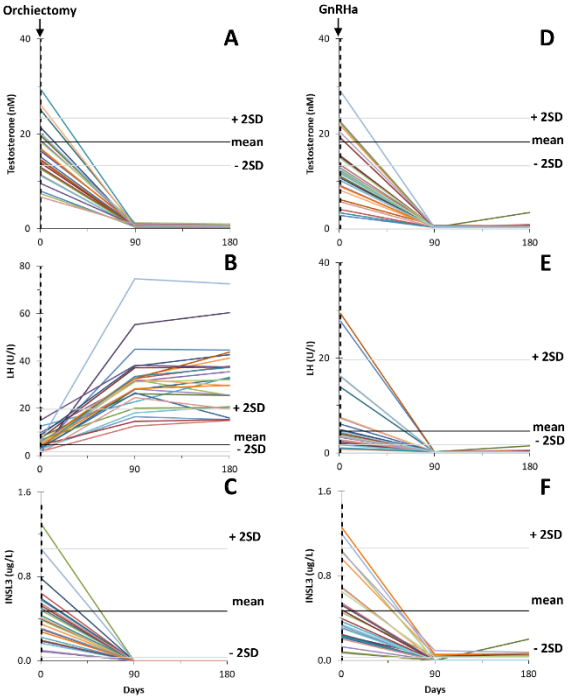
2 **Figure 3**



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