Effects of acute hCG stimulation on serum INSL3 and 25-OH vitamin D in Klinefelter syndrome

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Article type : Original Article

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Abstract

Background: It has recently been suggested that the hypergonadotropic hypogonadism characterizing Klinefelter syndrome (KS) might not be due to a steroidogenic dysfunction *per se*, but mainly to an altered testosterone (T) secretion into the bloodstream. However, the Leydig cell functionality remains incompletely studied in KS, and new markers should be considered. Previous data indicated that chronic hCG stimulation influence the production of both Insulin-like peptide 3 (INSL3) and 25-hydroxy-vitamin D (25-VD) in eugonadal men.

Aim of the study: To evaluate INSL3 and 25-VD serum levels, as markers of Leydig cell functionality, in association with sex steroids, after an acute hCG test in a group of KS patients and healthy volunteers.

Methods: A retrospective analysis of a prospective, case-control, clinical trial was carried out. KS patients (n=11) and age-matched healthy controls(n=11) provided a basal blood sample (V0) immediately followed by a single intramuscular injection of hCG 5000 IU. Blood samples were taken in the following five days(V1-V5).

Results: At baseline, INSL3 was lower in KS patients compared to controls (p=0.007). When adjusted for INSL3 levels, the production of steroids was similar between KS patients and controls. 25-VD was in the insufficient range both in KS patients and controls and was not different (p=0.064). Acute hCG stimulation increased neither INSL3 nor 25-VD in both KS patients and controls. In controls, an inverse correlation was detected between INSL3 levels and body mass index (p=0.020) and waist circumference (p=0.020).

Conclusions: INSL3 secretion is independent from steroidogenesis and its production is mostly not influenced by acute hCG stimulation both in KS men and controls. INSL3 serum levels should be considered as a marker of Leydig cell differentiation and numbers rather than steroidogenesis. 25-VD serum levels are also not increased by a single acute hCG administration, which was not able to restore the normal concentrations of 25-VD.

Introduction

Klinefelter syndrome (KS) is a clinical condition characterized by chromosome aneuploidy, generally 47,XXY, with hypergonadotropic hypogonadism and testicular alterations, such as fibrosis accompanied by hyalinization of seminiferous tubules and Leydig cell hyperplasia [1-3]. Historically, reduced testosterone (T) production has been confirmed by several authors for KS men following stimulation with human chorionic gonadotropin (hCG) [4-8]. These observations have sustained for many years the notion that hypergonadotropic hypogonadism is due to an enzymatic defect in testicular steroidogenesis. Recently, this paradigm has been challenged by showing that hCG stimulation is able to activate the first steroidogenic steps normally in KS Leydig cells, thereby increasing serum T levels as in healthy subjects [9]. Thus, the reduced serum T levels typical of KS patients are probably not due to a disruption of steroidogenesis but rather to a reduced secretion of T from the testis into the circulation [10]. With this in mind, the real Leydig cell functionality in KS remains unclear. Other testicular hormones and functions should be evaluated to understand better Leydig cell functionality in this context [11,12].

Insulin-like peptide 3 (INSL3) is a member of the relaxin-insulin family of peptide hormones and in male mammals is produced by the mature Leydig cells of the testes [13]. INSL3 is known to have a role in gubernacular differentiation and testicular descent during embryonic development [14,15]. Its role in adults is less well understood and several authors have suggested that INSL3 serum levels could be considered as a serum biomarker of Leydig cell function and a peripheral marker of intratesticular T levels [16,17]. These hypothetical roles come from the demonstration that serum INSL3 drastically declined after chronic gonadotropin deprivation and could be partly restored after four days of hCG stimulation in healthy subjects [18]. However, in the same time-frame, the hCG administration considerably increased serum T levels, rather than INSL3, suggesting that INSL3 secretion is independent of T production and not acutely stimulated by hCG [18]. Thus, INSL3 should probably be considered less as a marker of steroidogenesis and intratesticular T, but rather as an indirect marker of Leydig cell functional capacity (Leydig cell numbers x differentiation status) [19].

Leydig cell functionality may be further evaluated considering the recently-proposed cross-talk between testis and bone. Leydig cells seem able to modulate bone metabolism, directly influencing osteoblast function through INSL3 production [19,20], and indirectly modulating

the expression of the *CYP2R1* gene [21,22]. This gene encodes the enzyme involved in 25-hydroxylation of vitamin D (25-VD), which is a key regulatory factor of bone mineralization and calcium homeostasis [21,22]. This novel link between testis and bone is further underscored by the reduced levels of both INSL3 and 25-VD serum levels [23,24] in hypogonadal patients. However, whether VD 25-hydroxylase expression and *CYP2R1* transcription in Leydig cells is regulated by gonadotropin (hCG) stimulation has so far been poorly investigated, particularly in KS patients . Indeed, *in vitro* and *in vivo* studies showed that CYP2R1 expression in Leydig cells appeared to be hCG dependent [25] and long-term treatment with hCG in hypogonadal men with hypovitaminosis D is able to restore 25-VD levels [26].

In this study, we evaluated Leydig cell function in KS patients , considering INSL3 and 25-VD as new functional markers. In particular, we evaluated the ability of the Leydig cells to respond to acute hCG stimulation, comparing KS patients to healthy age-matched controls.

Materials and Methods

Study design

A retrospective evaluation of a previous, prospective, case-control clinical trial [9] was carried out.

In brief, the study design included six visits. At 8:00 a.m. of the first visit (V0), all subjects provided a basal blood sample immediately followed by a single intramuscular injection of hCG, 5000 IU. During the first visit (V0), the subjects underwent physical examination (height, weight, body mass index (BMI), arm span, and upper segment measurement) and testicular ultrasound for the calculation of testicular volume. Further visits were performed in the five days following hCG injection (V1, V2, V3, V4 and V5). A blood sample was taken at each visit after an overnight fast. Blood samples were centrifuged at 3600 rpm for 15 minutes. Sera were transferred into plain polypropylene tubes and stored at -20°C until assayed.

Thirteen KS patients and 12 age-matched, healthy control subjects were enrolled in the original study. Of these patients, blood samples were available for INSL3 and 25-VD measurements in 11 KS patients and 11 controls. For this retrospective analysis not all samples were available in sufficient volume for the measurement of INSL3 at all time-points

Inclusion criteria for the KS patients were: (i) genetic diagnosis of KS by karyotype analysis showing non mosaic 47,XXY karyotype on 50 metaphases, (ii) age between 18 and 45 years, and (iii) no current or past androgen replacement therapy (naïve KS patients). Inclusion criteria for the control group were: (i) 46,XY karyotype, (ii) normal testosterone and gonadotropin serum levels, (iii) age between 18 and 45 years, and (iv) no history of pubertal delay, chronic disease and absence of risk factors for testicular dysfunction.

Hormonal evaluation

INSL3 serum levels were measured by a well validated time-resolved fluorescent immunoassay [13].

Progesterone, 17-hydroxy-progesterone (17-OHP), Androstenedione, dehydroepiandrosterone (DHEA), and T were determined by liquid chromatography, tandem mass spectrometry (LC–MS/MS) at the laboratory of the Centre for Applied Biomedical Research of the S. Orsola-Malpighi Hospital, Alma Mater Studiorum, University of Bologna, Bologna, Italy, as previously reported [9].

Finally, 25-VD was measured by a chemiluminescent immunoassay on a fully automated platform (LIAISON XL 25 Dihydroxyvitamin D, DiaSorin S.p.A Italy). The assay was performed in the laboratory of endocrinology, belonging to the Department of Laboratory Medicine and Pathology, AUSL Modena.

Statistical analysis

Data are shown as means and standard deviation. Data distribution was evaluated by Kolmogorov-Smirnov test. First, to better understand the trending profile of INSL3 after hCG stimulation and to confirm its constitutive production, V1 and V2 data were also pooled together, as well as V4 and V5, reducing the data variance, since the dynamics of any INSL3 increase are not known. Moreover, data were expressed as percentage variation relative to baseline (V0) levels. Then, the comparison between KS patients and controls was performed considering patients at baseline (V0). The T on INSL3 ratio (T/INSL3) and 17-OHP on INSL3 ratio (17-OHP/INSL3/) were also calculated using INSL3 values to correct for possible variations in Leydig cell functional capacity.

ANOVA univariate and Mann-Whitney U test were used for normally and not-normally distributed parameters, respectively. The INSL3 and 25-VD response to hCG stimulation was evaluated comparing data among visits using the Kruskal-Wallis test. The Tukey test was used to perform post hoc analyses. Moreover, INSL3 serum levels were compared between KS patients and controls at each visit, using ANOVA univariate or Mann-Whitney U test for normally and not-normally distributed parameters, respectively. The correlation between INSL3 and other parameters collected were evaluated by Pearson and Rho's Spearman correlation tests, for normally and not-normally distributed parameters, respectively.

Statistical analysis was performed using the 'Statistical Package for the Social Sciences' software for Windows (version 21.0; SPSS). For all comparisons, p-values of <0.05 were considered to be statistically significant.

Results

Baseline Leydig cell functionality

The steroid profiles after hCG stimulation in KS patients and controls have been previously published [9,27].

On average, baseline INSL3 levels (V0) were lower in KS patients (444 + 350 pg/mL) than in controls (780 + 190 pg/mL) (p=0.007) (Table 1).

T serum levels were significantly lower in KS patients compared to controls (9.35 + 3.34 *vs* 15.16 + 4.77 nmol/L, p=0.004), whereas 17-OHP serum levels were not significantly different between KS patients and controls (2.97 + 0.80 *vs* 4.68 + 2.59 nmol/L, p=0.052). When Leydig cell steroid production was adjusted for INSL3 levels, as a measure of the number and differentiation status of the Leydig cells, the T/INSL3 and 17-OHP/INSL3 ratios were not significantly different between KS patients and controls (p=0.251 and p=0.282, respectively). This result suggests that the Leydig cell steroidogenic potential is preserved in this genetic syndrome.

At baseline, 25-VD serum levels were not significantly different between KS patients and controls (22.76 + 5.84 *vs* 17.14 + 6.84 ng/mL, p=0.064), although below the normal ranges (i.e. >30 ng/mL) [28] in both groups (Table 1).

In KS patients , basal INSL3 levels did not significantly correlate with serum T levels (Rho 0.575, p=0.064). Moreover, no correlations were found between INSL3 and 17-OHP (Rho 0.409, p=0.212), androstenedione (Rho 0.238, p=0.482) and 25-VD (Rho 0.118, p=0.729). However, although the KS patients' testes were small, INSL3 significantly correlated with testicular volume (Figure 1), but not with patients' age or anthropometric parameters. Basal 25-VD serum levels were not related to T (Rho -0.218, p=0.519), 17-OHP (Rho -0.155, p=0.650), androstenedione (Rho -0.173, p=0.612), testicular volume, patients' age, or anthropometric parameters.

In controls, INSL3 was inversely related to BMI (Rho -0.750, p=0.020) and waist circumference (Rho -0.750, p=0.020), while no significant correlations were found between INSL3 and serum testosterone (Rho 0.202, p=0.663), 17-OHP (Rho -0.550, p=0.125), androstenedione (Rho 0.171, p=0.660), or 25-VD (Rho 0.588, p=0.074). Moreover, no correlations were found among INSL3 and testicular volume, controls' age and other anthropometric measures. Similarly, basal 25-VD did not correlate with T (Rho -0.079, p=0.829), 17-OHP (Rho -0.212, p=0.556), androstenedione (Rho -0.248, p=0.249), testicular volume, controls' age or anthropometric parameters.

Human chorionic gonadotropin (hCG) stimulation

HCG stimulation did not significantly change INSL3 and 25-VD serum levels, neither in KS patients nor in controls (Table 1). Interestingly, the INSL3 variation after hCG administration is heterogeneous among patients in both groups (Figure 2). To confirm the constitutive pattern of INSL3 production, the secretion pattern was compared between INSL3 and 17-OHP (Figure 3). When the data were pooled across subjects for V1 and V2 as well as for V4 and V5, there was no significant effect of hCG stimulation on INSL3 for either controls or KS patients (Figure 3). These results suggest that hCG stimulation rapidly increases 17-OHP serum levels and, consequently, T, without changes in INSL3 serum levels. However, a small INSL3 increase at V1 and V2 upon hCG stimulation was observed in two KS patients and four controls (Figure 2). Taken together, these results confirm that INSL3 production and secretion are acutely independent from steroidogenesis and are not significantly influenced by a single bolus hCG stimulation in both KS patients and controls.

Considering each visit, INSL3 remained significantly higher in controls compared to KS patients at V1, V4 and V5 (Table 1). On the other hand, INSL3 serum levels were similar between KS patients and controls at V2 and V3 (Table1). Similarly, both T/INSL3 and 17-OHP/INSL3 ratios remained similar between KS patients and controls at each visit (data not shown). Finally, 25-VD did not differ between KS patients and controls at any visit.

Discussion

Here, we confirm that KS patients' Leydig cells on average secrete INSL3 into the blood circulation at a slightly reduced rate compared to healthy subjects [29]. On average, INSL3 serum levels are 45% lower than in healthy age-matched controls [29,30], in line with previous reports [23,24]. In our small group of patients , we first confirm that INSL3 production is independent from steroidogenesis and is constitutively represented. Interestingly, although the average INSL3 serum levels are lower in KS patients than controls, a high variability is observed, and some KS patients have INSL3 serum levels within the reference range. Indeed, also T levels in KS patients are normal in about 50% of cases, although the mean values are lower than normal. Acute hCG stimulation is not able to increase the INSL3 production rate, which remains stable and is probably related to cell differentiation status and number.

Here, we confirm low T serum levels in KS patients , as previously reported [1,31]. However, the old paradigm of low T levels in KS men due to steroidogenic dysfunction has been recently changed. In 2010, an experimental mouse model of KS (41,XXY) was generated, showing hyperplastic Leydig cells hyper-reactive to hCG *in vitro*, with intratesticular T levels comparable to wild type mice [32-34]. In 2014, high intratesticular T levels were demonstrated for the first time in KS men, despite reduced serum T levels [10]. Accordingly, in 2016, we demonstrated that steroidogenesis starts normally in KS patients after acute hCG stimulation [9]. This evidence suggests that Leydig cell function, at least in regard to steroidogenesis, is not impaired in KS and that the low T levels detected in the blood could be due to lower T release into the blood circulation [10]. Here, we have not evaluated intratesticular T, thus we could not speculate on the real T production in KS patients . However, we show a constitutive INSL3 production, independent from steroidogenesis and acute hCG stimulation and probably reflecting the Leydig cell functional capacity [30,35,36]. When steroid production (i.e. T and 17-OHP) was adjusted for

Leydig cell functional capacity (i.e. INSL3 serum levels), no differences are detected between KS patients and controls, confirming that the Leydig cell capability is preserved in KS patients.

Several authors have evaluated INSL3 serum levels after chronic hCG stimulation. First, when gonadotropic suppression is achieved in eugonadal men by GnRH agonist [37] or antagonist [17] administration, the Leydig cell activity is impaired, with a consequent reduction in INSL3 and T levels [38-40]. Then chronic Leydig cell stimulation with endogenous gonadotropins leads to inhibition of the hypothalamic-pituitary-gonadal axis, with a consequent Leydig cell de-differentiation and INSL3 reduction [38-40]. Second, other authors suggested that chronic hCG stimulation could elicit INSL3 serum increase by inducing a further differentiation of less immature Leydig cells [30]. Here, we have evaluated an acute hCG stimulation that should not affect the Leydig cell differentiation state and thus should not be reflected in a change of INSL3 serum level. HCG acute administration activates a specific signal transduction pathway, which acutely stimulates steroidogenesis but not INSL3 production [30]. It is well known that hCG activates signal transduction pathways partly different from LH, although they act on the same membrane receptor (LHCGR) [41,42]. In particular, hCG mainly leads to intracellular cyclic adenosine mono-phosphate (cAMP) increase, which in turns acutely stimulates T production [41,42]. On the other hand, less is known about the transcriptional pathways at the basis of INSL3 production, and only few transcription factors have been found to regulate Insl3 promoter *in vitro*, such as NUR77, KLF6, COUP-TFII and SF1 [43-45]. HCG stimulation of the murine MA-10 Leydig cell line *in vitro* resulted in a transient robust increase in Nur77 mRNA, which, however, does not lead to INSL3 intracellular mRNA increase [46]. Other reports indeed showed that cAMP is involved in INSL3 production from the MA-10 Leydig cells through interaction with another transcription factor, NR4A1 [45]. Nevertheless, it is now accepted that the acute hCG stimulation of the LHCGR does not regulate INSL3 production, which, in contrast, is constitutively regulated in Leydig cells depending on their differentiation and maturity.

In our small group of healthy subjects, INSL3 is not related to T serum levels, but only to anthropometric variables, such as BMI and waist circumference. Previous reports showed negative correlation between INSL3 levels and BMI in obese men, suggesting that obesity might alter the functionality of the Leydig cells [47]. These data have been also confirmed in obese adolescent boys and a negative correlation between INSL3 and leptin was found [48]. Similarly, in

men with type 2 diabetes mellitus, waist circumference negatively correlates with INSL3 [49], again suggesting an overall Leydig cell dysfunction caused by visceral adiposity. Furthermore, Overvad and colleagues demonstrated a negative correlation between INSL3 and metabolic parameters in healthy men, such as serum glucose levels and the homeostasis model assessment (HOMA) index [29]. Therefore, anthropometric parameters could influence global Leydig cell function (INSL3 and T production). However, this is not true in our KS patients, where anthropometric characteristics are probably influenced more by the genetics-related phenotype and by the longstanding untreated hypogonadism. Although there is no evident relationship between testis size and INSL3 production in control subjects, there is indeed a significant correlation between these two parameters in KS patients. The small average size of the testes in KS patients, in spite of near normal INSL3 levels, emphasizes that the condition predominantly affects the seminiferous compartment, thereby explaining why the proportionately larger interstitial compartment can correlate with overall testis size in KS patients but not in controls. Considering that the Leydig cells of several KS patients were secreting INSL3 in the normal range, this strongly suggests that the chromosomal abnormality of KS patients is influencing the interstitial compartment far less than the seminiferous compartment, at least for some KS patients. Indeed, the testicular damage in KS starts in general from mid-puberty, and it involves both compartments, as evidenced by increased levels of FSH and LH invariably in all KS patients. However, the extent of this damage is variable among KS patients and we can speculate that the Leydig cell differentiation that normally occur during puberty could proceed variably among KS patients, therefore determining different INSL3 levels in adults as marker of the Leydig cell global activity and maturity.

We confirmed here that KS patients have low-normal 25-VD serum levels, similarly to healthy subjects who also had subnormal values. Both in mouse models and humans, the 25-hydroxilating enzyme *CYP2R1* mRNA is expressed in many tissues, with the highest relative expression in the testis [50,51]. Moreover, several authors have suggested a reduced vitamin D activation in patients with testiculopathy [20,52]. These demonstrations led to the interesting suggestion that 25-VD serum levels could be altered in KS men and related to bone mineral density and risk of osteoporosis [53]. Here, the number of patients enrolled is limited, aiming at describing only the effects of hCG stimulation on 25-VD levels. Moreover, it was suggested that *CYP2R1* expression is stimulated in the Leydig cell by hCG and LH administration in men with

hypogonadotropic hypogonadism [25,26,50,54,55]. Here, 25-VD serum levels do not increase after acute hCG administration, suggesting that the acute LHCGR activation *in vivo* is not able to stimulate testicular *CYP2R1* expression and VD 25-hydroxylation. However, these results should be cautiously evaluated, considering the small sample size in both groups and the reduced 25-VD serum levels detected at baseline in both KS patients and controls.

Our study shows important limitations. First, this is a retrospective analysis that should be cautiously evaluated. Second, the sample size is limited, although it is in line with the KS prevalence. Third, we are not able to clearly evaluate Leydig cell functionality, since intratesticular T measurement was not available. Fourth, 25-VD serum levels vary among and within subjects, and were low in all subjects, including controls. Finally, this study was not designed to evaluate the usefulness of INSL3 measurement in clinical practice. Indeed, currently due to methodological limitations the measurement of INSL3 does not find a clear clinical application in KS men, and future studies are needed.

In conclusion, this study demonstrates that INSL3 secretion is independent from steroidogenesis and its production seems to be not influenced by acute hCG stimulation both in KS men and controls. Although INSL3 serum levels should be considered as a marker of Leydig cell differentiation and numbers, its implication in clinical practice should be further analyzed with well-designed clinical trials.

Acknowledgement

We are grateful to Dr Hiroaki Soyama for help in the preliminary stages of this work.

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Table 1.

INSL3 (pg/mL)	**KS patients**	**controls**	*p-value*
Visit 0	440 + 350	780 + 190	**0.007**
Visit 1	520 + 320	830 + 190	**0.024**
Visit 2	580 + 340	790 + 290	0.133
Visit 3	460 + 340	760 + 320	0.079
Visit 4	410 + 250	780 + 310	**0.010**
Visit 5	300 + 230	720 + 260	**0.002**
p-value	0.447	0.951	
25-VD (ng/mL)			
Visit 0	22.68 + 6.98	17.14 + 6.84	0.101
Visit 1	22.97 + 5.45	16.83 + 6.73	0.060
Visit 2	22.31 + 4.06	16.92 + 6.34	0.056
Visit 3	21.85 + 4.70	17.03 + 6.62	0.076
Visit 4	21.66 + 4.48	17.27 + 6.73	0.076
Visit 5	22.04 + 4.89	16.98 + 6.12	0.056
p-value	0.999	0.999	

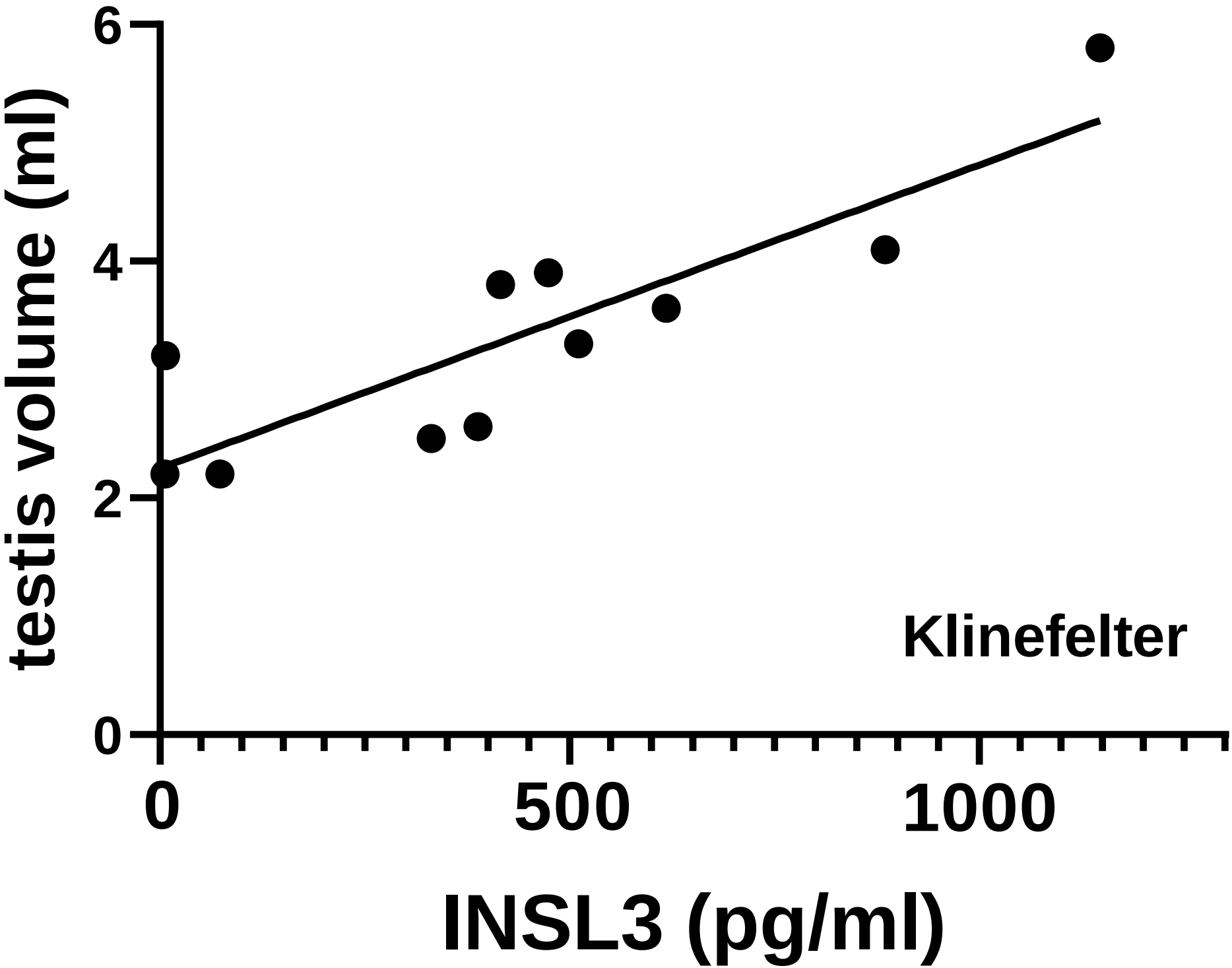
[Footnotes to Table 1]: INSL3: Insulin like peptide 3, KS: Klinefelter syndrome, VD: vitamin D.

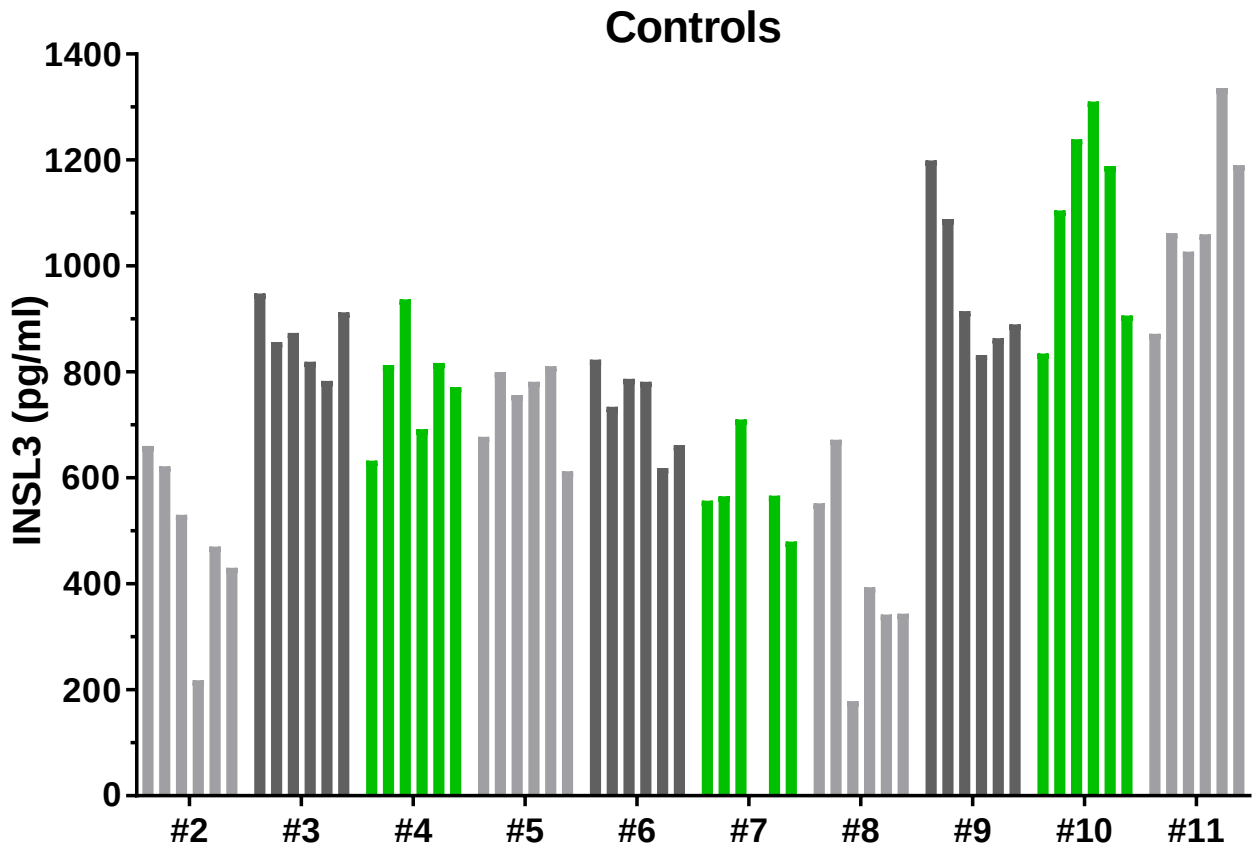
Figure legend

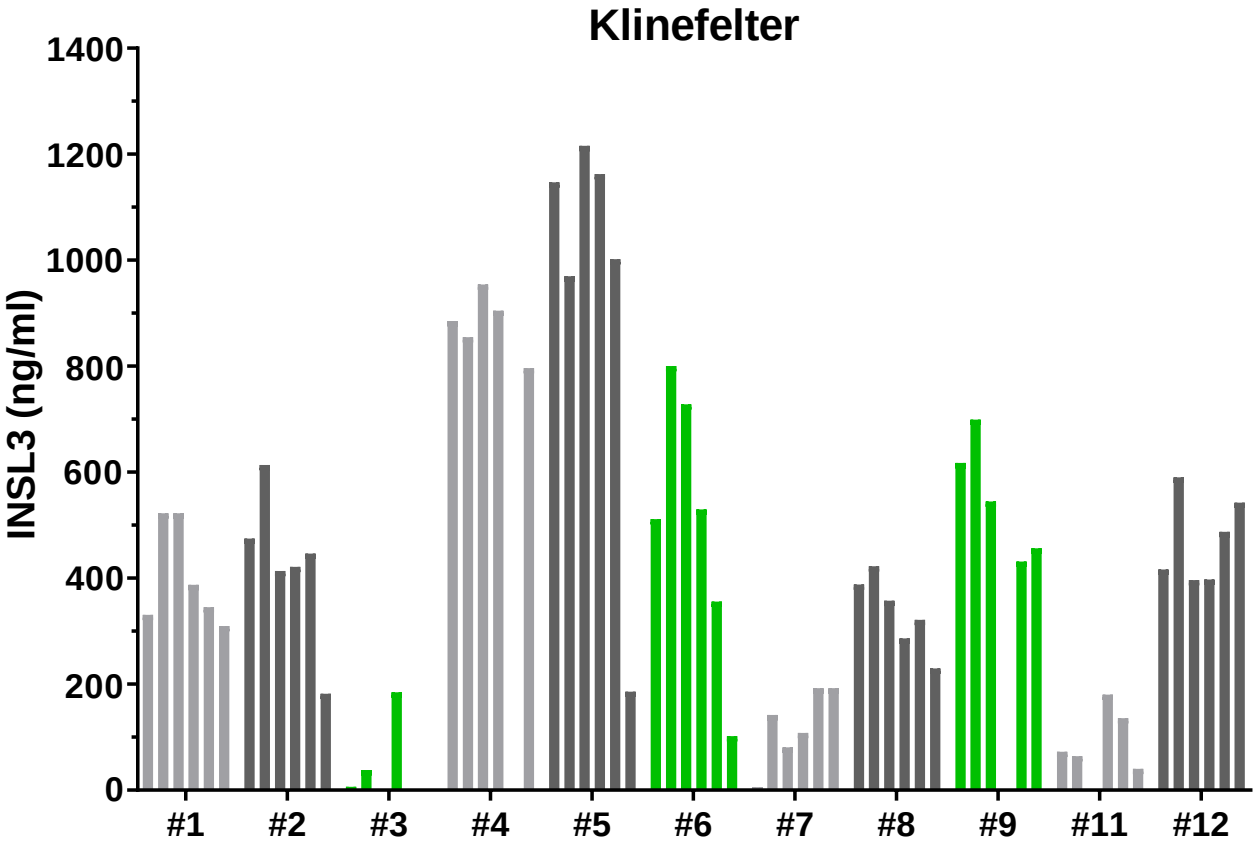
Figure 1. Linear regression between basal INSL3 and testicular volume in KS men.
[Footnotes to Figure 1]: INSL3: Insulin like peptide 3, KS: Klinefelter syndrome.

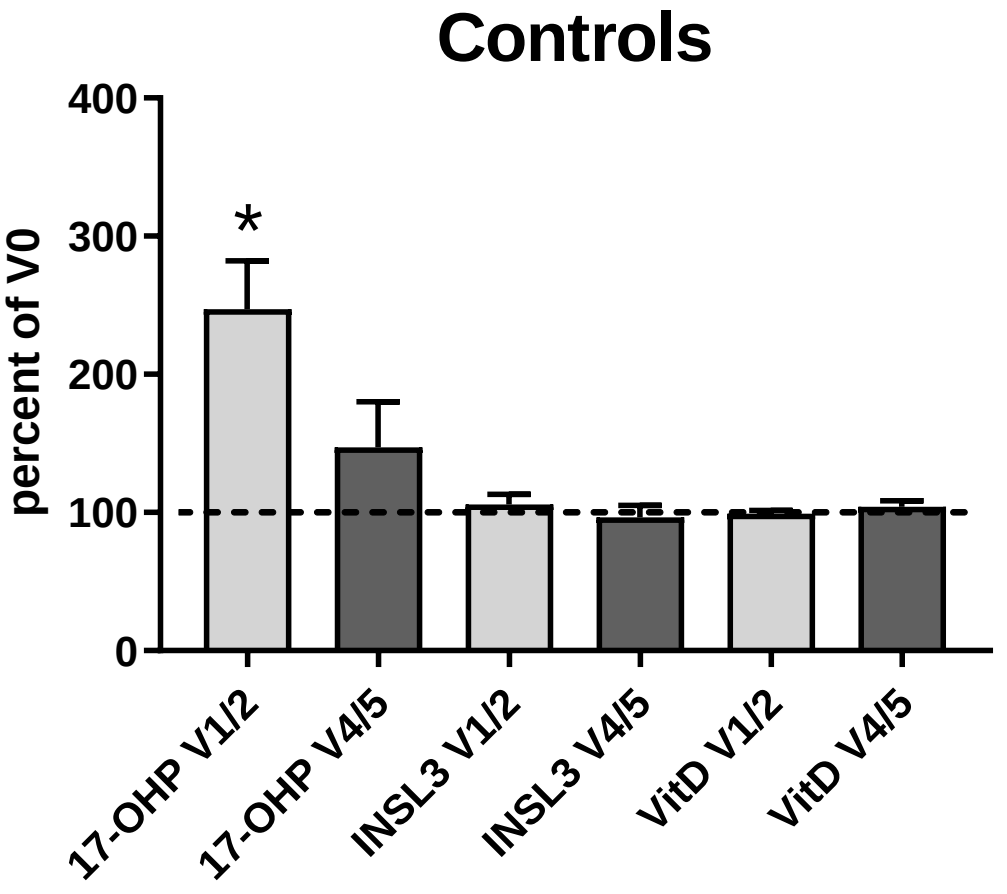
Figure 2. INSL3 serum levels at each visit in KS men (panel A) and controls (panel B). Gaps in the histograms represent missing samples
[Footnotes to Figure 2]: INSL3: Insulin like peptide 3, KS: Klinefelter syndrome.

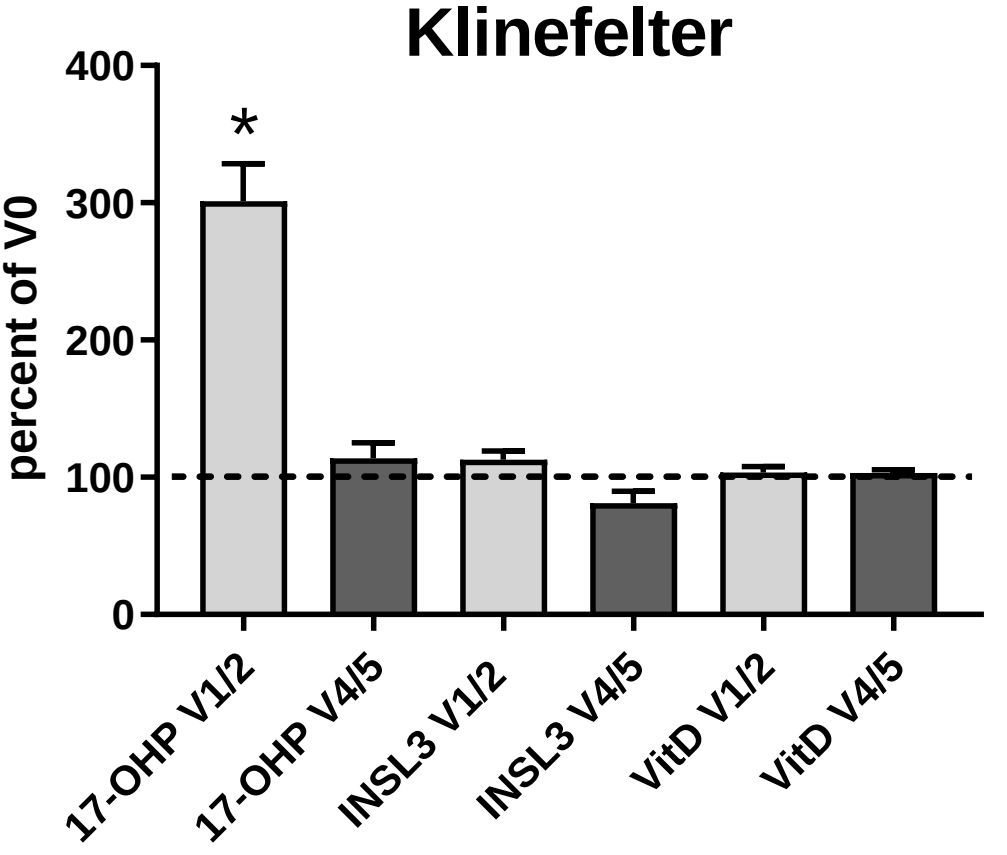
Figure 3. INSL3 and 17-OHP serum levels after hCG stimulation, aggregating V1 with V2, and V4 with V5 in KS men (panel A) and controls (panel B).
[Footnotes to Figure 2]: 17-OHP: 17-hydroxy-progesterone, avg: average, INSL3: Insulin like peptide-3, KS: Klinefelter syndrome.


testis volume (ml)
6
4
2
0
0
500
1000
INSL3 (pg/ml)
Klinefelter


Controls
INSL3 (pg/ml)
1400
1200
1000
800
600
400
200
0
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11


Klinefelter
INSL3 (ng/ml)
1400
1200
1000
800
600
400
200
0
#1
#2
#3
#4
#5
#6
#7
#8
#9
#11
#12


Controls
400
300
200
100
0
percent of V0
*
17-OHP V1/2
17-OHP V4/5
INSL3 V1/2
INSL3 V4/5
VitD V1/2
VitD V4/5


Klinefelter
400
300
200
100
0
percent of V0
*
17-OHP V1/2
17-OHP V4/5
INSL3 V1/2
INSL3 V4/5
VitD V1/2
VitD V4/5