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Low INSL3 in Klinefelter syndrome is related to osteocalcin, testosterone treatment and body composition, as well as measures of the hypothalamic–pituitary–gonadal axis

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SUMMARY

Klinefelter syndrome (KS) is characterized by infertility and hypogonadism associated with increased prevalence of osteoporosis, diabetes and metabolic syndrome. Insulin-like factor 3 (INSL3) is produced in the Leydig cells. INSL3 has been suggested to play a role in bone health. Here, we studied INSL3 in relation to bone markers, body composition, the metabolic syndrome and diabetes. This was a case–control study. Sex hormones, anthropometric measures, vitamin D metabolites, parathyroid hormone, growth factors, muscle strength, maximal oxygen consumption and BMD were measured. We included 70 adult KS patients and 71 age-matched controls. INSL3 was lower in testosterone-treated KS compared with untreated KS. Correlation analyses showed a positive correlation between INSL3 and osteocalcin among KS, but not in controls; a significant positive correlation between INSL3 and testosterone in controls and in untreated KS, but not in treated KS men. Among controls a negative correlation was found between INSL3 and lipids, and glucose, but not in KS. HOMA2-B and impaired fasting glycaemia was positively correlated with INSL3 in controls. Among KS males we found a negative correlation between INSL3 and BMI, weight and waist/hip ratio, as well as positive correlations between INSL3 and FSH, LH, SHBG and testis volume. Multivariate analyses showed that age, testosterone and HDL cholesterol were the principal independent variables among healthy controls, whereas the determinants of INSL3 concentration among KS were age, LH, current testosterone treatment and testicular volume. INSL3 in KS is influenced by testosterone treatment and INSL3 is correlated with measures of bone metabolism, body composition and the metabolic syndrome. This may suggest that low INSL3 concentration is related to the pathogenesis behind an unfavourable change in body composition and bone metabolism among KS patients.

INTRODUCTION

The male reproductive system takes action in a wide range of endocrine functions. Androgens have widespread effects on both reproductive and non-reproductive tissues. Because androgens play a vital role in many tissues, changes in androgen signalling are associated with a broad range of diseases. Recently, attention has been drawn to the relation between testosterone and bone metabolism. Hypogonadism is a well-known cause of secondary osteoporosis in both females and males, but newly published studies propose a connection between regulation of bone, energy metabolism and reproduction (Confavreux, 2011), suggesting that the skeleton is truly an endocrine organ.

Osteocalcin (OC) is a protein produced by osteoblasts and odontoblasts and is known as a marker of bone turnover. OC is carboxylated post-translationally and both the fully carboxylated OC (cOC) part, considered biologically inactive, and the under-carboxylated OC (ucOC) which is regarded as biologically active, are distributed in the systemic circulation (Schwetz *et al.*, 2012). OC stimulates testosterone production in Leydig cells and testosterone again contributes to bone growth (Schwetz *et al.*, 2012). It has been demonstrated that androgens acting on adipose tissue increase the expression of both forms of OC. One study found that knock-out mice missing OC in osteoblasts had low testes weight, low sperm count and lower testosterone levels (Oury *et al.*, 2011).

Currently, clinical management of patients with hypogonadism is evaluated using serum testosterone, LH and FSH, with testosterone seen as a marker of Leydig cell function. But is testosterone the best marker of androgen status? Recently, attention has been drawn to insulin-like factor 3 (INSL3) as a new biomarker of Leydig cell function. INSL3 is a small peptide hormone secreted uniquely by mature Leydig cells in the testes of all mammals. Importantly, this expression and secretion appears to be constitutive. Healthy individuals show minimal diurnal or within-individual fluctuation, even over several weeks or months. Therefore, it reflects the differentiation status and number of the Leydig cells present, differing thereby from testosterone which is acutely and homeostatically regulated by the hormones of the hypothalamus–pituitary–gonadal (HPG) axis, mainly LH (Ivell *et al.*, 2013), as well as by other factors like acute exercise (Hejazi & Hosseini, 2012). INSL3 binds to its receptor, relaxin family receptor 2 (RXFP2), and plays an important role in the correct transabdominal descent of the testis during intrauterine development. Testosterone circulates bound to plasma proteins, with only 2–3 % present as the free hormone. Around 60–70 % is bound to SHBG and the remainder to albumin and it is still not clear whether only the free fraction or also SHBG-bound testosterone is active. Intratesticular testosterone (IT-T) produced upon stimulation by LH, is found in very high concentrations, 100- to 1000-fold higher than the concentrations of testosterone in the circulation (Roth *et al.*, 2013).

The concentration of INSL3 circulating in the blood initially parallels the mRNA levels within the testes, suggesting again that it is more or less constitutively secreted as soon as it is synthesized (Ivell *et al.*, 2013). In the male INSL3 production depends on LH activity, but INSL3 only changes slowly and not similarly to testosterone, where acute changes in LH leads to immediate changes in the level of testosterone, and is therefore not so dependent on the hypothalamus–pituitary–testicular axis as testosterone (Bay *et al.*, 2006). Therefore, INSL3 could be a more ideal biomarker of Leydig cell function. Nevertheless, determination of INSL3 serum levels has not yet been standardized, and reference ranges and cut-off values for diagnosis of hypogonadism are not available (Ferlin *et al.*, 2013). INSL3 has been shown to be related to bone metabolism, and studies have concluded that mutations in RXFP2 are associated with osteoporosis (Ferlin *et al.*, 2008, 2011a). However, the precise physiological role of INSL3 is not clear and the role it plays in KS is even less studied.

All the above findings led us to examine men with Klinefelter syndrome (KS), who usually exhibit hypergonadotropic hypogonadism (Nielsen & Wohlert, 1990; Smyth & Bremner, 1998; Bojesen *et al.*, 2003; Lanfranco *et al.*, 2004), and lower circulating levels of INSL3 (Bay *et al.*, 2005). We aimed at determining whether there is a connection between serum level of INSL3 and bone and body composition, as well as the metabolic syndrome and diabetes, and how testosterone treatment affects these parameters.

MATERIALS AND METHODS

Subjects

A total of 70 KS patients were recruited from endocrine clinics, fertility clinics and other sources, thus comprising a mixture of patients referred for different causes and diagnosed at different ages. Data from this study regarding glucose metabolism, bone metabolism and genetics have been reported earlier (Bojesen *et al.*, 2006, 2011a,b). Inclusion criteria were as follows: age above 18 years, diagnosed KS and signed informed consent. Exclusion criteria were untreated hypothyroidism or hyperthyroidism, present or past malignant diseases, clinical liver disease or treatment with drugs other than testosterone known to interfere with bone homeostasis (e.g. glucocorticoids). Thirty-five (50%) of the 70 KS patients received testosterone treatment at the time of investigation [intramuscular testosterone injections (n = 20), oral testosterone undecanoate (n = 14) and mesterolon (n = 1)] and nine had received testosterone treatment in the past, but not during the last year before examination. Because of the inability of some of the KS patients to recall the date of last injection, dose or label, and since we did not have access to all patients files, we do not have this information in the treated KS patients. We also recorded whether patients had ever received testosterone and age at diagnosis. A healthy age- and sex-matched subject group was recruited by advertising for healthy volunteers at the University of Aarhus and at the Blood Bank at Aarhus University Hospital. KS patients were age matched one-to-one with their own healthy subject. None of the healthy subjects received any kind of steroid therapy.

All received oral and written information concerning the study prior to giving written informed consent. The protocol was approved by the Aarhus County Ethical Scientific Committee (# 20010155) and the Danish Data Protection Agency.

All participants were examined in the morning after an overnight fast. Blood was drawn at 8 o'clock in the morning, and serum and plasma were immediately separated and stored at −20°C in multiple vials for later analysis. INSL3 was measured by a time-resolved fluoroimmunoassay as described previously (Bay *et al.*, 2005).

Maximal oxygen consumption (VO_2 max) test and the isometric strength of the right biceps and quadriceps muscles were measured, testicular size was measured in males with KS using Praders orchidometer and bitesticular volume is given, DXA scans by Hologic 2000/w osteodensitometer (Hologic Inc., Bedford, MA, USA), assays and CAG repeat length of the androgen receptor was determined as described previously (Bojesen *et al.*, 2006, 2011b).

Statistics

All statistics were calculated using SPSS (Version 21; SPSS Inc., Chicago, IL, USA). p values less than 0.05 were regarded as significant. Few of the variables were normally distributed and non-parametric tests were used to test for differences between groups unless otherwise described. All results are shown as medians and range unless else described. Spearman correlation analysis with INSL3 as the variable of interest was used to describe correlations between variables to select principal independent variables for later use in multivariate analyses. Based on the correlation analyses we then performed stepwise multivariate regression analysis to evaluate the impact of independent variables on the dependent variables (BMD at different sites and muscle strength) in the KS group and healthy subject group separately. Significance level for entering and for removal of variables from the model was $p < 0.05$ and $p < 0.10$ respectively.

RESULTS

Klinefelter syndrome and healthy controls were matched by age. BMI, weight, waist circumference, TBF and BFtr were all significantly greater in KS, as described previously (Table S1) (Bojesen *et al.*, 2006). Fasting plasma glucose and fasting serum insulin were significantly higher among KS males, whereas insulin sensitivity (HOMA2-S) was significantly reduced. Triglycerides, total cholesterol and LDL cholesterol were significantly increased, and HDL cholesterol significantly reduced in KS males. More KS males had the metabolic syndrome, impaired fasting glycaemia and diabetes, as reported previously. KS males had significantly lower maximal oxygen uptake (VO_2 max). CAG repeat length was similar in both groups (Bojesen *et al.*, 2011b).

Testosterone, free testosterone, SHBG and INSL3 were significantly lower, and FSH and LH were significantly higher in KS males, with a considerable overlap in INSL3 values for KS and controls (Fig. 1). There was no significant difference in 17β-estradiol between KS and healthy controls. Within the group of KS, testosterone-treated males had significantly lower levels of INSL3, LH and FSH compared with untreated KS (Fig. 1), while testosterone was similar between groups (Table S2). There was also a considerable overlap between INSL3 values in treated and untreated KS patients.

As previously described (Bojesen *et al.*, 2011a), we found a statistically significant difference between BMD (bone mineral density) in the cases compared with the healthy controls measured in the hip ($p = 0.01$), spine ($p = 0.01$) and forearm ($p = 0.01$). We found that 25-hydroxy vitamin D was significantly lower among KS compared with healthy controls ($p < 0.0001$) (Table S1).

INSL3 and relation to other variables

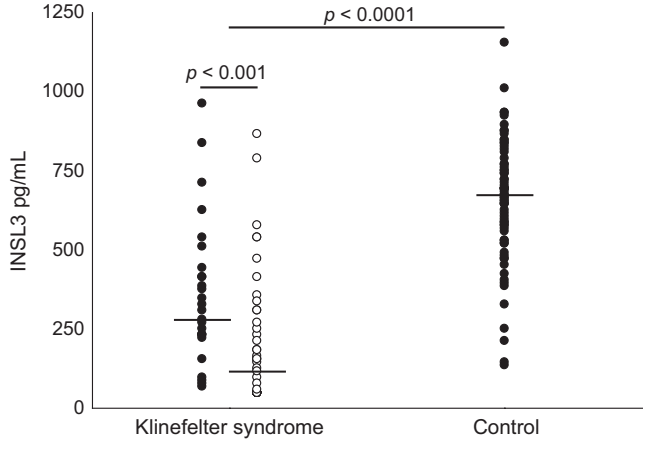
Among the healthy controls a negative correlation was found between INSL3 and HDL cholesterol and plasma glucose (Table 1 and Fig. 2), while a statistically significant correlation was not present in KS. HOMA2-B and impaired fasting glycaemia was found to be positively correlated with INSL3 in controls but not in KS. We found a significant positive correlation between INSL3 and both testosterone ($p = 0.029$) (Fig. 2) and free testosterone (p less than 0.0001) in the healthy controls, but not in males with KS ($p = 0.169$). However, when splitting the KS group in untreated and treated, we found a significant positive correlation with testosterone ($r = 0.48$, $p = 0.004$) and free testosterone ($r = 0.44$, $p = 0.009$) in untreated KS and a significant positive correlation with LH ($r = 0.62$, $p < 0.001$) and FSH ($r = 0.63$, $p < 0.001$) in treated KS (Table 1). There was no significant correlation between INSL3 and CAG repeat length among KS patients or controls (results not shown).

Among testosterone-treated males with KS we found correlations between INSL3 and measures of body composition (BMI, waist/hip ratio) and glucose homeostasis (plasma glucose, insulin and presence of diabetes) as well as maximal oxygen uptake and C-reactive protein (CRP), while such relations were not present in untreated KS. INSL3 was correlated with testicular size in both treated and untreated KS (Fig. 3) and with gynaecomastia among untreated KS. Furthermore, a positive correlation was found between INSL3 and osteocalcin (Fig. 3), which was due to a relation only seen among untreated KS, when splitting the KS group in treated and untreated. No correlation was found between INSL3 and vitamin D or BMD form any region (results not shown). For both healthy controls and KS males we found a negative correlation between INSL3 and age as expected.

Multivariate models to predict independent variables of INSL3

Based on correlation analyses, we then performed separate multiple linear regression analyses employing the variables that proved significant in bivariate correlations as possible independent variables. Among KS patients age, LH, current testosterone treatment and testicular volume were independent variables accounting for 61% of the variance in INSL3 (Table 2). Among healthy subjects age, testosterone and HDL cholesterol were the principal independent variables accounting for about 31% of the variance of INSL3 (Table 2).

Figure 1 Difference in insulin-like factor 3 (INSL3) measured in Klinefelter syndrome and healthy controls and among Klinefelter syndrome because of testosterone substitution therapy. Black circles indicate untreated Klinefelter syndrome males and controls. Open circles indicate treated Klinefelter syndrome males. Medians are indicated as horizontal bars for treated and untreated Klinefelter syndrome males and for controls. Level of significance is indicated in the figure.



DISCUSSION

This study shows that INSL3 is about 70% lower among males with KS compared with normal males. In addition, we show that INSL3 is correlated with testosterone, testicle volume, gynaecomastia and osteocalcin in untreated KS, whereas INSL3 was correlated with LH, FSH, measures of body composition and glucose homeostasis among testosterone treated KS patients. Furthermore, and as previously shown (Bay *et al.*, 2005), testosterone-treated males with KS had lower levels of INSL3 than untreated KS males and simultaneously lower levels of LH, suggesting that testosterone treatment, probably through the effect on the pituitary with lowering of LH, leads to lower constitutive production of INSL3. Thus, although INSL3 is clearly a Leydig cell function marker also in KS it is affected by testosterone treatment, although we do not know if the INSL3 level would rise if treatment was withdrawn in treated KS males. This apparent suppression of INSL3 during testosterone treatment seems most likely to be through lowering of LH (Bay *et al.*, 2006), as indicated by study of normal males subjected to a GnRH antagonist and subsequent treatment with either hCG, testosterone or placebo (Roth *et al.*, 2013). This study showed a complete suppression of INSL3 with GnRH antagonist and placebo or testosterone supplementation, but rescue of INSL3 production when hCG

Table 1 Spearman correlation analyses with INSL3 as the dependent variable in and variables of interest in treated and untreated KS patients and in healthy controls

	Healthy controls		Klinefelters syndrome patients					
	INSL3		INSL3					
			All		Treated		Untreated	
	R	*p*-value	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value
Age (years)	**−0.265**	**0.01**	**−0.440**	**<0.0001**	**−0.478**	**0.004**	**−0.473**	**0.004**
Testosterone (nmol/L)	**0.227**	**0.03**	0.116	0.2	−0.096	0.6	**0.476**	**0.004**
Free testosterone (nmol/L)	**0.399**	**<0.0001**	0.062	0.3	−0.141	0.4	**0.437**	**0.009**
SHBG (nmol/L)	−0.132	0.1	**0.210**	**0.04**	0.287	0.09	−0.014	0.9
LH (IU/L)	0.059	0.3	**0.465**	**<0.0001**	**0.622**	**<0.0001**	−0.013	0.9
FSH (IU/L)	−0.640	0.3	**0.508**	**<0.0001**	**0.629**	**<0.0001**	0.211	0.2
17β-estradiol (pmol/L)	0.080	0.3	−0.071	0.3	0.301	0.08	−0.250	0.1
Testicle volume (mL)	NA	NA	**0.456**	**<0.0001**	**0.441**	**0.009**	**0.401**	**0.02**
BMI (kg/m^2)	0.009	0.5	**−0.289**	**0.008**	**−0.478**	**0.004**	−0.239	0.2
Waist/hip ratio	0.123	0.2	**−0.312**	**0.004**	**−0.457**	**0.006**	−0.255	0.1
HDL cholesterol (mmol/L)	**0.232**	**0.03**	0.142	0.1	0.242	0.2	−0.090	0.6
Serum insulin (pmol/L)	0.077	0.3	**−0.204**	**0.05**	**−0.382**	**0.02**	−0.020	0.9
Plasma glucose (mmol/L)	**0.240**	**0.02**	−0.15	0.1	**0.372**	**0.03**	−0.065	0.7
C-reactive protein (mg/dL)	−0.147	0.2	−0.198	0.1	**−0.452**	**0.006**	−0.154	0.4
Gynaecomastia	NA	NA	**−0.327**	**0.003**	−0.260	0.1	**0.353**	**0.04**
VO_2 max (mL O_2/kg/min)	0.052	0.3	**0.299**	**0.01**	**0.488**	**0.007**	0.073	0.7
HOMA2-B (%)	**0.208**	**0.04**	−0.125	0.2	−0.157	0.4	0.033	0.9
Osteocalcin (U/L)	0.059	0.6	**0.337**	**0.004**	0.316	0.06	**0.370**	**0.03**
Diabetes c (%)	−0.169	0.08	**−0.308**	**0.005**	**−0.402**	**0.02**	−0.212	0.2
Impaired fasting glycaemia (%)	**−0.204**	**0.04**	−0.168	0.08	**−0.342**	**0.04**	−0.030	0.9

NA, not available. Bold figures indicate significant correlations and these results from the Spearman correlation analyses was subsequently used in multivariate linear regression analyses.

was administered. However, INSL3 production also correlated with intratesticular testosterone at the end of study and therefore, a direct effect of exogenous testosterone, which lowers intratesticular testosterone, on the Leydig cell and INSL3 production cannot be excluded (Roth *et al.*, 2013).

In healthy males INSL3 was primarily correlated with testosterone and lipids, and in addition INSL3 was correlated with measures of the metabolic syndrome, and a similar situation was present in treated KS. This positive relation between INSL3 and testosterone in the healthy controls has been reported before, supporting the theory that INSL3 is a good marker of Leydig cell mass and thus testicular function (Roth *et al.*, 2013), at least in scenarios where LH is not modulated by exogenous testosterone treatment.

Along with INSL3, testosterone and free testosterone were significantly reduced in KS compared with the concentrations in the healthy controls. These findings are expected and caused by the hypergonadotropic hypogonadism present in KS, and emphasizes that not only steroidogenesis, but rather global Leydig cell function is compromised in KS, in spite of the compensating increased levels of FSH and LH. These findings support the results from former studies (Bay *et al.*, 2005; Cabrol *et al.*, 2011). The fact that INSL3 is reduced to a greater extent than testosterone supports the theory that mild testicular dysfunction may be associated with low concentrations of INSL3 but concentrations of testosterone within the normal range, albeit with elevated LH (Ferlin *et al.*, 2013). This is actually the opposite of the situation seen in Prader–Willi syndrome where INSL3 levels are normal in many, while testosterone is low – thus indicating that if one wants to fully understand any condition where Leydig cell function is subnormal, it is necessary to study both INSL3 and testosterone (Hirsch *et al.*, 2013). Interestingly, INSL3 was positively related to testis volume in both treated and untreated KS and could therefore possibly (albeit speculatively) also be a marker of preserved spermatogenic foci in the testes – a theory that would need testing in other KS populations, where many males currently opt for fertility treatment with aspiration of spermatids from spermatogenic foci. There was a considerable overlap between INSL3 values in KS and controls, contrary to the situation in congenital hypogonadotropic hypogonadism where a recent study showed complete separation of INSL3 with very low values in congenital hypogonadotropic hypogonadism (Trabado *et al.*, 2014). Here, it was also shown that therapy with hCG very effectively increased INSL3 (and testosterone) levels, a situation which might well be different in KS, where hCG treatment would probably not lead to the same increase in INSL3 and thus Leydig cell function. Evidence suggests that INSL3 is a more sensitive Leydig cell function marker than testosterone, and recently it was also suggested that normal circulating levels of 25-hydroxy vitamin D levels are necessary for normal Leydig cell function and thus production of testosterone (Ivell & Anand-Ivell, 2011; Lee *et al.*, 2012). Although the precise nature of the relationship between 25-hydroxy vitamin D and normal functioning of the Leydig cell is still elusive (Ferlin *et al.*, 2013; Jensen, 2014), one study suggested that increasing levels of 25-hydroxy vitamin D that were in the low range in overweight males also lead to an increase in testosterone (Pilz *et al.*, 2011). And knowing that males with KS notoriously have low levels of 25-hydroxy vitamin D (Bojesen *et al.*, 2011a; Ferlin *et al.*, 2011b), as also shown here, supplementation of 25-hydroxy vitamin D might increase testosterone also in KS. Currently, we treat patients with KS with testosterone and although at present treatment with INSL3 is not available, it may well be relevant to consider such treatment, if it becomes available. Along these

Figure 2 Correlations between insulin-like factor 3 (INSL3) and measures of glucose homeostasis (plasma glucose), (A) testosterone (B) and HDL cholesterol (C) in healthy controls. Correlation coefficient and levels of significance are indicated in the figures.

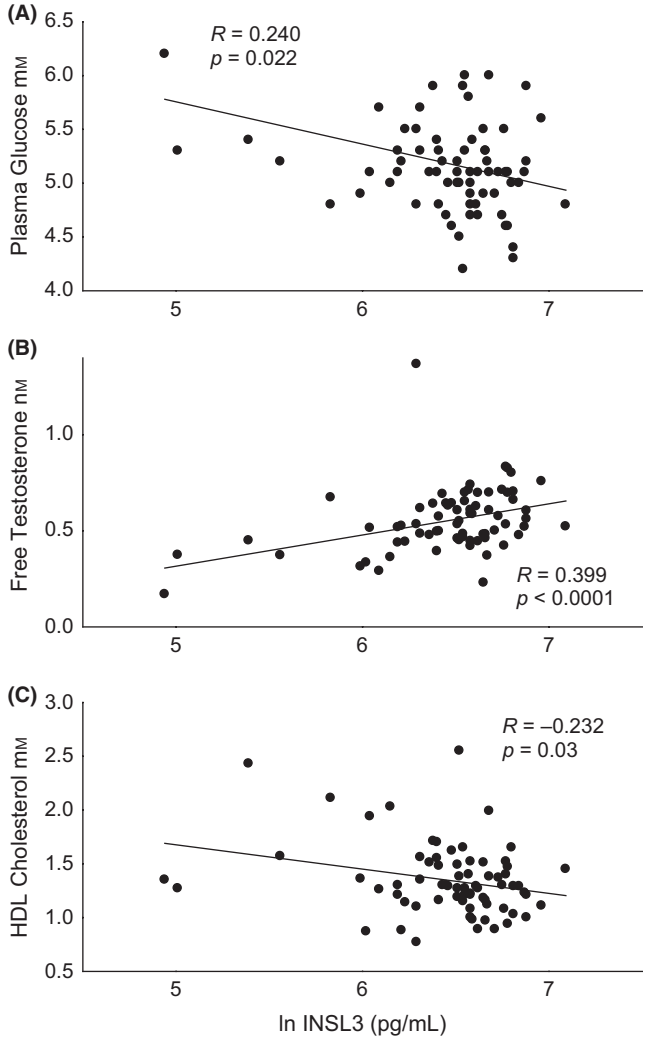
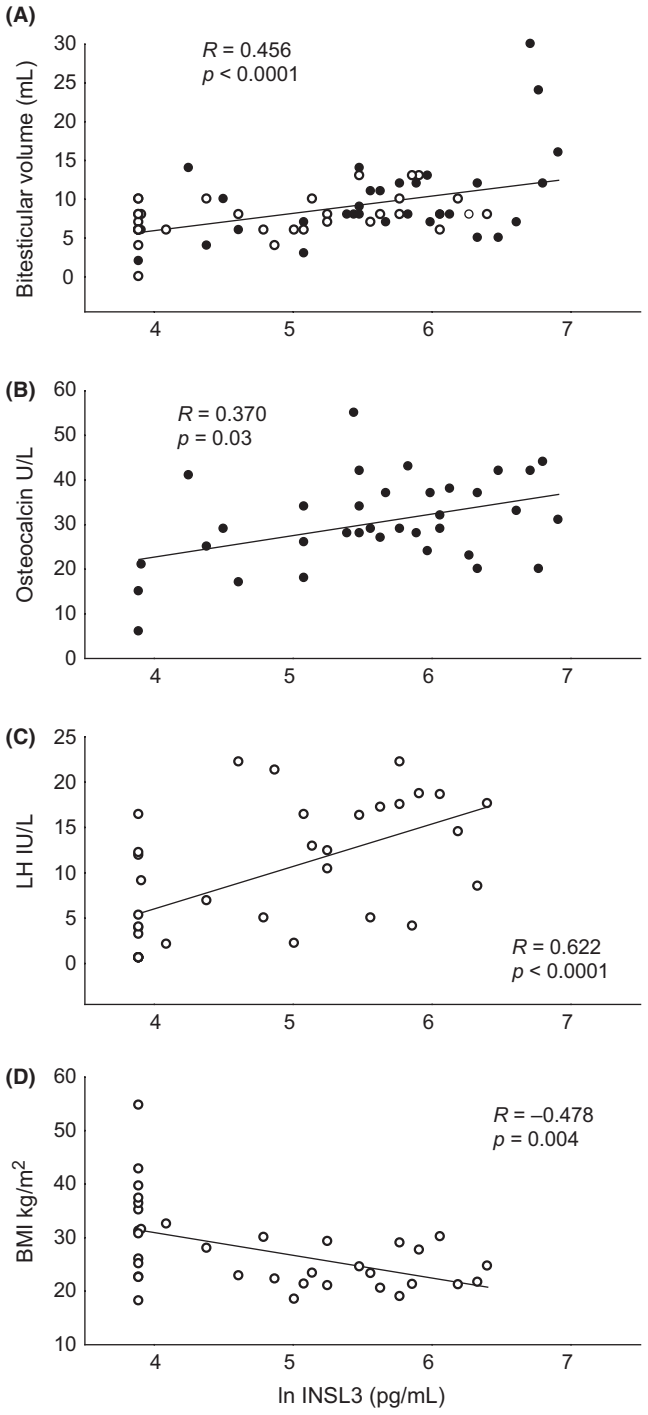


Figure 3 Correlations between insulin-like factor 3 (INSL3) and bitesticular volume (A), osteocalcin (B), LH (C) and BMI (D) in testosterone treated (open circles) and untreated (filled circles) Klinefelter syndrome.



lines, other types of treatment to increase endogenous testosterone, such as aromatase inhibitors, may also prove to increase INSL3 production.

Former studies have shown a positive correlation between testosterone and both vitamin D and osteocalcin (Ferlin *et al.*, 2013). Here, we found a correlation between osteocalcin and INSL3 among untreated males with KS, while this was not present in controls and testosterone-treated KS. In KS males many mechanisms are possible contributors to reduced bone mass. Low INSL3 levels could very well be one of them, in addition to the well-known low levels of testosterone, low vitamin D levels and unfavourable fat/muscle ratio. All of which can contribute to reduced BMD (Ferlin *et al.*, 2011c). In the past years, new information on the crosstalk between testis and bone function has emerged, increasing the understanding that testicular function is linked to normal bone health (Ferlin *et al.*, 2013).

We also saw interesting inverse correlations between INSL3 and measures of body composition in treated KS but not in normal control males. Whether these correlations are because of the fact that INSL3 clearly is linked to both LH and testosterone, and that especially testosterone in KS is closely linked to these same measures is indeed possible. However, a number of studies have linked testosterone with different measures of body composition and it is evident that obesity is related to lower levels of serum testosterone and on the other hand that treatment with testosterone can diminish fat stores, although the direct effector seems to be 17β-estradiol produced by aromatization of testosterone (Nielsen *et al.*, 2007; Finkelstein *et al.*, 2013; Tchernof & Després, 2013). In type 2 diabetic males matched on BMI and age with

Table 2 Parameters related to INSL3 in KS and in healthy subjects (backward multiple linear regression analysis)

Parameters	Regression coefficient and SD	β	p	R^2
Klinefelter syndrome				
LnINSL3				0.611
Age	38.6 ± 12.4	−0.36	<0.001	
LH	13.4 ± 7.6	0.47	<0.001	
Current testosterone treatment[a]	0.5 ± 0.5	−0.18	0.059	
Testicular volume	8.6 ± 4.4	0.30	0.001	
Healthy subjects				
LnINSL3				0.312
Age	39.09 ± 12.27	−0.40	<0.001	
Testosterone	23.08 ± 7.11	0.22	0.049	
HDL cholesterol	1.35 ± 0.34	−0.26	0.019	

[a]Current testosterone treatment had the value '0' for no treatment and '1' for current treatment.

controls, it has recently been shown that INSL3 levels are lower among T2DM males and that the sole determinant of INSL3 in multiple linear regression analysis was a measure of body composition (waist circumference) (Ermetici *et al.*, 2009). Also, in obese males it has recently been reported that INSL3 levels are lower than in controls and also that INSL3 was inversely correlated with BMI (Foresta *et al.*, 2009). The available evidence thus suggests that measures of body composition are inversely linked with INSL3 and Leydig cell function and one possible mediator may be the presence of chronic low-grade inflammation present in obesity and also present in KS, here exemplified by CRP (Bojesen *et al.*, 2006; Thomsen *et al.*, 2013).

The current study represents a quite heterogenous group of KS patients with respect to age, distribution of BMI and treatment with testosterone (50% received substitution therapy), which could be seen as a drawback. However, we believe that the study cohort quite well illustrates the clinical situation in the outpatient clinic with a diverse population, some already being treated with testosterone, whereas others are not receiving substitution therapy. Likewise, we thought it of value to include males with KS with a wide age range and BMI, rather than restricting the study cohort to specific more narrow age ranges and within specific BMI limits. This should also increase the external validity of the study. Another limitation is of course the observational nature of the study, which precludes firm conclusions as to cause and effect, and therefore new studies, preferably randomized, placebo-controlled studies or at least observational studies before and during testosterone treatment are needed to fully elucidate the relationship among INSL3, testosterone, testicular size, fertility and pituitary function.

In conclusion, we find lower INSL3 concentrations in KS males and even lower in testosterone-treated KS patients. INSL3 is related to testicular volume and measures of body composition, but the relation is impacted to a large degree by whether or not KS males receive testosterone treatment. To restore BMD in men with hypogonadism and osteoporosis, it has been shown that testosterone replacement therapy alone is not sufficient (Ferlin *et al.*, 2013), which suggests that alternative therapeutic approaches should be evaluated in future studies, such as the use of vitamin D supplements and INSL3, if such treatment ever becomes available. To do so, we need to clarify the relative role of INSL3 as well as testosterone, 17β-estradiol and vitamin D on osteoclast function.

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DISCLOSURE

The authors have no conflicts of interest to declare. ClinicalTrials.gov #NCT 00523835.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Data on anthropometrics and parameters related to metabolism, sex hormones and bone in KS and healthy controls.

Table S2. Data on anthropometrics and parameters related to metabolism, sex hormones, lipids and bone in treated and untreated KS