



# Serum insulin-like factor 3 quantification by LC–MS/MS in male patients with hypogonadotropic hypogonadism and Klinefelter syndrome

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## Abstract

**Purpose** Insulin-like factor 3 (INSL3) is an emerging testicular marker, yet larger studies elucidating the clinical role of INSL3 in patients with hypogonadism are lacking. The aim was to describe serum INSL3 concentrations analyzed by LC–MS/MS methodology in males with hypogonadotropic hypogonadism (HH) and Klinefelter syndrome (KS).

**Methods** This was a combined study from two tertiary centers in Denmark and France analyzing INSL3 concentrations by LC–MS/MS. In total, 103 patients with HH and 82 patients with KS were grouped into treated (HH:  $n = 96$ ; KS:  $n = 71$ ) or untreated (HH:  $n = 7$ ; KS:  $n = 11$ ). Treatment modalities included testosterone and hCG. Serum concentrations and standard deviation (SD) scores of INSL3, total testosterone, and LH according to age and treatment were evaluated.

**Results** In both HH and KS, INSL3 concentrations were low. In HH, INSL3 was low regardless of treatment, except for some hCG-treated patients with normal concentrations. In untreated HH, testosterone was low, while normal to high in most testosterone- and hCG-treated patients. In untreated KS, INSL3 and testosterone concentrations were low to normal, while in testosterone-treated KS, serum INSL3 was low in most patients. INSL3 SD scores were significantly lower in untreated HH than in untreated KS ( $p = 0.01$ ).

**Conclusions** The dichotomy between lower INSL3 and higher testosterone concentrations, particularly observed in hCG-treated patients with HH, confirms that INSL3 is a different marker of Leydig cell function than testosterone. However, the clinical application of INSL3 in males with hypogonadism remains unclear.

**Keywords** INSL3 · Hypogonadotropic hypogonadism · Klinefelter syndrome · LC–MS/MS

## Introduction

Insulin-like factor 3 (INSL3) is a peptide hormone produced by mature testicular Leydig cells in males. The hormone

plays a role in the testicular descent [1] and may also play a role in germ cell survival [2]. In postnatal life, INSL3 increases transiently in minipuberty [3] and again in puberty [4]. INSL3 has been suggested as a circulating biomarker of

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the testicular Leydig cell capacity as the concentration reflects the number of Leydig cells in the testis as well as the degree of differentiation of the cells [5–7]. The utility of INSL3 as a clinical biomarker has further been indicated by the fact that it appears to be more sensitive than testosterone to Leydig cell impairment [8], and it appears to be a direct marker of intratesticular testosterone [9]. INSL3 secretion by the Leydig cells is probably stimulated by luteinizing hormone (LH) and seemingly with LH as the only endogenous stimulant [10], although it is not acutely regulated by LH [11].

The clinical investigation and implementation of INSL3 has been impeded by the lack of commercially available, sensitive and robust assays [12]. However, recently, we developed and validated a state-of-the-art liquid chromatography–tandem mass-spectrometry (LC–MS/MS) method for INSL3 measurements [13] and produced male reference ranges for INSL3 according to age [14]. Quantification of INSL3 serum concentrations may be clinically relevant in some patients suspected of primary or secondary hypogonadism. The most common congenital form of primary hypogonadism is seen in patients with Klinefelter syndrome (KS, 47,XXY) in whom the gonadotropins are typically elevated, while testosterone is in the low-normal range [15]. Adults with KS are clinically characterized by varying degrees of abdominal obesity, osteopenia, gynecomastia, and azoospermia, and most patients with KS need testosterone treatment from adolescence onwards.

Patients with so-called secondary or central hypogonadism (i.e. hypogonadotropic hypogonadism, HH), characterized by low gonadotropin secretion, can be categorized into subgroups according either to genotype, clinical causes, or age of presentation. They are classically grouped into congenital HH (CHH), acquired HH (AHH), and idiopathic or isolated HH (IHH) [16]. In general, patients with HH have profound impairment in the secretion of gonadotropins. Thus, in adult life, all patients need testosterone treatment, which may be changed to combined hCG/FSH treatment to induce spermatogenesis, while maintaining normal testosterone concentrations [16]. Importantly, reversal of HPG-axis activity, even after decades of testosterone therapy, has been described in a certain proportion of patients with genetically verified CHH [17], implying that testosterone substitution would no longer be required. Altogether, this highlights the importance of regular re-evaluation of the endogenous hormones in such patients.

Serum INSL3 has previously been evaluated in both KS and HH using immunoassays; however, its clinical application has yet to be identified. Thus, based on INSL3 quantification via a sensitive and specific LC–MS/MS method, the aim of this study was to add a piece to the

INSL3-puzzle by evaluating INSL3 concentrations in a larger cohort of male patients with HH or KS.

## Material and methods

### Study population

In total, 103 males with HH and 82 males with KS were included in this study from the two participating centers as outlined below. The median ages were 37.4 years (range: 9.2–79.3 years) in males with HH and 35.4 years (range: 9.9–73.7 years) in males with KS.

### Danish patients with HH

In total, 63 male patients with HH were followed at the Department of Growth and Reproduction, Rigshospitalet, Copenhagen: CHH:  $n = 17$ ; AHH:  $n = 12$ ; IHH:  $n = 31$ ; and multiple pituitary hormone deficiency (MPHD):  $n = 3$ . Causes of AHH included macroadenoma ( $n = 2$ ), trauma ( $n = 3$ ), and medication ( $n = 7$ ). In all Danish patients with HH, diagnoses were verified through patient files either clinically or mutation based. Six Danish patients had verified mutations as previously published [18]. Those denoted as ‘HH’ in the files (i.e. no an- or hyposmia and no genetic identified cause) were categorized as IHH. In all patients with CHH and IHH, other pituitary functions were normal.

### French patients with HH

Forty male patients with HH were identified at the Department of Endocrinology, Bicêtre Hospital, Paris: CHH:  $n = 37$ ; AHH:  $n = 1$ ; and panhypopituitarism:  $n = 2$ . In these patients, all diagnoses were verified through patient files, or diagnoses were genetically or clinically verified as previously published [19, 20].

For patients with HH (Danish and French), blood samples were taken either prior to ( $n = 7$ ) or after initiation of hormone treatment ( $n = 96$ ). Treatment included different formulations of testosterone (intramuscular, dermal, and oral) or different regimens of human chorionic gonadotropin (hCG) stimulation.

### Danish patients with KS

We identified a total of 82 male patients with KS including mosaic variants followed at the Department of Growth and Reproduction, Copenhagen. Diagnoses were verified through patient files. Blood samples were taken either prior to ( $n = 11$ ) or after initiation of hormone treatment ( $n = 71$ ). Testosterone was administered by different regimens (intramuscular, transdermal, or oral).

## Healthy participants

INSL3, LH, and testosterone concentrations were measured in serum samples from 302 Danish, healthy men aged 31–61 (mean 46.7) years participating in a population-based cross-sectional study (Health2008). The study was conducted at the Research Centre for Prevention and Health, Glostrup University Hospital, Denmark and included randomly selected participants recruited from Copenhagen [21]. Results regarding INSL3, T and LH according to age have previously been published [14, 22].

## Hormone assays

### Danish patients

Serum was stored at  $-20^{\circ}\text{C}$  (up to 3 months) before analysis. INSL3 was determined by LC–MS/MS as described previously [13, 14] with a limit of detection (LoD) of  $0.03\ \mu\text{g/L}$ , and inter-assay coefficients of variation (CV's) below 9%. Serum concentrations of LH were analyzed by a time-resolved fluoro-immunometric assay (AutoDELFIA, Perkin Elmer, Turku, Finland) with an LoD of  $0.05\ \text{IU/L}$  and an inter-assay CV below 5%. Testosterone was measured either by a chemiluminescence immunoassay (Access 2, Beckman Coulter, Brea, CA, USA) with an LoD of  $0.35\ \text{nmol/L}$  and an inter-assay CV below 5%, or by LC–MS/MS-method as previously described [23] with an LoD of  $0.10\ \text{nmol/L}$  and an inter-assay CV below 6%. Based on internal method comparison between these two methods ( $n = 58$  samples), all T measurements obtained from the Access method were re-calculated to the corresponding LC–MS/MS-values. All analyses from Denmark were accredited by The Danish Accreditation Fund for medical examination according to the standard DS/EN 15189.

### French patients

Serum was stored at  $-20^{\circ}\text{C}$  (up to 9 months) before analysis. INSL3 was determined in Copenhagen as reported above, as well as in Paris by a radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA) [10, 19] with an LoD of  $0.011\ \mu\text{g/L}$ , and inter-assay CVs below 9%. Serum concentrations of LH were analyzed by a chemiluminescence immunometric assay (Centaur, Siemens, Deerfield, USA), at Department of Hormonology, Bicêtre Hospital, Paris, France, with an LoD of  $0.07\ \text{IU/L}$  and CVs below 2.3%. Testosterone was measured as reported [19] by a radioimmunoassay using Orion Diagnostica (Spectria, Espoo, Finland) with an LoD of  $0.02\ \text{ng/mL}$  (corresponding to  $0.07\ \text{nmol/L}$ , see equation below) and CVs below 4.8%. In patient samples, testosterone concentrations were reported

in  $\text{nmol/L}$  (DK) and  $\text{ng/mL}$  (FR). Based on the molar mass of T of 288 Daltons, all values were standardized to  $\text{nmol/L}$  ( $\text{nmol/L} = \text{ng/mL} \times 3.47$ ). Forty French patients included in this study previously had INSL3 analyzed using an immunoassay [19].

## Healthy participants

Serum was stored at  $-20^{\circ}\text{C}$  (up to 20 years) before analysis. INSL3 was determined as described under the Danish patients. Data on LH in the Danish, healthy population has been published previously [24].

Reference ranges and LODs plotted for INSL3, LH, INSL3 as a function of LH, and testosterone were based on the assays used at the Department of Growth and Reproduction, Copenhagen University Hospital, Copenhagen, Denmark, of which INSL3, LH, and testosterone have previously been published [13, 23, 24]. All values from patients and healthy participants below the local LODs were plotted as  $\text{LOD}/2$ .

## Statistical methods

The bivariate plot of the interrelationship between LH and INSL3 in healthy participants above 20 years of age was established as previously described [25]. The reference line is defined by  $A = \{(x, y) \mid P(\text{LH} > x, \text{INSL3} < y) = 0.025\}$ , and it allows for distinction between individuals with normal and abnormal LH-INSL3 combinations. The 2.5th percentile of INSL3 and the 97.5th percentile of LH are represented as the horizontal and vertical parts of the reference line, respectively. Only patients above 20 years of age were plotted on the bivariate LH/INSL3 plot.

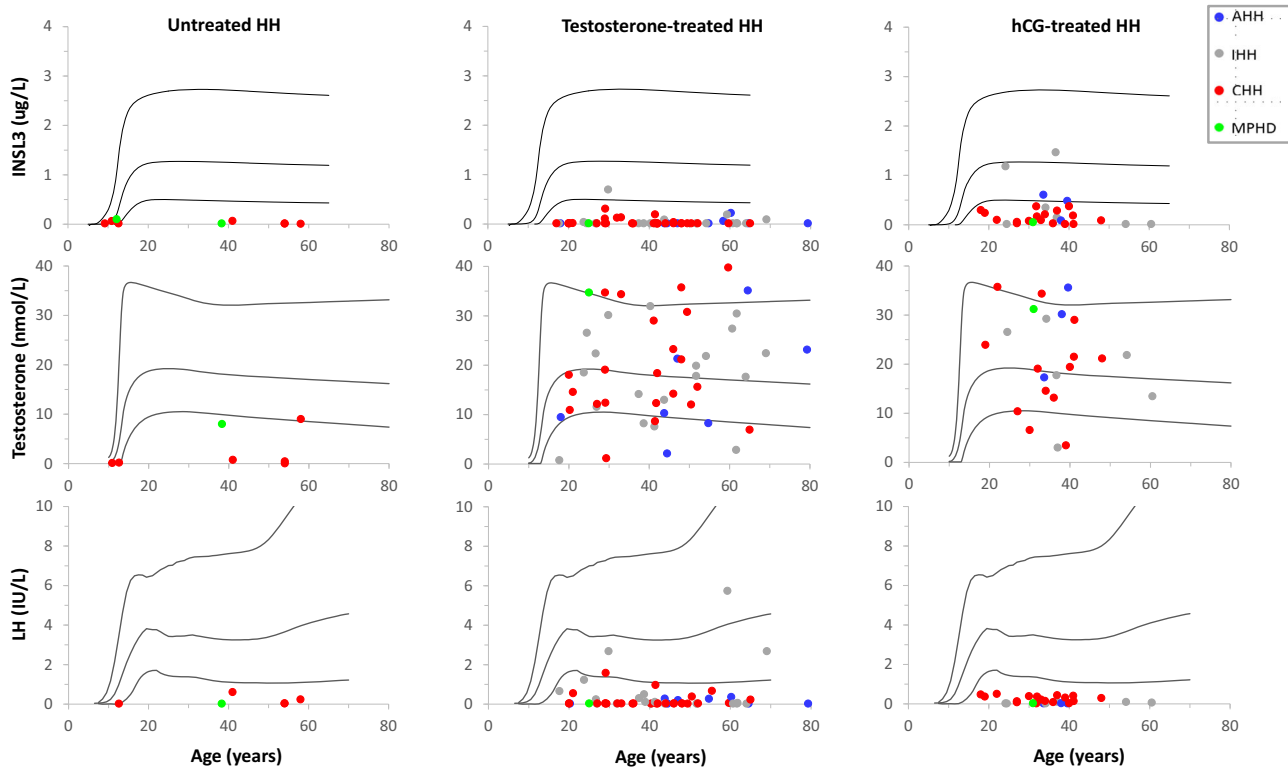
A Generalized Additive Model for Location, Scale, and Shape was used to create reference ranges in a previous study [14]. These were used to calculate standard deviation (SD) scores for INSL3 concentrations in patients using the following equation:  $\text{SD score} = ((X/M)^L - 1) / (L \times S)$ , where X is the measurement and  $L \neq 0$ .

The Mann–Whitney *U*-test was applied to compare INSL3 SD scores in untreated patients with HH and KS. Due to the low number of patients, data were reported as medians and inter-quartile ranges (IQRs). A  $p$ -value  $< 0.05$  was considered statistically significant.

## Results

### INSL3 in HH

Serum concentrations of INSL3, testosterone, and LH in patients with HH (untreated, testosterone-treated, and hCG-treated) are shown according to age, reference ranges, and



**Fig. 1** Serum concentrations of insulin-like factor 3 (INSL3), testosterone, and luteinizing hormone (LH) in untreated (left panel), testosterone-treated (middle panel), and human chorionic gonadotropin (hCG)-treated patients (right panel) with hypogonadotropic

hypogonadism (HH). Patients are sub-grouped into acquired HH (AHH, blue), idiopathic HH (IHH, gray), congenital HH (CHH, red), and multiple pituitary hormone deficiency (MPHD, green)

HH subtype (CHH, AHH, IHH, and MPHD) in Fig. 1. INSL3 was generally low in HH regardless of treatment and treatment modality, except for some hCG-treated patients. The hCG-treated patients with CHH appeared to have higher levels compared to untreated patients. Some hCG-treated patients with AHH and IHH even had normal INSL3 concentrations, although a large proportion still had INSL3 concentrations below  $-2$  SD. Similarly, patients with HH had low levels of LH independent of treatment. The testosterone concentrations were low in untreated patients, while within the reference range in the majority of testosterone- and hCG-treated patients with HH.

### INSL3 in KS

Serum concentrations of INSL3, LH, and testosterone in patients with KS (untreated and testosterone-treated) are shown according to age and reference ranges in Fig. 2. Untreated patients with KS had low to normal INSL3 and testosterone concentrations and normal to elevated LH concentrations. In testosterone-treated KS, INSL3 concentrations were below  $-2$  SD for the majority of patients, albeit a few were within the normal range. LH was either low or normal to elevated and testosterone was mostly within the normal range.

Median SD scores for INSL3 in untreated and treated HH (all subtypes) and KS are visible in Fig. 3. INSL3 SD scores were significantly lower in untreated HH (median SD score:  $-3.16$ ; IQR:  $-5.89$  to  $-1.20$ ) than in untreated KS ( $-1.00$ ;  $-1.99$  to  $-0.06$ ),  $p = 0.01$ .

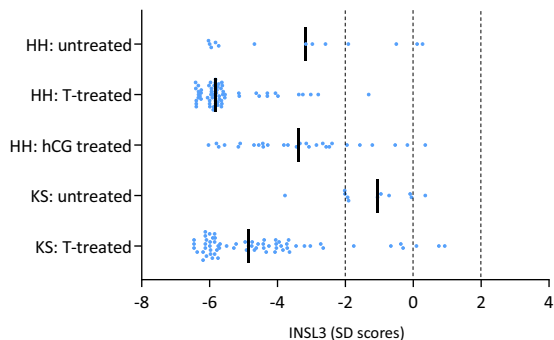
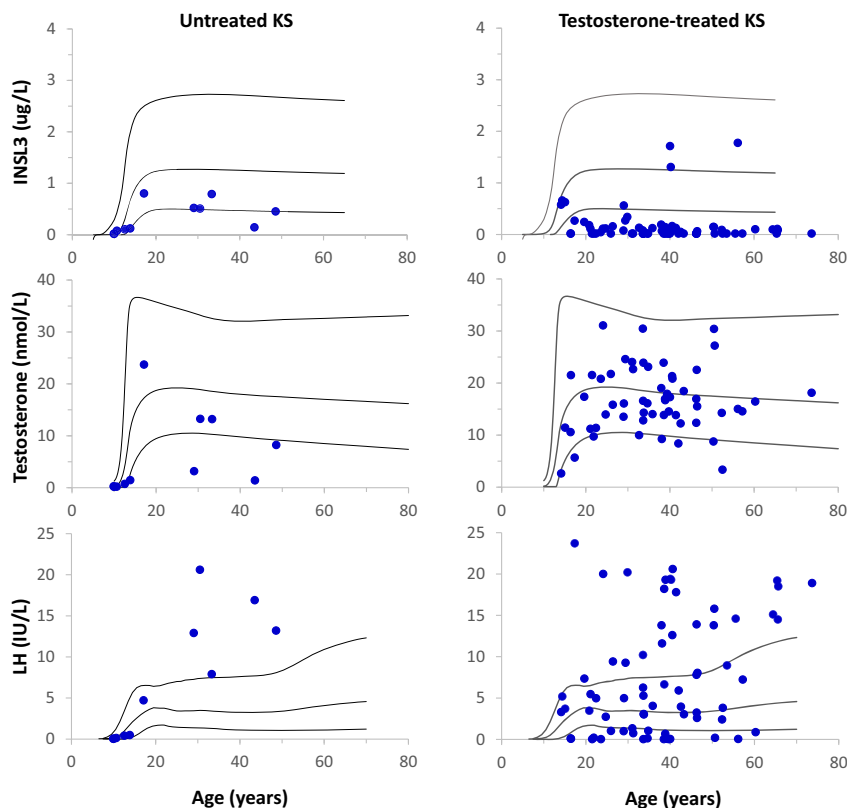
Corresponding serum concentrations of INSL3 as a function of LH from healthy, adult men were graphically visualized in a bivariate plot demarcated with the 97.5th percentile, Fig. 4. All untreated patients with HH had values outside the reference line on the bivariate plot. Similarly, all patients with KS had values outside the reference line.

### Discussion

In this study, we evaluated INSL3 by a sensitive and robust assay in a larger cohort of 83 males with KS and 103 males with HH. In both conditions, we confirmed that INSL3 is a different marker of Leydig cell function than testosterone. This descriptive study with its state-of-the-art methodology is therefore a piece of the puzzle to the clinical application of INSL3 in males with hypogonadism.

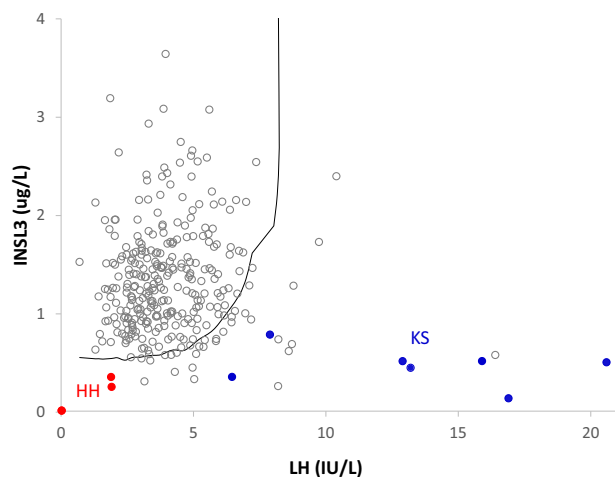
Overall, INSL3 concentrations were low in both HH and KS, particularly in patients undergoing testosterone treatment. In patients with HH, the low INSL3 levels would seemingly

**Fig. 2** Serum concentrations of insulin-like factor 3 (INSL3), testosterone, and luteinizing hormone (LH) in untreated (left panel) and testosterone-treated (right panel) patients with Klinefelter syndrome



**Fig. 3** Standard deviation scores (SDS) for serum concentrations of insulin-like factor 3 (INSL3) in untreated, testosterone (T)-treated and human chorionic gonadotropin (hCG)-treated patients with hypogonadotropic hypogonadism (HH) and Klinefelter syndrome (KS). Each blue dot represents one patient and his SDS. Bold, black bars indicate medians. Dashed lines indicate  $-2$  SDS,  $0$  SDS, and  $+2$  SDS, respectively

be due to the lack of stimulation of the Leydig cells. In line with this, the hCG-treated patients with HH (except those with CHH) had higher concentrations of INSL3 than the non-hCG-treated patients with HH. In fact, some hCG-treated patients had INSL3 concentrations within the normal range. Interestingly, despite normal testosterone levels, all hCG-treated patients with CHH had concentrations of INSL3 below  $-2$  SD. This dichotomy highlights the difference between INSL3 and testosterone. It could indicate that Leydig cell



**Fig. 4** Bivariate plot of serum concentrations of luteinizing hormone (LH) and insulin-like factor 3 (INSL3) with reference line (black) and untreated patients with hypogonadotropic hypogonadism (red) and Klinefelter syndrome (blue), respectively. Each dot represents one patient

number, capacity, and differentiation—which INSL3 is believed to reflect [5–7]—are developed prior to pubertal onset. This would explain why hCG-treated patients with CHH had lower INSL3 levels than those with AHH and IHH, supporting previous findings of both INSL3 and testosterone in CHH [26]. In line with this, an increased LH:INSL3 ratio was observed in persistently cryptorchid boys during



minipuberty [3], and in men suspected of hypogonadism, low baseline INSL3 concentrations were associated with low hCG-induced INSL3 [14]. Interestingly, in this study, postpubertal testosterone synthesis did not appear to be similarly sensitive to early maturation of Leydig cells as indicated by the normal and high-normal testosterone values in the hCG-treated patients regardless of HH subtype. It is important to note that the variation in testosterone concentrations may reflect the hCG dosage, which could blur the observed discrepancy between INSL3 and testosterone concentrations.

In patients with KS, the low INSL3 concentrations observed in this study confirm previous findings in adults with KS [11, 27]. Similar to HH, there was also a dichotomy with lower INSL3 and higher testosterone concentrations in KS. However, several patients with untreated and testosterone-treated KS had INSL3 concentrations within the normal range, some even high-normal. Similar to our results, higher concentrations of INSL3 have been observed in untreated KS not needing treatment compared to those undergoing treatment [28], probably explained by a relatively preserved Leydig cell function in patients with KS not in need of treatment. Comparing untreated KS and HH, INSL3 and testosterone levels were higher in KS, which further indicates the relatively good Leydig cell function in untreated KS. While the exact relevance of INSL3 in KS remains elusive, it has been speculated that the low concentrations may be involved in the pathogenesis of the osteopenia and osteoporosis observed in KS [29]. However, bone health in KS was beyond the scope of this study.

As LH is believed to chronically regulate INSL3 by stimulating the Leydig cells, studying their interrelationship in healthy individuals through a bivariate plot of INSL3 as a function of LH was of interest. The bivariate plot indicates the INSL3 concentration above which a patient should be for a given LH concentration to be within the normal range. To our knowledge, this is the first normative plot of its type to be published. The bivariate plot of stimulant and product has been deemed highly useful in the instance of FSH and inhibin B when evaluating Sertoli cell function and spermatogenesis [30, 31] and for LH and testosterone in hypogonadal men [32]. Thus, the LH and INSL3 bivariate plot is intended to aid the clinician when evaluating the Leydig cell function. However, further studies are needed to ascertain the clinical application.

The strengths of this study included: (1) INSL3 was measured by a newly implemented, highly sensitive LC–MS/MS method; and (2) it was a large cohort of patients with KS and HH. However, the limitations of this study included: (1) different assays were used for LH and testosterone in the French and Danish patients, respectively, and no method comparison was performed across countries between the assays used to quantify these two hormones; (2) many patients were hCG or testosterone-treated; (3) all

healthy individuals were Caucasians; (4) the bivariate plot on INSL3 and LH is restricted to the specific analytical methods used and may not be applicable to other laboratories; and (5) the samples from healthy individuals were stored for up to 12 years which may affect the durability.

In conclusion, we present serum concentrations of INSL3, testosterone, and LH in patients with HH and Klinefelter syndrome. Divergent concentrations of INSL3 and testosterone confirm that INSL3 provides additional information about Leydig cell quality and quantity compared to testosterone alone. However, the clinical application of INSL3 remains unclear.

### Data availability

Restrictions apply to some or all the availability of data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethics approval** Denmark: The study was approved by the ethics committees of the Capital Region of Denmark (H-KA20060011). All patient samples were taken as part of the clinical follow-up. Access to Danish patient data was approved by the Danish Patient Safety Authority (no. 3–3013–1376/1/) and the Danish Data Protection Agency (no. 2015–235, I-Suite no. 04204). In Denmark, the permission for INSL3 measurements was approved as part of a quality assurance project at Rigshospitalet, University Hospital of Copenhagen (approval no. 20012618). France: The study was approved by Assistance Publique-Hôpitaux de Paris and the institutional review boards at Bicêtre teaching hospital (Comité de Protection des Personnes Ile de France, Hôpital Bicêtre, Programme Hospitalier de Recherche Clinique [PHRC-2009, Hypoproteo]).

**Consent to participate** France: All participants gave written informed consent according to the French Bioethics Law and the Declaration of Helsinki.

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