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REVIEW ARTICLE

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Insulin-like peptide 3 (INSL3) as an indicator of leydig cell insufficiency (LCI) in Middle-aged and older men with hypogonadism: reference range and threshold

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ABSTRACT

Insulin-like peptide 3 (INSL3) is a circulating biomarker for Leydig cell functional capacity in men, also indicating Leydig Cell Insufficiency (LCI) and potential primary hypogonadism. Using results from large cohort studies we explore sources of biological and technical variance, and establish a reference range for adult men. It is constitutively secreted with little within-individual variation and reflects testicular capacity to produce testosterone. The main INSL3 assays available indicate good concordance with low technical variance; there is no effect of ethnicity. INSL3 declines with age from 35 years at about 15% per decade. Like low calculated free testosterone, and to a lesser extent low total testosterone, reduced INSL3 is significantly associated with increasing age-related morbidity, including lower overall sexual function, reflecting LCI. Consequently, low INSL3 ($\leq$0.4 ng/ml; ca. $<$2 SD from the population mean) might serve as an additional biochemical marker in the assessment of functional hypogonadism (late-onset hypogonadism, LOH) where testosterone is in the borderline low range. Excluding individuals with low LCI (INSL3 $\leq$ 0.4 ng/ml) leads to an age-independent ($>$ 35 years) reference range (serum) for INSL3 in the eugonadal population of 0.4 – 2.3 ng/ml, with low INSL3 prospectively identifying individuals at risk of increased future morbidity.

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Introduction

Insufficient production of testosterone (T) by the testes and its clinical consequences is referred to as hypogonadism [1–3]. Hypogonadism has been categorized as primary, if the reason for the low T production is testicular in origin, or secondary, where the cause is due to a hypothalamic or pituitary insufficiency. Clinically, these have been defined by the concentrations of circulating T and luteinising hormone (LH) (e.g. primary: $T < 10.5$ nmol/L, LH $\geq$ 9.4 U/l; secondary: $T < 10.5$ nmol/L, LH $<$ 9.4 U/l [4]). Additionally, there is a third category, referred to as compensated (primary) hypogonadism, where T levels are maintained within the normal range due to an increased pituitary production of LH ($T > 10.5$ nmol/L, LH $>$ 9.4 U/l [4]). Low T alone, however, is insufficient to justify endocrine intervention.

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Many men with low T do not have symptoms of androgen deficiency and conversely, many men with apparently normal T do show symptoms suggestive of hypogonadism. Attempts to improve diagnostic accuracy of hypogonadism, especially in middle-aged and older men, include proposals that low total T (and low free T – see below) should be syndromically associated with at least three androgen-dependent sexual symptoms [3]. To improve the biochemical diagnostic precision, several additional parameters have been considered. Primary amongst these is the inclusion of circulating SHBG (steroid-hormone binding globulin), which varies with age and other physiological and pathological conditions [5, 6], in the construction of calculated free testosterone (cFT) [7], postulated to represent the portion of circulating T which is bioavailable for interaction with androgen receptors. Indeed, cFT does indicate a better correlation with morbidity incidence in aging men than T alone [8–10].

A key factor in this discussion is that low T may have multiple causes, either primary or secondary, influencing different levels within the HPG axis. Moreover, it is now known that morbidity itself, particularly conditions leading to systemic inflammation, such as obesity, may also affect T production [11]. Furthermore, circulating T is subject to considerable biological (diurnal and other) variation within an individual, together leading to substantial natural variance in this biochemical parameter and hindering its usefulness as a diagnostic indicator of testicular (Leydig cell) function. With this background, we have been investigating the possible application of a new testicular peptide hormone, insulin-like peptide 3 (INSL3), as a biochemical parameter in the assessment of androgen deficiency in older men.

In the adult, INSL3 is a circulating hormone secreted into the bloodstream exclusively from the steroidogenic Leydig cells of the testes [12]. Studies in humans and other species support the view that it is mostly present in the blood as a small, approximately 6000 Dalton, A-B heterodimeric peptide, like insulin, following intracellular processing of a longer precursor polypeptide and removal of a connecting C-peptide domain [12, 13]. Both *in vivo* (human) and *in vitro* (rodent Leydig cell culture) experiments indicate that INSL3 is secreted in a constitutive manner, without significant modulation by LH or hCG in the short term [14–16]. In long term studies of men with relatively low Leydig cell functional capacity, LH or hCG, administered for weeks or longer, may stimulate the differentiation or proliferation of the Leydig cells, and hence their overall capacity for INSL3 production [17]. INSL3 is a biomarker of the functionally mature, fully differentiated Leydig cell, and hence only appears in the male concomitantly with the onset of puberty and adolescence, with blood levels gradually increasing through puberty, as the Leydig cells mature, to attain a maximum in young men at around 18-24 years of age [18, 19]. Additionally, in pregnancy INSL3 is made by a separate population of fetal Leydig cells where it promotes the first phase of testicular descent [20]. There may also be a small postnatal rise in circulating INSL3 during what is called the "mini-puberty" at age 2-4 months [21], following which INSL3 is undetectable in children until the advent of pubertal development at around 9 to 11 years of age [18].

In adult men, because of the constitutive nature of its secretion, INSL3 in the blood can be seen as an accurate measure of the so-called "functional capacity" of the population of Leydig cells in the two testes, i.e. the product of total Leydig cell number and their average differentiation status, thereby reflecting also their capacity to produce steroids, such as T [22]. However, T differs from INSL3 as a parameter of Leydig cell function since the former is acutely and continuously regulated by the gonadotropin LH from the pituitary and exhibits high intra-individual biological variability. In contrast, INSL3 shows only very low within-individual variance over a 24-h period, or even over periods of months or several years [12, 19, 23, 24]. However, there does appear to be relatively high between-individual variance in blood INSL3, with most population studies suggesting at least a 10-fold variation between the highest and lowest values in community-living adult men [24–26]. We have very little understanding of the sources of such variation, although, unadjusted, INSL3 does indicate small though significant correlations with obesity, bone health, cardiovascular disease, diabetes, and related age-dependent morbidity [10]. The extent to which INSL3 may be causally related to these conditions, or merely reflects levels of androgen production, is also not clear. Nor do we know the extent to which Leydig cell functional capacity is itself modulated by these morbidities.

We have recently shown that low circulating INSL3 not only correlates closely with hypogonadism but is able prospectively to predict morbidity in aging men several years later [10]. The present study has two objectives: firstly, to use previously published data, their re-analysis, and some new findings to assess potential sources of technical and biological variance in INSL3 measurement. This is essential when considering the utility of INSL3 as a biomarker of Leydig cell functional capacity, and hence of Leydig cell insufficiency (LCI), thereby providing an alternate and additional biochemical measure of primary hypogonadism.

A second objective is the definition of a reference range for INSL3 in adult men, and a threshold below which LCI or primary hypogonadism pertains.

Methods and materials

Human subjects and studies

Table 1 lists published studies carried out on community-dwelling adult men, including studies where these are represented as control groups of greater than 25 individuals. The present analysis focuses on population studies where INSL3 has been measured using time-resolved fluorescent immunoassay (TRFIA); these include the Florey Adelaide Male Aging Study (FAMAS) and the European Male Aging Study (EMAS), for both of which the principal methods and data are already published [24, 25]. Additional data derive from a cohort of 18-year-old military conscripts from Sweden [26] and unpublished data from an additional analysis of the HUSERMET (Human Serum Metabolome) cohort comprising middle aged and elderly men from the Greater Manchester area of the UK [27]. This includes 116 men of Afro-Caribbean and 133 men of South Asian descent, as well as 281 Caucasian men. Full descriptive statistics of these men are provided in Eendebak et al. [27]; there were no significant differences in mean anthropometric variables between ethnicities.

Measurement of INSL3

As outlined in Table 1, most population studies for which circulating INSL3 has been measured have employed one of three different assay systems. Phoenix Pharmaceuticals (Burlingame, CA) has produced essentially the same assay, but in two different formats: as a competitive radio-immunoassay (RIA) using ^{125}I-labelled human INSL3 as tracer and as an enzyme-linked immunometric assay (EIA). Both use the same single primary anti-human INSL3 polyclonal antibody raised in rabbits. Details of these immunoassays are provided by the manufacturer. Secondly, there is a time-resolved fluorescence immunoassay (TRFIA) developed in the Anand-Ivell & Ivell laboratory which also uses a single primary antibody competitive format with Europium-labelled human INSL3 as tracer [14, 24, 25]. Whilst an earlier version of this assay used an anti-human INSL3 polyclonal antibody raised in rats (version 1) the current version uses a different anti-human INSL3 polyclonal antibody raised in rabbits (version 2). Full details of these assays are provided in the respective publications (see Table 1). Finally, a new assay based on LC-MS/MS separation of the A-B heterodimeric human INSL3 hormone was developed by Albrethsen et al. [28]. A comparative analysis of individual samples showed that this gave virtually identical results to the TRFIA described above [28].

Whilst most studies have assayed INSL3 directly in serum, a few have instead used blood plasma; an initial report [12] had indicated no significant difference between the two matrices. To explore this further, blood samples were made available from six European centres of the EMAS cohort (Leuven, Lodz, Malmö, Manchester, Santiago de Compostela, and Tartu). Of these, the majority were plasma samples from individuals in the six community-dwelling populations, while paired plasma and serum samples (collected at the

Table 1. INSL3 Concentrations in diverse control populations of community-dwelling adult men measured using different assays.

code	matrix	number of subjects	age (mean ± SD/range)	ethnicity	country	assay type/source	INSL3 (mean ± SD/range)	reference
1	serum	40	29.4 ± 3.4	Caucasian	Italy	RIA (Phoenix)	0.56 ± 0.16	[17]
2	serum	135	19 (18-25)[a]	Caucasian	Denmark	TRFIA version 1	0.99 (0.55-1.73)[b]	[14]
3	serum	1183	55.0 ± 11.6	Caucasian	Australia	TRFIA version 1	35-44 yrs: 1.29 ± 0.47 75-80 yrs: 0.79 ± 0.39	[25]
4	serum	30	54.4 ± 7.0	Caucasian	Italy	EIA (Phoenix)	1.5 ± 0.7	[30]
5	serum	32	24.4 ± 3.0	Caucasian	France	EIA (Phoenix)	0.75 (0.39-1.13)[c]	[31]
6	serum	71	30.0 ± 9.0	Caucasian	Italy	RIA (Phoenix)	0.66 ± 0.28	[32]
7	serum	500	29.1 (23.0-38.0)[b]	Chinese	China	RIA (Phoenix)	0.94 + 0.30	[33]
8	plasma	35	32.3 ± 0.6[d]	Chinese	Taiwan	RIA (Phoenix)	0.64 ± 0.06[d]	[34]
9	serum	111	19-50	Caucasian	New Zealand	EIA (Phoenix)	young: 1.8 ± 1.1	[35]
		98	70-90	Caucasian			old: 1.0 ± 1.0	
10	plasma	37	32.7 ± 0.6[d]	Chinese	Taiwan	RIA (Phoenix)	0.64 ± 0.43[d]	[36]
11	serum	60	33.7 ± 9.7	Caucasian	Italy	RIA (Phoenix)	0.46 ± 0.18	[37]
12	serum	675	17.8 – 60.9[a]	Caucasian	Denmark	LC-MS/MS	19-40 yrs: 1.3 (0.9-2.7)[b] 51-60 yrs: 1.2 (0.9-2.5)[b]	[19]
13	serum	302	18.2 ± 0.4	Caucasian	Sweden	TRFIA version 2	2.15 ± 0.86	[26]
		43	39.0 ± 6.1	Caucasian	Australia		1.78 ± 0.82	
14	serum	886	31.3 ± 3.8	Chinese	China	EIA (Phoenix)	1.67 (0.11-4.77)[a]	[38]
15	serum	2283	63.0 ± 10.5	Caucasian	mixed European	TRFIA version 2	0.99 ± 0.50	[24]

[a]range.
[b]95% CI.
[c]5/95th percentiles.
[d]geometric mean.

same time) from 8 individuals and duplicate serum samples from 13 individuals were also available. All samples of serum or plasma were stored frozen at −80 °C until analysis. Independent control experiments had previously shown no effect of freezing over extended periods on INSL3 concentration and up to at least 3 complete freeze-thaw cycles [12].

Assessment of hypogonadism

Besides the hormones of the HPG axis, as a surrogate for the consequences of functional hypogonadism in the EMAS cohort, we applied the *Overall Sexual Function* (OSF) parameter from the EMAS Sexual Function Questionnaire [29]. This represents multiple attributes of erectile dysfunction and loss of libido on a scale from 1 to 33. Men with high T (>97.5% CI) or high cFT (>97.5% CI) registered a mean of 17 ± 6 or 20 ± 5 on this scale, respectively; similarly, those aged 40-49 years registered a mean of 20 ± 5. Both represent eugonadal states. In contrast, those with very low T (<2.5% CI) or cFT (<2.5% CI), and thus likely hypogonadal, indicated means of 12 ± 7 or 11 ± 7, respectively. Here an OSF of ≤16 (25th percentile) was used as discriminant for poor sexual function.

Statistical analysis

All data derive from the Swedish, FAMAS and EMAS cohorts as described previously [24–26] or were analysed for INSL3 as in Anand-Ivell et al. [24]. Where indicated, for comparative purposes some data are represented as means and 95% confidence intervals (CI) for an "average man of 65 years", derived by interpolation from INSL3 vs age regressions. All new regressions, interpolations, and descriptive statistics made use of Graphpad Prism (version 10.1), or for non-parametric Receiver Operated Curve (ROC) analysis SPSS (version 29.0.20) was used, as indicated in the text and figure legends.

Results and discussion

In any discussion of a new diagnostic it is important to understand the sources of technical and biological variance encountered and how these might influence results.

Available INSL3 assays

The majority of studies worldwide have used one of three different assays to measure INSL3 in community-dwelling adult men. These assays are listed in Table 1 together with their principal attributes, literature references, and approximate reference ranges based on simple population means, standard deviations, 95% confidence intervals or range. The studies indicating smaller numbers of subjects (codes 4-6,8,10,11 from Table 1) represent control groups of men used for comparison, with no evident attributes likely to affect INSL3 levels. Those data using either the TRFIA or LC-MS/MS assays show good concordance with one another. In contrast, those using the Phoenix assays appear to be more variable. Some (codes 1, 6, 8, 10 and 11; Table 1; means 0.46 – 0.66 ng/ml) are markedly lower in the estimated mean INSL3 concentration, whereas others (codes 4, 5, 7 and 14; Table 1; means 0.75 – 1.80 ng/ml) indicate values relative to age (see below), which are close to those measured by the TRFIA and LC-MS/MS assays (Table 1).

All assays have been validated to show no cross-reaction with any other insulin-like peptides or other measured substances, and none indicate a concentration for INSL3 greater than approximately 0.2 ng/ml in healthy female blood samples. One anorchic man was measured in the context of the FAMAS study and indicated no detectable INSL3 [25]. Unlike in men, in women there are no major sources of INSL3 contributing to blood levels. The ovarian theca interna cells, which are the female equivalent of Leydig cells, only produce a small amount of INSL3 sufficient for local paracrine actions [39] and only in pre-menopausal women give rise to very low circulating concentrations fluctuating around the limit of assay detection [40]. This is important to note, since some less well validated INSL3 assays erroneously suggest that female blood may have similar concentrations to male blood (e.g. [41]).

Plasma or serum

Most studies have consistently used conventional blood serum rather than plasma, although earlier control samples suggested no significant difference in the assayed INSL3 concentration based on a small number of samples [12]. We have re-evaluated this using a larger number of samples from the EMAS cohort (Suppl. Figure 1). Firstly, duplicate serum samples drawn from the same individuals collected at the same time, though measured at different times, show excellent concordance (Suppl. Figure 1A), demonstrating the low level of technical variance inherent in the TRFIA assay. For the 8 samples from the same individuals processed separately for plasma or serum (Suppl.

Figure 1B), there is greater variance, and although the mean values for the paired plasma and serum samples are not significantly different ($p = 0.72$; paired t test), it does suggest that plasma INSL3 on average represents approximately 90% of the concentration in serum. Finally, when population means are calculated for the different EMAS centres (total 2170 samples), adjusting for age by interpolation of age vs INSL3 regressions for a man of 65 years (Suppl. Figure 1C), then it is evident that use of plasma underestimates INSL3 concentration by approximately 10% (slope [serum INSL3] = 1.13 x [plasma INSL3]). The reason for this is unclear though may have to do with a differential partitioning of the peptide hormone between the aqueous and non-aqueous phases. When post-menopausal female serum, which has no detectable INSL3, is spiked with pure INSL3 peptide, recovery rates are approximately 100%, implying that serum is not over-estimating INSL3 measurement [12] (and unpublished).

Effect of subject age

Although a recent study using an MS-based INSL3 assay concluded that any effect of age on INSL3 concentration in men was negligible between 30 and 60 years [19], we have consistently found a significant age-dependent decline in INSL3 concentration from 18 to 90 years of age [24, 25]. For this reason, and for comparison in population studies, we prefer to normalize the INSL3 mean to a representative individual of a standard age, such as 65 years [10, 24]. Consequently, putative reference ranges (means and 95% CI) for INSL3 were also reported on a per decade basis (Figure 1), though see later. Figure 1 also illustrates the excellent concordance between the two large population cohort studies, FAMAS and EMAS, although measured 15 years apart using two different versions of the TRFIA assay. The results are also in good agreement for the equivalent age ranges of the Danish study measured using LC-MS/MS (Figure 1; horizontal red dashed lines).

Ethnicity

To date, most studies using the INSL3 assays listed in Table 1 have involved almost exclusively white Caucasians, with just 3 involving ethnic Chinese subjects. Although the assays used for these studies suggested a high level of technical disparity (see above), it is difficult at this time to determine whether there may be any population differences in circulating INSL3 concentration due to ethnicity. Partly to address this issue, we have retrospectively analysed two populations of South Asian and Afro-Caribbean extraction from the Greater Manchester area of the UK in comparison with the Manchester EMAS cohort, of Caucasian origin [27]. The age-adjusted results indicate no significant difference in means and 95% confidence intervals between the three ethnic groups (Suppl. Figure 2).

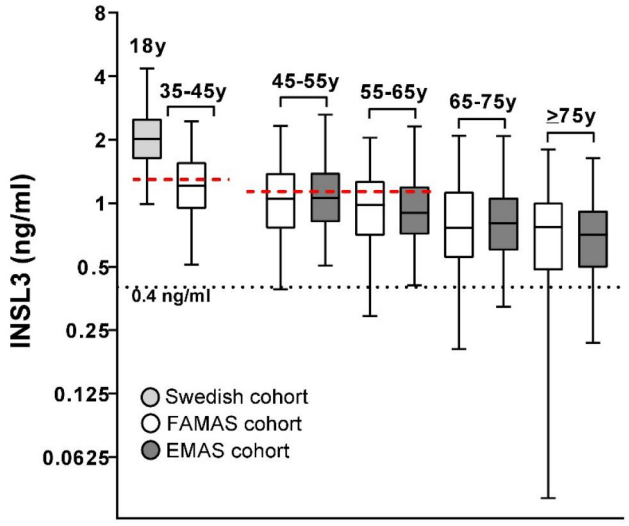

Figure 1. Means and 95%CI for different age groups derived from the Swedish [26], FAMAS [25] and EMAS [24] cohorts, as indicated. Each age-range is non-overlapping, i.e. 35-45 y represents subjects aged from 35.0 to 44.9 years, etc. The horizontal black dotted line represents the 0.4 ng/ml threshold below which indicates hypogonadism. The horizontal red dashed lines indicate the means assessed by the LC-MS/MS method for a Danish cohort [19]. INSL3 concentration is represented as a Log_2 scale.

It was previously noted in the context of the EMAS cohort that there may be considerable variation in population means based on geography, despite essentially similar ethnicity [24]. In that study, the difference between geographic centres could be attributed in large part to differences in health and lifestyle, with BMI or waist circumference and smoking being key contributory factors [24].

INSL3 in hypogonadal men

Hypogonadism is a clinical condition where Leydig cells are unable to produce sufficient T to maintain androgen-dependent physiology. In childhood this is manifest as CHH (congenital hypogonadotropic hypogonadism) or constitutional delay of growth and puberty (CDGP) whereby mostly a failure of the HPG axis prevents puberty from progressing normally. In adult-onset hypogonadism, varying degree of loss of androgen-dependent sexual, anabolic and psychological functions and

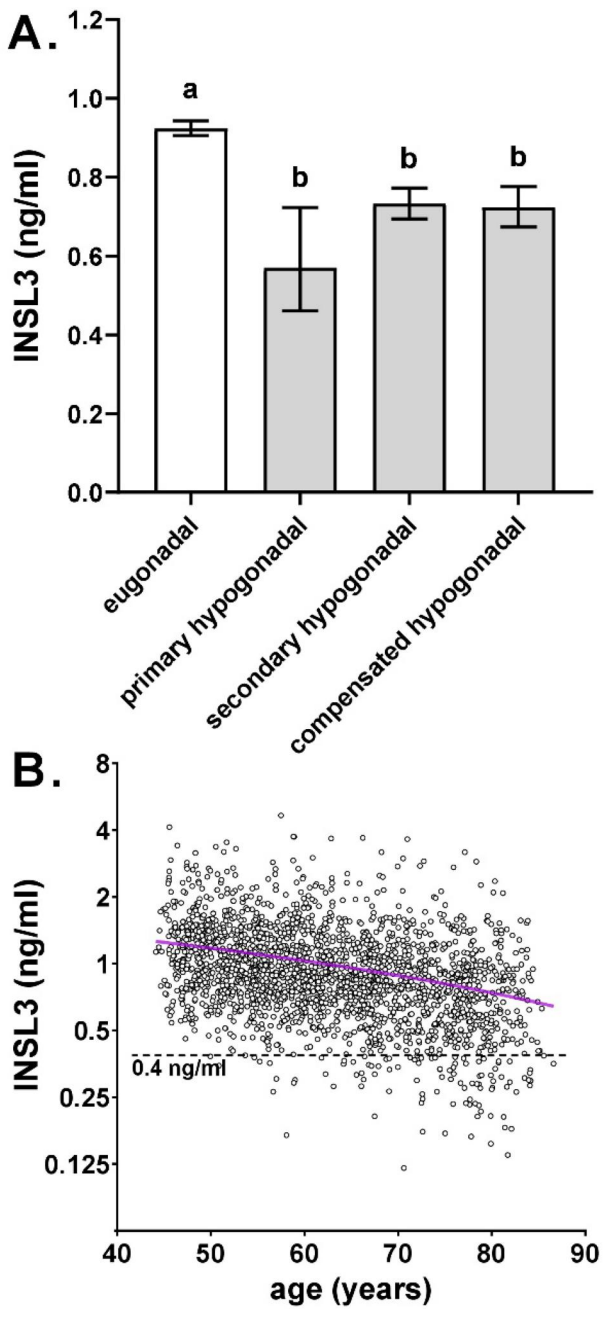

Figure 2. A. Interpolated 65-year-old means and 95% CI calculated from age vs INSL3 regressions for subjects assessed as being eugonadal ($T > 10.5$ nmol/l; LH $\leq$ 9.4 nmol/l), primary hypogonadal ($T \leq 10.5$ nmol/l; LH $>$ 9.4 nmol/l), secondary hypogonadal ($T \leq 10.5$ nmol/l; LH $\leq$ 9.4 nmol/l), or compensated hypogonadal ($T > 10.5$ nmol/l; LH $>$ 9.4 nmol/l) according to the criteria of Tajar et al. [4]. Different lowercase lettering indicates significant difference at $p < 0.05$ (*from* [10]). **B**. Individual INSL3 values from the second phase of the EMAS cohort from all centres plotted against age. INSL3 concentration is represented as a Log_2 scale. The magenta slope indicates the significant regression approximating a decline in INSL3 of approximately 14% per decade. The black horizontal dashed line indicates the proposed 0.4 ng/ml threshold below which hypogonadism is prevalent (*from* [24]).

some secondary sexual characteristics are the hallmarks. Classical hypogonadism can be differentiated into primary testicular (e.g. Klinefelter syndrome) or secondary hypothalamic-pituitary (e.g. pituitary tumours) hypogonadism depending on the nature and site of the pathology. INSL3 as well as T are low in these subjects [31, 42, 43]. In Klinefelter syndrome, INSL3 is also reduced during adolescence and young adulthood [44].

Hypogonadism may also develop in a minority of adult men as they become older (>65 – 70 years); this has been referred to as LOH (late-onset hypogonadism) or functional hypogonadism when low (usually borderline low rather than in the frankly pathological hypogonadal range) T is associated with (non-specific) symptoms compatible with androgen deficiency but no recognisable pathology, as opposed to classical hypogonadism [1–3]. To improve understanding of the aging-related changes in HPG axis function, men with circulating morning T concentration below 10.5 nmol/l in EMAS were categorized as having primary or secondary biochemical hypogonadism where LH concentration is either above or below normal (>9.4 U/l), respectively [4]. Compensated hypogonadism refers to the situation where T is in the normal range but LH is elevated ($T > 10.5$ nmol/l; LH $>$ 9.4 U/l). In all three categories of biochemical hypogonadism, there is a significant Leydig cell dysfunction indicated by low circulating INSL3 concentration (Figure 2A) compared to the eugonadal state [10]. However, a clinical diagnosis of LOH is problematic due to the weak relationship between T concentration and the presence or absence of symptoms. To increase the precision and specificity for the diagnosis of functional hypogonadism, it has been proposed that not only low total T, but also low cFT, should co-exist with multiple (three) sexual symptoms, since these appear to be most dependent on T [1–3]. However, part of the difficulty in using T to define functional hypogonadism is due to its biological variability within an individual, the wide range of variance between individuals, as well as the uncertainty surrounding the threshold level indicative of a deficiency state, with a large borderline grey zone. Could the less variable INSL3 concentration offer a supporting parameter to define hypogonadism especially in elderly men with low T?

A significantly reduced INSL3 indicates that all types of hypogonadism, even if compensated (normal total T), have discernible Leydig cell functional insufficiency (LCI) (Figure 2A). Secondly, low INSL3 appears to be significantly associated with a greater number of age-dependent morbidities, when these are assessed

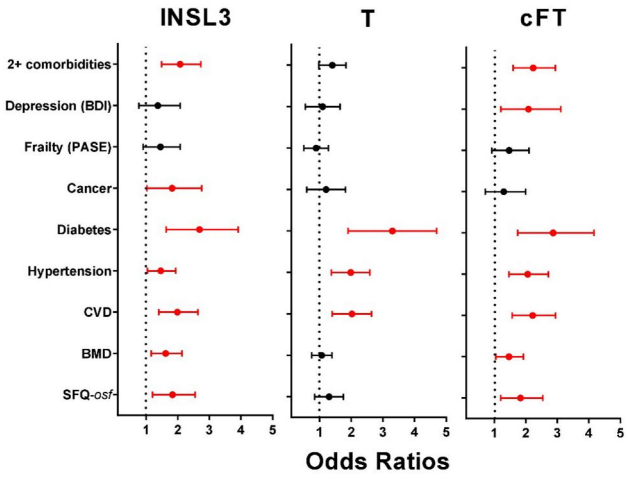

Figure 3. Unadjusted Odds Ratios for the association of low phase 1 INSL3, T, or cFT concentrations with the incidence of various morbidities, as indicated, in the EMAS cohort in phase 2, mean 4.3 years later (*adapted from* [10]). Abbreviations: BDI, Beck Depression Index; PASE, Physical Activity Scale for the Elderly; CVD, cardiovascular disease; BMD, bone mineral density; SFQ-*osf*, sexual function questionnaire – overall sexual function.

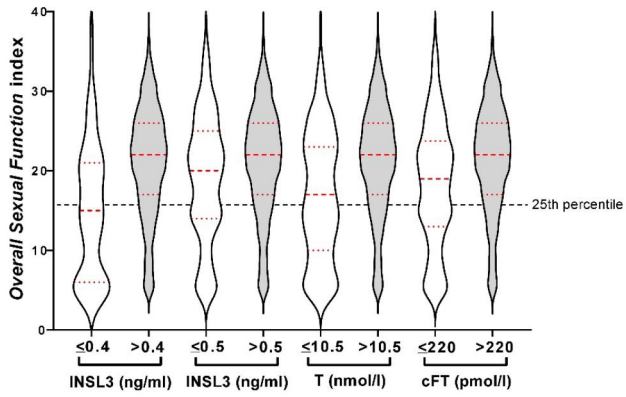

Figure 4. Violin plots of the Overall Sexual Function (OSF) index [29], with means and quartiles (red dashed and dotted horizontal lines within the violins), for phase 2 of the EMAS cohort comparing different thresholds of INSL3, testosterone (T), or calculated free T (cFT) (as indicated on the x-axis). No shading indicates subjects below the threshold (hypogonadal), grey shading above the threshold (eugonadal). The black horizontal dashed line shows the 25th OSF percentile for all subjects. See text for further details.

4-5 years later, than does T, suggesting that INSL3 may be a better predictor of such illness [10] (Figure 3). cFT is similar to INSL3 in this regard [10]. This is particularly true for overall sexual dysfunction where T is not at all predictive at a population level [10] (Figure 3).

A serum concentration of 0.4 ng/ml INSL3 represents the lower 95% CI (2 SD) for men aged 60-65 years, the average age of the EMAS cohort (Figures 1 and 2B) and is close to the mean for those identified as having primary biochemical hypogonadism (0.53 ng/ml; Figure 2A). The mean circulating T and cFT concentrations of those individuals in the EMAS cohort ($n = 117$) with INSL3 $\leq$ 0.4 ng/ml were 11.9 ± 6.4 nmol/l and 191 ± 132 pmol/l, respectively; i.e. close to the biochemical criteria used to help identify functional hypogonadism in the EMAS cohort ($T < 10.4$ nmol/l and cFT $<$220 pmol/l) [3]. Within the EMAS cohort, none of the community-dwelling men aged $\leq$50 years exhibited INSL3 levels lower than 0.4 ng/ml, whereas 11.3% showed such low INSL3 in the age group $\geq$70 years, and only 5% in the cohort overall. We therefore suggest $\leq$0.4 ng/ml INSL3 as a threshold to define clinical LCI and hence a primary biochemical hypogonadism (Figure 4). Interestingly, if such individuals are discounted from the EMAS cohort, the remainder no longer exhibits a significant age-dependent decline in INSL3, allowing this residual cohort to have a single reference range (95% CI; serum) for men aged $>$35 years of (0.4-2.3 ng/ml),

Table 2. Comparisons of INSL3, testosterone (T), and calculated free T (cFT) as leydig cell biomarkers to discriminate men with functional hypogonadism (hypogonadism with sexual symptoms) from the eugonadal population (using data from phase 2 of the EMAS cohort). The OSF threshold was set at the 25th percentile (OSF index $\leq$16).

discriminator	% hypogonadal	OSF threshold	false positive	false negative
INSL3 ($\leq$0.4 ng/ml)	5.0%	25% (16)	41.0%	24.1%
INSL3 ($\leq$0.5 ng/ml)	10.8%	25% (16)	49.3%	22.8%
T ($\leq$10.5 nmol/l)	14.3%	25% (16)	62.4%	23.3%
cFT ($<$220 pmol/l)	14.7%	25% (16)	59.0%	21.5%

which is comparable to that proposed for the younger and smaller Danish cohort using the MS methodology (0.9-2.7 ng/ml; age 18-60 years) [19].

ROC analysis (Suppl. Figure 3) for INSL3, T and cFT using an OSF value of $\leq$16 (25th percentile) as discriminator for reduced sexual function and surrogate for the physiological effects of hypogonadism, confirms that INSL3 is as good as cFT, and considerably better than T, in diagnosing reduced sexual function (see also Figure 4). However, the Area Under the Curve (AUC) results (Suppl. Figure 3), like those for the false positive and false negative rates (Table 2), emphasize that no hormone parameter individually is good at identifying the physiological consequences of hypogonadism. If we inspect the borderline "grey zone" of T values 10.5 $<$ 15.0 nmol/l from the EMAS cohort, then while cFT $\leq$220 pmol/l could recruit a further 10% of those EMAS subjects with an OSF $\leq$16, INSL3 at 0.4 ng/ml or 0.5 ng/ml fails to recruit more than a further 5%, implying that INSL3 appears less suitable as

an additive discriminating parameter for functional hypogonadism, though may serve as an alternative to T or cFT.

In extreme situations such as recovery from anabolic steroid misuse, or application of a male steroidal contraceptive regimen, where even though the endogenous T concentration may become normalized after several weeks' cessation of steroid application, INSL3 concentration remains significantly below pre-treatment levels even after many months [45, 46]. This indicates that although LH can acutely compensate to increase T levels, there is insufficient effect on Leydig cell functional capacity to restore these cells to their pre-treatment differentiation status and/or numbers. In young men this may be only a matter of time; in older men or those with incipient morbidity, such loss of Leydig cell functional capacity may be longer-lasting or even permanent and may therefore premise increased morbidity as these individuals age. This could be particularly concerning in those middle-aged and elderly men undertaking T-therapy. Future studies will need to consider potential long-term loss of Leydig cell functional capacity and LCI in this context.

A further situation of relevance is the application of lifestyle change and weight-loss programs to improve health outcomes; although circulating T may be restored to normal levels, the lack of change in INSL3 resulting from such interventions suggests that LCI may persist [24, 47], with associated increased risk of later morbidity. Moreover, Leydig cells not only produce T, but also promote circulating INSL3 and vitamin D hydroxylation, both independently important for bone and skeletal muscle metabolism [48, 49], besides supporting spermatogenesis and fertility, and other possible functions [50].

Concluding remarks

INSL3 offers a direct assessment of Leydig cell functional capacity, independent of hypothalamic-pituitary feedback regulation. Hence low INSL3 or LCI can potentially contribute to the diagnosis of primary hypogonadism. Low circulating INSL3 correlates with age-dependent morbidity (Figure 3), including sexual symptoms (see also ROC analysis), and suggests that INSL3 could act as a further discriminator of functional hypogonadism. Even where T levels are considered normal as in compensated hypogonadism, INSL3 levels may still be significantly low, as also in secondary hypogonadism, and thus predictive of future age-related morbidity (Figure 3). Further research is needed, particularly in large clinical trials and cohort studies, to elaborate how INSL3 might best be used as a diagnostic or prognostic biomarker in the evaluation of hypogonadism and its treatment by androgen therapy.

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