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Male seminal parameters are not associated with Leydig cell functional capacity in men

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Abstract

Background: Insulin-like peptide 3 (INSL3) is a constitutive, secreted peptide produced in the male uniquely by the Leydig cells of the testes. It is a biomarker for Leydig cell functional capacity, which is a measure of the numbers and differentiation status of these steroidogenic cells and lacks the biological and technical variance of the steroid testosterone. This retrospective study was carried out to examine the relationship between seminal parameters and the Leydig cell compartment, and secondarily to assess other factors responsible for determining Leydig cell functional capacity.

Methods: INSL3 was assessed together with seminal, anthropometric, and hormonal parameters in a Swedish cohort of 18-year-old men, representing the average population, and in a smaller, more heterogeneous cohort of men visiting an Australian infertility clinic.

Results and discussion: Average INSL3 concentration at 18 years is greater than that reported at younger or older ages and indicated a large 10-fold variation. In neither cohort was there a relationship between INSL3 concentration and any semen parameter. For the larger, more uniform Swedish cohort of young men, there was a significant negative relationship between INSL3 and BMI, supporting the idea that adult Leydig cell functional capacity may be established during puberty. In both cohorts, there was a significant relationship between INSL3 and FSH, but not LH concentration. No relationship was found between INSL3 and androgen receptor trinucleotide repeat polymorphisms, reinforcing the notion that Leydig cell functional capacity is unlikely to be determined by androgen influence alone. Nor did INSL3 correlate with the T/LH ratio, an alternative measure of Leydig cell functional capacity, supporting the view that these are independent measures of Leydig cell function.

KEYWORDS
hypothalamic-pituitary-gonadal axis, insulin-like peptide 3, Leydig cell functional capacity, male fertility

1 | INTRODUCTION

Recent publications have highlighted a possible link between Leydig cell functionality and male fertility.[1–7] Whilst it is well established that testosterone produced by Leydig cells is essential for the progression of spermatogenesis via its effects on Sertoli cells, what is not clear is whether a moderate disruption or defect in the Leydig cell compartment independently of testosterone might be partly responsible for reduced sperm numbers and/or motility. The situation is confused since both the interstitial and seminiferous compartments are under the homeostatic control of the HPG axis via a common GnRH pathway and the anterior pituitary hormones LH and FSH, respectively.[8] So, for example, in classical hypothalamic hypogonadism, a reduction of gonadotropin production leads to loss of both spermatozoa and testosterone. Moreover, the integrity of the two compartments could be developmentally linked via their joint and/or mutual relationship in the context of the testicular dysgenesis syndrome, whereby it is postulated that prenatal endocrine disruption might impact the differentiation of both spermatogenic and steroidogenic components.[9] Extensive results of semen and hormonal analyses in young men and infertility patients show that within the normal range there appears to be no correlation between circulating testosterone (T) and any semen parameter.[4] However, there does appear to be a negative association between circulating LH and sperm concentration.[4] This would imply that the resulting normalized testosterone concentration may be the result of a compensated Leydig cell dysfunction, which is reflected by the ratio of T/LH. The latter parameter has been referred to elsewhere as Leydig cell functional capacity and is thought to reflect the number and functionality of the Leydig cells.[1,2] For example, dysfunctional Leydig cells might have impaired LH receptor signalling, leading to a reduced T biosynthesis for a given concentration of LH.

Insulin-like peptide 3 (INSL3) is also a hormone synthesized and secreted into the bloodstream uniquely by testicular Leydig cells. Importantly, its production is largely constitutive and acutely independent of the hypothalamic-pituitary-gonadal (HPG) axis. Unlike steroids, such as testosterone, whose synthesis and secretion is regulated within minutes by LH, INSL3 is not altered by LH (or hCG) within this time frame[10] but only after days or weeks, when the gonadotropin may influence the proliferation and/or differentiation of Leydig cells.[11] Consequently, INSL3 is a reliable biomarker for the number and mature differentiation status of the Leydig cells.[11] In other words, it is also a measure of Leydig cell functional capacity. In order to explore whether the Leydig cell compartment rather than the HPG axis could indeed be directly influencing spermatogenesis in adult men, two independent cohorts were analysed retrospectively for circulating INSL3, together with other reproductive hormones and a range of seminal parameters. One cohort comprised 303 young men aged 18 years and of unknown fertility, called up for military service in Sweden, and representing a cross-section of the Swedish male population.[12,13] A second cohort comprised 43 men (aged 26–53 years) attending an Australian infertility clinic with an average duration of infertility of 2.5 ± 0.6 years. These men had been recruited for a trial of an antioxidant preparation[14] and were similarly assessed for seminal, hormonal and anthropometric parameters.

The Swedish cohort of 18-year-old men also allowed a secondary aim of comparing INSL3 values at 18 years with those estimated in older and younger individuals of similar ethnic and geographic background. Detailed studies in rats have shown that circulating INSL3 concentration reaches a peak at around 6 weeks (the time when mature spermatozoa are first evident in the epididymis) and subsequently declines to stable lower mean levels by about 3 months.[10] For the human, several studies of boys undergoing puberty show that maximal INSL3 values have probably not been attained by Tanner stage 5 (aged ca. 14–17 years).[15–17] Also, another study has indicated that INSL3 concentration declines steadily from age 35–40 to old age (80+ years).[18] There is little detailed information about mean INSL3 concentration at an intermediate age between puberty and established adulthood, though one recent study of Danish men has supported higher INSL3 values in young adulthood.[19]

Whilst the primary aim of this retrospective study was to assess the relationship between seminal parameters and Leydig cell functional capacity, the parameters available also allowed a third aim of determining possible factors linked to the large inter-individual variation in circulating INSL3 measured. Besides seminal, anthropometric and hormonal parameters, the Swedish cohort of 18-year-old men was also assessed for androgen receptor polymorphisms to elucidate a further possible interaction with androgen action. In this association study, INSL3 was compared with testosterone, since it has recently been suggested that INSL3 is an important new clinical measure of Leydig cell function, which differs from and complements testosterone.[20]

2 | MATERIALS AND METHODS

2.1 | Subjects

The Swedish cohort of 305 young men (Table 1A) called up for military service and representing the southern Swedish population has been described in detail before.[12] They are representative of 95% of the male population and exclude only individuals with severe medical conditions. Of these 305 subjects, average age 18.2 years and mean BMI (weight/height^2) of 22.6 + 3.2 kg/m^2, the majority (248) were born and raised in Sweden. Serum samples were only available for INSL3 analysis from 303 of them. Smoking habits were ascertained from a questionnaire.[12] Physical examination also included the estimation by ultrasound of left and right testis volume. Scanning made use of a 7.5-MHz transducer connected to an Aloka 900 SSE scanner (Aloka, Tokyo, Japan) in two projections; volume was calculated as length × width × depth × 0.52.[21] Mean and median parameters are indicated in Table 1A.

Additionally, 271 serum samples from older men within the general population and without severe illness were available for the measurement of INSL3 from the second phase of the European Male Aging Study, all collected at the same centre in Sweden as the

TABLE 1 Average descriptive, semen and hormone parameters

Parameter	Mean ± SD (*n*)	Median (25%, 75% quartiles)	Reference limits
A. Swedish cohort of military conscripts			
Age (years)	18.2 ± 0.4 (305)	18.2 (18.1, 19.2)	NA
BMI	22.56 ± 3.17 (303)	22.20 (20.80, 23.60)	18.5–24.9
Sperm concentration ($\times 10^6$/ml)	72.17 ± 66.44 (301)	53.80 (28.15, 94.90)	$>15 \times 10^6$
Sperm motility (CASA, %)	50.8 ± 22.0 (286)	51.0 (35.0, 68.0)	>32%
Semen volume (ml)	3.21 ± 1.31 (302)	3.2 (2.3, 4.0)	>1.5
DNA fragmentation index (%)	13.60 ± 8.99 (278)	11.14 (7.64, 15.98)	<20%
Left testis vol (US, ml)	14.19 ± 3.98 (305)	13.5 (11.5, 16.8)	NA
Right testis vol (US, ml)	14.84 ± 4.14 (304)	14.5 (11.7, 17.8)	NA
INSL3 (ng/ml)	2.15 ± 0.86 (302)	2.02 (0.74, 2.52)	NA
Total testosterone (nmol/L)	22.95 ± 5.33 (304)	23.00 (19.4, 27.0)	8.7–33.0
LH (IU/L)	4.3 ± 1.6 (304)	4.14 (3.18, 5.28)	1.2–9.6
FSH (IU/L)	3.4 ± 1.7 (304)	3.15 (2.20, 4.20)	1.0–10.5
T/LH ratio	5.96 + 2.39 (304)	5.56 (4.28, 7.19)	NA
SHBG (nmol/L)	28.46 ± 9.65 (304)	28.0 (22.0, 34.0)	13–50
Inhibin B (ng/L)	209.1 ± 61.6 (304)	200.0 (166.3, 251.0)	100–240
AR no. CAG repeats	22.19 ± 2.96 (298)	22.0 (20.0, 24.0)	NA
AR no. GGN repeats	16.63 ± 2.49 (224)	17.0 (17.0, 18.0)	NA
B. Swedish cohort of men enrolled in the second phase of the EMAS study			
Age (years)	63.3 ± 10.6 (271)	61.6 (54.7, 72.4)	NA
BMI	27.31 ± 4.13 (252)	27.0 (2.4, 29.9)	18.5–24.9
INSL3 (ng/ml)	0.96 ± 0.49 (271)	0.84 (0.63, 1.16)	NA
Total testosterone (nmol/L)	15.93 ± 6.31 (271)	14.71 (11.77, 19.71)	8.7–33.0
LH (IU/L)	6.39 ± 4.78 (229)	5.32 (3.87, 7.18)	1.2–9.6
T/LH ratio	3.21 ± 1.98 (229)	2.81 (1.97, 3.95)	NA
C. Australian men attending an infertility clinic			
Age (years)	39.0 ± 6.1 (40)	38.0 (35.0, 43.0)	NA
BMI	29.31 ± 3.86 (30)	28.6 (26.7, 32.7)	18.5–24.9
Sperm concentration ($\times 10^6$/ml)	32.54 ± 40.53 (40)	19.3 (12.0, 33.9)	$>15 \times 10^6$
Sperm motility (%)	36.2 ± 12.5 (40)	38.5 (29.0, 45.0)	>32
Semen volume (ml)	3.07 ± 1.83 (40)	2.65 (1.80, 4.00)	>1.5
Sperm normal morphology (%)	6.35 ± 4.88 (40)	5.00 (2.00, 9.75)	>4
DNA fragmentation index (%)	22.55 + 8.52 (40)	22.55 (16.53, 25.93)	<20
ROS (μg formazan/10^7 sperm)	81.20 + 61.33 (40)	66.5 (45.5, 94.0)	NA
INSL3 (ng/ml)	1.78 ± 0.82 (43)	1.52 (1.25, 2.16)	NA
Total testosterone (nmol/L)	13.84 ± 3.93 (40)	14.1 (10.9, 15.6)	8.7–33.0
LH (IU/L)	4.33 ± 1.53 (40)	4.00 (5.00, 5.23)	1.2–9.6
FSH (IU/L)	5.52 ± 2.81 (40)	5.45 (3.18, 7.08)	1.0–10.5
T/LH ratio	3.53 ± 1.46 (40)	3.10 (2.48, 4.68)	NA
AMH (pmol/L)	63.42 ± 29.65 (38)	64.10 (37.28, 88.88)	5–140

Note: Average relevant descriptive, semen and hormone parameters for (A) the Swedish cohort of military conscripts, (B) the Swedish component of the European Male Aging Study (second phase), (C) the Australian cohort of men attending an infertility clinic. Relevant reference limits are also included. NA, not applicable.

military conscripts, and representing the same southern Swedish population.[22,23] Relevant mean and median parameters are presented in Table 1B. The data for INSL3 included in Figure 1B for boys going through puberty are redrawn from Johanssen et al.[17] and represent a longitudinal pilot cohort from the Copenhagen area of Denmark. Data were obtained from 10 boys of Caucasian ethnicity

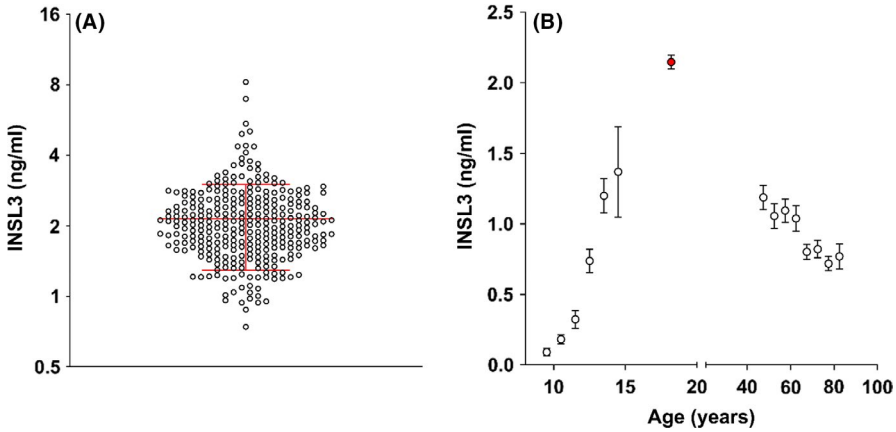

FIGURE 1 (A) Scatterplot of peripheral INSL3 concentration in 303 young men aged 18 years called up for military service in southern Sweden. INSL3 expressed using Log_2 scale (mean ± SD). (B) Age distribution for circulating INSL3, based on three independent cohorts (aged <16 years from the Danish puberty pilot study[17]; aged 18 years from A; aged >40 years from phase 2 of the Malmo subcohort of the EMAS study[22,23]) using the same TRFIA assay and controls (means ± SEM; for details see text)

followed for several years from before and during puberty with regular blood sampling, as described previously.[17]

Serum was also available from 43 participants at entry from a study involving men recruited at an Australian infertility clinic to investigate the effects of antioxidant treatment on semen parameters.[14] Altogether 50 men had been recruited with entry criteria of at least one million sperm per millilitre in their ejaculate and evidence of seminal oxidative stress. The average age of participants was 39 years (range 26–53 years) with an average duration of infertility of 2.5 years. The relevant mean and median parameters are presented in Table 1C.

Full ethical approval and patient consent had been obtained for all studies, as published,[12,14,22,23] including later use of archived blood samples for retrospective analyses.

2.2 | Semen samples and analyses

For the Swedish cohort, semen collection followed 48–72 hs abstinence and analysis followed WHO guidelines,[24] employing strict morphological criteria, and has been reported in detail previously.[13,21,25] Sperm concentration was estimated using a Neubauer chamber and percentage sperm motility was assessed by CASA using the CRISMAS system as previously described.[25] Sperm DNA damage was assessed by the DNA fragmentation index (DFI), as described,[13] based on the proportion of acridine orange red fluorescence (single-stranded DNA) measured by FACS. The Australian samples measured sperm concentration and semen volume as above. Sperm motility and morphology were assessed microscopically according to WHO guidelines,[24] as above. TUNEL was used to assess DNA integrity employing the In situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) to detect single-strand breaks.[14] The DNA fragmentation index (DFI) was calculated as the percentage of TUNEL-positive nuclei (FITC-labelled, green) per total number of sperm nuclei (propidium iodide, red). Reactive oxygen species (ROS) was measured by a modified colorimetric nitroblue tetrazolium assay,[14] recording results as the intracellular product formazan in μg per 10^7 spermatozoa.

2.3 | Androgen receptor trinucleotide repeat analysis

The N-terminal domain of the androgen receptor encoding the transactivation region is subject to marked polymorphism due to two trinucleotide repeat elements. Extremely long and short lengths of the poly-glutamine encoding CAG repeat are linked to Kennedy's disease with reduced androgen responsiveness and male infertility[26] and in vitro even modest variation in length can affect androgen receptor function.[27] The more downstream GGN repeat element has not been well studied and does not appear to associate with any phenotypic trait.[28] Since Leydig cell function may be directly influenced by androgen action, both CAG- and GGN repeat lengths were determined by nested PCR using flanking gene-specific oligonucleotide primers, followed by sequencing of PCR products, as previously described in detail,[13,28] and retrospectively compared to Leydig cell functional capacity as represented by INSL3 concentration in the circulation.

2.4 | Hormone analyses

For the Swedish cohort of young men, FSH, LH, total testosterone (T) and SHBG were measured by automated fluorescent immunoassay (Autodelfia; Wallac Oy, Turku, Finland) at the routine clinical chemistry laboratory at Uppsala University Hospital.[13] Intra- and total assay variation for these parameters was <4.0% and <7.5%, respectively. Free testosterone (FT) was estimated from the concentrations

of total testosterone and SHBG[29] and Inhibin B made use of a specific immunometric assay with a detection limit of 15 ng/L and intra-assay and total assay variation coefficients <7%.[30] For the Australian cohort, with the exception of AMH (see below), all hormone assays used the automated ADVIA Centaur chemiluminescent immunoassay system (Bayer Australia Ltd; Pymble, NSW, Australia). For LH and FSH, the limit of detection was 0.1 and 0.3 IU/L, respectively, with intra- and total assay coefficients of variation of <4.0%. For testosterone, the limit of detection was 0.35 nmol/L, with intra- and total assay coefficients of variation of <6% and <9%, respectively. Serum AMH concentration was measured by Immunotech high-sensitivity immuno-enzymetric assay (Beckman Coulter, Marseille, France). The sensitivity of this assay was 0.7 pmol/L; intra- and total assay coefficients of variation were ≤12.3% and ≤14.2%, respectively.

INSL3 was measured in all cohorts by time-resolved fluorescent immunoassay, as previously described.[11] We have previously shown that the concentration of this peptide hormone is quantitatively stable over many years when stored frozen at −20°C and subjected to several freeze-thaw cycles.[11] Importantly, for all the assays reported here, the same internal standards were used, as well as identical external control male serum samples. The limit of detection of this assay, as used, was 20 pg/ml with intra- and total coefficients of variation of <3% and <8%, respectively. Recently, this assay was directly compared with a new LC-MS/MS procedure with almost identical results.[31]

2.5 | Statistical analysis

Data were first explored for outliers and associations using scatter plots and simple correlation analysis, and descriptive statistics computed for each variable. INSL3 and total testosterone (T) were each considered as response (dependent) variables. Multiple linear regression analysis was fitted for each of the response variables using the parameters listed in Table 1 as independent variables. Known essential variables and potentially useful subsets of explanatory variables were first selected from the list. A combination of stepwise regression, forward selection and backward elimination was used to identify the best final model, with best fit being assessed using the adjusted R-square criterion. Multicollinearity among variables was checked using the variance inflation factor (VIF). Consequently T, but not FT, nor the T/LH ratio were included in the models. Assumptions of multiple regression were checked using residuals. Variables were transformed when assumptions of normality and constancy of variance were violated. Only INSL3 needed to be log-transformed as dependent variable. Parameter estimates of variables with statistically significant contribution at the 5% level of significance to the prediction of the dependent variable are reported. Few variables with 10% level of statistical significance were left in the final model to inform the reader of the potential significance of the variables in further study. An independent variable with a negative model coefficient indicates that it has an inverse association with the dependent variable (that is, when one increases, the other decreases). The standardized regression beta coefficient compares the strength of the effect of each individual independent variable to the dependent variable. The higher the absolute value of the beta coefficient, the stronger the effect. For a proper interpretation of a model fitted on a log-transformed response variable, the parameter estimates should be first back-transformed to the original units by taking the exponent of the model coefficient for each variable. The adjusted coefficient of determination (R^2) is reported to show the amount of variation in the dependent variable that is explained by the independent variables of the model, along with the *p*-values of the ANOVA showing the statistical significance of the final model. Data were analysed using Graphpad Prism version 8.2 and SPSS (IBM SPSS Statistics 26) software. The data for trinucleotide repeats were compared by ANOVA followed by Neumann-Keuls post hoc test.

3 | RESULTS

3.1 | Descriptive parameters for the Swedish and Australian cohorts

Table 1A shows mean and median data for the Swedish cohort of military conscripts. These are of a very close age range and predominantly healthy, with mostly low BMI, modest smoking prevalence (28.8% smokers), and seminal parameters within the normal range. Similarly, hormonal parameters are also within the normal range. For the Australian men attending an infertility clinic (Table 1C), these are older men, with higher BMI, mixed to poor semen parameters, including a higher mean DNA fragmentation index and evidence of oxidative stress. Testosterone is reduced compared to the Swedish cohort and gonadotropins are elevated. Table 1B shows parameters available for the second phase cohort of older men recruited as part of the EMAS study and used for comparison only.

3.2 | INSL3 concentration in 18-year-old men represents peak post-pubertal levels

Applying the same validated INSL3 assay that has been used for studies of puberty and ageing (including identical positive control blood samples), we show here that mean INSL3 concentration in 18-year-old Caucasian men is substantially greater at 2.15 ± 0.86 ng/ml (mean ± SD) than in late adolescence (1.37 ± 0.55 ng/ml at Tanner stage 5), or after 40 years (1.18 ± 0.49 ng/ml at mean 47.5 years; Figure 1A and B). The data for ageing men in Figure 1B represent men from the same district of Sweden analysed as part of the EMAS study[23] (R Anand-Ivell & R Ivell, unpublished). This suggests a profile of Leydig cell development largely similar to that observed in rats, with maximal Leydig cell functional capacity evident in young adulthood (ca. 18 years). Also evident from this cohort is that there is a similar approximately 10-fold range of INSL3 values (0.74–8.2 ng/ml) in 18-year-old men as is observed in the larger ageing male population.[18]

3.3 | Lack of relationship between INSL3 and seminal parameters

Both in the Swedish cohort of 18-year-old young men, as well as in the Australian men attending an infertility clinic, initial correlation analysis showed that there was no significant association between INSL3 and any sperm-related parameter (sperm concentration, total sperm number, ejaculate volume, progressive motility and DNA fragmentation index). For testosterone as dependent variable, the final multiple regression model suggested, for the larger Swedish cohort of young men, a significant negative association with sperm concentration ($p = 0.011$) though for no other sperm-related parameter (Table 2); for the final multiple regression model of the smaller Australian cohort, testosterone failed to show this association, though did indicate a significant positive relationship to the DNA fragmentation index (DFI; $p = 0.017$; Table 3). Substituting free testosterone as dependent or independent variable in the multiple regression analysis had no qualitative effect on the relationships described above (not shown).

TABLE 2 Multiple regression analysis of the Swedish cohort of young men

Model	B	Std. Error	Beta	*t*	*p*-value
(A) Dependent variable: lnINSL3					
(Constant)	0.306	0.227		1.350	0.178
Smoking	−0.071	0.042	−0.091	−1.679	0.094
T	0.017	0.004	0.260	4.048	<0.001
Inhibin B	0.001	<0.001	0.141	2.180	0.030
FSH	0.040	0.013	0.194	3.063	0.002
BMI	−0.016	0.006	−0.145	−2.488	0.013
Avg. testis vol.[a]	0.016	0.005	0.175	3.026	0.003
(B) Dependent variable: T					
(Constant)	15.965	3.175		5.029	<0.001
LH	0.887	0.180	0.261	4.915	<0.001
SHBG	0.264	0.031	0.480	8.607	<0.001
BMI	−0.212	0.090	−0.132	−2.359	0.019
Avg. testis vol.[a]	0.211	0.078	0.154	2.689	0.008
No. GGN[b]	−0.210	0.121	−0.094	−1.766	0.079
lnINSL3	2.177	0.834	0.140	2.609	0.010
Sperm conc.	−0.011	0.004	−0.141	−2.555	0.011

Note: (A) This optimal model accounts for 14.0% (adjusted R^2) of variability in lnINSL3 ($p < 0.001$). (B) This optimal model accounts for 40.9% (adjusted R^2) of variability in total testosterone ($p < 0.001$). Optimal coefficients for the final multiple regression models of the Malmo cohort of 18-year-old young men with either lnINSL3 (log-transformed INSL3) or total testosterone (T) as dependent variables.

Abbreviations: Avg. testis vol, represents the mean of left and right testis volumes determined by ultrasound; BMI, body mass index; No. GGN, represents the sequence length of the GGN trinucleotide repeat in the androgen receptor gene; SHBG, serum hormone-binding globulin.

[a]Avg. testis vol. represents the mean of left and right testis volumes determined by ultrasound.

[b]No. GGN represents the sequence length of the GGN trinucleotide repeat in the androgen receptor gene.

3.4 | Correlation between INSL3 and systemic hormonal and clinical parameters

For the Swedish cohort of 18-year-old men from the general population, as expected, the best model showed a significant association between INSL3 and total T ($p < 0.001$; Table 2). There was also a significant positive association with FSH ($p = 0.002$; Table 2), though not with LH. In the preliminary correlation analysis, INSL3 concentration showed no correlation with the T/LH ratio, and a negative trend with reported smoking incidence, which was kept in the final model ($p = 0.094$; Table 2). INSL3 was also significantly negatively associated with BMI ($p = 0.013$; Table 2), Inhibin B ($p = 0.030$; Table 2), and positively with average testis size measured by ultrasound ($p < 0.003$; Table 2), even though the Leydig cell compartment comprises less than 10% of testis volume, the bulk being made up of the spermatogenic compartment. Note that the model was fitted to the transformed values of INSL3, and hence, the coefficients should be first back-transformed using exp(B) for the purpose of interpretation. For total T, for instance, this becomes exp(0.017) = 1.02. That means, if all other variables in the model are held constant, for a unit increase in total T, the value of INSL3 increases by 2%. For BMI, exp(−0.016) = 0.98, indicating a 2% reduction in INSL3 concentration for each unit increase in BMI.

In the final multiple regression model, T was significantly associated with LH in this study (Table 2; $p < 0.001$), though showed no relationship towards FSH. In the final model, total T was also

TABLE 3 Multiple regression analysis of Australian men attending an infertility clinic

Model	B	Std. Error	Beta	*t*	*p*-value
(A) Dependent variable: lnINSL3					
(Constant)	−0.544	0.214		−2.536	0.016
T	0.053	0.014	0.496	3.840	<0.001
FSH	0.059	0.019	0.398	3.088	0.004
(B) Dependent variable: T					
(Constant)	13.923	4.851		2.870	0.008
lnINSL3	4.033	1.299	0.416	3.104	0.005
BMI	−0.348	0.151	−0.313	−2.304	0.030
AMH	0.063	0.019	0.451	3.307	0.003
DFI	0.190	0.074	0.359	2.560	0.017

Note: (A) This optimal model accounts for 40.5% (adjusted R^2) of variability in lnINSL3 ($p < 0.001$). (B) This optimal model accounts for 50.5% (adjusted R^2) of variability in total testosterone ($p < 0.001$). Optimal coefficients for the final multiple regression models of the Australian cohort of men attending an infertility clinic with either lnINSL3 (log-transformed INSL3) or total testosterone (T) as dependent variables.

Abbreviations: AMH, Anti-Mullerian Hormone; BMI, body mass index; DFI, DNA fragmentation index measured by TUNEL.

associated with SHBG ($p < 0.001$), BMI ($p = 0.019$), average testis size ($p = 0.008$), lnINSL3 ($p = 0.010$) and with an association trend in regard to the number of GGN trinucleotide AR repeats ($p = 0.079$).

Assessing all baseline (pre-antioxidant therapy) parameters in the smaller, more heterogeneous Australian cohort showed that, as for the Swedish cohort, INSL3 in the final model was strongly associated with total T (Table 3; $p < 0.001$). INSL3 was also associated with FSH (Table 3; $p = 0.004$), though not with any other parameter. Apart from an association with AMH (Table 3; $p < 0.003$), T showed no significant association with any other parameter except INSL3 (Table 3; $p = 0.005$), BMI (Table 3; $p = 0.030$) and the sperm DNA fragmentation index measured by TUNEL (Table 3; $p = 0.017$).

As indicated previously,[13] whereas 3 months of antioxidant treatment appeared to improve some seminal parameters dependent on oxidative stress, it had no effect on any hormonal parameter, also now including INSL3 concentration, which was not different within individuals between pre- and post-antioxidant treatment (mean ± SEM: pre: 1.78 ± 0.13 ng/ml; post: 1.77 ± 0.12 ng/ml). In fact, reflecting what had been shown previously for the within-individual consistency of INSL3 over time,[10] the individual values measured at entry and completion of the antioxidant trial after 3 months were very similar ($r = 0.656$, $p < 0.001$).

3.5 | Lack of relationship between INSL3 expression and androgen receptor variants

For the Swedish cohort, data were also available in regard to the presence of common androgen receptor polymorphisms, represented by two trinucleotide repeat regions within the androgen receptor gene. Longer or shorter repeat regions have been associated with suboptimal androgen responsiveness[32,33] and some reproductive parameters.[27,34,35] No association was evident between circulating INSL3 concentration and shorter or longer trinucleotide repeat variants of the androgen receptor (Figure 2). The CAG repeat number did indicate an independent negative correlation with total sperm number ($r = -0.141$, $p = 0.015$; not shown) as well as positively with LH concentration ($r = 0.114$, $p = 0.046$; not shown), though with no other parameter in this cohort, whereas the GGN repeat number, other than with the CAG repeat number ($r = -0.240$, $p < 0.001$; not shown), did correlate with semen volume ($r = 0.187$, $p = 0.005$; not shown) and indicated a negative trend in regard to T (Table 2; $p = 0.079$), allowing it to be included in the final predictive multiple regression model.

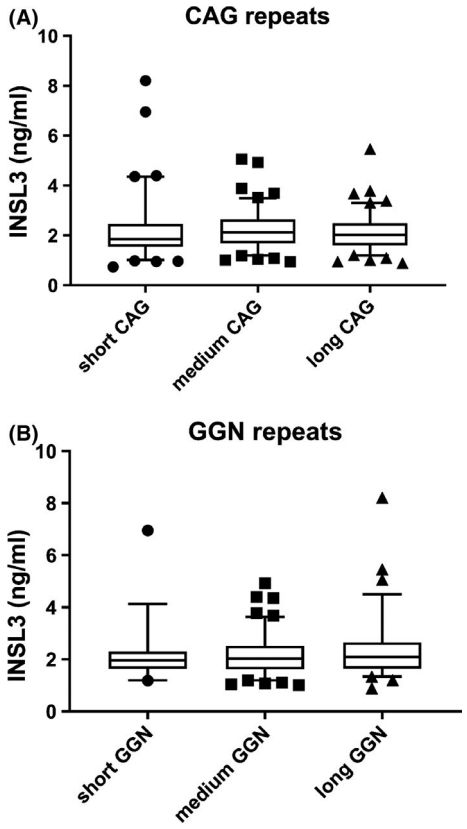

FIGURE 2 Relationship between peripheral INSL3 concentration and length of the CAG- and GGN repeats within the 5′ region of the androgen receptor gene. CAG repeat no.: small 13–20 ($n = 90$), medium 21–23 ($n = 110$), long 24–30 ($n = 102$); GGN repeat no.: small 4–16 ($n = 33$), medium 17 ($n = 114$), long 18–20 ($n = 75$). Box and whisker plot represents median and 5/95 percentiles

4 | DISCUSSION

4.1 | Maximal Leydig cell functional capacity is achieved in early adulthood

Whilst individual Leydig cells mostly appear neither to divide nor to die during normal adult life, there does appear in vivo to be a decline in 3betaHSD2 stainable interstitial cells and overall INSL3 gene expression with increasing age,[36] suggesting either a reduction of Leydig cell numbers or their differentiation status, even though in vitro such older cells still appear to be as steroidogenic and responsive to gonadotropins as younger ones.[36] The functional capacity of Leydig cells to produce hormones like testosterone or INSL3 in vivo will reflect the total Leydig cell mass and their average level of differentiation, which will only change slowly over adult lifetime. Both the ratio of naturally circulating T to LH, as well as the acute T response to LH or hCG injection, have also been suggested as indicators of in vivo Leydig cell functional capacity. The biomarker INSL3 is a constitutive and relatively robust indicator also of Leydig cell functional capacity, acutely independent of the HPG axis, with low within-individual and technical variance,[10,37] and effectively measuring the product of Leydig cell numbers and their average differentiation status. It therefore differs from testosterone whose measurement is associated with relatively high technical and biological variance, and whose circulating level is under constant acute feedback correction through the phasic hormones of the HPG axis. One consequence of this is the peripubertal profile of testosterone in most species, which with increasing LH rises to a maximum at the end of puberty, attaining a level which is then homeostatically maintained into adulthood[38]

and is compensated by increasing LH as men age.[39] INSL3, in contrast, rises to a maximum at the end of puberty concomitant with the massive increase in LH, but then settles in post-puberty to a lower steady-state concentration as pituitary LH declines to more stable adult levels.[14] During puberty, LH stimulates both the proliferation and differentiation of Leydig cells which appear to attain their maximum number in late adolescence. This chronic effect of LH is in stark contrast to the acute phasic stimulation by LH of testosterone production and secretion. In rats, we have shown that peak INSL3 coincides with the first appearance of spermatozoa in the epididymis, but in humans until recently, we have not had such information. Conventionally, in boys, we identify the attainment of puberty with Tanner stage 5 at around 14–17 years. But the present study shows that Leydig cell functional capacity continues to increase to at least 18 years, before its biomarker INSL3 reduces to a lower value at some time within the next decade or so.

Recently, Albrethsen et al.[19] published the results of an assessment of INSL3 in several Danish cohorts, using a new LC/MS-MS method earlier shown to give very similar results to the INSL3 immunoassay used here.[31] They showed a similar pattern of INSL3 increase during puberty and young adulthood, as well as a negative association with smoking and increasing age. However, in the Danish study, the mean and confidence intervals illustrated for young men at approximately age 20 appear to be somewhat lower than those values reported here for the Swedish 18-year-old men. It should be recalled that there appear to be substantial differences in some andrological parameters between otherwise comparable Danish and Swedish men,[12] with the first having significantly worse semen parameters. Since Sertoli and Leydig cell numbers in the human testis appear to correlate closely,[36] with the former largely responsible for supporting spermatogenesis, this would imply that the INSL3 reference values given in the article by Albrethsen et al.[19] may only be appropriate for Danish subjects. Further research on INSL3 geographic variation will be important to help clarify some of these issues.

The uniform age of the Swedish cohort means that any associations evident between parameters can be assessed free of any age-dependent confounding factors, which can add substantial variance to this kind of analysis. For the Swedish cohort of 18-year-old men, INSL3 did not correlate significantly with the T/LH ratio, nor was there was any relationship between these parameters for the smaller, more heterogeneous Australian cohort, even after correcting for age and/or BMI, implying that other factors are influencing the T/LH ratio compared to INSL3. Moreover, and in agreement with Toppari,[20] INSL3 appears to provide a different measure of Leydig cell function compared to T or the T/LH ratio, perhaps reflecting chronic rather than acute Leydig cell functional capacity.

4.2 | Lack of association between Leydig cell functional capacity and semen parameters

A key finding of the present study, involving two separate cohorts of men, is that we find no relationship at all between Leydig cell functional capacity, reflected by circulating INSL3 concentration, and male fertility potential evidenced in standard seminal parameters. There is substantial inter-individual variance in INSL3 concentration in young men, and elsewhere, it has been shown that this is a good predictor of morbidity as men age, since it correlates not only with testosterone, but also with smoking, obesity, diabetes, cardiovascular disease and bone density[40] (R Anand-Ivell & R Ivell, unpublished). Interestingly, seminal parameters have also recently been considered to be good predictors of morbidity, such as the metabolic syndrome.[41,42] Logically, therefore, one might have expected to see a correlation between Leydig cell parameters and those of the seminiferous compartment, especially given the significant association between Sertoli and Leydig cell numbers.[36] That this is not the case suggests that the associations with morbidity are relatively independent events, or that such a relationship might only pertain to older subjects. Moreover, the very high intra-testicular T concentration in eugonadal men would suggest that only in extreme hypogonadism could androgen receptors in the seminiferous compartment remain unoccupied and be affected by changing T concentration.

One reason for this discussion is that it has been shown that male fertility parameters may partly be determined early during foetal life as a consequence of a postulated testicular dysgenesis syndrome, which shares its origins also with the development of the Leydig cell compartment and associated androgen production.[9] Foetal or perinatal exposures to environmental endocrine disruptors, maternal pharmaceuticals or other early developmental insults appear to impact on stem and progenitor cells within the developing gonads, leading to impairment of spermatogenesis as well as of Leydig cell-dependent physiology, for example, resulting in cryptorchidism.[43] Moreover, in recent Chinese studies it has been shown that both INSL3 and sperm parameters may mutually interact under the negative influence of adult phthalate exposure.[44,45] The findings from the two studies presented here imply that poor sperm parameters and Leydig cell functional capacity in young adulthood are likely to have a separate postnatal aetiology from that caused by a common association in early life, and that lifestyle and/or exposures in childhood and adolescence are likely to be playing a significant role. In particular, the Swedish study highlights the marked negative influence of BMI on chronic Leydig cell functional capacity, reinforcing preliminary findings from a recent study looking at late adolescent boys,[46] as well as other more diverse cohorts.[19,40] How BMI affects circulating INSL3 levels is not known, but unlike for testosterone where the increased levels of aromatase in fat tissue will have a directly modifying effect on the circulating androgen, it appears more likely that adipose tissue factors may be influencing Leydig cell function. Moreover, a recent study in bulls has shown a positive effect of a prepubertal high-protein diet on both later INSL3 production and the timing of puberty.[47] Together, such results support a view that healthy prepubertal nutrition may positively influence chronic adult Leydig cell functional capacity, though a negative influence of other environmental and lifestyle stressors cannot be excluded.

The lack of association between INSL3 and male seminal parameters appears to contradict studies which suggest that INSL3, acting via specific RXFP2 receptors on spermatocytes and spermatids, may act anti-apoptotically to prevent germ cell loss. However, this relationship has been revealed only in extreme situations of INSL3 immunization in pigs,[48,49] INSL3 antagonist treatment of rats,[50] or hormonal suppression of the GnRH axis in men.[51] For mice in which RXFP2 expression has been specifically suppressed in adult male germ cells, there was no evidence for any such positive effect of INSL3 on spermatogenesis.[52] It thus appears likely that a role for INSL3 to promote germ cell survival may only be relevant in such extreme situations, or maybe during the first wave of spermatogenesis in early prepuberty, when intra-testicular INSL3 levels are lower and RXFP2 receptors are likely to be not desensitized by the later high intra-testicular concentration of INSL3.

4.3 | Role of FSH in determining Leydig cell functional capacity

One of the more interesting findings from the present studies was the marked influence of FSH, rather than LH, on INSL3 concentration. Within the testes, FSH receptors are only found on Sertoli cells within the seminiferous compartment. Yet there is no relationship between semen parameters and INSL3, nor between the Sertoli cell product AMH and INSL3 in the Australian study (not shown). One explanation could be simply the lower biological variance associated with circulating FSH compared to LH or testosterone, both of which exhibit a much more marked pulsatility of expression. But there is also the possibility that FSH may be involved in a more direct link to Leydig cell function, possibly through the agency of Sertoli cell activity. This notion would be supported by the association of INSL3 with testis size, as well as with Inhibin B, which is largely due to the action of FSH on Sertoli cells to promote spermatogenesis. There appears to be mutual paracrine regulation of the interstitial and seminiferous compartments,[5] and it is known that Sertoli cells secrete a number of factors, including PDGF,[53] which can influence Leydig cell function directly through specific PDGF receptors.[53] This observation is also strongly supported by a recent study in mice, which showed a direct link between Leydig cell number and the size of the adult Sertoli cell population.[54] Similarly, in the human testis, Sertoli and Leydig cell numbers appear to correlate.[36] An earlier study had shown in mice that specific Sertoli cell expression of the androgen receptor also affects Leydig cell numbers and proliferation,[55] even though in the present study we found no relationship between androgen receptor polymorphism and INSL3 expression. However, in agreement with Lundin et al.[34] as well as with von Eckardstein et al.[56] we do see a significant relationship between the number of androgen receptor trinucleotide repeats and some seminal parameters. Although they had not measured INSL3, the lack of relationship between AMH and Leydig cell parameters in the Australian men was also shown by Aksglaede et al.[57] for a Danish cohort, suggesting that AMH may not be instrumental in this context.

4.4 | Concluding remarks

In conclusion, we have made use of two mutually supportive cohort studies in men to investigate the relationship between the highly specific Leydig cell biomarker INSL3 and male fertility, reflected in a range of seminal parameters as well as by the hormones of the HPG axis. Whereas there appears to be no association between Leydig cell functional capacity and spermatogenesis, yet surprisingly there is a strong correlation between INSL3 and FSH, rather than LH. Given the observed relationship between testis parameters and morbidity, it will be important for future studies to dissect the pathways of causality involved here, thus allowing a better understanding and a more precise interpretation of these parameters.

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CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR CONTRIBUTIONS

RAI and RI conceived the study, supervised the UK analytical work, and largely wrote the manuscript. KT was responsible for the Australian study and analysis. HS contributed to the UK analyses, and DE supported the statistical work.

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