

ORIGINAL ARTICLE

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Insulin-like factor 3, luteinizing hormone and testosterone in testicular cancer patients: effects of β -hCG and cancer treatment

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SUMMARY

Background: Primary hypogonadism (low testosterone and high luteinizing hormone, LH) is present in approximately 20% of testicular cancer (TC) survivors after orchidectomy with or without chemotherapy.

Objectives: We investigated insulin-like factor 3 (INSL3), a novel marker of Leydig cell function, in TC patients.

Materials and Methods: We analyzed: (I) a cross-sectional cohort of TC patients after orchidectomy with or without chemotherapy (1988–1999) at long-term follow-up (median 36 and 35 years of age at follow-up, respectively) and healthy men of similar age; (II) a longitudinal cohort of chemotherapy-treated TC patients (2000–2008), analyzed before and 1 year after chemotherapy (median 29 years of age at chemotherapy). INSL3, testosterone, and LH were compared between groups and over time and related to pre-chemotherapy β -hCG levels.

Results: In the cross-sectional cohort, TC patients at median 7 years after orchidectomy and chemotherapy ($n = 79$) had higher LH ($p < 0.001$), lower testosterone ($p = 0.001$), but similar INSL3 as controls ($n = 40$). After orchidectomy only ($n = 25$), higher LH ($p = 0.02$), but no differences in testosterone or INSL3 were observed compared to controls. In the longitudinal cohort, patients with normal pre-chemotherapy β -hCG (≤ 5 mU/L, $n = 35$) had increased LH 1 year after chemotherapy compared to pre-chemotherapy ($p = 0.001$), and no change in testosterone or INSL3. In contrast, patients with high β -hCG pre-chemotherapy ($n = 42$) had suppressed LH, markedly elevated testosterone, and low INSL3 at start of chemotherapy, with increased LH, decreased testosterone, and increased INSL3 1 year later (all $p < 0.001$).

Discussion: Changes in LH show that gonadal endocrine function is disturbed before chemotherapy, 1 year later, and at long-term follow-up in chemotherapy-treated TC patients.

Conclusion: Pre-chemotherapy, β -hCG-producing tumors affect the gonadal endocrine axis, demonstrated by increased testosterone and decreased LH. INSL3 did not uniformly follow the pattern of testosterone.

INTRODUCTION

Testicular germ cell cancer (TC) is the most frequent malignancy in men between 20 and 40 years of age. Therapy consists of orchidectomy and, in case of disseminated disease, platinum-based chemotherapy. Since this treatment has high cure rates, detection and management of late therapy-related toxicity in survivors is important (Hanna & Einhorn, 2014).

Primary hypogonadism is a toxic effect of chemotherapy in TC patients. Low testosterone (< 9 – 10 nmol/L) or testosterone replacement therapy are reported in 11–19% of long-term

survivors (Gerl *et al.*, 2001; Huddart *et al.*, 2003; Haugnes *et al.*, 2010). In stage I TC patients treated with orchidectomy alone, luteinizing hormone (LH) is higher than in healthy men (Nord *et al.*, 2003; Nuver *et al.*, 2005; Sprauten *et al.*, 2014). Patients with metastatic disease treated with orchidectomy and chemotherapy have both elevated LH and reduced total and free testosterone (Nuver *et al.*, 2005; Sprauten *et al.*, 2014). These findings suggest a negative effect of orchidectomy and potentially the presence of TC itself on the number of functional Leydig cells, while chemotherapy seems to induce further impairment.

Unfortunately, interpreting testosterone levels is challenging due to large inter- and intraindividual variations and the lack of a generally accepted lower limit of normal. Testosterone levels react acutely to changes in the hypothalamic–pituitary–gonadal (HPG) axis, and repeated measurements are recommended to exclude transient testosterone decreases (Wang *et al.*, 2008). Furthermore, only a modest correlation exists between total testosterone and symptoms of hypogonadism (Diver, 2006; Wang *et al.*, 2008; Collier *et al.*, 2010). Moreover, no convincing data are available regarding the harmful effects of low or low-normal testosterone in young men over longer time periods.

Increased cardiovascular risk in TC survivors is a concern, given a standardized incidence ratio for myocardial infarction or angina pectoris of 2.06 in 5-year TC survivors after chemotherapy compared to the general population (van den Belt-Dusebout *et al.*, 2006). In TC patients, total testosterone is negatively associated with prevalence of the metabolic syndrome (Nuver *et al.*, 2005; Haugnes *et al.*, 2007; de Haas *et al.*, 2013). Similarly, low testosterone is associated with the metabolic syndrome and increased mortality in the general population (Ohlsson *et al.*, 2011; Kelly & Jones, 2013; Brand *et al.*, 2014).

To better define hypogonadism and investigate its effects, a marker of Leydig cell function with less fluctuation than testosterone would be valuable (Giannetta *et al.*, 2012). Such a marker might better indicate low Leydig cell function and predict the metabolic syndrome and increased cardiovascular risk in TC patients. Insulin-like factor 3 (INSL3) is a peptide that is almost exclusively produced by men in the Leydig cells (Ivell *et al.*, 2013, 2014). INSL3 reaches relatively stable levels in adulthood, slightly declining with increasing age (Anand-Ivell *et al.*, 2006; Atlantis *et al.*, 2009). According to previous reports, INSL3 expression is less dependent on acute regulation by the HPG axis than testosterone, and may better reflect the number and function of Leydig cells and their differentiation status than testosterone (Bay & Andersson, 2011; Giannetta *et al.*, 2012; Ivell *et al.*, 2013), with no overt diurnal pattern (Chong *et al.*, 2015).

In the present study, we investigated INSL3 as a novel marker of Leydig cell function in TC patients following orchidectomy only or orchidectomy and chemotherapy.

MATERIALS AND METHODS

Cross-sectional and longitudinal cohorts

Measurements were carried out in a cross-sectional and a longitudinal cohort. The cross-sectional cohort consisted of three groups: TC patients after orchidectomy and chemotherapy for disseminated disease, TC patients after orchidectomy alone for stage I disease, and healthy males of similar age. These men had participated in a previous study on hormone levels, as described elsewhere (Nuver *et al.*, 2005). Briefly, patients had been treated for non-seminoma at the University Medical Center Groningen (UMCG) between 1988 and 1999. Extragenadal TC, radiotherapy, testosterone replacement therapy, and age > 55 years at chemotherapy were exclusion criteria. In addition, participants were excluded if stored serum was not available ($n = 20$) or INSL3 could not be measured due to technical issues ($n = 3$).

The longitudinal TC cohort consisted of patients treated with unilateral orchidectomy and at least three courses of chemotherapy. Between 2000 and 2008, these patients had participated in various prospective studies on cardiometabolic effects of TC

treatment in the UMCG. Patients with age >50 years at chemotherapy, extragonadal TC, cardiovascular disease before chemotherapy, or testosterone replacement therapy before or within 2 years after chemotherapy were excluded. Patients were selected based on availability of stored serum obtained at start (after orchidectomy and before chemotherapy) and at 1 year after chemotherapy (365 ± 100 days); a serum sample at 2 years after chemotherapy (730 ± 100 days) was optional. The different study protocols were approved by the local medical ethical review committee, and each participant gave written informed consent.

Assessment of INSL3, gonadal endocrine function, and the metabolic syndrome

Insulin-like factor 3 was measured in serum samples, stored at -20°C , using a commercial enzyme immunoassay research kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). Serum was diluted 1 : 3. The serum samples used in the cross-sectional analysis were measured in duplicate; in our hands, intra- and interassay variation coefficients (over four assays) were 4.3% and 8.3%. Considering this low variation, INSL3 measurements in the longitudinal cohort were not duplicated. For INSL3 measurements below the detection threshold (<0.32 ng/mL), 0.32 ng/mL was used for statistics. Low INSL3 was defined as below percentile 2.5 of INSL3 in the healthy men from the cross-sectional analysis (1.06 ng/mL).

Measurements of total testosterone, LH, and follicle-stimulating hormone in morning blood samples were described previously (Nuver *et al.*, 2005). Primary hypogonadism was defined biochemically as total testosterone <10 nmol/L and/or LH >10 U/L. For the last follow-up time point in the longitudinal cohort, hypogonadism was also considered present if a patient had started testosterone replacement therapy (if started more than 2 years after chemotherapy).

From previous studies, history of cardiovascular disease, body mass index (BMI), and blood pressure were recorded. In the TC patients, the β -subunit of human chorionic gonadotropin (β -hCG, IU/L) was routinely measured during treatment and follow-up. Triglycerides, total cholesterol, high-density and low-density lipoprotein cholesterol, and glucose had been measured in fasting blood samples. The metabolic syndrome was defined according to the NCEP-ATPIII criteria (2005 update) (Grundy *et al.*, 2005) and considered absent if ≥ 3 criteria were not met.

Statistical analysis

We determined if data were normally distributed by graphical evaluation of the distribution and by formal normality testing using Shapiro–Wilk test. In the cross-sectional analysis, continuous variables were compared between all three groups using Kruskal–Wallis tests (with additional post hoc comparison as indicated using Dunn test), or between the two TC groups only using Mann–Whitney U-tests. Differences in categorical variables were tested with Fisher's exact test. For correlations, Spearman's correlation coefficient was determined.

In the longitudinal cohort, given possible gonadal endocrine activity of β -hCG similar to that of LH, TC patients were stratified between TC tumors with or without β -hCG production (>5 IU/L) pre-chemotherapy. Paired variables were compared between the two time points using Wilcoxon signed-rank test or McNemar's test. For the subset of patients with INSL3

measurements at 2 years after chemotherapy, differences in INSL3 between the three time points were compared using Friedman test. Spearman's correlation coefficient was determined for correlation between change in INSL3 and other variables. To test if INSL3, total testosterone and LH predict hypogonadism or the metabolic syndrome at long-term follow-up, univariate logistic regression analysis was performed. All tests were two-sided and unadjusted for multiple comparisons. A $p < 0.05$ was considered significant. Patients with missing variables were excluded on a per test basis. Statistics were calculated with R 3.3.4 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Cross-sectional cohort

The cross-sectional cohort consisted of 79 patients treated with orchidectomy and chemotherapy for disseminated TC, 35 patients after orchidectomy alone for stage I TC, and 40 healthy men (Table 1). The groups had comparable age ($p = 0.92$), and time since treatment did not differ between TC patient groups ($p = 0.61$). Antihypertensive drugs ($n = 8$), statins ($n = 5$), antidiabetic drugs ($n = 1$), and antiplatelet agents (for stroke, $n = 2$, and myocardial infarction, $n = 1$) were only reported in the chemotherapy group.

Insulin-like factor 3 was lower in chemotherapy-treated compared to orchidectomy-only patients ($p = 0.01$, Table 2, Fig. 1), but there were no differences between orchidectomy-only or chemotherapy-treated patients and healthy men ($p = 0.38$ and $p = 0.12$, respectively). No correlation existed between INSL3 and serum storage duration ($p = 0.47$). At follow-up, all β -hCG levels in the chemotherapy-treated group were < 1.5 IU/L. In all groups combined, INSL3 correlated with total testosterone ($p < 0.001$, $r_s = 0.31$), but not with LH ($p = 0.27$) or age ($p = 0.24$). In the 114 TC patients, similar correlations between

INSL3 and total testosterone were found, with no correlation between INSL3 and time since treatment ($p = 0.18$). INSL3 did not differ between TC patients with hypogonadism ($n = 18$, median 1.60 ng/mL, interquartile range (IQR) 1.07–2.00 ng/mL) or without hypogonadism ($n = 96$, 1.74 ng/mL, IQR 1.35–2.08 ng/mL, $p = 0.34$).

Longitudinal cohort

All 81 TC patients in the longitudinal cohort had been treated with orchidectomy and chemotherapy. At start of chemotherapy, 35 patients had a normal β -hCG (≤ 5 IU/L) and 46 patients had an elevated β -hCG (> 5 IU/L). All patients were treated with at least three cycles of chemotherapy; an additional fourth cycle was administered to 21 (60%) of the patients with normal β -hCG pre-chemotherapy and to 38 (83%) in patients with elevated β -hCG pre-chemotherapy. INSL3 at start of chemotherapy did not correlate with age ($p = 0.63$) or interval between orchidectomy and chemotherapy ($p = 0.13$).

In the 46 TC patients with elevated β -hCG pre-chemotherapy, testosterone was high and LH suppressed at start of chemotherapy, with 74% ($n = 34$) having a LH below 1 IU/L (Table 3, Fig. 2). One year later, testosterone had decreased with median -9 nmol/L (IQR -14 to -2 nmol/L, $p < 0.001$) and LH levels had increased with median 5.9 IU/L (IQR -4.8 to 4.5 IU/L, $p < 0.001$). Contrary to the decrease in testosterone, INSL3 increased with median 0.31 ng/mL (IQR 0.12–0.53 ng/mL, $p < 0.001$) in the patients with elevated β -hCG pre-chemotherapy.

In contrast, in the 35 TC patients with normal β -hCG pre-chemotherapy, all LH levels at start of chemotherapy were above 1 IU/L, and LH increased with median 3.0 IU/L (IQR 0.1–4.8 IU/L) between start of chemotherapy and 1 year later ($p = 0.001$). However, testosterone and INSL3 did not change between start of chemotherapy and 1 year later ($p = 0.81$ and $p = 0.99$, respectively).

For a subset of 39 patients, an additional blood sample was available at 2 years after chemotherapy. Within this subset,

Table 1 Baseline characteristics of the cross-sectional and longitudinal patient cohorts.

Variable	Cross-sectional cohorts		Longitudinal cohort ($n = 81$)
	Orchidectomy and chemotherapy ($n = 79$) Median (IQR)	Orchidectomy-only ($n = 35$) Median (IQR)	Median (IQR)
Age at therapy ^a , years	29 (25–35)	28 (23–35)	29 (24–36)
Age at follow-up ^b , years	37 (31–45)	36 (33–46)	–
Duration of follow-up, years	7 (4–11)	8 (5–10)	1, 2, 8.6 (6.3–10.0)
Histology, n (%)			
Seminoma	1 (1%)	0 (0%)	4 (5%)
Non-seminoma	78 (99%)	35 (100%)	77 (95%)
Stage, n (%)			
I	0 (0%)	35 (100%)	0 (0%)
II	43 (54%)	0 (0%)	57 (70%)
III	2 (3%)	0 (0%)	6 (7%)
IV	34 (43%)	0 (0%)	18 (22%)
IGCCCG prognosis group, n (%)			
Good	48 (61%)	–	56 (69%)
Intermediate	26 (33%)	–	23 (28%)
Poor	5 (6%)	–	2 (3%)
Interval orchidectomy–chemotherapy, days	77 (41–149)	–	69 (35–145)
Chemotherapy, n (%)			
BEP	62 (79%)	–	81 (100%)
EP	8 (10%)	–	–
Other ^c	9 (11%)	–	–

BEP, bleomycin, etoposide, cisplatin; EP, etoposide, cisplatin; IGCCCG, International Germ Cell Cancer Consensus Group classification; IQR, interquartile range. ^aAge at start of chemotherapy for groups treated with orchidectomy and chemotherapy, or age at orchidectomy for the orchidectomy-only group. ^bThe cross-sectional cohort of 40 healthy men had a similar median age of 37 years (IQR 29–42 years). ^cBEP followed by etoposide, ifosfamide, and cisplatin (VIP) in four patients; bleomycin, vincristin, and cisplatin (BOP) followed by VIP in four patients; and BOP followed by VIP and carboplatin, etoposide in one patient.

Table 2 Gonadal endocrine function and cardiovascular risk factors in the cross-sectional cohorts

Variable	Orchiectomy and chemotherapy (<i>n</i> = 79) Median (IQR)	Orchiectomy-only (<i>n</i> = 35) Median (IQR)	Healthy men (<i>n</i> = 40) Median (IQR)	<i>p</i> (overall)
Gonadal endocrine function				
Luteinizing hormone, U/L	6.3 (4.4–8.4)	5.0 (2.8–7.0)	3.7 (3.0–4.5)	<0.001
Total testosterone, nmol/L	18 (15–23)	20 (16–24)	23 (19–25)	0.005
INSL3, ng/mL	1.64 (1.28–1.94)	1.89 (1.55–2.20)	1.78 (1.47–2.09)	0.04
Low INSL3 ^a , <i>n</i> (%)	9 (11%)	1 (3%)	1 (3%)	0.17
Follicle-stimulating hormone, U/L	17.8 (9.6–23.8)	8.8 (7.6–11.3)	3.9 (2.4–4.9)	<0.001
Hypogonadism ^b , <i>n</i> (%)	16 (20%)	2 (6%)	0 (0%)	0.001
Cardiovascular risk factors				
Body mass index ^c , kg/m ²	24.7 (23.4–27.7)	25.2 (24.2–28.0)	23.9 (21.3–25.4)	0.01
Total cholesterol, mmol/L	5.46 (4.80–6.24)	5.04 (4.53–5.65)	4.97 (4.18–5.84)	0.05
HDL cholesterol, mmol/L	0.93 (0.77–1.09)	0.91 (0.80–1.03)	1.01 (0.86–1.05)	0.47
LDL cholesterol, mmol/L	3.72 (3.20–4.53)	3.45 (2.98–3.96)	3.32 (2.87–3.95)	0.11
Triglyceride, mmol/L	1.41 (0.90–2.24)	1.48 (0.92–2.18)	0.98 (0.73–1.43)	0.01
Metabolic syndrome ^d , <i>n</i> (%)	23 (29%)	15 (43%)	5 (13%)	0.01

Values were compared across the three groups. HDL, high-density lipoprotein; INSL3, insulin-like factor 3; IQR, interquartile range; LDL, low-density lipoprotein. ^aBelow p2.5 in healthy men (1.06 ng/mL). ^bTestosterone <10 nmol/L in one chemotherapy patient, all other patients had LH ≥10 U/L with testosterone >10 nmol/L.

^cMissing in eight chemotherapy patients and one healthy man. ^dMissing in one chemotherapy patient and one healthy man.

INSL3 increased between start of chemotherapy and 1 year and 2 years later in the 26 patients with elevated β-hCG pre-chemotherapy (Friedman test, $p < 0.001$), but not in the 13 patients with normal β-hCG pre-chemotherapy ($p = 0.19$) (Table S1).

Longitudinal INSL3 and long-term gonadal endocrine function

Long-term follow-up after chemotherapy was available for 80 TC patients from the longitudinal cohort (median follow-up 8.6 years, IQR 6.3–10.0 years) (Table 3). At last follow-up, twelve patients (15%) had hypogonadism (of whom two on testosterone replacement therapy).

To explore if the risk of long-term hypogonadism can be predicted early during follow-up, the relation between INSL3, LH and testosterone at 1 year after chemotherapy and hypogonadism at median 8.6-year long-term follow-up was analyzed with univariate logistic regression. Higher LH at 1 year after chemotherapy increased the likelihood of hypogonadism at last

follow-up in patients with normal β-hCG pre-chemotherapy (odds ratio (OR) 1.6, 95% confidence interval (CI) 1.2–2.9), but not in patients with elevated β-hCG pre-chemotherapy (OR 1.1, 95% CI 1.0–1.3). INSL3 and testosterone at 1 year after chemotherapy did not affect the likelihood of hypogonadism at last follow-up (Table S2). In both patients with normal or elevated β-hCG pre-chemotherapy, LH at 1 year after chemotherapy was correlated with LH at last follow-up ($r_s = 0.51$, $p = 0.007$, and $r_s = 0.50$, $p = 0.001$, respectively). In the patients with elevated β-hCG at start of chemotherapy, the β-hCG level at start of chemotherapy did not affect the likelihood of hypogonadism at last follow-up (OR 1.0, 95% CI 1.0–1.0).

INSL3, BMI, and the metabolic syndrome

In the 106 TC patients of the cross-sectional cohort with documented BMI, INSL3 did not correlate with BMI ($p = 0.33$). INSL3 did not differ between TC patients with ($n = 38$; median

Figure 1 Insulin-like factor 3 (INSL3, (A), total testosterone (B), and luteinizing hormone levels (C) in the cross-sectional cohorts of patients after orchiectomy and chemotherapy ($n = 79$), orchiectomy-only patients ($n = 35$), and healthy men ($n = 40$). p values are for overall comparison over the three cohorts using Kruskal–Wallis test and for post hoc comparisons with Dunn test. Gray areas mark INSL3 <1.06 ng/mL (below p2.5 in healthy men), total testosterone <10 nmol/L, and luteinizing hormone ≥10 U/L.

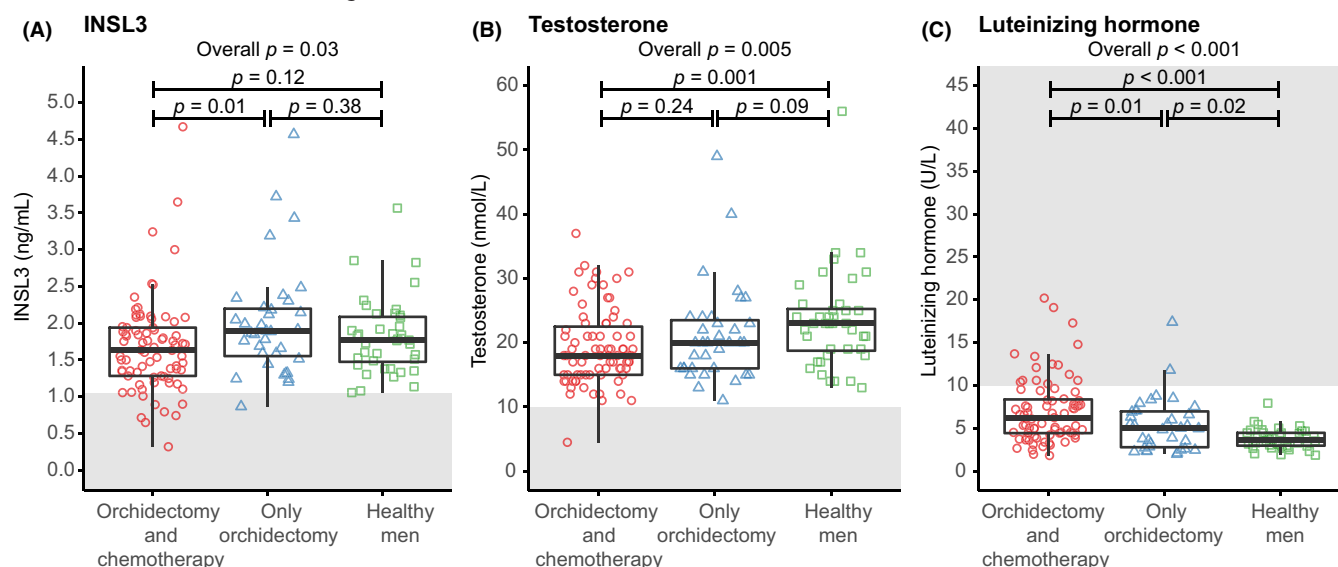


Table 3 Gonadal endocrine function at start of chemotherapy and 1 year after chemotherapy, and at long-term follow-up in the longitudinal cohort, stratified by pre-chemotherapy β -hCG level.

Variable	Time point	Normal β -hCG (≤ 5 IU/L) ($n = 35$)			Elevated β -hCG (> 5 IU/L) ($n = 46$)		
		Median (IQR)	Missing	p	Median (IQR)	Missing	p
Pre-chemotherapy β -hCG ^a , IU/L	Start	1.2 (1.0–1.6)	–	0.001	114 (31.3–529)	–	<0.001
	Start	5.6 (3.2–7.1)	–		0.1 (0.1–0.9)	–	
	1 year	8.5 (4.6–11.7)	–		6.8 (5.1–11.9)	7	
	Follow-up ^c	5.0 (3.3–7.0)	–		5.6 (4.2–8.5)	3	
Total testosterone, nmol/L	Start	20 (15–25)	–	0.81	23 (19–32)	–	<0.001
	1 year	21 (16–23)	9		15 (9–18)	7	
	Follow-up ^c	18 (16–22)	–		16 (13–20)	6	
	Start	1.19 (0.97–1.43)	–	0.99	0.71 (0.47–0.97)	–	<0.001
INSL3, ng/mL	1 year	1.25 (0.95–1.47)	–		1.13 (0.81–1.44)	–	
	Follow-up ^c	1.25 (0.95–1.47)	–		1.13 (0.81–1.44)	–	
	Start	12 (34%)	–	0.68	38 (83%)	–	<0.001
Low INSL3 ^b , n (%)	1 year	10 (29%)	–		22 (48%)	–	
	Follow-up ^c	7 (20%)	–		5 (12%)	4	
	Start	3 (9%)	–	0.03	1 (2%)	–	0.001
Hypogonadism ^d , n (%)	1 year	11 (42%)	9		13 (33%)	7	
	Follow-up ^c	7 (20%)	–		5 (12%)	4	
	Start	6.9 (4.8–8.7)	–	<0.001	0.14 (0.03–1.4)	–	<0.001
FSH, U/L	1 year	18.5 (14.1–26.7)	9		20.0 (13.2–33.9)	7	
	Follow-up ^c	8.0 (6.1–9.7)	–		10.6 (7.6–22.3)	2	
	Start	6.9 (4.8–8.7)	–		0.14 (0.03–1.4)	–	

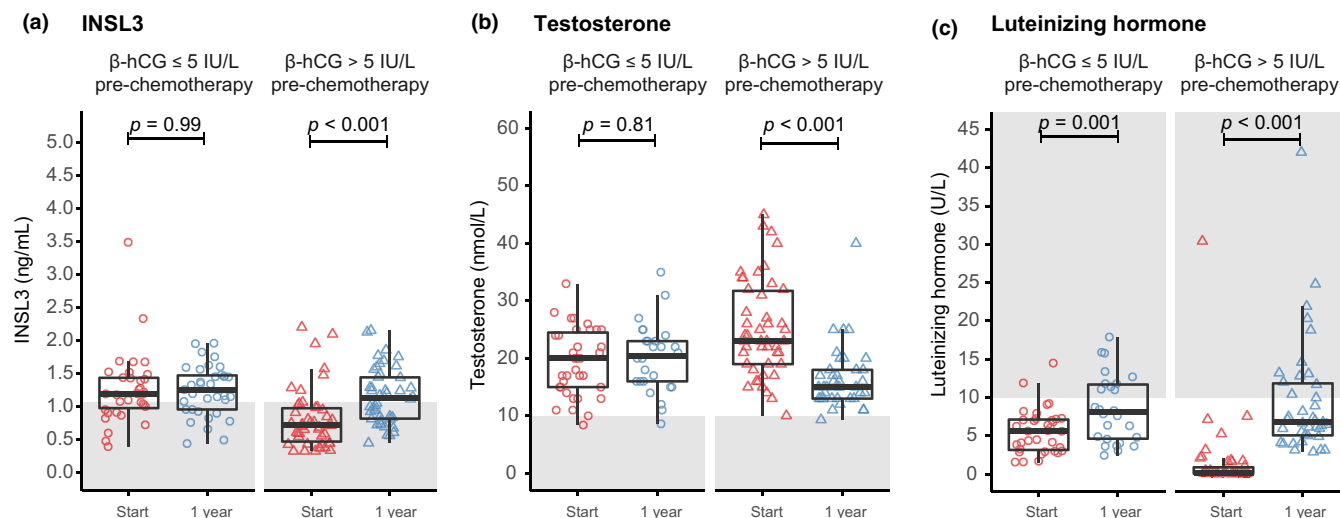
p values are for pair-wise comparisons between start of chemotherapy and 1 year later (excluding cases with a missing value at either time point). β -hCG, β -subunit of human chorionic gonadotropin; FSH, follicle-stimulating hormone; INSL3, insulin-like factor 3; IQR, interquartile range; LH, luteinizing hormone. ^aAfter chemotherapy, β -hCG levels were <1.5 IU/L for all patients. ^bINSL3 below p2.5 in the cross-sectional cohort of healthy men (1.06 ng/mL). ^cMedian follow-up: 8.6 years (range 6.3–10.0 years). One of the 81 patients was excluded from analysis at the follow-up time point, because of acute myeloid leukemia before 2 years of follow-up. Two patients were censored at 6 and 10 years of follow-up because of an astrocytoma and TC recurrence, respectively. ^dHypogonadism was based on testosterone replacement therapy in two patients at follow-up. Other hypogonadic patients had either low testosterone (<10 nmol/L; $n = 1$ at start of chemotherapy, $n = 2$ at 1 year, $n = 3$ at follow-up) or normal testosterone with elevated LH (≥ 10 U/L; $n = 3$ at start of chemotherapy, $n = 22$ at 1 year, $n = 8$ at follow-up).

1.74 ng/mL, IQR 1.26–1.91 ng/mL) vs. without the metabolic syndrome ($n = 75$; median 1.74 ng/mL, IQR 1.37–2.12 ng/mL, $p = 0.25$). In the longitudinal cohort, BMI at follow-up was median 25.1 kg/m² (IQR 24.3–26.3 kg/m²) in patients with normal β -hCG and 26.0 kg/m² (23.7–27.7 kg/m²) in patients with elevated β -hCG pre-chemotherapy. INSL3 at 1 year after chemotherapy was inversely correlated with BMI at follow-up ($r_s = 0.3$, $p = 0.007$) in the patients with elevated β -hCG at start of chemotherapy, but not in the subgroup with normal β -hCG at start of chemotherapy ($p = 0.6$). At last follow-up, the metabolic syndrome was found in seven patients (20%) with normal β -hCG

at start of chemotherapy and in 13 patients (28%) with elevated β -hCG at start of chemotherapy. INSL3 at 1 year after chemotherapy did not influence the odds of metabolic syndrome at follow-up in the patients with normal β -hCG (OR 0.8, 95% CI 0.2–3.7) or in those with elevated β -hCG pre-chemotherapy (OR 0.5, 95% CI 0.0–4.6).

DISCUSSION

We investigated INSL3 as a marker of Leydig cell function in relation to testosterone and LH in TC patients following orchidectomy and chemotherapy. Chemotherapy influences

Figure 2 Insulin-like factor 3 (INSL3, (A), total testosterone (B), and luteinizing hormone levels (C) in the longitudinal chemotherapy cohort ($n = 81$), stratified by pre-chemotherapy β -hCG level. Total testosterone and luteinizing hormone were missing in 16 patients. p values are for paired comparisons between the two time points. Gray areas mark INSL3 < 1.06 ng/mL (below p2.5 in healthy men), total testosterone <10 nmol/L, and luteinizing hormone ≥ 10 U/L. β -hCG: β -subunit of human chorionic gonadotropin.

gonadal endocrine function as shown by changes in LH but not INSL3 1 year after chemotherapy or at long-term follow-up. INSL3 1 year after chemotherapy was not related to the development of hypogonadism at long-term follow-up. In the long-term TC survivors of the cross-sectional cohort 3–13 years after treatment, INSL3 was clearly higher than in patients from the longitudinal cohort with INSL3 measurements at 1 year after treatment. This suggests that INSL3 in chemotherapy-treated TC patients may return to (near) normal over the course of several years and that Leydig cell recovery might be a prolonged process.

Insulin-like factor 3 is almost exclusively produced by the Leydig cells of the testes and may, therefore, accurately indicate gonadal endocrine function. INSL3 levels reflect Leydig cell number and differentiation status (Bay & Andersson, 2011; Ivell *et al.*, 2013). After unilateral orchidectomy, the number of Leydig cells is halved and the remaining testis needs to compensate to maintain adequate sex hormone levels. We hypothesized that early changes in INSL3 after orchidectomy reflect Leydig cell residual capacity, potentially indicating a patient's long-term risk to develop hypogonadism, as defined by a total testosterone <10 nmol/L and/or LH \geq 10 U/L. However, in our longitudinal analysis, INSL3 at 1 year after chemotherapy did not influence the odds of developing hypogonadism at a median 8.6-year follow-up. Therefore, INSL3 does not seem to be a suitable marker to predict hypogonadism as a late effect of chemotherapy in TC patients.

Interestingly, we also found interference of the tumor marker β -hCG with Leydig cell function. We showed that elevated β -hCG levels affect the gonadal endocrine axis and Leydig cell function, as demonstrated by high testosterone and suppressed LH and INSL3 in patients with elevated β -hCG at start of chemotherapy. Changes in INSL3, LH, and testosterone 1 year after chemotherapy likely result, for a large part, from successful cancer treatment and elimination of the β -hCG stimulus in these patients. In contrast, in patients who had not been exposed to elevated β -hCG levels, INSL3 did not change between start of chemotherapy and 1 year later. However, these patients did have a significantly higher LH 1 year after chemotherapy than before chemotherapy, indicating gonadal endocrine dysfunction at 1 year after chemotherapy without interference from high β -hCG levels.

Various explanations for the discrepant effect of β -hCG on gonadal hormones may be suggested. Although TC produces the intact hCG molecule, serum assays generally assess the β subunit of hCG, as the α subunit of hCG is also found in LH, FSH, and thyroid-stimulating hormone (TSH). Indeed, clinical hyperthyroidism due to similarity of hCG and TSH has been reported in TC patients with hCG levels exceeding 50,000 IU/L (Oosting *et al.*, 2010). Likewise, similarity between hCG and LH likely resulted in the upregulation of testosterone levels and subsequent downregulation of LH in the patients with elevated β -hCG levels in the longitudinal cohort. Surprisingly and contrary to the high testosterone production by the hCG-stimulated Leydig cells in these patients, INSL3 as a marker of Leydig cell number and differentiation was relatively low at start of chemotherapy. Possible explanations may be that this low INSL3 reflects the halved number of Leydig cells after orchidectomy, that hCG may not be a full substitute for LH when it comes to Leydig cell multiplication and differentiation and therefore INSL3 expression, that the

low INSL3 reflects exhaustion of the Leydig cells after prolonged hCG stimulation, or that the duration of the hCG stimulus was too short to observe an increase in INSL3 secretion accompanying Leydig cell multiplication and differentiation.

We did not find a clear association between INSL3 and BMI or the metabolic syndrome, although gonadal endocrine dysfunction has been associated with development of cardiovascular risk factors and the metabolic syndrome in TC patients (Nuver *et al.*, 2005; Haugnes *et al.*, 2007; de Haas *et al.*, 2013). In literature, data are scarce on the relationship between INSL3 and cardiovascular morbidity. Obese men have been reported to have significantly lower INSL3 than non-obese men, in addition to lower total testosterone (Foresta *et al.*, 2009). Moreover, subnormal INSL3 levels were demonstrated in obese men with normal testosterone levels (Foresta *et al.*, 2009). Notably, lower INSL3 levels have also been found in men with diabetes mellitus type 2 compared to BMI-matched controls (Ermetici *et al.*, 2009). However, low INSL3 did not prove to be a valuable marker to distinguish between TC patients with and without the metabolic syndrome in the present study.

To our knowledge, this is the first study measuring INSL3 in a large group of TC patients. By chance, a large Australian general population study investigating INSL3 included eleven men after orchidectomy (Anand-Ivell *et al.*, 2006). They reported lower INSL3 in a single subject after bilateral orchidectomy (0.04 ng/mL) and in ten men after unilateral orchidectomy (mean 0.77 ng/mL, standard deviation (SD) 0.47 ng/mL) compared to the remaining study population (1.08 ng/mL, SD 0.49 ng/mL, $n = 1160$). Within their small sample after unilateral orchidectomy, the investigators found a negative correlation between INSL3 and both LH and testosterone. The discrepancy with our results may be simply due to differences in sample size.

This study investigated the role of INSL3 in relation to the clinically relevant issue of hypogonadism in two well-defined cohorts of TC patients. Important strengths of our study are the longitudinal measurements and the long-term clinical follow-up. However, some limitations need to be addressed. First, the sample size is too small to draw any definitive conclusions on the relation between INSL3 and long-term outcome in terms of symptomatic hypogonadism or cardiovascular events. Second, while storage duration did not correlate with INSL3, an effect of storage duration on INSL3 cannot be excluded, although this effect is expected to have similar and equal influence on INSL3 in all cross-sectional groups. Third, no serum was available before orchidectomy, making it difficult to confidently differentiate between low INSL3 due to orchidectomy, chemotherapy, or TC itself. Indeed, INSL3 has been implicated in testicular descent and cryptorchidism (Bay & Andersson, 2011), which is an established risk factor for TC (Hanna & Einhorn, 2014). Furthermore, aberrant heterogeneous INSL3 expression was observed in histological samples from hCG-producing testicular tumors (Lottrup *et al.*, 2014).

Finally, use of different measurement methods hinders comparisons between absolute INSL3 levels in our and previous studies. However, our INSL3 measurements fit into the ranges found by Ermetici *et al.* (mean 1.1 ± 0.3 ng/mL in 30 diabetes mellitus patients and 1.5 ± 0.7 ng/mL in 30 controls) (Ermetici *et al.*, 2009) and by Chong *et al.* (1.6 ± 1.2 ng/mL and 2.4 ± 0.9 ng/mL in two groups of 13 and 20 healthy men, respectively) using the same assay (Chong *et al.*, 2015).

In conclusion, changes in LH show that gonadal endocrine function is disturbed before chemotherapy, 1 year later and at long-term follow-up in chemotherapy-treated TC patients. Pre-chemotherapy, β -hCG-producing tumors affect the gonadal endocrine axis, demonstrated by increased testosterone and decreased LH. Remarkably, INSL3 as a marker of Leydig cell activity and differentiation is simultaneously suppressed. INSL3 does not seem to be a suitable marker to predict hypogonadism as a late effect of chemotherapy in TC patients. After longer follow-up, INSL3 levels are comparable to levels in healthy men, implicating potentially ongoing Leydig cell recovery more than 2 years after treatment.

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DISCLOSURE OF INTEREST

The authors report no conflicts of interest.

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Table S1. Gonadal endocrine function at start of chemotherapy, 1 year and 2 years after chemotherapy, and at follow-up in a subset of the longitudinal cohort with available serum samples at 2 years after chemotherapy, stratified by pre-chemotherapy β -hCG level.

Table S2. Gonadal endocrine function at 1 year after chemotherapy in relation to hypogonadism at long-term follow-up in the longitudinal cohort, stratified by pre-chemotherapy β -hCG level.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.