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Quantification of the Leydig cell compartment in testicular biopsies and association with biochemical Leydig cell dysfunction in testicular cancer survivors

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SUMMARY

A simple histological method to evaluate the Leydig cell compartment is lacking. We aimed to establish such a method and to investigate if Leydig cell hyperplasia of the biopsy contralateral to the tumour-bearing testicle in patients with testicular germ cell cancer is associated with biochemical signs of Leydig cell dysfunction after long-term follow-up. A case group of 50 long-term testicular germ cell cancer survivors without human chorionic gonadotropin elevation, 10 testicular germ cell cancer patients with elevated human chorionic gonadotropin and 10 controls without testicular malignancy were included. For each subject, 2–4 representative sections from their testicular biopsies were selected for analysis. Using the image processing program ImageJ (V.1.48, NIH), an area with a minimum of 50 tubules was selected and delineated (total selected area) and the total Leydig cell area was calculated by adding up every delineated Leydig cell group within the total selected area. Four different methods were tested for the ability to quantify the Leydig cell compartment. In the 50 testicular germ cell cancer survivors, associations between the area of the Leydig cell compartment and serum levels of testosterone and luteinising hormone were investigated using linear regression analysis. The Leydig cell compartment was best quantified by the total Leydig cell area/total selected area index, which was significantly larger in the human chorionic gonadotropin-positive patients than in controls ($P = 0.00001$). In the 50 human chorionic gonadotropin-negative testicular germ cell cancer survivors, increasing total Leydig cell area/total selected area was significantly associated with decreased levels of total testosterone and decreased total testosterone/luteinising hormone ratio after a median of 9-year follow-up. In conclusion, a new simple method, total Leydig cell area/total selected area, was established to estimate the Leydig cell compartment in testicular biopsies. The index identified Leydig cell hyperplasia in the contralateral biopsy in patients with testicular germ cell cancer, and it was associated with long-term biochemical Leydig cell dysfunction. Although in testicular germ cell cancer survivors, the clinical value is limited because the contralateral biopsies are not commonly available, we propose a closer andrological follow-up in any patient with an increased total Leydig cell area/total selected area index.

INTRODUCTION

An enlarged Leydig cell compartment, commonly described as Leydig cell hyperplasia (LCH), is associated with poor testicular function and has been found in infertile men, men with Klinefelter syndrome and patients with testicular germ cell cancer (TGCC) (Ahmad *et al.*, 1969, 1971; Holm *et al.*, 2003). In these studies, the area of the Leydig cell compartment was quantified using stereological or histological methods (Ahmad *et al.*, 1971; Holm *et al.*, 2003). However, in clinical practice, stereological evaluation is not feasible, and simple histological assessment has been questioned as non-reproducible. Leydig cell function is currently assessed in the clinical setting by measuring serum levels of testosterone and luteinizing hormone (LH). A more objective method to assess the Leydig cell compartment would be a useful tool for clinicians in andrology centres, where testicular biopsies are commonly performed to assess spermatogenesis or the presence of pre-invasive germ cell neoplasia *in situ* (GCNIS).

Patients with TGCC are at risk of biochemical Leydig cell dysfunction manifested as increased serum levels of LH with or

without corresponding low levels of testosterone and many of them will undergo treatment that can further impair the function of the remaining testicle (Sprauten *et al.*, 2014; Bandak *et al.*, 2016; Isaksson *et al.*, 2017). We hypothesized that patients with Leydig cell hyperplasia (LCH) at the time of diagnosis were at increased risk of long-term Leydig cell dysfunction. We were able to address this hypothesis as we have access to archived biopsies from the testicle contralateral to the tumour-bearing one, a procedure which is routinely performed in Denmark (and electively in several other European centres) to screen for the presence of GCNIS (Berthelsen *et al.*, 1982; Kier *et al.*, 2015), previously known as testicular carcinoma *in situ* (CIS) (Berney *et al.*, 2016). Furthermore, in a previous study, we have collected detailed data on testicular function, including reproductive hormone profiles, in a series of long-term TGCC survivors (Bandak *et al.*, 2017a).

Thus, there were two aims of the present study: Aim 1: to establish a simple, reproducible method to quantify the Leydig cell compartment in testicular biopsies in order to define LCH, and Aim 2: to test the clinical value of this method by investigating if LCH identified by this method in biopsies from the contralateral testicle in patients with TGCC is associated with biochemical signs of Leydig cell dysfunction during long-term follow-up.

MATERIALS AND METHODS

Study subjects

We included 50 patients with hCG-negative TGCC treated from 1984 through 2012 at Copenhagen University Hospital, Rigshospitalet. These patients were included among the 158 participants of the clinical study 'Symptoms and Clinical Signs of Hypogonadism in Testicular Cancer Survivors' (ClinicalTrials.gov number, NCT02240966) which was conducted from 2014 through 2016. Inclusion and exclusion criteria for NCT02240966 are described elsewhere (Bandak *et al.*, 2017a). For this study, additional selection criteria were as follows: the availability of a contralateral biopsy of good technical quality, absence of GCNIS in the biopsy and normal hCG concentration in blood at the time of diagnosis. The final number of the patients (50) was also influenced by feasibility, because the histological evaluation, slide scanning and manual delineation of Leydig cells required a long time. The histological tumour type was recognized by experienced pathologists as seminoma in 29 patients and non-seminoma in 21 patients (Table 1). The absence of GCNIS in the biopsy was confirmed by immunohistochemical staining for at least one GCNIS marker (Rajpert-De Meyts *et al.*, 2015; Rajpert-De Meyts & Grigor, 2017).

As a control group of men expected to have a normal Leydig cell area, we included 10 men without testicular malignancy in whom bilateral testicular biopsies had been performed as a part of the clinical workup for infertility due to obstructive azoospermia. These men had serum levels of testosterone and LH within the normal range. There were not many of such biopsies available, because the diagnosis of obstructive azoospermia can be established without an open biopsy. Only biopsies with normal testicular histology with complete spermatogenesis could be included, hence further limiting the size of the control group. Finally, in order to investigate a group of men expected to have a substantially enlarged Leydig cell compartment due to stimulation by hCG (Asa *et al.*, 1984; Mendis-Handagama, 1997; Lottrup *et al.*, 2014), we included 10 patients with TGCC whose serum

Table 1 Clinical characteristics and serum levels of hormones reflecting Leydig cell function in 50 long-term hCG-negative testicular germ cell cancer survivors with or without Leydig cell hyperplasia of the non-tumour-bearing testicle at the time of diagnosis

	Patients WITHOUT Leydig cell hyperplasia (n = 28)	Patients WITH Leydig cell hyperplasia (n = 22)	P-value
Age at diagnosis*	31 (27–37)	32 (26–37)	0.9
Follow-up since biopsy	7 (4–13)	13 (5–17)	0.2
Age at follow-up	43 (36–48)	48 (40–51)	0.4
Histology			
Seminoma	17 (61%)	12 (55% and 45%)	
Non-seminoma	11 (60% and 40%)	10 (46%)	0.3
Treatment			
Orchiectomy alone	10 (36%)	10 (45.5%)	
Chemotherapy	13 (46%)	10 (45.5%)	
Abdominal radiotherapy	5 (18%)	2 (9%)	0.3
Pre-orchiectomy markers of Leydig cell function	(n = 17)	(n = 12)	
LH (IU/L)	2.5 (1.8–5.5)	5.0 (3.4–6.2)	0.1
LH (% of expected according to age)	77 (48–164)	152 (100–186)	0.1
Total testosterone (nmol/l)	16.3 (13.9–22.4)	12.9 (10.4–14.4)	0.05
TT (% of expected according to age)	95 (81–116)	74 (61–83)	0.04
Total testosterone/LH	4.8 (3.4–10.8)	2.7 (1.8–5.5)	0.3
Leydig cell function at follow-up			
LH (IU/L)	5.7 (4.0–9.0)	8.9 (5.9–12.8)	0.1
LH (% of expected according to age)	165 (120–279)	258 (172–370)	0.1
Total testosterone (nmol/l)	12.9 (11.4–16.4)	11.8 (7.5–13.6)	0.06
TT (% of expected according to age)	87 (75–110)	77 (49–92)	0.08
Total testosterone/LH	2.3 (1.3–3.4)	1.2 (0.7–2.4)	0.02
Changes in Leydig cell function	(n = 17)	(n = 12)	
LH (percentage point)	57 (2–92)	82 (29–122)	0.1
TT (percentage point)	−3 (−21–12)	2 (−28–21)	0.7
Ultrasound characteristics at follow-up			
Size of the testicle (ml)	19 (11–24)	10 (8–14)	0.002
Presence of microlithiasis	5 (18%)	5 (23%)	0.7

*Median, (interquartile range).

levels of hCG were highly elevated (designated in this study as 'patients with hCG-positive TGCC'). Selection criteria of the patients with hCG-positive TGCC were as follows: (1) a preoperative serum level of hCG >1000 IU and (2) availability of a contralateral biopsy of good size and technical quality. From 14 available biopsies fulfilling these selection criteria, we randomly selected 10 biopsies to match the number of controls. The study was approved by the Regional Ethical Committee (H-3-2013-175).

Evaluation of the Leydig cell compartment in testicular biopsies

For each testicular biopsy, we had 10 archived haematoxylin and eosin (HE)-stained microscopy slides. In the two groups of TGGC patients in whom a unilateral biopsy was taken from the testicle contralateral to the tumour-bearing testis, we selected every other slide, to have a minimum distance of 100 um between evaluated sections, thus avoiding overlapping Leydig cell micronodules. This resulted in four slides pr. biopsy. In the control group, bilateral biopsies were available; hence, two slides from each of the two testicular biopsies (and four in total) were analysed in the control group. A representative section from 6 to 8 serial sections on each slide was selected for analysis, and care was taken to avoid sections that were small or mechanically traumatized. An area with a minimum of 50 cross sections of seminiferous tubules was selected for analysis. The selected sections were scanned using a slide scanner, NanoZoomer 2.0 HT (Hamamatsu Photonics, Herrsching am Ammersee, Germany) and analysed using the software NDPview version 1.2.36 (Hamamatsu Photonics). Using the open-source image processing program ImageJ (V.1.48, NIH), the total selected area (TSA) and the area of every single Leydig cell group were manually delineated on the selected sections (Fig. 1). The total Leydig cell area (TLCA) was calculated by adding up the areas of all Leydig cell groups. To further account for heterogeneity of a histological section due to the presence of tubules with decreased spermatogenesis or Sertoli cell-only pattern, which have smaller diameter than tubules with complete spermatogenesis, the total number of tubules (Ntub) in the four sections was calculated. Subsequently, the following four methods were evaluated for the ability to quantify the Leydig cell compartment and identify the samples with obvious Leydig cell hyperplasia: (1) the mean Leydig cell area of all delineated Leydig cell groups 'mean Leydig cell area' (2) TLCA/TSA, (3) (TLCA/TSA)/Ntub and (4) TLCA/Ntub. The index that best separated the patients with hCG-positive TGCC from the controls was selected.

Two independent evaluations of a subset of the biopsies were performed by MGT and ERDM to validate the methods. The two investigators were blinded to the initial pathology reports' evaluation of the Leydig cell compartment (LC hyperplasia vs. no LC hyperplasia) when they evaluated the biopsies.

Treatment and follow-up for the 50 patients with hCG-negative TGCC

Stage I patients with TGCC (n = 20) were treated with unilateral orchiectomy alone, while patients with disseminated disease (n = 30) were treated with unilateral orchiectomy and 3–4 courses of cisplatin-based chemotherapy; bleomycin, etoposide and cisplatin (BEP) (n = 23), except for some patients with seminoma stage II who were treated with unilateral orchiectomy and abdominal radiotherapy (n = 7). The follow-up visit was conducted at a median follow-up of 9 years (interquartile range (IQR) 4–17 years) since diagnosis. At the follow-up visit, blood samples were drawn for analysis of reproductive hormones, systemic inflammation and metabolic profile (details described in (Bandak *et al.*, 2017a)). Testicular size and the presence of microlithiasis were determined at the follow-up visit using ultrasound equipment (Esaote myLab25 Gold, Genova, Italy).

Evaluation of reproductive hormones in the 50 patients with hCG-negative TGCC

Leydig cell function was evaluated pre-orchiectomy and at the follow-up visit, with blood samples drawn between 07.30 and 11.00 A.M. Serum concentration of LH was measured by time-resolved immunofluorometric assay (Delfia; Perkin Elmer, Turku, Finland) with intra-assay and interassay coefficients of variation (CV) below 5% and detection limits of 0.05 IU/l. Sex hormone-binding globulin (SHBG) and total testosterone (TT) were measured with chemiluminescent immunoassay (Access2; Beckman Coulter) with intra-assay and interassay CVs below 5% and detection limit of <0.35 nmol/l. At the time of TGCC diagnosis, total testosterone was measured by radioimmunoassay (Siemens Coat-a-count, Cruinn Diagnostics Limited, Dublin, Ireland) with a detection limit of <0.23 nmol/l and intra-assay and interassay CVs on 17% and 12.8%, respectively. The hormone measurements were performed at the certified hormone laboratory at the Department of Growth & Reproduction, Rigshospitalet, Copenhagen, Denmark.

Statistical evaluation of associations between Leydig cell histology and function

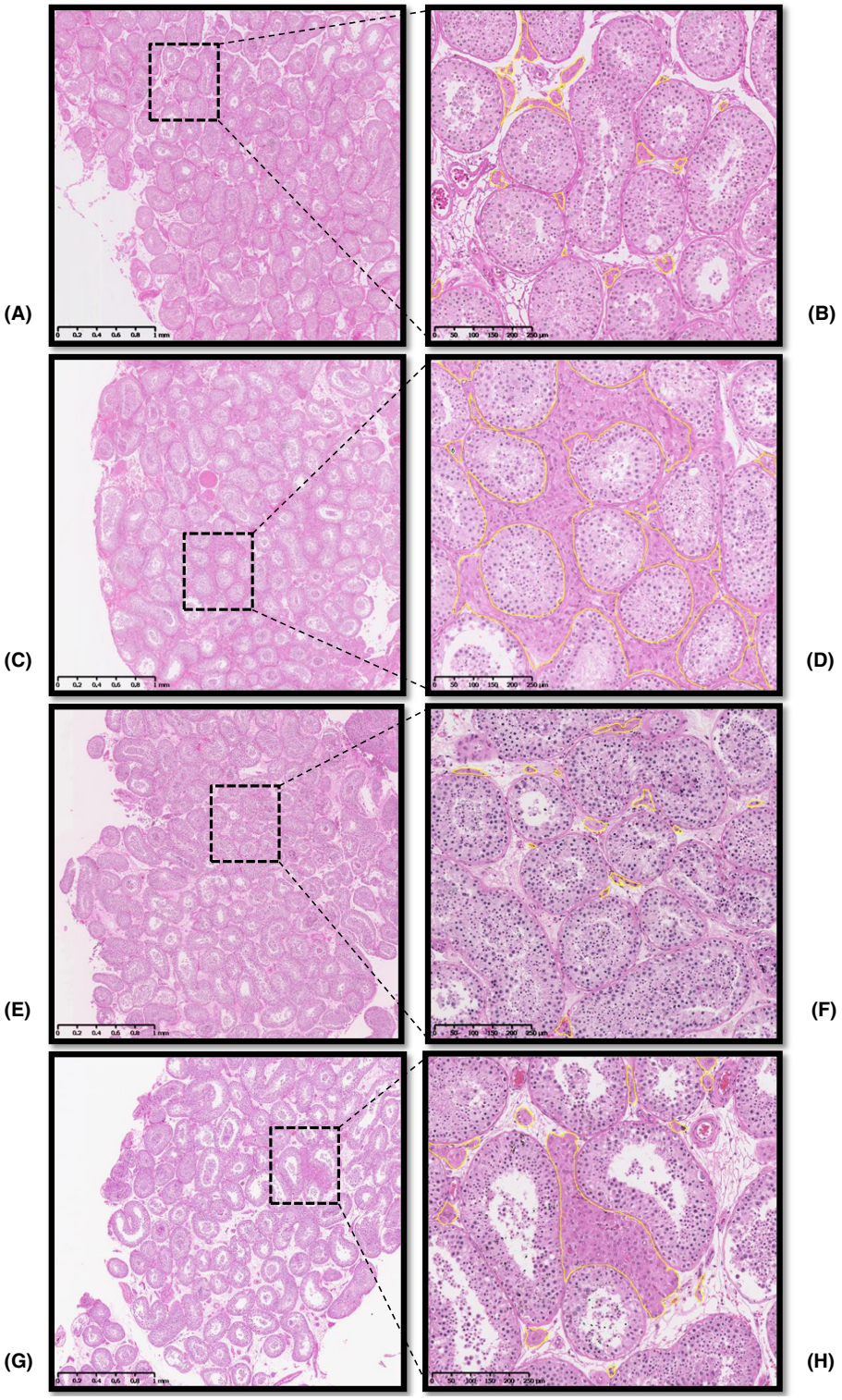
Continuous outcomes are presented as median with interquartile range (IQR), while categorical outcomes are presented as total number and percentages. The Leydig cell compartment as quantified by each of the four different methods mentioned above was compared between the control group and the hCG-positive TGCC patients with independent samples t-test, and the method which separated the two groups with the greatest accuracy was chosen for investigating aim 2.

Subsequently, in the 50 patients with hCG-negative TGCC, linear regression analysis was performed with (1) pre-orchiectomy levels, (2) follow-up levels and (3) changes in LH and TT, and TT/LH as dependent variable and Leydig cell area in the contralateral biopsy according to the new method for quantification of the Leydig cell compartment as independent variable. To adjust for age, we calculated percentage of predicted values of TT and LH by dividing each individual value with the mean value of age-matched controls. The method of calculation percentage of predicted values and the controls have been described in a previous publication (Bandak *et al.*, 2017b).

We then compared LH, TT, TT/LH and other characteristics of the 50 hCG-negative TGCC patients with and without LCH of the contralateral testicle according to the new method for quantification of the Leydig cell compartment using chi-squared test or independent samples t-test.

Finally, to further validate our new method for quantification of the Leydig cell compartment, we performed receiver

Figure 1 Examples of histology and Leydig cell area delineation in contralateral testicular biopsies in the studied TGCC groups and controls. The images on the left (A, C, E, G) show overview of the section, and the images on the right (B, D, F, H) show a higher magnification of the sections, with Leydig cell areas delineated in yellow using ImageJ software. (A, B) A control subject with obstructive azoospermia without testicular malignancy and normal testicular histology, including normal Leydig cell distribution. (C, D) A TGCC patient with highly elevated human chorionic gonadotropin (hCG) and Leydig cell hyperplasia. (E, F) An example of a contralateral biopsy from a patient with TGCC from the follow-up group without elevation of hCG and with a normal Leydig cell distribution. (G, H) An example from a patient with TGCC from the follow-up group without elevation of hCG but with Leydig cell hyperplasia.



operating characteristic (ROC) curve analysis testing the new method against the evaluation of the Leydig cell compartment (LCH vs. no LCH) performed at the time of diagnosis.

All statistical tests were computed in IBM SPSS Statistics, version 22.0. Armonk, NY: IBM Corp, and a two-sided *P*-value <0.05 was considered statistically significant.

RESULTS

Quantification of the Leydig cell compartment and definition of Leydig cell hyperplasia (aim 1)

The ability of each of the four methods to quantify the Leydig cell compartment is shown in Fig. 2. The Leydig cell area was significantly larger in patients with hCG-positive TGCC than in the control group using the two methods, TLCA/TSA ($P = 0.00001$) and (TLCA/TSA)/Ntub ($P = 0.001$). On the contrary, the Leydig cell area was significantly smaller in patients with hCG-positive TGCC than in the control group when using TLCA/Ntub ($P = 0.01$), while there was no difference in the mean Leydig cell area between the two groups. In addition, we found that the total number of tubules in the evaluated areas was significantly higher in the hCG-positive group and lower in controls than in the patients with hCG-negative TGCC (Supplementary Table 1).

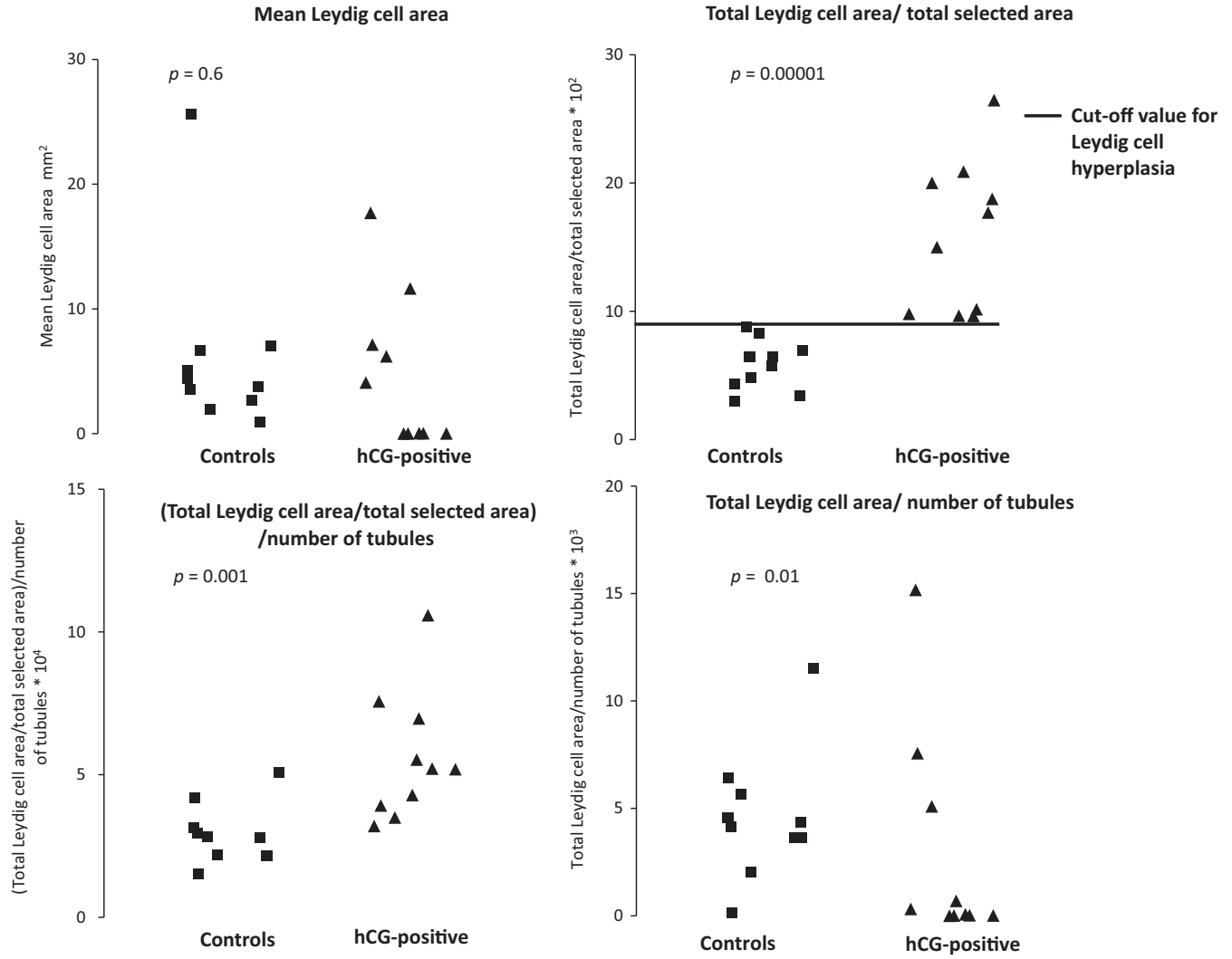
As the index TLCA/TSA was able to completely separate the controls and the hCG-positive patients with the lowest *P*-value (Fig. 2), we decided to use this method to address aim 2. Using a cut-off value for definition of LCH of TLCA/TSA > 9%, there was no overlap between controls and the hCG-positive patients which is shown with the horizontal line in Fig. 2.

To test our new definition of LCH against LCH as determined by the evaluator at the time of diagnosis, we performed ROC curve analysis for TLCA/TSA with LCH according to the initial pathology report as golden standard. The area under the curve was 0.85 (95% confidence interval 0.73–0.96) ($P < 0.001$). When using LCH according to the initial pathology report as golden standard, we found a sensitivity of 84% and specificity of 81% of the new definition of LCH (TLCA/TSA > 9%). When using the new definition of LCH as golden standard, we found a sensitivity of 73% and a specificity of 93% of LCH according to the initial pathology report.

Association between Leydig cell hyperplasia and biochemical Leydig cell dysfunction in patients with TGCC (aim 2)

Using the new definition of LCH (TLCA/TSA > 9%), 22 of the 50 patients with hCG-negative TGCC had LCH identified in the biopsy of the contralateral testicle at the time of diagnosis (Table 1). Patients with LCH had significantly lower TT

Figure 2 Scatter plots evaluating the ability of the four investigated methods to quantify the Leydig cell compartment. Each *P*-value represents comparison between the 10 controls with obstructive azoospermia and the 10 hCG-positive testicular germ cell cancer patients.



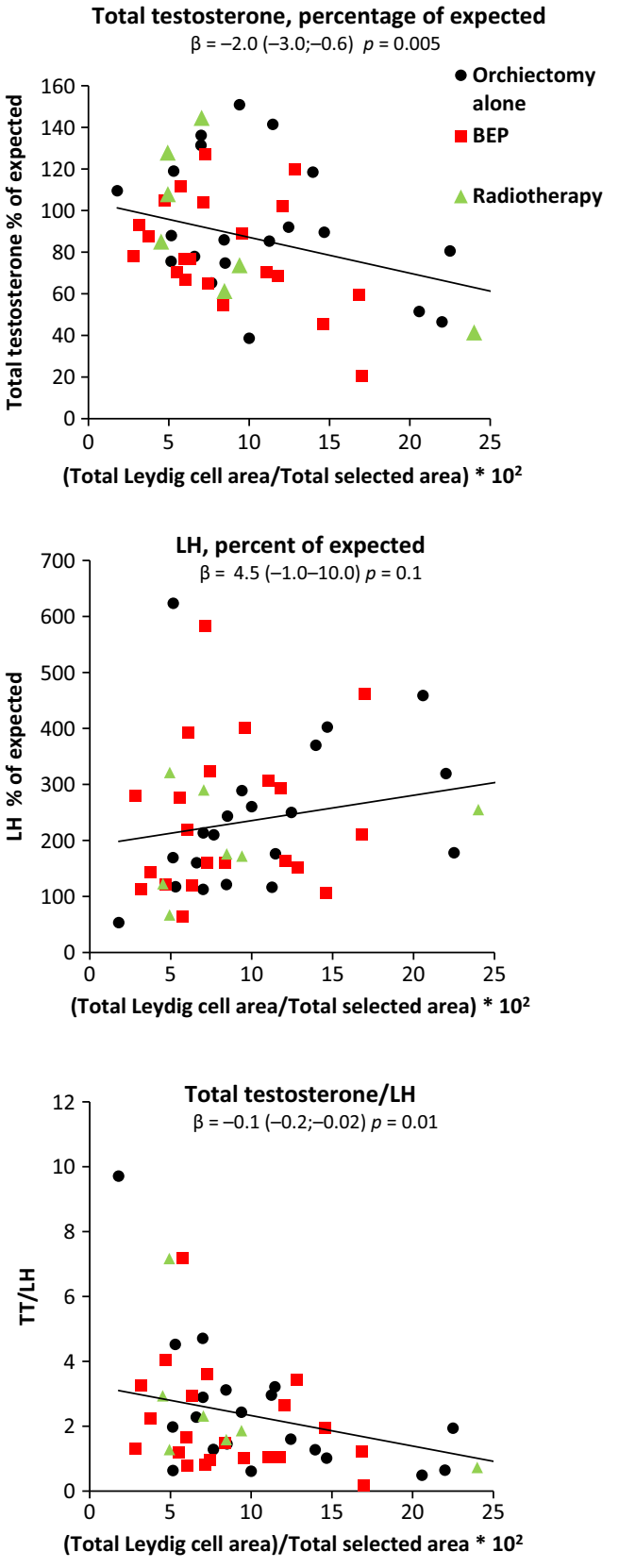


Figure 3 Results of a linear regression analysis with Leydig cell area of the non-tumour-bearing contralateral testicle (Total Leydig cell area/total selected area) as independent variable and serum total testosterone (percentage of expected according to age) serum LH (percentage of expected according to age) and total testosterone/LH *at follow-up* as dependent variable in 50 long-term testicular germ cell cancer survivors. (● represents patients treated with orchiectomy alone, ▲, represents patients treated with radiotherapy, and ■ represents patients treated with bleomycin, etoposide and cisplatin).

(percentage of predicted according to age) at the time of diagnosis, and a significantly lower TT/LH ratio at follow-up. Furthermore, testicular size at follow-up was significantly smaller in TC patients with LCH.

Increasing degree of the TLCA/TS index in the contralateral biopsy was significantly associated with biochemical signs of Leydig cell dysfunction at follow-up with decreased TT (percentage of expected according to age) as well as decreased TT/LH (Fig. 3). The trend appeared to be the same independently of treatment modality. There were no significant associations between increasing Leydig cell area and biochemical Leydig cell dysfunction at the time of diagnosis, as well as increasing Leydig cell area and changes in Leydig cell function between diagnosis and follow-up (Figure S2–S3).

DISCUSSION

In this study, we attempted to establish a semiquantitative and clinically applicable method to estimate the Leydig cell area in testicular biopsies, as the histological description of the Leydig cell compartment is currently based on the subjective impression of the evaluator. We found that it is possible to quantify the Leydig cell compartment by manual delineation using the publicly available image processing program ImageJ. Using the index (TLCA/TSA), which reflects the fraction of the total area of a biopsy section occupied by Leydig cells, we were able to separate controls with a normal Leydig cell compartment and a group of TGCC patients with highly elevated serum hCG levels who had an enlarged Leydig cell area.

Secondly, in patients with hCG-negative TGCC, we found that increased area of the Leydig cell compartment and LCH of the testicle without malignancy was associated with long-term biochemical signs of Leydig cell dysfunction, reflected by decreased serum levels of total testosterone and decreased total testosterone/LH ratio. Although the androgen status is easily evaluated by simply measuring hormone levels in blood, this finding could be of importance for clinicians in cases when testicular biopsy has been performed. The presence of LHC in patients with TGCC at the time of diagnosis might alert clinicians to perform closer follow-up for early detection of biochemical signs and symptoms of Leydig cell dysfunction. We are currently investigating if TC survivors with biochemical Leydig cell dysfunction without obvious symptoms would benefit from testosterone substitution (Bandak *et al.*, 2017b).

There were previous attempts to quantify the Leydig cell compartment in testicular biopsies. In a cross-sectional study of 95 patients, including 66 patients with infertility, Holm *et al.* investigated the Leydig cell compartment using semistereological methods and examined possible associations with serum levels of reproductive hormones (Holm *et al.*, 2003). In 66 infertile men, they found a significant correlation between the presence of Leydig cell micronodules defined as clusters with more than 15 Leydig cells *and* increasing serum levels of LH and decreased TT/LH. Our results using a simple semiquantitative method to determine the Leydig cell area are in line with these findings and further support the association between LCH and biochemical Leydig cell dysfunction.

Adding a semiquantitative evaluation of the Leydig cell compartment would require some extra time for the pathologist/andrologist that provides a report on the testicular biopsy. Based on our experience, we estimate that manual delineation and calculating of the areas would take approximately 30 min per patient, which could possibly decrease to 15 min in experienced hands. Future development of the method could include immunohistochemical staining for a Leydig cell marker, for example steroidogenic enzyme 3-beta-hydroxysteroid dehydrogenase (3BHCD), followed by an automatic calculation of the area of stained cells by image software, which would eliminate the need for manual delineation of the Leydig cell groups. However, regardless of the LCA calculation method, this test ought to be performed primarily in andrology centres and in certain cases where the evaluation of Leydig cell function is essential, for example in patients suspected of testosterone deficiency and in patients with TGCC, in whom a contralateral biopsy is available.

Several factors such as small testicular size and presence of microlithiasis (Eberhard *et al.*, 2008; Pühse *et al.*, 2011), biochemical Leydig cell dysfunction at the time of diagnosis (Bandak *et al.*, 2017c; Eberhard *et al.*, 2008) and chemotherapy and radiotherapy have been associated with long-term risk of Leydig cell dysfunction and manifest testosterone deficiency in patients with TGCC (Bandak *et al.*, 2016). In the present study, ultrasonic size of testicle without a tumour evaluated at the follow-up was significantly smaller in patients with LCH, while there was no difference in the prevalence of microlithiasis among patients with or without LCH. A correlation between small testicular size and Leydig cell hyperplasia has been reported in other studies (Ahmad *et al.*, 1971; Holm *et al.*, 2003). However, in the present study, ultrasonic evaluation was performed many years after the testicular biopsy was performed, and the initial data were available only in a handful of patients, so it cannot be excluded that testicular size at follow-up is not comparable to ultrasonic size at the time when the biopsy was taken. The association between LCH and biochemical Leydig cell dysfunction appeared to be the same across treatment groups (orchiectomy alone, BEP, RT), although the sample size was too small to test this in a statistically meaningful manner. As measuring of serum levels of LH and TT and ultrasonic evaluation of the testicle are easily performed, it remains to be seen if LCH according to our proposed method adds additional prognostic value for estimating the risk of long-term Leydig cell dysfunction. However, to answer this question, a prospective study is needed, including systematic ultrasonic evaluation combined with hormone profiles and a registration of early signs of androgen insufficiency.

The main strength of the present study is a detailed andrological characterization of the patients with TGCC, including testis size and reproductive hormones, as well as an access to a unique material of the contralateral biopsy. This study confirms the value of this procedure, which gives an insight not only into the presence of GCNIS, but also into the function of the remaining testicle at the time of orchiectomy, including Leydig cell function and quality of spermatogenesis, although screening for GCNIS remains the main indication (Rajpert-De Meyts & Grigor, 2017). However, the LHC is a warning sign, while the presence of at least some tubules with late spermatids is a positive finding, especially for the patients in whom semen cryopreservation could not be performed before orchiectomy.

The limitations of this study include a retrospective set-up, the modest sample size and the heterogeneity of treatment modalities among patients with hCG-negative TGCC. As only approximately half of the patients had Leydig cell function evaluated before orchiectomy, the study is possibly underpowered to evaluate these associations as well as changes in Leydig cell function. In addition the different methods used for analysis of serum levels of testosterone at diagnosis and at follow-up are a limitation. However, as we calculated percentage of predicted value based on reference values from age-matched controls analysed with the same methods, we have tried to adjust for the change in method. Another problem is the histological heterogeneity and different quality of spermatogenesis, which can affect the calculations of the TLCA/TSA index. By choosing four representative slides with a minimum of 50 tubules and counting the number of tubules within TSA, we attempted to account for tissue heterogeneity and different diameters of the tubules. We found a higher total number of tubules in the evaluated slides of hCG-positive patients, suggesting increased tissue heterogeneity or impaired spermatogenesis in this group of patients. We tried to adjust for these differences by dividing TLCA/TSA with the total number of tubules and by evaluation the index TLCA/Ntub; however, these adjustments did not improve the ability to separate the patients with hCG-positive TGCC from the control group. Future studies should further investigate methods to account for diameter of tubules when estimating the Leydig cell compartment. Finally, our test is of limited clinical value for patients with TGCC, because systematic screening for contralateral GCNIS in patients with TGCC is not routine in most countries. Clinical guidelines suggest a contralateral biopsy in patients <40 years of age with testicular volume <12 ml, history of cryptorchidism or poor spermatogenesis (Albers *et al.*, 2011). These patients already carry an increased risk of poor Leydig cell function and primary hypogonadism; hence, it remains to be seen if presence of LCH of the contralateral testicle has additional prognostic value in predicting patients at high risk of developing symptoms of testosterone deficiency.

CONCLUSION

A new semiquantitative method allowing a quick and objective evaluation of the Leydig cell compartment in a testicular biopsy was found and assessed for clinical application. The index, based on a calculation of the fraction of the total area of a biopsy occupied by Leydig cells, was able to accurately distinguish patients with Leydig cell hyperplasia from those with a normal Leydig cell compartment. In patients with testicular germ cell cancer, the presence of an enlarged Leydig cell compartment in the biopsy contralateral to the tumour-bearing testicle was associated with biochemical Leydig cell dysfunction during the long-term follow-up period. Closer andrological follow-up of these patients might be suggested. Further prospective studies should validate this method, and future perspectives include automatic delineation of the Leydig cell area.

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DISCLOSURE OF INTEREST

The authors have declared no conflict of interests.

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SUPPORTING INFORMATION

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

Figure S1 Scatter plots showing the number of tubules in the evaluated slides. Each p-value represents comparison between the 10 controls with obstructive azoospermia and the 10 hCG-positive testicular germ cell cancer patients.

Figure S2 Results of a linear regression analysis with Leydig cell area of the non-tumour bearing testicle *(Total Leydig cell area/total selected area)* as independent variable and serum total testosterone (percent of expected according to age where 100% corresponds to the mean value of an age-matched man) serum LH (percent of expected according to age) and total testosterone/LH *at the time of diagnosis* as dependent variable in 29 long-term testicular germ cell cancer survivors with available reproductive hormones.

Figure S3 Results of a linear regression analysis with Leydig cell area of the non-tumour bearing testicle *(Total Leydig cell area/total selected area)* as independent variable and changes in serum total testosterone (percent of expected according to age) and serum LH (percent of expected according to age where 100% corresponds to the mean value of an age-matched man) as dependent variable in 29 long-term testicular germ cell cancer survivors with available reproductive hormones. (● represents patients treated with orchiectomy alone, ▲, represents patients treated with radiotherapy and ■ represents patients treated with bleomycin, etoposide and cisplatin).