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ORIGINAL ARTICLE

Leydig cell dysfunction is associated with post-transcriptional deregulation of CYP17A1 in men with Sertoli cell-only syndrome

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STUDY QUESTION: Is the expression of steroidogenic enzyme 17α-Hydroxylase/17,20-Lyase (CYP17A1) down-regulated in Leydig cells (LCs) of men with spermatogenic failure and compensated impairment of LC function, i.e. a low testosterone to LH (T/LH) ratio?

SUMMARY ANSWER: Although the transcriptional expression of *CYP17A1* is increased, its protein expression is decreased, in isolated LCs of men with spermatogenic failure and reduced serum T/LH.

WHAT IS KNOWN ALREADY: Primary spermatogenic defects have been associated with functional and morphological abnormalities of LCs, characterized by decreased serum testosterone (T) levels, decreased T/LH, increased 17β-estradiol (E2) and E2/T ratio, and larger clusters of LCs (LC hyperplasia). CYP17A1 is a key enzyme in the testosterone pathway and has been implicated in the steroidogenic lesion produced by E2 stimulation.

STUDY DESIGN, SIZE, DURATION: We studied 18 azoospermic patients with Sertoli cell-only syndrome (SCOS) and signs of LC dysfunction (cases) and 10 obstructive azoospermic/oligozoospermic men with normal spermatogenesis (controls). The SCOS patients were sub-grouped into 9 cases with T/LH <2 and 9 cases with T/LH ≥2. All of the men underwent testicular biopsy for sperm retrieval at the Reproductive Unit of a University Hospital.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The transcriptional expression of *CYP17A1* and *SF-1* (steroidogenic factor 1) was quantified by SYBR®Green-based qPCR in LCs isolated by laser capture microdissection (LCM), and relative expression to the control pool was assessed. CYP17A1 protein expression was semi-quantified by indirect immunofluorescence (IFI) using Image-Pro Plus v7.0 (Media Cybernetics) in testicular tissue. FSH and LH serum concentrations, and serum and intratesticular T (ITT) and E2 (ITE2) were measured by IRMA and RIA, respectively.

MAIN RESULTS AND THE ROLE OF CHANCE: Relative *CYP17A1* mRNA expression was increased in cases with T/LH <2 compared to cases with T/LH ≥2, by a mean of 3.3-fold ($P = 0.002$). No corresponding increase in protein expression was found; in fact, CYP17A1 immunostaining intensity assessed by the Integrated Optical Density (IOD) parameter was lower in the cases with T/LH <2 compared to controls ($P = 0.008$). Relative *SF-1* mRNA expression was similar in both case subgroups. *CYP17A1* mRNA expression correlated with ITE2 and intratesticular E2/T ($r = 0.536$; $P = 0.026$ and $r = 0.542$; $P = 0.016$, respectively), while an inverse association was observed for ITE2 and protein level expression ($r = -0.421$; $P = 0.05$).

LARGE SCALE DATA: Not applicable.

LIMITATIONS REASONS FOR CAUTION: We should interpret the results of the semi-quantification of immunofluorescent staining by Image-Pro Plus software with caution, because it is a semi-quantitative method that may have certain difficulties regarding the disposition of

protein in the cells. However, it is not influenced by variations in the number of cells that express the protein, as could be the case of western blot analysis in testicular tissue.

WIDER IMPLICATIONS OF THE FINDINGS: Dysfunctional LCs of men with SCOS show post-transcriptional deregulation of CYP17A1, with increased mRNA and decreased protein expression, which may be modulated by increased ITE2 levels. In addition, transcriptional expression of CYP17A1 was not associated with changes in *SF-1* mRNA expression.

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Key words: spermatogenic failure / Leydig cell dysfunction / CYP17A1 / Sertoli cell-only syndrome / steroidogenic factor 1

Introduction

Studies in men with primary spermatogenic failure have reported that subjects with severe deterioration of spermatogenesis, such as Sertoli cell-only syndrome (SCOS) or mixed atrophy, show elevated luteinizing hormone (LH) levels but testosterone (T) levels that are low or near the lower limit. These findings suggest a state of compensated Leydig cell (LC) dysfunction (Andersson *et al.*, 2004; Lardone *et al.*, 2013; Jorgensen *et al.*, 2016). Additionally, some men with a reduced serum T to LH ratio (T/LH) have 17β-estradiol (E2) levels in the normal-to-high range and elevated intratesticular E2 to T ratio (ITE2/T). These data suggest increased testicular aromatase activity, which in turn may contribute to the androgenic dysfunction of the LC. At the histological level, large LC clusters are a common finding, indicative of true LC hyperplasia (LCH) (Holm *et al.*, 2003; Lardone *et al.*, 2013). It is hypothesized that the etiology of testicular dysgenesis syndrome (TDS) is associated with impaired androgen production during fetal development. This early damage may have health consequences in adults, including compromised adult LC function, given the evidence of compensated LC failure in infertile patients (Skakkebaek *et al.*, 2001; Sharpe and Skakkebaek, 2008).

The steroidogenic enzyme cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), encoded by *CYP17A1*, is a key enzyme in the T biosynthetic pathway in the testis. This enzyme catalyzes the conversion of pregnenolone to 17-OH pregnenolone and then to dehydroepiandrosterone (DHEA) (Δ5 pathway) or the conversion of progesterone (P) to 17-OH progesterone (17OH-P) and finally to androstenedione (A) (Δ4 pathway), through to its 17α-hydroxylase and 17,20-lyase enzymatic activities. Finally, A is converted to T by the enzymatic action of 17β-hydroxysteroid dehydrogenase (17BHSD). The Δ5 pathway is preferentially used by the testicle to produce T, however there is a moderate production of P and 17OH-P that is of exclusive testicular origin (Miller and Auchus, 2011). Therefore, CYP17A1 appears to be fundamental in the progression of the steroidogenic pathway. Apart from the clear consequences of *CYP17A1* mutations and complete CYP17A1 deficiency in men with absent or incomplete development of the external genitalia (Miller and Auchus, 2011), milder forms of testicular CYP17A1 deficiency may be associated with idiopathic infertility or hypospadias (Marsh and Auchus, 2014). In addition, there is some evidence of impaired CYP17A1 enzymatic activity, which does not preclude sexual differentiation, in patients with Klinefelter syndrome (Belli *et al.*, 2016).

Steroidogenic factor 1 (SF-1), a member of the orphan nuclear receptor family NR5A, is primarily responsible for regulation of tissue-specific expression of *CYP17A1* and other genes involved in steroidogenesis, such as *CYP11A1* (P450scc) and *CYP19A1* (P450 aromatase) (Morohashi *et al.*, 1992; Parker and Schimmer, 1995; Schimmer and White, 2010). SF-1 has also been reported to regulate the expression of the StAR protein (Sugawara *et al.*, 1996; Leers-Sucheta *et al.*, 1997) and non-P450 hydroxylases, such as HSD3B2 (Mizutani *et al.*, 2015). Mutations in the gene encoding SF-1, *NR5A1*, are found in approximately 1–4% of men with unexplained severe spermatogenic failure, but normal sexual or adrenal development (Bashamboo *et al.*, 2010; Ropke *et al.*, 2013).

We have recently shown that SCOS patients with low T/LH ratios and LCH have LCs that overexpress *CYP19A1*, leading to increased intratesticular E2 levels and E2/T ratios (Lardone *et al.*, 2017). Based on these findings, we postulate that impaired androgenic production in SCOS is associated with depressed CYP17A1 expression, possibly regulated by E2. In this study, we measured the steroidogenic function of LCs in patients with SCOS and compared the results with values for controls with normal spermatogenesis, by quantifying transcriptional expression of *CYP17A1* and *SF-1* in LCs isolated by laser capture microdissection (LCM). To evaluate protein expression, we performed a semi-quantitative study of CYP17A1 expression in testicular tissue using indirect immunofluorescence (IFI). In addition, the enzymatic activity of CYP17A1 was measured as the ratio of specific-testicular products and initial substrates in the serum using liquid chromatography-mass spectroscopy (LC–MS), and compared the results for patients with SCOS and controls. Finally, we evaluated whether altered CYP17A1 and/or SF-1 expression is associated with differences in intratesticular concentrations of T (ITT) and E2 (ITE2).

Materials and Methods

Subjects, histology and ethical considerations

We studied 28 subjects described in our former study (Lardone *et al.*, 2017). Of these subjects, 18 were azoospermic cases with SCOS (14 complete and 4 focal) and signs of LC dysfunction and 10 were obstructive controls (9 azoospermic and 1 severe oligozoospermic: 290 000 spermatozoa/mL) with normal spermatogenesis (Johnsen score ≥8). Semen analysis was performed following the World Health Organization semen analysis guidelines (WHO, 1999, 2010). Azoospermia was diagnosed when no spermatozoa were observed after centrifugation of two separate seminal samples at 1000 × *g* for 5 min.

Taking into account previous criteria for decreased T/LH ratio and LCH (Lardone *et al.*, 2017), the selected cases had more than 11 LCs per cluster and T/LH was <2 in 9 cases and ≥2 in the other 9, with each group including 7 complete SCOS and 2 focal SCOS. Briefly, the cut off of 11 LCs per cluster was determined as the 97.5 percentile of a population of 68 subjects with histological diagnosis of normal spermatogenesis (Lardone

et al., 2013). The T/LH was calculated as the ratio between serum T (nmol/L) and LH (IU/L) concentrations. The normal range of T/LH ratio was defined in a cohort of 127 subjects with preserved spermatogenesis by determining that 95% of the subjects had a T/LH ratio greater than 2 (Lardone et al., 2013).

Additional 12 secretory azoospermic cases (11 complete and 1 focal SCOS) with low T/LH ratio (T/LH <2) and 14 obstructive azoospermic controls, were selected to estimate the testicular enzymatic activity of CYP17A1 in the serum.

All patients were referred at the Institute of Maternal and Child Research or José Joaquin Aguirre Clinical Hospital of the University of Chile for infertility during 2012–2015. The study was accepted by the Institutional Review Boards of both institutions. The patients had indications of testicular biopsy for the purpose of sperm recovery and all accepted to provide testicular tissue and blood samples after informed consent. Testicular tissues samples were obtained through biopsy from adjacent portions of parenchyma for histology and molecular analysis. Bilateral biopsy was performed in all cases. The histological analysis consisted of the qualitative and quantitative evaluation of the germinal epithelium and the calculation of the Johnsen score in 20 seminiferous tubules as previously described (Johnsen, 1970; Jezek et al., 1998; Lardone et al., 2013). In this sense, bilateral biopsy showed the same diagnosis in 93% (28/30) of the samples; in the remainder, one testicle was diagnosed as complete SCOS and the contralateral testicle had a diagnosis of focal SCOS. Focal SCOS was defined in five patients who showed 30%, 21%, 15%, 14% and 13% of tubules with germ cells, giving Johnsen Scores for these tubules of 5.3, 4.6, 8.0, 3.1 and 5.2, respectively. Spermatic retrieval was achieved in only three of the cases with focal SCOS, while there was no sperm recovery in any of the cases with complete SCOS. A similar pattern both at the seminal and histological level and in the hormonal presentation between subjects with complete and focal SCOS allowed us to classify them into the same group (Behre et al., 2000; McLachlan et al., 2007; Lardone et al., 2013).

The histological analysis also included the counting of LCs per cluster in 5–10 clusters. The mean number of LCs/cluster in cases and controls is showed in Table I. As previously described, larger cluster of LCs are observed in cases with SCOS compared to controls, mainly due to greater number of LCs per cluster and not to an increase in the size of the LC (Holm et al., 2003; Lardone et al., 2013, 2017)

Hormone analysis

Serum hormone concentrations were determined in blood samples obtained through venous puncture between 8 and 10 am. LH, FSH, SHBG, T and E2 were measured as previously described (Lardone et al., 2010, 2017). Serum P, 17OH-P, A and total T were determined by LC–MS using a Shimadzu Agilent 1260 coupled to an AB Sciex 5500 Quantum Ultra Triple Quad mass spectrometer with ion capture. Liquid chromatography separation was carried out on a 150 mm long × 300 μm internal diameter column packed with 4 μm Synergi Hydro-RP particles and maintained at 40°C. To process the samples, we used the Chromsystems kit (Chromsystems, Germany). Unknowns, calibrators, and quality controls were prepared according to the manufacturer's instructions and all samples were measured in each experiment. Briefly, 500 μL of serum sample, 450 μL of precipitation reagent and 50 μL of internal standard were mixed and, after 10 min incubation, samples were centrifuged at 3000 × *g* for 5 min. Then, 10 μL were injected into the HPLC-MS/MS system. Total run time was 10.5 min. The sensitivities for T, P, 17OH-P and A were 0.01 ng/mL, 0.02 ng/mL, 0.05 ng/mL and 0.03 ng/mL, respectively. The corresponding intra- and interassay coefficients of variation (CV) were 2.6% and 5.2%; 3.4% and 6.0%; 2.0% and 6.8%; 4.0% and 6.0%; and 1.2% and 5.5%, respectively.

Testicular T and E2 were obtained from testicular tissue and measured by radioimmunoassay as previously described (Lardone et al., 2010).

Table I Clinical and hormonal features in cases and controls.

	All Cases (*a*)	Cases T/LH < 2 (*b, c*)	Cases T/LH ≥ 2	Controls	a	b	c
N	**18**	**9**	**9**	**10**	***P-values***		
Age (years)	35.5 (32.2–37.5)	36 (36–38)	33 (33–35)	39 (33–41.7)	0.170	0.110	0.512
BMI (Kg/m^2)	26.8 (25.1–28.9)	28.3 (26.2–32)	25.6 (24.8–27.7)	28.2 (25.1–30.2)	0.810	0.093	0.540
FSH (mIU/mL)	20 (13.4–26.1)	26.5 (19–30.2)	14.6 (11.4–21)	3.5 (2.4–6.5)	**0.000**	**0.024**	**0.001**
LH (mIU/mL)	5.8 (3.2–6.8)	6.9 (6.1–8.9)	3.1 (2.3–5.2)	2.1 (1.8–2.6)	**0.002**	**0.002**	**0.000**
SHBG (nmol/L)	20 (14.4–29)	20 (16–29)	17.9 (11.8–24.7)	22.2 (19–40.5)	0.291	0.554	0.513
T (nmol/L)	10.6 (7.3–14.7)	7.7 (6.9–12.5)	11.2 (10.1–15.7)	11.7 (9.7–12.2)	0.502	0.111	0.165
E2 (pmol/L)	117 (77–168)	113.8 (58.7–186.3)	117.5 (84.4–143.2)	54.7 (46.4–72.1)	**0.003**	0.659	0.050
T/LH (nmol/IU)	2.2 (1.2–2.9)	1.1 (0.9–1.7)	3 (2.7–4.1)	5.2 (4.6–7.3)	**0.002**	**0.000**	**0.000**
E2/T (pmol/nmol)	11.4 (6.7–17.5)	13.5 (6.7–18)	10 (6.7–16)	5.2 (4.6–7.3)	**0.014**	0.757	**0.041**
ITT (nmol/mg protein)	10.5 (5.1–15.6)	12.6 (4.7–15.1)	8.15 (6–20.4)	2.2 (1.8–5.7)	**0.005**	0.923	**0.010**
ITE2 (pmol/mg protein)	395.4 (137.9–497.8)	451.1 (183–826.2)	238.3 (124.5–397.6)	100.6 (40.6–117.8)	**0.003**	0.068	**0.003**
ITE2/T (pmol/nmol)	32.7 (23.1–53)	48 (32.7–65.7)	24.9 (11.3–33)	24 (19–42.2)	0.446	**0.021**	0.101
LCs/cluster	13.6 (12.6–16.5)	14.5 (13.5–17.1)	12.6 (11.9–15.5)	6.2 (4.9–6.8)	**0.000**	0.157	**0.000**
Test.volume (cm^3)$^{\#}$	12 (11–13.5)	12 (10–12)	13.5 (10–13.5)	20 (14.6–25)	**0.000**	0.206	**0.001**
Testicular alterations	Mild varicocele (2/18) Cryptorchidism (1/18)			Testicular cancer (1/10)			

Values are presented as median (interquartile range). BMI, body mass index; ITT, intratesticular testosterone; ITE2, intratesticular E2; ITT/E2, intratesticular T/E2 ratio. $^{\#}$Average of both testicles measured with Prader orchidometer. *a* indicates comparison between all cases to controls, *b* and *c* indicate comparison to cases T/LH≥2 and to controls, respectively, by Mann–Whitney test. Significant *P*-values are shown in bold. Reference ranges (commercial kit): FSH 1.0–8.0 mIU/mL; LH 1.0–8.0 mIU/mL; Testosterone 6.9–27.7 nmol/L; Estradiol <183.5 pmol/L; SHBG 10–80 nmol/L.

Briefly, 6 µm-thick cryosections of testicular tissue were homogenized with phosphate-buffered saline (PBS) and steroids were extracted with diethyl ether by vortexing. Dried extracts were resuspended in PBS for subsequent radioimmunoassay. The intra- and interassay CV were 5.1 and 6.4% for T, and 5.7 and 7.9% for E2, respectively.

Laser capture microdissection and RNA extraction

The methodology, outcomes and purity of LC isolation from testicular tissues using LCM were described in our previous report (Lardone *et al.*, 2017). Briefly, testicular tissue 10 µm-thick cryosections were stained with Mayer's hematoxylin (HistoGene® Frozen Section Staining kit, Applied Biosystems, USA) to visualize the LC clusters. LCM was achieved through an infrared (IR) laser in an Arcturus XT instrument (Applied Biosystems, USA). RNA was extracted from the capture of available clusters in 12–16 cryosections of the same patient using one pre-conditioned RNA purification column (PicoPure RNA isolation kit, Applied Biosystems, USA), obtaining approximately 100 ng of RNA. To eliminate DNA contamination, samples were treated with RNase-Free DNase Set (Qiagen, Germany) during RNA purification following the manufacturer's instructions. The RNA integrity was assessed in a fragment analyzer (Agilent 2100 Bioanalyzer. USA) using the Agilent RNA 6000 Pico kit (Agilent, USA). RNA samples with RNA integrity number (RIN) ≥6 were accepted for further RT-qPCR. Complementary DNA (cDNA) was synthesized using the RevertAid H Minus Reverse Transcriptase (Thermo Scientific, USA) with 0.5 µg oligo (dT) primer (Invitrogen, USA), following the manufacturer's instructions.

Cellular purity was controlled by microscopic observation of the residual tissue on the slice. Moreover, the expression of germ cell (SpermatoGenesis-Related Gene, *SGRG*) and Sertoli cell (Follicle-stimulating hormone receptor, *FSHR*) specific transcripts was almost undetectable in the RNA extracted from isolated LC clusters (Lardone *et al.*, 2017). In addition, a low expression of *EMR1* (Human epidermal growth factor module-containing mucin-like receptor) and *CMA1* (Thymosin-b4, chymase) transcripts were found, suggesting low contamination with macrophages and mast cells, respectively, when capturing LC clusters (Lardone *et al.*, 2017).

Quantitative real-time PCR (qPCR)

In a final volume of 30 µL, 15 ng of cDNA were amplified for the quantification of *CYP17A1*, *SF-1* or *GAPDH* transcripts using SYBR Green-based real-time PCR (Platinum SYBR® Green qPCR–SuperMix-UDG with ROX, Invitrogen, USA) in a Step One Plus thermocycler (Applied Biosystems, USA). The primer sequences for amplification of *CYP17A1* (Hilscherova *et al.*, 2004), *SF-1* (Sirianni *et al.*, 2002) and *GAPDH* (Lardone *et al.*, 2010) transcripts were previously reported. The amplification of *GAPDH* mRNA was used to control for variation in RNA input quantities and reverse transcription efficiency. The relative expression levels of *CYP17A1* and *SF-1* transcripts were calculated using the Pffafl formula (Pfaffl, 2001), using a pool of cDNAs from 10 control samples as a calibrator. Expression levels were defined as fold changes (Rq) compared to the expression of the pool of control samples. To calculate PCR efficiencies, the same fragment amplified by the specific pair of primer, and inserted in a plasmid, was serially diluted from 1×10^6 to 1×10^2 copies/µL and amplified in every experiment generating a standard curve where the slope of the curve represents PCR efficiency. Percentages of PCR efficiencies no less than 90% and no more than 105% were accepted. All reactions were accomplished in triplicate, and variations ≥0.5 Ct from the triplicate mean were not accepted.

Indirect immunofluorescence

Immunofluroresce was performed following a standardized procedure in 4 µm-thick section of testicular tissues embedded in paraffin and mounted in xylanized slices, as previously described (Lardone *et al.*, 2017). For the detection of CYP17A1 protein, we used a primary antibody against human CYP17A1 (sc-46 084, Santa Cruz Biotechnology, USA), diluted 1:400 with PBS/BSA 2%, and incubated at 37°C for 1 h. As a secondary antibody, we used a F(ab')2-rabbit anti-goat IgG (H + L) (A-21 222, Life Technologies, USA) diluted 1:300 and incubated at room temperature for 20 min. Non-specific immunostaining was controlled by omitting the first antibody in one slice of every experiment. The analysis and semi-quantification of positive immunoreaction was performed using the Image-Pro Plus v7.0 software (Media Cybernetics Inc., USA) in images captured with a Cool Snap-Proof Color camera (Media Cybernetics Inc. USA), at a magnification of 400× in an Olympus BX51 fluorescent microscope. Approximately, 10 images of representative LC clusters were captured for every sample with the same conditions of light intensity and exposure time. The average of the Integrated Optical Density parameter (mean IOD) for each subject was used as the semi-quantitative IOD value (arbitrary units). To reduce possible deviations in the analysis, we introduced case and control tissues in each experiment. The interassay CV of the standardized method in our laboratory was 11.6%.

Statistical analysis

Statistical analysis was performed using SPSS software v21 (IBM Corp) and Graph Pad software PRISM 5.01 (GraphPad Software). To assess differences between groups and for correlation analyses, we used the Kruskal-Wallis and Mann–Whitney tests and the Spearman's rank correlation coefficient (r), respectively. The Relative Expression Software Tool (REST©) was used for the relative comparison of gene expression (Ct) between cases with T/LH <2 and cases with T/LH ≥2. For all tests, the statistical significance was established with P-values <0.05.

Results

Clinical and hormonal profiles

We have previously reported the hormonal profile of SCOS patients and described a subgroup of these cases with a more pronounced steroidogenic dysfunction of the LC, defined as T/LH <2 (Lardone *et al.*, 2017). The clinical and hormonal findings for cases and controls are summarized in Table I. As in previous studies, serum FSH and LH levels were higher in cases with spermatogenic failure than in controls ($P < 0.001$). Testosterone levels did not differ between cases and controls, although the cases with lower T/LH ratios showed slightly lower T levels. In addition, cases with T/LH <2 showed a significantly higher ITE2/T than cases with T/LH ≥2 ($P = 0.021$) due to higher ITE2 concentrations.

Transcriptional expression of *CYP17A1* and *SF-1* in human Leydig cells

Transcriptional expression of *CYP17A1* and *SF-1* was more than two-fold higher in most cases as compared to the control pool sample (7.4 ± 2.1, range: 0.7–7.8; and 3.4 ± 1.4, range: 1.4–6.1, respectively) (Supplementary Fig. 1). Although no statistical significance was achieved, *CYP17A1* expression levels were directly related to *SF-1* expression ($r = 0.465$; $P = 0.052$). In assessing for an association between gene expression and morphological and/or hormonal variables of steroidogenic dysfunction of the LC, we found that *CYP17A1* expression levels were inversely correlated with T/LH ratio ($r = -0.678$; $P = 0.002$). When we compared relative mRNA expression in the groups of subjects according to T/LH level, we observed that *CYP17A1* mRNA levels were higher in the group of subjects with T/LH < 2 as compared to the group with T/LH ≥2, by a mean factor of 3.3 ($P = 0.002$), while *SF-1* mRNA levels

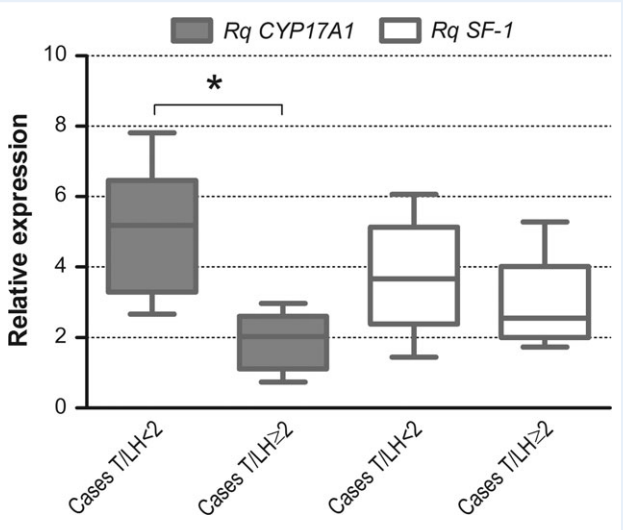

Figure 1 Comparison of CYP17A1 and SF-1 expression levels in cases sub-grouped by testosterone/LH ratio. Transcriptional expression measured as fold changes (Rq) compared to controls; *$P = 0.002$.

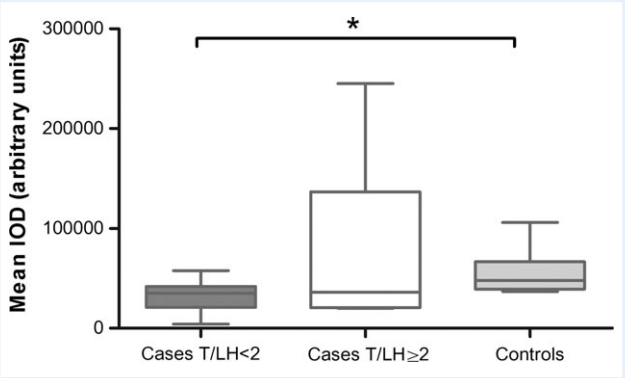

Figure 2 Semi-quantification of immunofluorescent intensity for CYP17A1 in testicular tissue from cases and controls. Analysis performed using Image-Pro Plus software to quantify the mean Integrated Optical Density (IOD) parameter; *$P = 0.008$.

were similar in the two groups ($P = 0.283$) (Fig. 1). Similarly, *CYP17A1* mRNA relative expression was higher in cases with T/LH <2 versus cases with T/LH ≥2 when only cases with complete SCOS were compared (mean factor of 3.015; $P = 0.009$).

Semi-quantification of CYP17A1 expression

CYP17A1 was detected exclusively in LCs from the testicular tissue of cases and controls and showed a homogeneous cytoplasmic pattern (Supplementary Fig. 2). The intensity of staining was measured in 12.6 ± 2.4 LC clusters per subject. Contrary to what was found in the transcriptional expression analysis, CYP17A1 specific immunostaining intensity evaluated by the IOD parameter tended to be lower in cases than controls, without reaching statistical significance ($P = 0.058$). In addition, we observed a positive correlation between the IOD parameter and T/LH levels ($r = 0.420$; $P = 0.033$). The consequent comparison between the subgroups of cases revealed that those with T/LH <2 had a lower staining intensity than controls ($P = 0.008$) (Fig. 2). This difference was maintained when we compared only complete SCOS with T/LH versus controls ($P = 0.005$).

Since T and E2 are the main final products of the metabolic pathway in which CYP17A1 participates, we investigated the correlation of the enzyme with intratesticular levels of these steroids. We observed that the *CYP17A1* transcription and CYP17A1 staining intensity did not correlate with ITT content. However, ITE2 showed a significant positive correlation with *CYP17A1* transcriptional expression levels ($r = 0.536$; $P = 0.026$), and a trend towards an inverse association with CYP17A1 staining intensity ($r = -0.421$; $P = 0.05$).

Activities of testicular steroidogenic enzymes

The enzymatic 17α-hydroxylase and 17,20-lyase activities of CYP17A1 were inferred from the ratio between the serum concentrations of 17OH-P to P and A to 17OH-P, respectively. The serum concentration levels of these hormones and their ratios in cases with T/LH <2 and controls are summarized in Table II. The group of cases with T/LH <2 and the controls consisted of 7 cases and 6 controls from the analysis of *CYP17A1* mRNA expression, and 12 additional cases (11 complete and 1 focal SCOS) and 14 additional controls with normal spermatogenesis, respectively. The complete hormonal profile of these groups is detailed in Supplementary Table I. As expected, the ratio of T (measured by LC–MS) to LH was lower in cases than in the controls ($P < 0.001$). Accordingly, T was significantly lower in cases than controls ($P = 0.022$). Meanwhile, the concentrations of androgen precursors were similar between the two groups, but the A/17OH-P ratio (the hormonal ratio representing 17,20-lyase activity) was significantly lower in cases with T/LH <2 as compared to controls ($P = 0.026$). No difference was observed in the T/A ratio denoting no changes in 17BHSD activity.

Discussion

This study investigated LC steroidogenic dysfunction in men with primary spermatogenic failure. A key finding of this study is that the LCs of SCOS patients with compensated LC dysfunction show *CYP17A1* transcript overexpression as well as decreased CYP17A1 protein expression.

We have recently reported that an overexpression of P450 aromatase in LCs from SCOS patients may lead to an increase in testicular E2, which in turn may affect androgen biosynthesis and account for the functional impairment of the LC (Lardone *et al.*, 2017). The participation of estrogens as intratesticular regulators of androgen biosynthesis was initially documented by Nozu *et al.* (1981a,b). These authors demonstrated that LCs from rats previously treated with large amounts of hCG showed decreased CYP17A1 expression and activity, with accumulation of 17OH-P and lower production of T after hCG stimulation in vitro. Comparable results were obtained in hypophysectomized rats treated with E2, whereas the effect was reversed by administering tamoxifen (a selective estrogen receptor modulator), suggesting that the 'steroidogenic lesion', is due to the inhibitory effect of endogenous intracellular estrogen and depends on the doses of hCG administered. Further in vitro studies with normal and dysfunctional human LCs

Table II Hormonal concentrations measured by LC–MS and their ratios.

	Cases T/LH < 2	Controls
N	**19**	**20**
Progesterone (nmol/L)	0.3 (0.2–0.5)	0.2 (0.2–0.3)
17OH-progesterone (nmol/L)	2.9 (2.3–3.6)	2.6 (2–3.4)
Androstenedione (nmol/L)	2.4 (1.7–3)	2.7 (2.3–3.4)
Testosterone (nmol/L)	10.4 (7.6–14.4)[a]	13 (11.7–19)
17OH-P/P	9 (6.2–13)	9.7 (7.2–13.5)
A/17OH-P	0.8 (0.7–1)[a]	1.1 (0.9–1.4)
T/A	4.1 (3.2–6.3)	4.7 (4–6.3)
T/LH[£]	1.4 (1.1–1.7)[a]	5.9 (4.4–8.5)

Values are medians (interquartile range). P, progesterone; 17OH-P, 17OH-progesterone; T, testosterone; A, androstenedione. [£]T was measured by LC–MS and LH was measured by IRMA. [a]$P < 0.05$, Mann–Whitney test.

exposed to increasing LH doses or E2 would be helpful to test this hypothesis.

We found an increase in the transcriptional expression of *CYP17A1* in cases with signs of LC dysfunction, but immunofluorescent intensity for CYP17A1 was lower in cases than in controls. Because we did not observe differences in the positive label distribution between cases and controls, nor in the ratio between the area of immunofluorescent staining with the area of LC clusters ($P = 0.439$), we assume that the decreased immunostaining intensity for CYP17A1 represents smaller amounts of the protein. This technical approach has been used in other studies demonstrating that immunostaining intensity is able to estimate protein expression (Wang *et al.*, 2009; Hu *et al.*, 2017).

Moreover, indirect evaluation of testicular CYP17A1 activity showed a decreased A/17OH-P ratio in cases with a low T/LH ratio, possibly due to 17OH-P accumulation, suggesting decreased 17,20-lyase activity of CYP17A1 consistent with the findings of Nozu *et al.* (1981a,b). Supporting these findings, repetitive administration of 1500 IU of hCG for 3 days to oligozoospermic patients resulted in an accumulation of 17OH-P and an elevated ratio 17OH-P to T in a group of oligozoospermic patients with lower T/LH ratios, reinforcing the idea of impaired 17,20-lyase or 17β-hydroxysteroid dehydrogenase activity (Anapliotou *et al.*, 1994). More recently, Belli *et al.* (2016) showed that LCs of men with Klinefelter syndrome have lower conversion rates of androgen precursors, with accumulation of P after hCG stimulation, suggesting that the impaired T production in these patients is related to decreased enzymatic activity in the steroidogenic pathway. Overall, our results imply that post-transcriptional regulation is a factor in the steroidogenic impairment of the LC in men with SCOS. These findings provide insights into the potential involvement of estrogens in this process.

In the present study, intratesticular E2 showed a positive correlation with transcriptional expression of *CYP17A1*, but a trend towards an inverse correlation with protein expression. In line with a role of estrogens as regulators of the steroidogenic pathway, there is evidence that estrogens regulate the expression of microRNAs (miRs) in steroidogenic cells and that their target genes participate in the steroid biosynthesis pathway (Hu *et al.*, 2013). For example, adrenal glands from rats treated with 17α-ethinyl estradiol (17α-E2) showed increased expression of miR-212, miR-183, miR-182, miR-132, miR-134, miR-370, miR-377 and miR-96, whereas miR-125b, miR-200b, miR-122, miR-466b, miR-138, miR-214, miR-342, miR-503, miR-542 and miR-27a were down-regulated (Hu *et al.*, 2013). Interestingly, expression of Srebp-1c (sterol regulatory element binding proteins-1c, expressed in Sertoli cells) and LDLR (low-density lipoprotein receptor, expressed in LCs) was down-regulated in vitro in the presence of miR-132 and miR-182/miR-214, respectively (Hu *et al.*, 2013). Additionally, in irradiated rat testicular tissue, the treatment with E2 has been shown to down-regulate the expression of several miRs and change the expression of potential target genes (Zhou *et al.*, 2011). Therefore, our results, along with evidence in other steroidogenic cells, suggest that the increased E2 levels observed in certain patients with SCOS may play a role in the deterioration of steroidogenic function in LC at the post-transcriptional level.

The induction of steroidogenesis is determined by the action of LH over its receptor in LCs, through the cAMP signaling pathway and the recruitment of transcription factors to the promoters of steroidogenic genes (Sewer and Jagarlapudi, 2009; Mizutani *et al.*, 2015). One of these factors, SF-1, has been proposed as crucial for the expression of these genes. Moreover, cAMP signaling may induce SF-1 phosphorylation and other post-translational modifications, such as acetylation and SUMOylation, which control the transactivation function of this receptor in steroidogenic cells (Chen *et al.*, 2004, 2005). In the current study, transcriptional expression of *SF-1* showed a weak correlation with transcriptional expression of *CYP17A1*. Although *SF-1* expression was relatively higher in some cases, these differences were not correlated with signs of steroidogenic dysfunction, suggesting that the mRNA expression of *SF-1* is independent of LH stimulation (Nomura *et al.*, 1998; Mamluk *et al.*, 1999). Therefore, it seems likely that men with spermatogenic failure and signs of steroidogenic dysfunction are exposed to persistently high levels of LH, increasing the activation and recruitment of the SF-1 protein to the promoters of *CYP17A1* and *CYP19A1*, stimulating their transcriptional expression.

In conclusion, we demonstrated that LCs of men with SCOS and signs of testicular steroidogenic dysfunction show discordance between transcriptional and protein expression of *CYP17A1*, associated with impaired T production and elevated intratesticular E2 levels. Abnormal transcriptional expression of *CYP17A1* does not appear to be determined by changes in the transcriptional expression of *SF-1*. Rather, the constitutive expression of SF-1 may be characteristic of the adequate differentiation of LCs in these patients. Alternatively, the increased transcriptional expression of *CYP17A1* could be explained by increased activation or recruitment of SF-1 attributable to the constant and elevated stimulation by LH. Our findings pave the way for future research on possible post-transcriptional mechanisms involved in the negative modulation of T biosynthesis by E2.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* Online.

Authors' roles

M.C.L. wrote the paper, performed qPCR experiments and contributed to the design, statistical analyses and interpretation of the results. F.A.

performed LCM and RNA extractions. M.L. performed IFI experiments and analysis. M.F. collaborated in the recruitment and clinical evaluation of patients and in sample collection. A.P. performed histological analysis of testicular biopsies. M.E and C.P. performed urological and andrological evaluations and testicular biopsies. A.C. designed the study and revised and gave final approval to the manuscript.

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Conflict of interest

None declared.

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