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Testicular steroid sulfatase overexpression is associated with Leydig cell dysfunction in primary spermatogenic failure

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Abstract

Background: Decreased testosterone (T) to LH ratio and increased 17β-estradiol (E2) serum concentrations represent a common finding among patients with severe spermatogenic failure, suggesting a concurrent Leydig cell steroidogenic dysfunction. Aromatase overexpression has been associated with increased serum and intratesticular E2 in these patients. However, it is unknown whether the sulfatase pathway contributes to the increased availability of active estrogens in patients with primary spermatogenic failure.

Objectives: To assess estrogen sulfotransferase (SULT1E1) and steroid sulfatase (STS) mRNA abundance in testicular tissue of patients with Sertoli cell-only syndrome (SCOS) and normal tissues, its association with serum and intratesticular hormone levels, and to explore the mRNA and protein testicular localization of both enzymes.

Materials and Methods: Testicular tissues of 23 subjects with SCOS (cases) and 22 patients with obstructive azoospermia and normal spermatogenesis (controls) were obtained after biopsy. *SULT1E1* and *STS* transcripts accumulation was quantified by RT-qPCR. For mRNA and protein localization, we performed RT-qPCR in Leydig cell clusters and seminiferous tubules isolated by laser-capture microdissection and immunofluorescence in testicular tissues. Serum and intratesticular hormones were measured by immunoradiometric assays.

Results: *SULT1E1* mRNA accumulation was similar in both groups. The amount of *STS* mRNA was higher in cases ($p = 0.007$) and inversely correlated with T/LH ratio ($r = -0.402$; $p = 0.02$). Also, a near significant correlation was observed with intratesticular E2 ($r = 0.329$, $p = 0.057$), in agreement with higher intratesticular E2 in cases ($p < 0.001$). Strong STS immunoreaction was localized in the wall of small blood vessels but not in Leydig cells. Both *SULT1E1* and *STS* mRNA abundance was similar in Leydig cell clusters and the tubular compartment, except for lower *SUTL1E1* mRNA in the seminiferous tubules of SCOS patients ($p = 0.001$).

Conclusions: Our results suggest that an unbalance of the STS/SULT1E1 pathway contributes to the testicular hyperestrogenic microenvironment in patients with primary spermatogenic failure and Leydig cell dysfunction.

KEYWORDS
steroid sulfatase, estrogen sulfotransferase, spermatogenic failure, intratesticular estradiol, Leydig cell dysfunction

1 | INTRODUCTION

Spermatogenic failure is associated with steroidogenic Leydig cell dysfunction, characterized by decreased testosterone (T) to LH ratio and increased serum and/or intratesticular 17β-estradiol (E2) levels.[1-3] In the testis, estrogens (E2 and estrone) are produced by the conversion of androgens (T and androstenedione), catalyzed by the enzyme aromatase encoded in the *CYP19A1* gene. Estrogen biosynthesis takes place mainly in Leydig cells, although adult human Sertoli cells have been shown to produce E2 under T stimulation in vitro,[4,5] and aromatase mRNA and protein expression has also been detected in germ and Sertoli cells.[2,6]

In addition, estrogen bioavailability is determined through the metabolic transformation of active estrogens into inactive estrogens through sulfonation. A biological system of estrogen sulfotransferases and steroid sulfatases catalyzes the transfer of the sulfonate group (SO_3^{-1}) from the sulfonate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a hydroxyl site on the estrogens, including E2 and estrone (E1), and the removal of this group, respectively.

The estrogen sulfotransferase (EST or SULT1E1) is a member of the sulfotransferase family. It has been detected in testicular and other reproductive tissues as well in the liver and white adipose tissue.[7,8] Steroid sulfatase (STS), also known as arylsulfatase C, is involved in steroid desulfonation being E1 sulfate (E1S), dehydroepiandrostenedione sulfate (DHEAS), pregnenolone sulfate (P5S), and cholesterol sulfate the principal hormone substrates for STS.[9] Similar to SULT1E1, STS is expressed in several reproductive tissues including the human testis.[7,8] In addition, efflux transporters and uptake membrane carriers for sulfated steroids are described to be highly expressed in the testis.[10] Overall, this evidence supports that, in addition to the provision of estrogens by the secretory activity of the testis, the "sulfatase pathway" might be responsible for supplying active estrogens in this tissue.

In the mouse, testicular disruption of *Sult1e1* gene produces disrupted spermatogenesis, hypertrophy/hyperplasia of Leydig cells, and decreased steroidogenic capacity in the mutant mouse testes, which could be explained by a chronic increase of local estrogen activity as a result of increased intracrine estrogen stimulation in the absence of intracellular SULT1E1.[11,12] The hypothesis that SULT1E1 plays a role protecting peripheral tissues from excessive estrogenic effects is supported by the inverse correlation between SULT1E1 immunoreactivity and breast tumor size or lymph node status,[13] and corroborated by reduced cell proliferation in the presence of E2 after inducing overexpression of SULT1E1 in a breast cancer cell line (MCF-7).[14]

On the other side, STS represents the counterpart of SULT1E1 action. Studies in estrogen-dependent breast cancer have shown that STS expression and activity are increased in breast carcinoma,[15,16] contributing to the local bioavailability of active estrogens. In conditions where estrogens have a protective function against cardiovascular events, a milder form of atherosclerosis has been associated with an increased expression of STS and a lower expression of SULT1E1 in the vascular smooth muscle cells of post-menopausal women, suggesting the relevance of STS to SULT1E1 ratio for the local regulation of estrogen activity.[17]

Therefore, our aim was to assess whether an unbalanced expression of the STS/SULT1E1 system is related to the intratesticular estrogenic environment in testicular tissue of patients with severe spermatogenic failure and correlates to signs of Leydig cell dysfunction. To this purpose, we quantified the level of STS and SULT1E1 mRNA in testicular tissues of patients with Sertoli cell-only syndrome (SCOS) compared with normal spermatogenesis tissues. Additionally, we evaluated the testicular localization of STS and SULT1E1 and measured serum and intratesticular E2 levels.

2 | MATERIALS AND METHODS

2.1 | Testicular samples

Testicular tissues were obtained from azoospermic or severe oligozoospermic patients (<5 million spermatozoa/ml) who underwent testicular biopsy for sperm retrieval at the Institute of Maternal and Child Research and at the José Joaquín Aguirre Hospital of the University of Chile. Patients with hypogonadotropic hypogonadism, Y-chromosome microdeletions, numerical or structural chromosomal aberrations, chronic diseases, or those undergoing present or recently past hormonal treatments or who reported alcohol or drugs abuse were excluded from the study. The participants went through a complete physical examination and semen analysis according to WHO criteria (2010).[18]

Histological analysis was performed in testicular tissue fixed in Bouin's solution through hematoxylin-eosin, PAS, Masson (trichromic) and orcein staining. The analysis comprised a qualitative and quantitative evaluation of the germinal epithelium in at least 20 seminiferous tubules, calculation of the modified Johnsen Score,[19,20] and the counting of Leydig cells per cluster as previously described.[21]

2.2 | RNA extraction and real-time polymerase chain reaction (qPCR)

Immediately after biopsy, an additional and contiguous piece of testicular tissue to that used for histology analysis was immersed in RNA stabilization solution (RNAlater™, Ambion), and stored at −80°C. RNA extraction was performed using a silica-membrane RNA binding column

(RNeasy Mini Kit, Qiagen) and DNase digestion (RNase-Free DNAase Set, Qiagen) for the removal of contaminant DNA. RNA was quantified by absorbance at 260 nm (ND-1000 Spectrophotometer, NanoDrop), and RNA integrity was assessed using the 2100 Bioanalyzer with the Agilent RNA 6000 Pico kit (Agilent). The synthesis of complementary DNA (cDNA) was performed using 1 µg RNA following the manufacturer's instructions (RevertAid H Minus M-MulV, MBI Fermentas, Burlington, ON, Canada) and stored at −20°C until use.

STS and *SULT1E1* mRNA abundance was quantified by qPCR in a StepOnePlus thermocycler (Applied Biosystems) using predesigned TaqMan® Gene Expression Assays for the amplification of *STS* (Hs00996676_m1) and *SULT1E1* (Hs00960941_m1) transcripts, and TaqMan Universal PCR Master Mix (Applied Biosystem) in a duplex reaction following manufacturer's instructions. Samples were tested in triplicate, and deviation ≥0.5 *Ct* (threshold cycle) from the triplicate mean were not accepted.

PCR efficiencies in the duplex reaction were calculated through a standard curve generated by the amplification on 5 different starting cDNA quantities from 100 ng to 1 ng. The expression level of *SULT1E1* and *STS* was calculated relative to the expression of the reference gene *GAPDH* using the ΔCt method ($2^{\Delta Ct}$). GAPDH mRNA was measured in the same cDNAs samples by means of SYBR Green-based real-time PCR using a pair of primers designed as previously described.[2]

In order to differentiate the transcriptional expression of *STS* and *SULT1E1* between seminiferous tubules and interstitium in control and SCOS tissues, we performed laser-capture microdissection (LCM) as previously described.[3] Briefly, 10-µm-thick cryosections were stained with Mayer's hematoxylin (HistoGene® Frozen Section Staining kit, Applied Biosystems). LCM was achieved in an Arcturus XT instrument (Applied Biosystems) capturing separately Leydig cell clusters or seminiferous tubules. RNA was extracted using PicoPure RNA isolation kit (Applied Biosystems). To eliminate DNA contamination, samples were treated with RNase-Free DNase Set (Qiagen). Complementary DNA (cDNA) was synthesized using the RevertAid H Minus Reverse Transcriptase (Thermo Scientific) with oligo (dT) primer (Invitrogen).

2.3 | Hormonal analysis

To measure serum LH, FSH, total T, and E2, we obtained blood samples drawn between 8 and 10 AM. Immunoradiometric assay (Izotop, Budapest, Hungary) and radioimmunoassay (DIAsource ImmunoAssays S.A. and Pantex) were used for gonadotropins and sex steroids, respectively. To assess intratesticular T and E2 concentrations, steroids were extracted from testicular tissues as previously described[2] and measured by radioimmunoassay using the commercial kits mentioned above. T antiserum has 0.28% cross-reactivity with 5α-dihydrotestosterone and 0.31% with androstenedione. Likewise, E2 antiserum has 1.4% cross-reactivity with α-estradiol and <0.018% with estriol. For the T immunoassay, the intra- and inter-coefficient of variation is 5.1 and 6.4%, and 5.7 and 7.9% for E2 determination, respectively.

2.4 | Indirect immunofluorescence

Indirect immunofluorescence (IIF) was performed in cryosections of testicular tissues which had been embedded in optimal cutting temperature (O.C.T) compound (Sakura Finetek, Torrance, CA, USA), chilled in dry ice-isopentane (2-methylbutane) vapors and stored at 80°C immediately after biopsy. Tissue cryosections were fixed with cold methanol for 20 min at −20°C, followed by permeabilization with 1% Triton X-100 in phosphate-buffered saline PBS-0.1% Tween 20 and 1 h of incubation at 4°C in a blocking solution consisting of PBS-0.1% Tween 20 with 2% bovine serum albumin. Alternatively, IIF was performed in 4-µm-thick sections of testicular tissue fixed with 4% formaldehyde in PBS and embedded in paraffin. After dewaxing, rehydrating, and blocking, the slides were incubated overnight at 4°C with anti-STS antibody diluted 1:100 (cat# ab154312, Abcam) or 1 h at 37°C with rabbit anti-SULT1E1 antibody diluted 1:40 (HPA028728, Sigma Aldrich), mouse anti-SULT1E1 (E-12) antibody diluted 1:50 (sc376009, Santa Cruz), or rabbit anti-SULT1E1 (H40) antibody diluted 1:50 (sc-292049, Santa Cruz). Alexa Fluor 546-conjugated F(ab')2-goat anti-rabbit IgG (H + L) (A-11071, Life Technologies) was used as secondary antibody for STS immunofluorescence. As secondary antibody for SULT1E1, first antibodies were biotin-conjugated $F(ab')_2$ fragment goat anti-rabbit (cat # 111–066–046, Jackson Immunoresearch) or biotin-conjugated $F(ab')_2$ fragment rabbit anti-mouse IgG (cat# 315-066-046, Jackson Immunoresearch), as appropriate, followed by incubation with Alexa Fluor 488-conjugated streptavidin (cat# 016-540-084 Jackson Immunoresearch). Nuclear staining was performed by mounting slides with ProLong® Gold antifade reagent containing DAPI (Molecular Probes). Images were acquired in a BX-51 microscope (Olympus, Tokyo, Japan) coupled with charge-coupled device (CCD) camera Infinity 2 (Lumenera) using the software Image-Pro Plus v7.0 (Media Cybernetics Inc.).

2.5 | Statistical analysis

Statistical analysis was performed with SPSS software version 21 (IBM Corp). To assess differences between groups, we used Mann-Whitney U test and the Spearman's rank correlation coefficient (*r*) for correlation analysis. For the comparison of fold changes of gene expression, the Relative Expression Software Tool (REST©) application was used. *p* values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Patients and hormonal profile

We studied 23 subjects with histological diagnosis of SCOS (20 complete and 3 focal) and 22 subjects with normal spermatogenesis (obstructive azoospermia) as controls. The reproductive hormonal profile, histological analysis, and clinical characteristics are

summarized in Table 1. Cases and controls had similar age and BMI. As expected, FSH and LH levels were significantly higher in cases compared with controls ($p < 0.001$). Serum T levels were similar in both groups, although the T to LH ratio (T/LH) was significantly lower in cases compared with controls ($p < 0.001$). Even though serum E2 level was similar in both groups, intratesticular E2 was significantly higher in cases ($p = 0.001$). Moreover, the number of Leydig cells per cluster was increased in cases compared with controls ($p < 0.001$).

3.2 | Quantification of testicular expression of *STS* and *SULT1E1*

We quantified the level of STS and SULT1E1 mRNA accumulation in the testicular tissue of 18 subjects with SCOS and 17 controls. The reaction for the amplification of *STS* and *SULT1E1* transcripts achieved similar efficiencies ($E \approx 90\%$) and, *STS* and *SULT1E1* mRNA expression was detectable in all samples. *SULT1E1* expression level was similar in SCOS and tissues with normal spermatogenesis ($p = 0.658$) (Figure 1). In contrast, *STS* expression level was significantly higher in SCOS than in tissues with normal spermatogenesis ($p = 0.007$), revealing that *STS* is overexpressed in cases compared with controls by a mean factor of 2.41 ($p = 0.035$ by REST©).

TABLE 1 Age, BMI, hormonal, and histological profile in cases and controls.

	Cases	Controls
N	23	22
Age (years)	35 (30–38)	37 (33–41)
BMI (kg/m^2)	28.1 (25.1–30.7)	29.1 (26.2–30.9)
FSH (mUI/ml)	16.4 (12.8–26.9)*	3.9 (2.5–5.7)
LH (mUI/ml)	6.9 (3.7–8.8)*	2.2 (1.8–2.8)
Total T (ng/ml)	2.7 (2–3.6)	3.2 (2.5–4.2)
Serum T/LH	1.7 (0.9–2.4)*	4.6 (3.3–7)
E2 (pg/ml)	30 (20.3–39)	23.2 (16.9–30.9)
SHBG (nmol/L)	21.5 (14.6–33.6)	19 (13.5–29.7)
Serum E2/T	11.4 (6.7–17.6)	6.9 (5.2–11.5)
IT-T (ng/mg protein)	3.8 (1.1–5.3)	1.5 (0.5–8.8)
IT-E2 (pg/mg protein)	49.4 (38.6–110)*	23.3 (11.1–33)
IT-E2/T	29.1 (11.8–51.6)	21.1 (5.6–41.9)
Testicular volume[a]	12 (10–13.5)*	20 (15–20)
Johnsen Score[a]	2 (1.9–2)	9.4 (8.9–9.5)
Leydig cells/cluster[a]	13.6 (11.1–15.9)*	6 (4.9–6.9)

Abbreviations: BMI, body mass index; IT, intratesticular.

Values are presented as median (interquartile range). Reference values: FSH 1–7 mIU/ml; LH 1–8 mIU/ml; Testosterone (T) 2–8 ng/ml; Estradiol (E2) 0–60 pg/ml; SHBG (Sex hormone binding globulin) 10–80 nmol/L. Testicular volume measured with Prader orchidometer.

[a]Average of both testicles.

*$p < 0.05$ compared with controls by Mann-Whitney test.

Our analysis showed that *STS* expression correlates with FSH levels and T/LH ratio ($r = 0.438$; $p = 0.001$ and $r = -0.402$; $p = 0.02$). Among SCOS patients, those with diminished T/LH ratio (T/LH <2) showed a higher *STS* mRNA accumulation compared with controls ($p = 0.01$). Moreover, in agreement with a higher level of intratesticular E2 in SCOS tissues ($p < 0.001$), a near significant positive correlation was observed between intratesticular E2 concentrations and *STS* mRNA ($r = 0.329$, $p = 0.057$).

In order to assess *STS* and *SULT1E1* expression in the different testicular compartments, we used LCM to isolate Leydig cell clusters from the interstitium and seminiferous tubules followed by qRT-PCR in 9 tissues with SCOS and 5 control tissues (Figure 2). *STS* and *SULT1E1* transcripts were detected in both compartments in normal and SCOS tissues, except in 2 SCOS patients where no *SULT1E1* transcripts were detected in the tubular compartment. In controls, similar quantification was observed between Leydig cell clusters and seminiferous tubules for both transcripts. Meanwhile, Leydig cells clusters isolated from the interstitium of SCOS patients showed a significant increase of *SULT1E1* mRNA ($p = 0.001$) and an almost significant increase of *STS* mRNA ($p = 0.06$). Compared with controls, seminiferous tubules of SCOS showed a lower level of *SULT1E1* mRNA accumulation ($p = 0.005$) (Figure 2).

3.3 | Localization of STS and SULT1E1 in human testicular tissue by IIF

In order to determine the localization of the STS and SULT1E1 protein expression in the testicular tissue, we performed IIF in testicular tissues with SCOS and normal spermatogenesis. For the detection and localization of STS, we were able to test a single commercial antibody in 2 tissues with SCOS and 2 tissues with normal spermatogenesis. Intense immunoreaction was observed in the wall of small blood vessels within the interstitium, whereas weak intensity was observed in the cytoplasm of Sertoli cell in the seminiferous epithelium and in the peritubular cells. No immunoreaction was observed in Leydig cells (Figure 3).

SULT1E1 was assessed in formaldehyde-fixed or frozen tissues in 5 tissues with normal spermatogenesis. Non-congruent results were observed when using three different commercial antibodies: The polyclonal antibody H-40 showed an intense reaction in the testicular interstitium and in an adjacent gross section of the peritubular compartment, while the second polyclonal antibody, HPA028728, showed weak positive immunostaining restricted to the Leydig cell clusters when testing frozen tissues and not in formaldehyde-fixed tissues. Moreover, the HPA028728 antibody showed an unexpected positive immunoreaction in the spermatocytes in frozen tissues, which was also observed with the mouse monoclonal E-12 antibody in frozen, as well as in formaldehyde-fixed tissues. On the other hand, small intestine tissue fixed in 4% formaldehyde was used as a positive tissue control for the expression of SULT1E1 using H-40, HPA02728, and E-12 antibodies. As expected, we observed specific

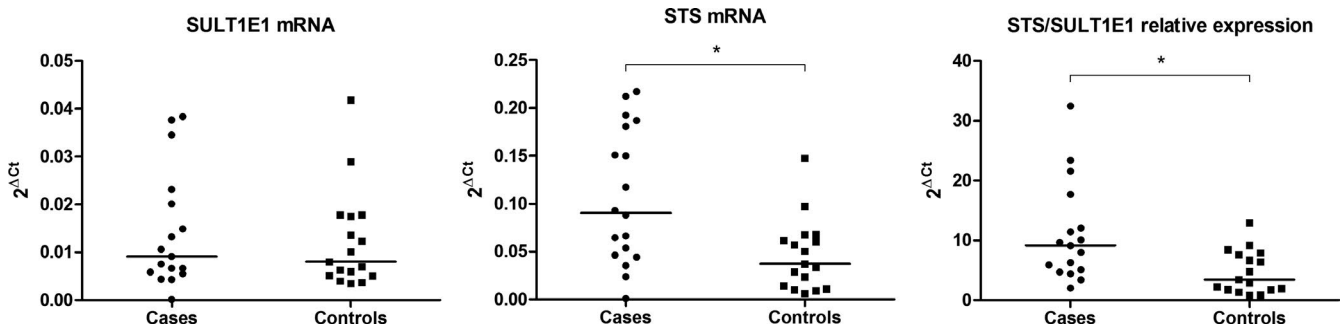

FIGURE 1 Quantification of *SULT1E1* and *STS* transcripts in testicular tissue of subjects with Sertoli cell-only syndrome (cases) and normal spermatogenesis (controls). Relative quantification normalized to the reference gene *GAPDH* using the ΔCt method. The line shows the median of the values. *$p < 0.05$, Mann-Whitney test.

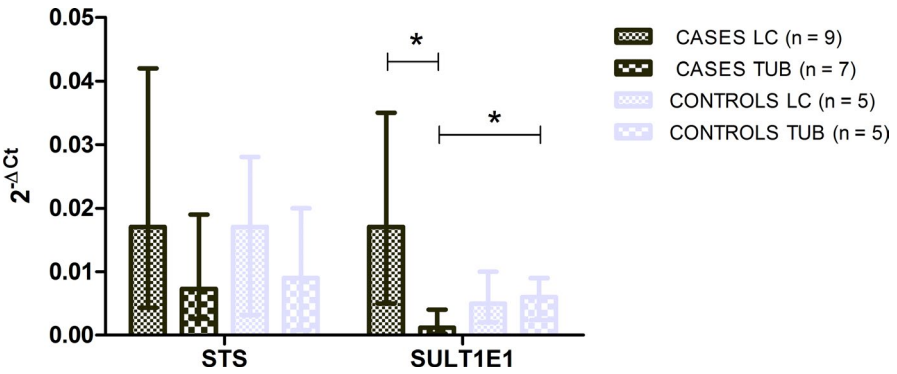

FIGURE 2 *STS* and *SULT1E1* expression in Leydig cell clusters and seminiferous tubules in SCOS and normal tissues. mRNA was obtained from isolated Leydig cell clusters and seminiferous tubules using laser-capture microdissection in 9 testicular tissues of patients with SCOS and 5 controls. STS and SULT1E1 mRNA quantification was normalized to the reference gene *GAPDH* using the ΔCt method. LC, Leydig cell clusters; Tub, Seminiferous tubules. The bars show the median and the range. *$p < 0.05$, Mann-Whitney test.

cytoplasmic immunoreaction in the epithelial cells of the intestinal mucosa.

4 | DISCUSSION

In this study, we observed overexpression of *STS* in testicular tissue of idiopathic infertile patients with SCOS, whereas *SULT1E1* did not change its expression with respect to tissues with normal spermatogenesis. In addition, transcriptional expression of *STS* correlated positively with the testicular content of E2, which supports the contribution of STS activity to the observed higher concentration of intratesticular E2 of SCOS patients.

It has been reported that the testis expresses the STS/SULT1E1 enzymatic system,[8] and the complementary uptake carriers and efflux transporters expressed in the Leydig, Sertoli, and germ cells support the transport of steroid sulfates from the Leydig cells across the seminiferous epithelium.[8,10] SULT1E1 has a high affinity for E2,[22,23] and E1S is one the principal substrates for STS.[9] Particularly in pig testicular tissues, it has been demonstrated that STS has approximately 6 times more activity with E1S than when incubated with DHEAS or P5S.[24] Therefore, the SULT1E1/STS system is likely to be an important source for active estrogens in the testis and principally in the seminiferous tubule, since we have observed a relatively high mRNA accumulation of *STS* in this testicular compartment in SCOS tissues.

Increased STS mRNA accumulation or activity has been demonstrated in steroid-dependent cancers such as breast cancer,[25] prostate cancer,[26] or endometrium cancer,[27] pointing to a dysregulation of the sulfatase pathway, and to a source of precursors for the local supply of estrogens in estradiol-dependent diseases. It has been shown that inflammatory cytokines such as TNFα, IL-1β, and IL-6 increase *STS* mRNA abundance or activity in MCF-7 and in other androgen-dependent cancer cells.[28,29] These findings are relevant for the spermatogenic failure model, which represents a pro-inflammatory state.[30-32]

Also, a potential role for E2 in the upregulation of *STS* expression through its nuclear receptor ERα has been described in breast cancer tissue.[33] On the other hand, the spermatogenic failure is associated with higher levels of serum and intratesticular E2.[1,34-36] Indeed, we have reported that mRNA aromatase level is upregulated in tissues with SCOS leading to higher E2 production and to the functional impairment of the Leydig cells in SCOS patients.[2,3] In this context, the higher concentrations of E2 in SCOS as a result of overexpression of aromatase might upregulate the expression of *STS* and increase the bioavailability of estrogens in these tissues. In accordance with this

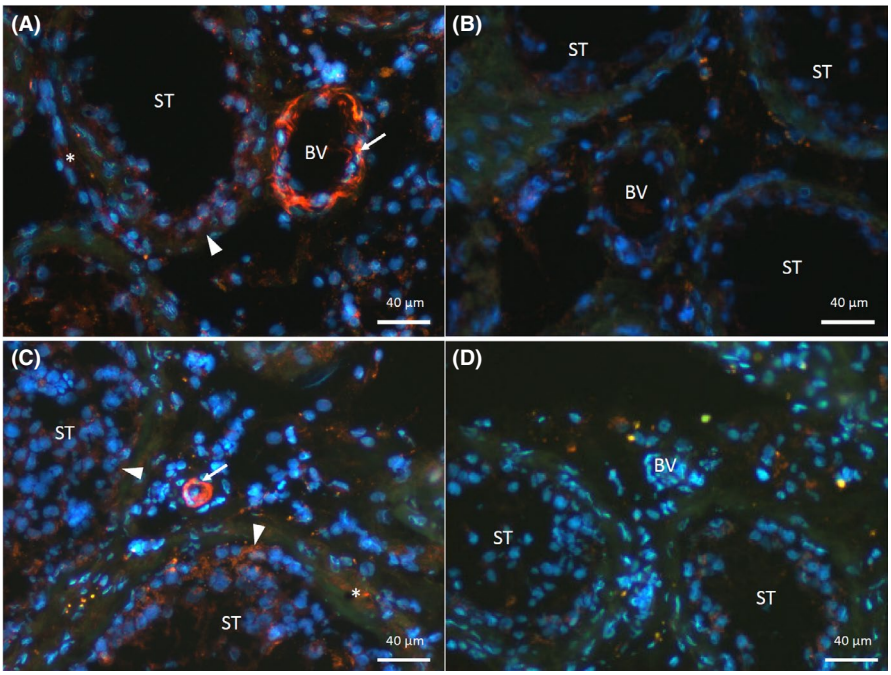

FIGURE 3 Localization of STS expression in human testicular tissue by indirect immunofluorescence. A, Positive reaction in testicular tissue of patients with Sertoli cell-only syndrome (SCOS). C, Positive reaction in testicular tissue with normal spermatogenesis. STS was localized in the wall of blood vessels (BV, arrows), in the seminiferous epithelium (arrowheads) and lamina propria (asterisk) of seminiferous tubules (ST) in control and SCOS patient. A–D, Secondary antibody is conjugated with Alexa Fluor®546 (red), and nuclear staining is performed with DAPI (blue). B and D, corresponding negative controls without first antibody.

hypothesis, in the present study we observed a positive tendency in the correlation between *STS* mRNA expression and intratesticular E2 content.

SCOS represents a common severe spermatogenic impairment in primary testicular failure with the absence of germ cells and is associated with major morphological and functional disturbance of the Leydig cell compartment.[21,37] In this study, we showed that *STS* transcript accumulation is correlated with markers of severity of spermatogenic failure and Leydig cell dysfunction, such as FSH and T/LH ratio, respectively. Moreover, those SCOS patients with signs of Leydig cell dysfunction showed the higher levels of STS mRNA accumulation. Similarly, we have reported that the abundance of aromatase mRNA is higher in the Leydig cells of this subgroup of patients,[3] and in the testicular tissue of severe spermatogenic failure histology, such as mixed atrophy.[2] Overall, these results suggest that the metabolism of estrogens in the testicular tissue of patients with severe spermatogenic failure is dysregulated, although it remains to be seen whether this is a cause or consequence of spermatogenic impairment. Hartmann et al.[8] found a diminished expression of *STS* in testicular tissues of patients with different forms of spermatogenic failure. The discrepancy with our findings could be attributed to the smaller number of SCOS patients studied and to the fact that the histological and hormonal characteristics of these patients were not described in detail. For example, it was not stated whether SCOS patients had spermatogenic foci or what was the proportion of subjects with reduced T/LH ratio. In this regard, we have shown that these histological and hormonal features are associated with the severity of the spermatogenic impairment and correlate with Leydig cell dysfunction and with the intratesticular estrogenic environment.[21]

Concerning the cellular localization of STS expression within the testicular tissue, we were able to detect intense positive immunoreaction in small blood vessels and to a lesser degree in the cytoplasm of Sertoli cells. In contrast to our mRNA quantification experiments, no immunoreaction was observed in Leydig cells; however, it is not possible to rule out contamination with small blood vessels during Leydig cell cluster isolation by laser-capture microdissection. No differences between tissues with SCOS and tissues with normal spermatogenesis were observed regarding the localization and intensity of STS expression. Conflicting evidence concerning the testicular expression of STS based on immunoreaction has been reported in a few studies. Miki et al.[7] described no expression of STS in adult testicular tissues using immunohistochemistry (IHC), although the mRNA was detected. Recently, Hartmann et al.[8] reported ubiquitous localization of STS immunoreaction in germ, Sertoli, and Leydig cells from adult testicular tissues by IHC using a non-commercial antibody. In agreement with our findings, STS expression has been localized in vascular smooth muscle cells from the human aorta and other arteries.[17] In pigs, a species exhibiting an abundant production of sulfated steroids, STS was predominantly identified in the cytoplasm of Leydig cells using IHC with a non-commercial anti-human STS antibody.[24] Also, the same study reports a weak intensity in the vascular system of the testis and epididymis.[24] A recently identified interstitial cell type named telocyte has been shown to locate in the

testicular tissue forming a network in close proximity with peritubular myoid, blood vessels, and Leydig cells, introducing a new player and further complexity in the communication of Leydig cells, blood vessels, and seminiferous tubules.[38] This leads us to speculate that the presence of STS in the peritubular cells and blood vessels may be responding to this interconnected system between the testicular compartments.

Concerning the discrepancy of STS localization in the testicular tissue using the IHC/IIF approaches, we aimed to assess *STS* transcriptional expression in seminiferous tubules and Leydig cells, separately. Our results suggest that *STS* is more abundantly expressed in the interstitium than in the tubular compartment in SCOS and normal tissues. This result is in agreement with the increased expression of *STS* observed in total testicular tissue of our patients with SCOS which have enlarged Leydig cell clusters.

In relation to the expression of SULT1E1, studies report it is mostly localized in Leydig cells of human and mouse testicular tissues.[7,8,39] Unfortunately, using 3 different commercially available polyclonal antibodies and formalin-fixed or frozen testicular tissues, we were not able to detect a consistent specific immunoreaction for SULT1E1. Alternatively, our LCM approach revealed relative higher mRNA levels in the cell fraction enriched in Leydig cells compared with the expression in the tubular compartment in SCOS tissues.

This study supports the existence of an unbalance in *SULT1E1/STS* expression in testicular tissues of SCOS patients. In this regard, it has been shown that murine Leydig cells deficient in SULT1E1 accumulates cholesterol esters and exhibit steroidogenic impairment with low T production as a result of reduced 17α-hydroxilase and 17–20 lyase (CYP17A1) expression and activity.[12] Recently, we reported that the immunoreaction of CYP17A1 is decreased in Leydig cells of patients with SCOS, low T/LH ratio, and high intratesticular concentration of E2, suggesting a negative effect of E2 over T biosynthesis.[40]

In conclusion, our findings show increased *STS* gene expression and higher concentration of E2 in testicular tissue of patients with SCOS. These results suggest that an unbalanced STS/SUL1E1 pathway, in addition to increased aromatase expression, contribute to a testicular hyperestrogenic microenvironment that leads to impaired steroidogenesis in Leydig cells of SCOS patients.

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AUTHOR CONTRIBUTIONS

MCL supervised qPCR and IIF protocols, analyzed the data, and wrote the manuscript. INR performed qPCR and IFF protocols, and analyzed data; EO performed IFF protocols. AP performed histological analysis. CP and ME recruited infertile patients and performed urological and andrological evaluations, and testicular biopsies. AC conceived and designed the study, supervised IFF protocols, and performed critical revision of the manuscript.

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