

Bone Mineral Density and Testicular Failure: Evidence for a Role of Vitamin D 25-Hydroxylase in Human Testis

Carlo Foresta, Giacomo Strapazzon, Luca De Toni, Lisa Perilli, Antonella Di Mambro, Barbara Muciaccia, Leonardo Sartori, and Riccardo Selice

Department of Histology (C.F., G.S., L.D.T., L.P., A.D.M., R.S.), Microbiology, and Medical Biotechnologies, Section of Clinical Pathology and Centre for Male Gamete Cryopreservation, University of Padova, 35128 Padova, Italy; Department of Histology (B.M.) and Medical Embryology, University of Rome "La Sapienza," 00161 Rome, Italy; Clinica Medica I (L.S.), Department of Medical and Surgical Sciences, University of Padova, 35128 Padova, Italy; and EURAC Institute of Mountain Emergency Medicine (G.S.), 39100 Bozen/Bolzano, Italy

Working Hypothesis: Mutations in the *CYP2R1* gene, highly expressed in the testis and encoding vitamin D 25-hydroxylase, result in a vitamin D deficiency and a defective calcium homeostasis leading to rickets.

Objective: Our aim was to investigate *CYP2R1* expression in pathological testis samples and relate this to vitamin D metabolism in testiculopathic patients.

Design, Patients, Setting: Testis samples for *in vitro* study and 98 young men were transversally evaluated at Padova's Center for Male Gamete Cryopreservation.

Methods: *CYP2R1* mRNA expression and protein production were evaluated by quantitative RT-PCR, Western blot analysis, and immunofluorescence. Hormonal and bone-marker levels, and bone densitometry by dual-energy x-ray absorptiometry, were determined in patients with Sertoli-cell-only syndrome and severe hypospermatogenesis.

Results: We found a lower gene and protein expression of *CYP2R1* in samples with hypospermatogenesis and Sertoli-cell-only syndrome ($P < 0.05$) and a colocalization with INSL-3, a Leydig cell marker, at immunofluorescence. In all testiculopathic patients 25-hydroxyvitamin D levels were significantly lower and PTH levels higher compared to controls ($P < 0.05$). Furthermore, testiculopathic patients showed osteopenia and osteoporosis despite normal testosterone levels compared with controls both with increased bone-marker levels and altered dual-energy x-ray absorptiometry in the femoral neck and lumbar spine (for all parameters, $P < 0.05$).

Conclusions: Our data show an association between testiculopathy and alteration of the bone status, despite unvaried androgen and estrogen levels and no other evident cause of vitamin D reduction. Further studies in larger cohorts are needed to confirm our results. (*J Clin Endocrinol Metab* 96: 0000–0000, 2011)

The balance between bone remodeling and bone resorption is essential for normal skeletal development and maintenance during adult life (1, 2). Vitamin D is a key regulatory factor of bone mineralization and of calcium homeostasis in both men and women, being activated in a two-step tightly regulated process at positions 25 and 1 (3).

Even if 25-hydroxyvitamin D concentration is regarded as the most reliable indicator of vitamin D status, the identity of 25-hydroxylase and its regulation is still poorly understood. Both mitochondrial and microsomal subcellular fractions have 25-hydroxylase activity (4–7). The mitochondrial enzyme *CYP27A1* (8) is a low-affinity

ISSN Print 0021-972X ISSN Online 1945-7197

Printed in U.S.A.

Copyright © 2011 by The Endocrine Society

doi: 10.1210/jc.2010-1628 Received July 14, 2010. Accepted December 21, 2010.

Abbreviations: AR, Androgen receptor; BMD, bone mineral density; DEXA, dual-energy x-ray absorptiometry; 1,25(OH)₂D, 1,25-Dihydroxyvitamin D; ICTP, carboxyl-terminal telopeptide of collagen type I; 25(OH)D, 25-hydroxyvitamin D; SCOS, Sertoli-cell-only syndrome.

high-capacity enzyme, whose deficiency or loss of enzymatic activity has an important effect on cholesterol levels but poor influence on vitamin D metabolism (9, 10). Interestingly, a known inactivating mutation in the microsomal form, *CYP2R1* (4, 10), results in a deficiency of vitamin D, defective calcium homeostasis, and classical bone lesions referred to as rickets (4, 11–14). In human adult and fetal tissues *CYP2R1* mRNA is ubiquitous, but its expression reaches the highest levels in the testis (3, 15–17).

Steroid sex-hormone status reflects bone mass, such that hormonal deficiency leads to progressive bone loss (2, 18) and to a sexually-dimorphic pattern. In fact, men are relatively protected from the development of osteoporosis by a higher peak bone mass compared with women, whereas bone-loss rates increase in women after the menopause (19). Nevertheless, a loss of bone mass already starts in men during early adult life, increasing with age (20), but the pathophysiological mechanisms linking male osteoporosis and bone pathophysiology are not completely understood.

In this study we investigated the expression of *CYP2R1* mRNA and protein expression in normal and pathological male human tissues. Furthermore, we aimed to determine vitamin D status and bone mass in a cohort of normotestosteronemic young men with severe hypospermatogenesis or idiopathic Sertoli-cell-only syndrome (SCOS).

Materials and Methods

Human samples

Adult testis samples were obtained from subjects undergoing biopsy for fertilization purposes after subscription of informed consent. Each sample was divided into fragments, which were snap-frozen at -80°C for RNA and protein extraction.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from the testicular biopsy with RNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA samples were then quantified by measuring the absorbance at 260 nm by means of Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA was used for first-strand cDNA synthesis by the use of Quantitect Reverse Transcription (Qiagen) according to the manufacturer's instructions. cDNAs were tested by PCR using specific oligonucleotide primers for the housekeeping gene β -actin (β -actin forward 5'-CACTCTTCCAGCCTTCCTTCC-3' β -actin reverse 5'-CGGACTCGTCATACTCCTGCTT-3'). Commercial liver cDNA library (Clontech, Palo Alto, CA) was used as calibrator sample.

Quantitative real-time PCR

Quantitative expression of *CYP2R1* in human testis samples was evaluated with predesigned assay (Hs01379776_m1 TaqMan Gene Expression Assays, Applied Biosystems, Milan, Italy).

Thermal cycling included initial steps at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The fluorescence intensity of the double-stranded products was monitored in real time. cDNA was amplified and quantified using the iQ5 real-time PCR detection system (Bio-Rad, Milan, Italy). Commercial liver cDNA library (Clontech, Mountain View, CA) was used as positive control for gene expression. Data were normalized to β -actin as internal housekeeping gene (4326315E, Applied Biosystems). Data elaboration was performed as relative quantification analysis using the $\Delta\Delta\text{Ct}$ method. Results are reported as the mean value of three independent experiments in triplicate.

Western blot analysis

Protein extraction was performed by physical procedure (freeze-thaw cycles in liquid nitrogen followed by a 37°C water bath) into lysis buffer (Bio-Rad) containing protease inhibitor. Samples were then denatured with SDS and 2- β -mercaptoethanol, boiled for 10 min and then fractionated using SDS-PAGE gel (Bio-Rad). As a reference sample, a commercial liver protein library (Abcam, Cambridge, UK) was used. After blotting onto Hybond ECL Nitrocellulose Membrane (Perkin-Elmer, Waltham, MA) and blocking with 5% nonfat milk in 0.1% PBS-Tween 20 (Bio-Rad), blots were incubated overnight at 4°C with the goat antihuman *CYP2R1* antibody (sc-48985, Santa Cruz Biotechnology, Santa Cruz, CA) at the proper dilution in 5% nonfat milk in 0.1% PBS-Tween 20 buffer. Primary immunoreaction was detected by incubation with a properly diluted donkey anti-goat IgG secondary antibody conjugated with horseradish peroxidase (sc-2020 Santa Cruz Biotechnology) and visualized using enzyme-linked chemiluminescence (ECL, Perkin-Elmer) with the Chemidoc XRS System (Bio-Rad). β -actin (1:500; sc-47778 Santa Cruz) served as an internal control.

Immunofluorescence

Paraffin-embedded sections of testis, used for immunohistochemistry, were a kind gift of Dr. Annalisa Rasi ("Santa Maria della Misericordia" Hospital of Rovigo, Italy). All specimens derived from testicular germ cell tumors and the surrounding tissue contained tubules with normal spermatogenesis and were regarded as normal after histological evaluation, although the normal tissue was adherent to pathological parts. Paraffin sections were deparaffinized and rehydrated. Antigen retrieval was accomplished by microwaving the sections for 15 min in 0.1 M citrate buffer (pH 7.9). Sections were then incubated with 5% BSA/5% Normal Donkey Serum (respectively from Sigma-Aldrich, Milano, Italy and Millipore, Vimodrone, Milano, Italy) in PBS for 30 min at room temperature to minimize cross-reactivity. Samples were then incubated in darkness for 1 h, at room temperature, with both goat antihuman *CYP2R1* (C-15, sc-48985) and rabbit antihuman INSL-3 primary antibodies (respectively from Santa Cruz Biotechnology Inc. and Phoenix Pharmaceuticals, Burlingame, CA) at the proper dilution in PBS. In the negative control primary antibodies were omitted. Primary immunoreaction was detected by incubation with FITC-conjugated antirabbit IgG (Vector Laboratories, Burlingame, CA) and biotin-conjugated anti-goat IgG secondary antibodies, followed by further incubation with Streptavidin-Texas Red (Vector Labs). Finally, sections were counterstained with DAPI, mounted with anti-fade buffer, and analyzed with Video-Confocal (VICO) fluorescence microscope (Nikon, Firenze, Italy).

Subjects

A total of 57 infertile patients were consecutively recruited in the Center for Male Gamete Cryopreservation at the University of Padova. Of 57 patients, 21 had azoospermia with testicular cytology characterized by SCOS (defined as the absence of germ cells in both testes) and 36 had severe oligozoospermia (defined as $<5 \times 10^6/\text{ml}$) with testicular cytology characterized by severe hypospermatogenesis (defined by a Sertoli Index, the ratio of Sertoli cells to spermatogenic cells, ≥ 300). All men presented a normal 46,XY karyotype and did not carry Y chromosome microdeletions or androgen receptor (AR) mutations. Each subject was evaluated with a clinical history, full physical examination, a venous blood and sperm sample, and bone densitometry. No subjects had acute or chronic pathologies, skeletal disorders, nutritional deficiencies, or urologic abnormalities, and all were free from drugs known to influence bone metabolism. All of them had normal body mass index, no anemia and erythrocyte sedimentation rate, serum glucose, aminotransferases, urea, uric acid, creatinine, prolactin (PRL), progesterone, cortisol, thyroid hormones (T3 and T4), TSH, GH, IGF-I levels were in the normal ranges. As a control group, we studied 41 subjects evaluated in our Center for Sexual Dysfunctions, such as erectile dysfunction or premature ejaculation, with no involvement of the testicular function.

Serum levels of total testosterone, estradiol, LH, FSH, PTH, 25-hydroxyvitamin D [25(OH)D], 1,25-dihydroxyvitamin D [1,25(OH)₂D], bone-specific alkaline phosphatase, and carboxyl-terminal telopeptide of collagen type I (ICTP) were measured in all subjects. All participants in the study, both controls and patients, were evaluated in the same period of the year between November 1, 2008 to February 28, 2009.

Measurement of bone densitometry was done by dual-energy x-ray absorptiometry (DEXA) in the femoral neck and lumbar spine (L₁–L₄), and T-score was calculated as the number of SD that the bone mineral density (BMD) was above or below the mean for young healthy adults of the same race and gender.

Semen samples were obtained by masturbation; after liquefaction at room temperature, semen volume, pH, sperm concentration, motility, and morphology were determined following World Health Organization guidelines for semen analysis (21).

The study was approved by the Hospital Ethics Committee, and each participant gave his written informed consent.

Fine needle aspiration cytology

The testicular structure was analyzed in all patients by means of bilateral fine needle aspiration cytology (FNAC) (22–24). The methods of aspiration and cytological analysis have been described previously in detail (22–24). Briefly, testicular aspiration was performed with a 23-gauge (0.6-mm) butterfly needle attached to a 20-ml syringe. The retrieved material was placed on two or more microscope slides for each testis, stained with May-Grunwald and Giemsa stains, and examined under a light microscope at magnifications of $\times 125$, $\times 400$, and $\times 1250$, counting at least 200 spermatogenic cells (spermatogonia, primary and secondary spermatocytes, early and late spermatids, and spermatozoa) per smear and the interposed Sertoli cells. The cell number was expressed as a percentage. All patients underwent ultrasound scanning of the testis to evaluate testicular size and morphology before undergoing testicular aspiration.

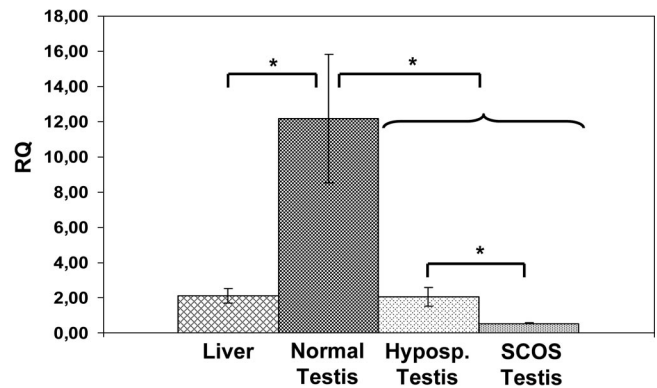


FIG. 1. Gene expression analysis. mRNA expression of *CYP2R1* in normal testis, testis affected by hypospermatogenesis (Hyposp. Testis), and testis affected by Sertoli cells only syndrome (SCOS Testis) compared with commercial liver cDNA library used as control for gene expression. Gene expression was normalized on β -actin. Significance (Student's *t* test): *, $P < 0.05$. Results are given mean \pm SD. RQ, relative quantity.

Hormone assays

Blood was collected in the fasting state between 0800 and 1000 h. Serum FSH, LH, total testosterone, and estradiol were evaluated by commercial electrochemiluminescence immunoassay methods (Elecsys 2010, Roche Diagnostics, Mannheim, Germany). For all parameters the intraassay and interassay coefficients of variation were $<8\%$ and 10% , respectively. PTH serum levels were determined with a direct, two-site, sandwich type chemiluminescent immunoassay (LIAISON N-TACT PTH, DiaSorin Inc. Stillwater, MN). 25(OH)D was determined with direct, competitive chemiluminescent immunoassay (LIAISON 25 OH Vitamin D TOTAL Assay, DiaSorin Inc). 1,25(OH)₂D was quantified by means of IDS 1,25-dihydroxyvitamin D RIA Kit (ImmunoDiagnosticSystem, UK), based on purification of 1,25(OH)₂D by immunoextraction and quantitation with ¹²⁵I RIA. Bone-specific alkaline phosphatase plasma levels were determined with OCTASE, a paramagnetic particles-linked chemiluminescent immunoassay (Access Immunoassay System Beckmann Coulter, Roma, Italy). ICTP were quantified by electrochemiluminescent immunoassay (β -CrossLaps/serum, Cobas, Roche diagnostics, Mannheim, Germany). All determinations were per-

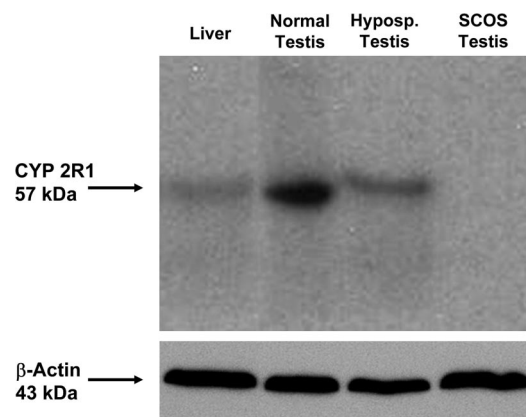


FIG. 2. Western blot analysis. Analysis of the protein expression of *CYP2R1* in normal testis, testis affected by hypospermatogenesis (Hyposp. Testis), and testis affected by Sertoli cells only syndrome (SCOS Testis) compared with commercial liver protein library used as control. β -actin was used as internal control.

formed according to manufacturer's instructions. 25(OH)D levels were considered deficient according to Institute of Medicine (IOM) Committee report 2011 (25).

Statistical analysis

Student's *t* test and ANOVA were used to compare means of gene expression analysis. Differences between three groups (SCOS, severe hypospermatogenesis, controls) were evaluated by unpaired two-sided Student's *t* test. Comparisons between the percentages were performed with χ^2 test. Relationships between continuous variables were assessed using nonparametric Spearman's ρ correlation test.

The significance level was set to $P < 0.05$. Variables are given as mean \pm SD.

Results

CYP2R1 expression

Gene expression of *CYP2R1* was evaluated on biopsies from subjects with diagnosis of, respectively, obstructive azoospermia with not significant alteration of testis parenchyma (normal testis), severe hypospermatogenesis, and SCOS (Fig. 1). Compared with liver tissue, used as reference, normal testes expressed approximately 5-fold higher levels of *CYP2R1* mRNA ($P < 0.05$). Significantly lower levels were found in testes of patients affected by both hypospermatogenesis and SCOS ($P < 0.05$). Interestingly, samples of SCOS testis showed lower expression than those of hypospermatogenesis ($P < 0.05$).

CYP2R1 expression was also investigated at translational level by Western blot analysis (Fig. 2). As observed in gene expression assay, compared with liver as reference, *CYP2R1* protein was markedly expressed in normal testis samples, whereas it was respectively decreased and undetectable in testis samples with hypospermatogenesis and SCOS. Cellular localization of *CYP2R1* protein was assessed by double-staining immunofluorescence assay, using INSL-3, a Leydig cell marker (26), and video-confocal technology to minimize the influence of background signal (Fig. 3, A and B). When observed at low magnification, the staining for *CYP2R1* appears essentially localized on Leydig cells with no other significant signal in cells of the seminiferous tubule (Fig. 3A). At higher magnification, *CYP2R1* staining appeared as specific dotted signals in the cytoplasm of Leydig cells compared with diffuse staining of INSL-3 (Fig. 3B).

Clinical evaluation

Clinical parameters and hormonal levels of our subjects, classified as affected by SCOS, by severe hypospermatogenesis and controls, are reported in Table 1. Age, weight, and height showed no statistical difference among all groups. Serum LH and FSH levels were significantly higher in SCOS and severe hypospermatogenesis groups compared with controls ($P < 0.001$), whereas total testosterone and estradiol levels resulted within normal range and no statistical difference was found among the three studied groups.

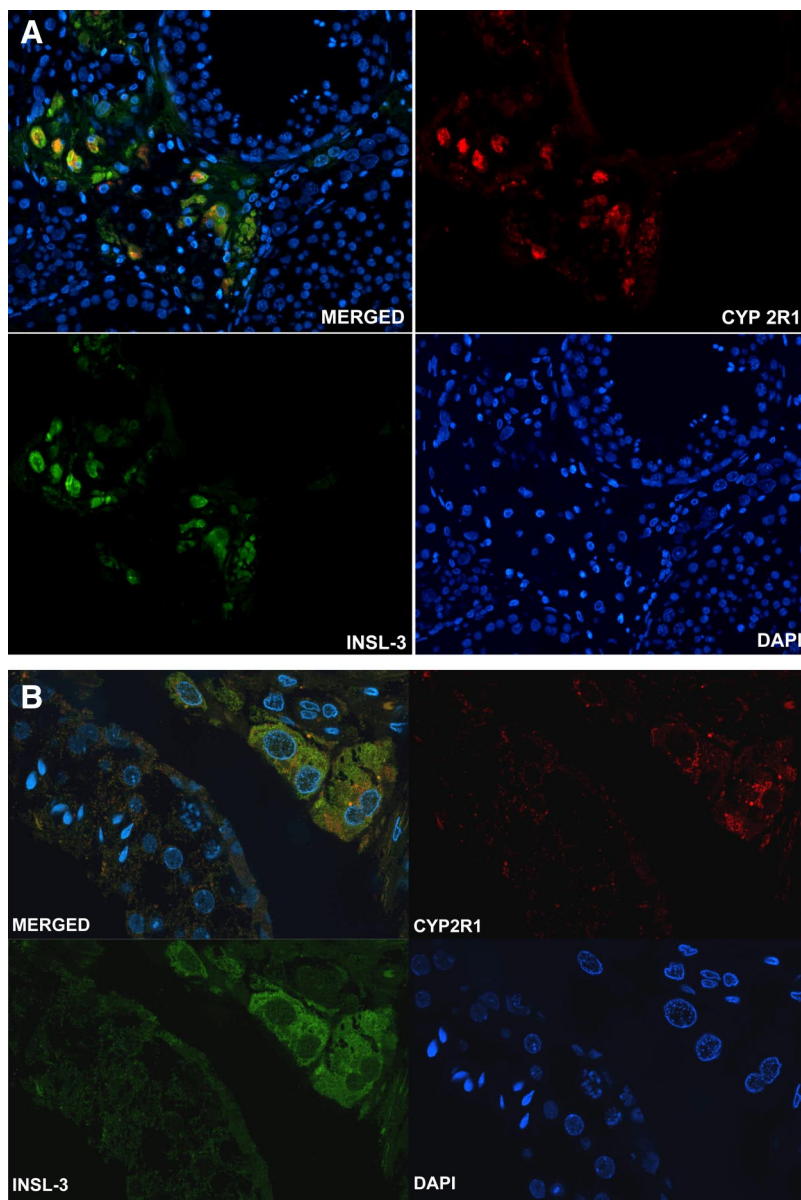


FIG. 3. Immunofluorescence. Analysis of the protein expression of *CYP2R1* in normal testis. Samples were counterstained with DAPI (blue). A, At lower magnification ($\times 200$), *CYP2R1* (red) essentially colocalizes with INSL-3 (green), a typical marker of Leydig cells. B, At higher magnification ($\times 1000$), the pattern for *CYP2R1* staining (red) appears dotted compared with the diffuse staining of INSL-3 (green).

TABLE 1. Clinical parameters and hormonal levels in testiculopathic patients compared to healthy controls

	SCOS	Severe hypospermatogenesis	Controls
Subjects	21	36	41
Age, yr	36.3 ± 5.7	35.4 ± 5.6	35.8 ± 6.2
Weight, kg	69.6 ± 7.2	71.4 ± 7.8	70.2 ± 6.9
Height, cm	174.1 ± 5.9	176 ± 6.3	174.9 ± 6.1
FSH, IU/liter (Reference value 1–8 IU/liter)	21.4 ± 9.8 [#]	17.2 ± 9.8 [#]	3.6 ± 1.6
LH, IU/liter (Reference value 1–9 IU/liter)	8.3 ± 3.3 [#]	8.2 ± 3.0 [#]	3.9 ± 2.0
Total testosterone, nmol/liter (Reference value 10–29 nmol/liter)	15.8 ± 4.4	15.6 ± 6.4	16.6 ± 5.7
17-β-estradiol, pmol/liter (Reference value 25–130 pmol/liter)	82 ± 20	86 ± 45	89 ± 32

[#], $P < 0.001$.

Values are shown as means ± SD.

Bone status parameters, reported as biochemical markers of bone metabolism and bone mass measurements, are shown in Table 2. 25(OH)D plasma levels were lower than 40 nmol/liter in 25/57 (43.8%) of testiculopathic patients and in 3/41 (7.3%) of controls ($P < 0.0001$). Furthermore, PTH serum concentrations were significantly higher in both SCOS and severe hypospermatogenesis groups compared with controls ($P < 0.005$). 1,25(OH)₂D plasma levels were found to be significantly lower only in patients affected by SCOS ($P < 0.05$ compared with controls). Bone-specific alkaline phosphatase and ICTP were found significantly higher in groups affected by testiculopathy compared with control subjects ($P < 0.05$), while serum levels of calcium and phosphorus were similar. Finally, bone densitometry by DEXA revealed that in SCOS patients the femoral neck and/or lumbar spine T-score was < -1 SD (osteopenia) in 8/21 subjects (38.1%) and < -2.5 SD (osteoporosis) in 4/21 subjects (19%). In the group of patients affected by severe hypospermatogenesis, DEXA values of osteopenia and osteoporosis were found, respectively, in 8/36 subjects (22.2%) and in 2/36 subjects (5.6%). None of 41 control subjects showed any significant alteration of BMD. Finally, in the SCOS group the percentage of subjects with bone disorders (osteopenia or

osteoporosis) was 57.1% (12/21), whereas in the severe hypospermatogenesis group it was 27.8% (10/36), significantly higher compared with the control group (0%, 0/41; $P < 0.0005$).

In the whole cohort (testiculopathic patients and healthy controls) plasma 25(OH)D was positively correlated with both femur and lumbar BMD values (respectively, $\rho = 0.39$, $P < 0.05$ and $\rho = 0.37$, $P < 0.05$) and negatively with PTH ($\rho = -0.33$, $P < 0.05$). FSH levels were negatively correlated with femur BMD ($\rho = -0.22$, $P < 0.05$), but not with lumbar BMD ($\rho = -0.11$). No significant correlations were found between LH and BMD values (respectively $\rho = -0.10$ for lumbar and $\rho = -0.08$ for femur BMD).

Discussion

Hypogonadism represents one of the most important causes of male osteoporosis. Testosterone regulates male bone metabolism both indirectly by aromatization to estrogens and directly through the AR on osteoblasts, promoting periosteal bone formation during puberty and reducing bone resorption during adult life (18). Fur-

TABLE 2. Results of bone markers and BMD in testiculopathic patients compared to healthy controls

	SCOS	Severe hypospermatogenesis	Controls
25(OH) D, nmol/liter (Reference value 50–125 nmol/liter)	49.1 ± 18.1*	45.2 ± 23.7*	74.9 ± 38
1,25(OH) ₂ D, pmol/ml (Reference value 43–148 pmol/ml)	75.2 ± 25.9*	104.2 ± 34.0	95.3 ± 31.8
PTH, ng/liter (Reference value 17–73 ng/liter)	66.3 ± 21.6 [#]	87.5 ± 39.4 [#]	49.5 ± 14.2
Serum calcium, mmol/liter (Reference value 2.10–2.55 mmol/liter)	2.4 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
Serum phosphorus, mmol/liter (Reference value 0.87–1.45 mmol/liter)	1.2 ± 0.1	0.9 ± 0.2	1.1 ± 0.2
BAP, μg/liter (Reference value 2.7–20.1 μg/liter)	14.8 ± 5.4*	14.2 ± 5.2*	11.6 ± 4.5
ICTP, pg/ml (Reference value <704 pg/ml)	418.0 ± 153.6*	391.8 ± 165.8*	317.9 ± 95.7
DEXA femoral neck BMD, g/cm ²	0.981 ± 0.115°	1.063 ± 0.172*	1.151 ± 0.128
DEXA lumbar spine L1–L4 BMD, g/cm ²	1.015 ± 0.137°	1.098 ± 0.169*	1.179 ± 0.119

*, $P < 0.05$ vs. controls; [#], $P < 0.005$ vs. controls; °, $P < 0.0001$ vs. controls.

Reference values are reported in brackets. Values are shown as means ± SD. BAP, Bone-specific alkaline phosphatase; ICTP, carboxyl terminal telopeptide of collagen type-I; PTH, parathyroid hormone.

thermore, recent studies suggest a possible relationship between vitamin D metabolism and testis function (17).

It has been demonstrated that the male reproductive tract expresses most of the enzymes involved in vitamin D activation through 25-hydroxylation (17). Inactivating mutations of *CYP27A1* gene, previously assumed to be the main vitamin D-activating enzyme, are actually associated with impairment of cholesterol metabolism (10). On the other hand, an inactivating mutation of the human *CYP2R1* gene has been shown to give rise to a bone phenotype (4, 10). Furthermore, a recent genome-wide association study, involving 33,996 individuals of European descent from 15 cohorts, evidenced that common variants at the *CYP2R1* locus are associated with alterations in circulating 25(OH)D concentrations (27) and our group demonstrated that bilateral orchiectomy results in 33% of reduction in 25(OH)D levels despite adequate testosterone-replacement therapy (28). Thus, our data are consistent with a role of *CYP2R1* and testis in vitamin D activation, showing a progressive decrease of *CYP2R1* expression in relationship to the severity of testiculopathy and suggesting that the Leydig cell could play a major role in this process.

It could be argued that high expression levels of *CYP2R1* in the testis *in vitro* do not reflect the 25(OH)D reduction observed *in vivo* in our orchiectomized (28) and testiculopathic patients. Vicarious effects of the remaining pool of both *CYP2R1* and of the other 25-hydroxylating enzymes, expressed in other tissues (3, 15–17), could explain this apparent discrepancy. Furthermore, the direct involvement of the Leydig cell is suggested by the expression of *CYP2R1* as we highlighted in the immunofluorescence assay (26).

Our patients displayed significantly reduced levels of 25(OH)D compared with controls. In particular, 43.8% of testiculopathic patients showed levels of 25(OH)D lower than 40 nmol/liter (25), despite the absence of nutritional derangements. Moreover, patients showed significant secondary increases in PTH levels compared to control values. A previous study demonstrated a central role of 25(OH)D in regulating PTH secretion (29), whose implication in the pathogenesis of osteoporosis is well established. Our results confirm their negative correlation and the pathogenetic link between 25(OH)D, PTH and BMD in altered bone metabolism.

Our patients affected by idiopathic SCOS and severe hypospermatogenesis were characterized by absence or reduced germ cells in the testis, normal testosterone, and increased LH and FSH levels. The altered LH levels depict a pattern of long-lasting sufferance of Leydig cells, partially compensated by a hyperactivation of the hypothalamic-gonadotropic-gonadal axis (30) that holds testos-

terone production at normal levels. Despite bone turnover is strictly affected by sex hormones, in our testiculopathic patients total testosterone and estrogens levels were in the physiological range. Moreover, we could not find AR mutations or altered CAG repeat polymorphism lengths (data not shown). However, Karin et al. reported how FSH and LH were better predictors of BMD than testosterone in osteoporotic men (31) and, accordingly, we found a significant and inverse correlation between FSH and femur BMD values in the cohort of testiculopathic patients. Another main actor in bone metabolism is 25(OH)D, which was positively correlated with both femur and lumbar BMD values in our population. Common risk factors for vitamin D deficiency include inadequate sun exposure, limited oral intake, malabsorption syndrome, kidney and proliferative diseases, and drugs (32). These conditions were excluded in our population, underlying a possible involvement of testiculopathy in bone metabolism.

Our study suffers limitations because a *CYP2R1* knock-out model is still lacking (33), however a clear pattern of low-circulating levels of 25(OH)D was described in a human model of *CYP2R1*-inactivating mutation (4). Furthermore, we did not have direct evidence of the production of 25(OH)D by Leydig cells. Such investigation is difficult because of the lack of a well-established experimental model of human mature Leydig cell culture (34). Finally, an analysis on a larger cohort could better disclose the involvement of gonadotropins and weight load on bone density modulation.

This is the first study, to the best of our knowledge, showing an association between testiculopathy, altered 25(OH)D levels, and alteration of the bone status, despite unvaried androgen and estrogen levels and no other evident cause of vitamin D reduction. Even if testicular-cell expression of *CYP2R1* suggests a possible influence on 25(OH)D levels, further studies on larger cohorts are needed to confirm our results.

Acknowledgments

We thank Dr. Roberta Venturini, Dr. Diego Faggian, and Flaviano Favaro for biochemical determinations, Dr. Maria Rosa Orlando for DEXA analysis, and the staff of the Center for Male Gamete Cryopreservation.

Address all correspondence and requests for reprints to: Prof. Carlo Foresta, Department of Histology, Microbiology, and Medical Biotechnologies, Section of Clinical Pathology and Centre for Male Gamete Cryopreservation, University of Padova, Via Gabelli 63, 35121 Padova, Italy. E-mail: carlo.foresta@unipd.it.

Disclosure Summary: The authors have nothing to declare.

References

- Cooper C, Westlake S, Harvey N, Javaid K, Dennison E, Hanson M 2006 Review: developmental origins of osteoporotic fracture. *Osteoporos Int* 17:337–347
- Bilezikian JP 2002 Sex steroids, mice, and men: when androgens and estrogens get very close to each other. *J Bone Miner Res* 17:563–566
- Strushkevich N, Usanov SA, Plotnikov AN, Jones G, Park HW 2008 Structural analysis of CYP2R1 in complex with vitamin D3. *J Mol Biol* 380:95–106
- Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW 2004 Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proc Natl Acad Sci USA* 101:7711–7715
- Bhattacharyya MH, DeLuca HF 1974 Subcellular location of rat liver calciferol-25-hydroxylase. *Arch Biochem Biophys* 160:58–62
- Björkhem I, Holmberg I 1978 Assay and properties of a mitochondrial 25-hydroxylase active on vitamin D3. *J Biol Chem* 253:842–849
- Madhok TC, Schnoes HK, DeLuca HF 1978 Incorporation of oxygen-18 into the 25-position of cholecalciferol by hepatic cholecalciferol-25-hydroxylase. *Biochem J* 175:479–482
- Li X, Hylemon P, Pandak WM, Ren S 2006 Enzyme activity for cholesterol 27-hydroxylase in mitochondria. *J Lipid Res* 47:1507–1512
- Pikuleva IA, Björkhem I, Waterman MR 1997 Expression, purification, and enzymatic properties of recombinant human cytochrome P450c27 (CYP27). *Arch Biochem Biophys* 343:123–130
- Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW 2003 De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxylase. *J Biol Chem* 278:38084–38093
- St-Arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH 1997 The 25-hydroxyvitamin D 1-alpha-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. *J Bone Miner Res* 12:1552–1559
- Fu GK, Lin D, Zhang MY, Bikle DD, Shackleton CH, Miller WL, Portale AA 1997 Cloning of human 25-hydroxyvitamin D-1 alpha-hydroxylase and mutations causing vitamin D-dependent rickets type 1. *Mol Endocrinol* 11:1961–1970
- Miller WL, Portale AA 2000 Vitamin D 1 alpha-hydroxylase. *Trends Endocrinol Metab* 11:315–319
- Demay MB 2006 Mechanism of vitamin D receptor action. *Ann NY Acad Sci* 1068:204–213
- Bièche I, Narjoz C, Asselah T, Vacher S, Marcellin P, Lidereau R, Beaune P, de Waziers I 2007 Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. *Pharmacogenet Genomics* 17:731–742
- Choudhary D, Jansson I, Stoilov I, Sarfarazi M, Schenkman JB 2005 Expression patterns of mouse and human CYP orthologs (families 1–4) during development and in different adult tissues. *Arch Biochem Biophys* 436:50–61
- Blomberg Jensen M, Nielsen JE, Jørgensen A, Rajpert-De Meyts E, Kristensen DM, Jørgensen N, Skakkebaek NE, Juul A, Leffers H 2010 Vitamin D receptor and vitamin D metabolizing enzymes are expressed in the human male reproductive tract. *Hum Reprod* 25:1303–1311
- Frenkel B, Hong A, Baniwal SK, Coetzee GA, Ohlsson C, Khalid O, Gabet Y 2010 Regulation of adult bone turnover by sex steroids. *J Cell Physiol* 224:305–310
- Stanworth RD, Jones TH 2008 Testosterone for the aging male; current evidence and recommended practice. *Clin Interv Aging* 3:25–44
- Scopacasa F, Wishart JM, Need AG, Horowitz M, Morris HA, Nordin BE 2002 Bone density and bone-related biochemical variables in normal men: a longitudinal study. *J Gerontol A Biol Sci Med Sci* 57:M385–M391
- World Health Organization 2010 WHO laboratory manual for the examination of human semen and sperm–cervical mucus interaction. Cambridge, UK: Cambridge University Press, 7–157
- Foresta C, Ferlin A, Bettella A, Rossato M, Varotto A 1995 Diagnostic and clinical features in azoospermia. *Clin Endocrinol* 43:537–543
- Foresta C, Varotto A 1992 Assessment of testicular cytology by fine needle aspiration as a diagnostic parameter in evaluation of the oligozoospermic subjects. *Fertil Steril* 58:1028–1033
- Foresta C, Varotto A, Scandellari C 1992 Assessment of testicular cytology by fine needle aspiration as a diagnostic parameter in evaluation of the azoospermic subjects. *Fertil Steril* 57:858–865
- Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs CS, Mayne ST, Rosen CJ, Shapses SA 29 November 2010 The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* 10.1210/jc.2010-2704
- Wikström AM, Bay K, Hero M, Andersson AM, Dunkel L 2006 Serum insulin-like factor 3 levels during puberty in healthy boys and boys with Klinefelter syndrome. *J Clin Endocrinol Metab* 91:4705–4708
- Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Koller DL, Peltonen L, Cooper JD, O'Reilly PF, Houston DK, Glazer NL, Vandenput L, Peacock M, Shi J, Rivadeneira F, McCarthy MI, Anneli P, de Boer IH, Mangino M, Kato B, Smyth DJ, Booth SL, Jacques PF, Burke GL, Goodarzi M, Cheung CL, Wolf M, Rice K, Goltzman D, Hidiroglou N, Ladouceur M, Wareham NJ, Hocking LJ, Hart D, Arden NK, Cooper C, Malik S, Fraser WD, Hartikainen AL, Zhai G, MacDonald HM, Forouhi NG, Loos RJ, Reid DM, Hakim A, Dennison E, Liu Y, Power C, Stevens HE, Jaana L, Vasani RS, Soranzo N, Bojunga J, Psaty BM, Lorentzon M, Foroud T, Harris TB, Hofman A, Jansson JO, Cauley JA, Uitterlinden AG, Gibson Q, Järvelin MR, Karasik D, Siscovick DS, Econs MJ, Kritchevsky SB, Florez JC, Todd JA, Dupuis J, Hyppönen E, Spector TD 2010 Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet* 376:180–188
- Foresta C, Selice R, Di Mambro A, Strapazzon G 2010 Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet* 376:1301
- Pepe J, Romagnoli E, Nofroni I, Pacitti MT, De Geronimo S, Letizia C, Tonnarini G, Scarpiello A, D'Erasmo E, Minisola S 2005 Vitamin D status as the major factor determining the circulating levels of parathyroid hormone: a study in normal subjects. *Osteoporos Int* 16:805–812
- Hammar M, Berg AA 1985 Impaired Leydig cell function in vitro in testicular tissue from human males with “Sertoli cell only” syndrome. *Andrologia* 17:37–41
- Karim N, MacDonald D, Dolan AL, Fogelman I, Wierzbicki AS, Hampson G 2008 The relationship between gonadotrophins, gonadal hormones and bone mass in men. *Clin Endocrinol (Oxf)* 68:94–101
- Holick MF 2007 Vitamin D deficiency. *N Engl J Med* 357:266–281
- Prosser DE, Jones G 2004 Enzymes involved in the activation and inactivation of vitamin D. *Trends Biochem Sci* 29:664–673
- Bilinska B, Kotula-Balak M, Sadowska J 2009 Morphology and function of human Leydig cells in vitro. *Immunocytochemical and radioimmunological analyses. Eur J Histochem* 53:35–42