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# Gender- and dose-related effects of cyclosporin A on hepatic and bone metabolism

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ABSTRACT

Previous data have shown gender-related differences in the skeletal effects of the immunosuppressive drug cyclosporin A (CsA) in rats. To test the hypothesis that the gender-related skeletal effects of CsA are caused by gender-specific metabolism of this drug, we treated aged male and female sham-operated, gonadectomized (GX) as well as sex hormone-supplemented GX rats with 5 mg/kg CsA three times per week for 2 months, and analyzed the bone phenotype as well as the concentrations of CsA and its major metabolites AM1, AM1c, AM9, and AM4N in blood, urine, and liver tissue. CsA treatment induced high turnover osteopenia in males, but not females. Male rats showed several-fold higher CsA and CsA metabolite blood levels compared with females. Renal clearance data revealed that CsA undergoes selective tubular reabsorption in male, but not female rats. However, a mathematical modeling approach demonstrated that the higher CsA blood levels in males were almost exclusively caused by a 6-fold lower hepatic clearance rate compared with females. In addition, we subcutaneously treated female rats with up to 6-fold higher doses of CsA. Similar to males, high dose CsA induced high turnover osteopenia in female rats. Our data show that the gender-related differences in the skeletal effects of CsA are caused by a higher hepatic clearance rate for CsA in female compared to male rats, and not by a differential skeletal response to CsA. Moreover, our study indicates that CsA blood levels of ≤200 ng/ml measured by HPLC do not induce high turnover osteopenia in aged rats.



## Introduction

The immunosuppressive drug cyclosporin A (CsA) is still one of the most important pillars of immunosuppressive therapy after organ transplantations and in autoimmune diseases. CsA is a fungal cyclical undecapeptide isolated from *Tolypocladium inflatum* [1] which inhibits T cell activation via transcriptional suppression of the interleukin-2 gene [2]. One of the potential untoward side effects of immunosuppressive therapy with CsA is a negative effect on the skeleton. The majority of experimental studies in rats have shown that CsA induces a high turnover osteopenia at higher doses [3–5]. However, the molecular mechanism of this effect is still obscure. Clinical studies examining the skeletal effects of CsA in transplant patients have yielded conflicting results. Studies in heart transplant patients [6,7], liver transplant patients [8,9], kidney transplant patients [10–12], or patients receiving bone marrow transplantation [13] indicated a deleterious effect of CsA on bone mass, while some trials in kidney transplant patients on monotherapy with CsA did not suggest CsA-induced bone loss [14–17]. Therefore, the clinical relevance of the potentially deleterious bony effect of CsA is unclear at present. In a previous study, we made the interesting observation that CsA is antiresorptive and bone-sparing in aged female rats but increases bone resorption and reduces bone mass in aged male rats [18]. The gender-specific skeletal effects of CsA were not modulated by sex hormones or gonadectomy [18]. This intriguing observation led us to hypothesize that there may either be gender-related differences in the response of bone cells or T lymphocytes to CsA, or gender-related differences in the metabolism of this drug leading to different skeletal responses.

There is good evidence suggesting that CsA metabolism may indeed be profoundly different in male and female rats [18–20]. After 4 months of chronic subcutaneous CsA administration we found that blood levels of CsA were several-fold higher in male compared with female rats, and that exogenous administration of supraphysiological doses of estradiol from estradiol-containing slow release pellets in ovariectomized (OVX) rats strongly reduced CsA blood levels [18]. Moreover, it has been shown that female rats clear CsA faster than male rats after a single intravenous injection [21] mainly due

*Abbreviations:* ANOVA, analysis of variance; BMD, bone mineral density; BW, body weight; CsA, cyclosporin A; $E_2$, 17β-estradiol; GX, gonadectomized; HRT, hormone replacement therapy; MCT, medium chain triglycerides; OATP, organic anion transporter; OVX, ovariectomized; ORX, orchiectomized; pQCT, peripheral quantitative computed tomography; RE, renal excretion; RIA, radioimmunoassay; SHAM, sham-operated; T, testosterone undecanoate.

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to increased biotransformation [19], and that the synthetic estrogen ethynylestradiol stimulates hepatic CsA metabolism through increased expression of the cytochrome P-450 dependent enzyme CYP3A9 in female rats [22]. In addition, a significant portion of CsA and it metabolites is excreted via the urine after short-term administration [19,23]. Therefore, gender-related differences in urinary excretion may also be involved in the sex-specific differences in CsA metabolism in rats. Experimental studies addressing the gender-related metabolism of CsA under steady state conditions are lacking. Thus, the underlying cause for the gender-related differences in CsA metabolism in chronically CsA-treated rats is still unknown.

In the current study we tested the hypothesis that the gender-related skeletal effects of CsA are caused by gender-specific metabolism of this immunosuppressive drug. To answer this question, we treated aged male and female sham-operated (SHAM) rats, ovariectomized (OVX) and orchiectomized (ORX) rats as well as sex hormone-supplemented OVX and ORX rats with clinically relevant doses of CsA for 2 months, and analyzed the bone phenotype as well as the concentrations of CsA and its major metabolites AM1, AM1c, AM9, and AM4N in blood, urine, and liver tissue. Here we show that the skeletal response of male and female rats to comparable blood concentrations of CsA is similar, and that the gender-related differences in the skeletal effects of CsA are caused by a 6-fold higher hepatic clearance rate for CsA in female compared to male rats.

## Methods

### *Animal procedures*

All animal procedures were approved by the Ethical Committees of the University of Munich and of the University of Veterinary Medicine Vienna, and by the local government authorities. Six-month-old female and 9-month-old male Fischer 344 rats (Charles River, Sulzfeld, Germany) were either sham-operated, OVX or ORX under ether or isofluorane anesthesia. The rationale for using 6-month-old female and 9-month-old male rats was that bone growth has reached very low levels in F344 rats at these ages, so that bony drug effects can be studied under non-growing conditions [18,24]. All experiments were designed using 6–10 rats for each treatment group. CsA treatment was started 5 days postsurgery. All rats received subcutaneous injections of vehicle or CsA at the dose of 5 mg/kg body weight (BW) three times per week. CsA was dissolved in medium chain triglycerides (MCT) containing 0.5% absolute ethanol in the final injection solution. All animals received the same injection volume per kg BW. In addition, male rats were subcutaneously treated during the whole experimental period with either vehicle (ricinus oil/benzyl benzoate, 1:2 v/v) or testosterone undecanoate (T, Bayer-Schering, Berlin, Germany) at the dose of 6 mg/kg once a week. Female rats were subcutaneously injected with either vehicle (ricinus oil/benzyl benzoate, 1:2 v/v) or 2.5 μg/kg 17β-estradiol ($E_2$, Sigma, Deisenhofen, Germany) 5 times per week. To reach higher blood levels of CsA in female rats, an additional experiment was performed in which intact female rats were treated with doses up to 30 mg/kg CsA dissolved in MCT three times per week. Urine was collected in metabolic cages during a 15-hour period overnight before necropsy. All rats were killed 2 months after the start of the experiments and 24 h after the last administration of CsA by exsanguination from the abdominal aorta under ketamine/xylazine anesthesia (50/10 mg/kg intraperitoneally). Liver samples were snap frozen in liquid nitrogen and stored at −80 °C. Whole blood and serum samples were stored at −80 °C until analysis. Urine samples were stored at −20 °C. At necropsy, the seminal vesicles in males and the right uterine horn in females were removed, rinsed in physiological saline, blotted dry, and weighed.

### *Clinical chemistry and analysis of CsA and CsA metabolites*

Serum and urinary creatinine was analyzed on a Hitachi 766 Autoanalyzer (Boehringer Mannheim, Mannheim, Germany). Blood, liver and urinary levels of CsA and its major metabolites AM1, AM1c, AM9, and AM4N were measured by HPLC as described in detail previously [22]. In brief, liver samples (300 mg) were homogenized in 1.2 ml of HPLC-grade water with an ultra-turrax (IKA Labortechnik, Staufen, Germany) and further centrifuged at 10,000 *g* for 20 min at 4 °C. One (1.0) ml of the clear supernatants, whole blood or urine samples were spiked with 50 μl of internal standard solution (5 μg/ml cyclosporine D in methanol) and the proteins precipitated by adding 2 ml of a mixture of acetonitrile/methanol/zinc sulfate (35:60:5, v/v/w). After centrifugation the supernatant was removed and further extracted with hexane (3 ml). The hexane layer was discarded, and the aqueous layer was passed through a C18-Bond-Elut column (Varian, Harbour City, CA, USA) equilibrated with 5 ml of methanol and water, respectively. The column was washed with acetonitrile in water (35% v/v; 2 ml), CsA and its metabolites were eluted with acetonitrile (100%; 2 ml), and dried under a stream of nitrogen. The residue was reconstituted with 200 μl of the mobile phase before injection (150 μl) onto the HPLC column. Chromatographic separation was performed on a Hypersil BDS-$C_{18}$ column (5 μm, 250×4.6 mm I.D., Astmoor, England), preceded by a Hypersil BDS-$C_{18}$ precolumn (5 μm, 10×4.6 mm I.D.), at a column temperature of 70 °C, using a mobile phase of acetonitrile/methanol/water/phosphoric acid (55:10:35, v/v/v), pH 3.0.

To compare the CsA blood levels measured by HPLC with those measured by RIA, methanol-extracted whole blood samples were additionally analyzed by RIA (Cyclotrac SP, Diasorin, Dietzenbach, Germany).

### *Bone mineral density (BMD) measurements*

BMD was measured by peripheral quantitative computed tomography (pQCT) using a XCT Research M + pQCT machine (Stratec Medizintechnik, Pforzheim, Germany). The measurements were made with a collimator opening of 0.2 mm on specimens stored in 70% ethanol. The voxel size was 100 μm. One slice in the mid-diaphysis of the tibiae located 2 mm proximal to the tibiofibular junction, and one slice in the tibial metaphysis located 2 mm distal from the growth plate was measured. For the measurement of trabecular BMD, we used contour mode 2 for detection of the outer bone edge, and peel mode 20 to separate trabecular from cortical-subcortical bone in the L4 vertebrae, while contour mode 1 and peel mode 20 was used in the proximal tibia. The percentage of trabecular bone was set to 50% of the cross-sectional area in the proximal tibia, and to 60% of the cross-sectional area in the L4 vertebra. A threshold of 710 mg/cm³ was used for calculation of cortical BMD. In the L4 vertebra, 3 slices were measured, one in a mid-transversal plane, and 2 located 2 mm rostral and caudal of the mid-transversal plane. BMD values of the L4 vertebral body were calculated as the mean over 3 slices.

### *Cancellous bone histology and histomorphometry*

Bone specimens were fixed in 40% ethanol at 4 °C for 48 h, embedded in methylmethacrylate, and quantitative cancellous bone histomorphometry was performed on median sections of the L1 vertebral bodies as described in detail elsewhere [25,26]. Sections were prepared using a HM 355S microtome (Microm, Walldorf, Germany), or a SM2500S microtome (Leica, Bensheim, Germany). In brief, structural data were measured with an automatic image analysis system (AxioVision 4.6, C. Zeiss, Jena, Germany) on sections stained with von Kossa, and cellular and fluorochrome-based measurements were made with a semiautomatic system (OsteoMeasure 3.0, Osteometrics, Decatur, GA, USA) and a microscope with a drawing attachment on unstained sections or sections stained with toluidine blue. The area within 0.5 mm from the