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## Bilateral testicular tumors resulting in recurrent Cushing's syndrome after bilateral adrenalectomy

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## SUPPLEMENTARY MATERIAL

### **Materials and Methods**

#### ***Serum Hormonal Assays***

Serum androstenedione, cortisol, 11-deoxycortisol, 17 $\alpha$ -OH progesterone, and testosterone, were assessed by an in-house SPE-LCMSMS. Samples underwent solid phase extraction (Oasis, HLB) after protein precipitation (ACN, 0.1% HCOOH). Analysis was performed by LCMSMS (Agilent 6490) with use of an Acquity BEH C18 1.7 $\mu$ M 2.1X50 mm column. Aldosterone, corticosterone, DHEA, dihydrotestosterone, 11 deoxy-corticosterone and estrone were assessed by in-house RIAs after extraction and chromatography with recovery correction. Inhibin B was measured by ELISA (gen II; Beckman-Coulter, Woerden, the Netherlands). ACTH, DHEAS, estradiol, LH and FSH were measured by ECLIA (E170, Roche). Plasma renin was measured by immunoradiometric assay provided by CIS Bio (Codolet, France). All methods used have been validated for use in routine clinical diagnostics.

#### ***Urine steroid metabolite profiling***

Measurement of 24-h urinary steroid metabolite excretion was carried out by a well-established method employing gas chromatography-mass spectrometry (GC/MS) in selected-ion-monitoring mode for quantification of 38 distinct steroid metabolites. Urine samples had been stored at -20 C before analysis, which was carried out within three months of collection. A detailed description of this methodology has been published previously (1). In summary, free and conjugated steroids were extracted from 1 ml urine by solid-phase extraction. Steroid conjugates were enzymatically hydrolyzed, re-extracted, and chemically derivatized to form methyloxime trimethyl silyl ethers. GC/MS was carried out on Agilent 5973 instrument operating in selected-ion-monitoring (SIM) mode to achieve sensitive and specific detection and quantification of 32 selected steroid metabolites. These represented important steroid groups, such as androgen metabolites, glucocorticoid metabolites, mineralocorticoid metabolites, and 3 $\beta$ -hydroxy- $\Delta$ 5 steroid precursors (2).

#### ***Spermatic vein sampling***

The patient underwent bilateral testicular tumor enucleation under general anesthesia. Spermatic vein sampling was done prior to enucleation as described before (3). Briefly, via an inguinal incision and after opening of the inguinal canal, the right spermatic cord was exposed. Care was taken not to manipulate the testes to prevent unwanted secretion of hormones into the circulation. The spermatic vein was cannulated and blood samples were collected to measure serum hormones. The same procedure was performed on the left side. Peripheral blood was collected from a cubital vein to measure the same hormones. All sera were stored at -20 C until measurements.

#### ***Tissue processing and qPCR***

All removed tumor tissue was investigated macroscopically and microscopically. Shortly after resection, a portion of the right testicular tumor sample was snap-frozen and kept in liquid nitrogen for mRNA analysis. Frozen tissue sections (30 x 30  $\mu$ m) were used for RNA isolation (Total RNA Purification Kit: Norgen Biotek Corporation, Thorold, Canada). Tissue sections were homogenized by addition of lysis buffer and pushing the homogenate through a 21 gauge needle using a 1 mL syringe. Further steps of the isolation were performed according to the manufacturer's protocol. RNA concentration and quality were determined using a NanoDrop 2000 Spectrophotometer (Thermo

Fisher Scientific, Waltham, USA). In addition, previous isolated RNA of TART samples of patients with CAH (4) and RNA of healthy control fibroblasts was included as a non-steroidogenic control.

Eight genes were selected for the characterization of the tumor tissue, including the genes encoding 5 key steroidogenic enzymes involved in the adrenocortical and testicular steroid synthesis. The steroidogenic pathways were divided in three parts: common to both adrenal cortex and Leydig tissue (*CYP17A1*, *HSD3B2*), adrenocortical-specific (*CYP11B1*, *CYP11B2*, *AGTR2*, *MC2R*), or Leydig-specific (*HSD17B*, *LHCGR*), as previously described (4). Gene-specific primers were obtained and tested as previously described (4). 0.5 µg of total RNA in a volume of 20 µL was used for cDNA synthesis using Superscript II (200U/µL, Gibco) random primers (0.25 µg/µg RNA, Promega), and oligo dTs (0.25 µg/µg RNA, Santa Cruz). cDNA synthesis was performed using the following cycle conditions: 10 min. 21°C, 45 min. 42°C, 15 min. 70°C, 10 min. 4°C, on a 2720 Thermal cycler (Applied Biosystems). For qPCR, the cDNA samples were diluted 5 times and 5 µL was added to 7.5 µL iQ™ SYBR® Green supermix (Bio-Rad Laboratories), in a total amount of 15 µL on a CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories). The PCR products were checked for quality by melting curve analysis.

### **Data analysis**

For the gene expression analysis, we calculated the mRNA expression of the genes using the delta-delta C<sub>t</sub> method ( $2^{-\Delta\Delta C_t}$ ). All expression values of the patient's tumor were normalized to the corresponding HPRT expression value and then relative to a non-steroidogenic control. Results, previously published, of patients with TART are also shown in Figure 1F for comparison (4).

For the urine steroid metabolites, the patient's results are presented with the median and interquartile ranges of a normal healthy control population, and we used the Cleveland algorithm implemented in SigmaPlot (Systat Software Inc., Chicago, IL).

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