

# Androgen Deficiency and Defective Intracrine Processing of Dehydroepiandrosterone in Salivary Glands in Sjögren's Syndrome

PAULIINA POROLA, LIISA VIRKKI, BEATA D. PRZYBYLA, MIKAEL LAINE, TUCKER A. PATTERSON, ANTTI PIHAKARI, and YRJÖ T. KONTTINEN

**ABSTRACT.** *Objective.* We hypothesized that in addition to dehydroepiandrosterone (DHEA) depletion, Sjögren's syndrome (SS) is characterized by local androgen deficiency in salivary glands and defects in local processing of DHEA.

*Methods.* Sex steroid levels in serum and saliva were measured using enzyme immunoassays. Androgen effects on salivary gland cells were analyzed using the cysteine-rich secretory protein-3 (CRISP-3) androgen biomarker.

*Results.* Serum and salivary concentrations of androgens were low in SS. Substrate to end-product ratios and correlations suggest that in SS salivary glands DHEA is effectively converted to testosterone, but that there are defects in converting testosterone further to dihydrotestosterone (DHT). In healthy controls no such phenomenon was seen, but testosterone is effectively converted to DHT. Salivary glands contained type I 5- $\alpha$ -reductase, and its inhibition with dutasteride completely blocked the upregulating effect of DHEA, but not of DHT, on CRISP-3 in human salivary gland acinar cells.

*Conclusion.* DHEA and DHT upregulate CRISP-3, which is reportedly low in SS. The effect of DHEA on CRISP-3 is indirect and is inhibited by dutasteride, showing that there is intracrine processing of DHEA in salivary glands. In healthy glands, but not in SS, DHEA is effectively taken up and converted to DHT. Sex steroid concentrations in saliva in part reflect glandular uptake of DHEA-sulfate and local intracrine DHEA metabolism, which seem to be defective in SS. Our study demonstrates a prominent androgen deficiency and a defect in intracrine production of active androgens in SS salivary glands, also suggesting that salivary DHT cannot be maintained at a normal level in this female-dominant autoimmune exocrinopathy. (First Release Oct 1 2008; J Rheumatol 2008;35:2229–35; doi:10.3899/jrheum.080220)

## Key Indexing Terms:

SJÖGREN'S SYNDROME ANDROGENS INTRACRINOLOGY SALIVARY GLANDS

From the Institute of Clinical Medicine, Biomedicum Helsinki, University of Helsinki; ORTON Orthopaedic Hospital of the Invalid Foundation, Helsinki, Finland; Institute of Aging, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA; COXA Hospital for Joint Replacement, Tampere, Finland; Division of Neurotoxicology, University of Arkansas for Medical Sciences; Helsinki City Health Department, Teaching Clinic; and Department of Medicine, Hospital District of Helsinki and Uusimaa, Helsinki, Finland.

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P. Porola, MSc, Institute of Clinical Medicine, Biomedicum Helsinki; L. Virkki, MSc, Institute of Clinical Medicine, Biomedicum Helsinki and ORTON Orthopaedic Hospital of the Invalid Foundation; B.D. Przybyla, PhD, Institute of Aging, University of Arkansas for Medical Sciences; M. Laine, MD, DDS, Institute of Clinical Medicine, Biomedicum Helsinki and COXA Hospital for Joint Replacement; T.A. Patterson, MD, Division of Neurotoxicology, University of Arkansas for Medical Sciences; A. Pihakari, MD, DDS, Helsinki City Health Department, Teaching Clinic; Y.T. Konttinen, MD, PhD, Professor of Medicine, ORTON Orthopaedic Hospital of the Invalid Foundation, COXA Hospital for Joint Replacement, and Department of Medicine, Hospital District of Helsinki and Uusimaa.

Address reprint requests to Dr. Y.T. Konttinen, Department of Medicine, Biomedicum Helsinki, PO Box 700 (Haartmaninkatu 8), 00029 HUS, Helsinki, Finland. E-mail: yrjo.konttinen@helsinki.fi

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Sjögren's syndrome (SS) is characterized by dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia), which are associated with focal sialadenitis and serum SSA and/or SSB autoantibodies<sup>1</sup>. Its prevalence in the adult population has been estimated at 3%–4%<sup>2</sup>. However, this high prevalence has not been confirmed in other studies, and the actual estimates fluctuate from 0.026% in women to 4.8% in a geriatric population<sup>3</sup>. This is probably due to the varying classification criteria previously used for this syndrome<sup>4</sup>. The reason for this relatively common autoimmune condition of lacrimal and salivary exocrine glands is not known; however, sex may be a factor, since 90% of patients with SS are women<sup>5</sup>. Therefore, SS may involve sex steroids, either estrogens or androgens. In systemic lupus erythematosus (SLE), estrogens promote autoimmunity, so that SLE often starts at the age of 20 years and can be triggered and/or aggravated by the use of contraceptive pills<sup>6</sup>. In contrast to SLE, patients with SS develop relatively diverse features of the syndrome, usually at the age of 40–50 years. At this age, ovaries degenerate and estrogen production diminishes,

which can lead to menopausal symptoms. These women have estrogen deficiency rather than estrogen excess. Estrogens are supposed to predispose women to autoimmune diseases, but androgens have been suggested to protect against autoimmunity<sup>7</sup>. This has been given as an explanation for why men are less affected by autoimmune rheumatic diseases such as SLE or SS. Indeed, it has been shown by Valtysdottir and colleagues, and confirmed by our laboratory, that patients with SS have low serum dehydroepiandrosterone (DHEA) levels in serum and saliva<sup>8,9</sup>. We have also shown that SS is characterized by low salivary gland and saliva concentrations of androgen-dependent cysteine-rich secretory protein-3 (CRISP-3)<sup>9</sup>. As CRISP-3 in human salivary gland (HSG) acinar cells and salivary glands is regulated by androgens, it could be that the low serum DHEA in SS and the low glandular and salivary CRISP-3 in SS are somehow associated, i.e., that low salivary androgen levels lead to low salivary CRISP-3 levels<sup>9</sup>.

These findings led us to hypothesize that the key factor behind SS is systemic and local androgen deficiency. Our hypothesis was that in addition to the decreased DHEA concentrations, patients with SS also have lower plasma concentrations of testosterone and dihydrotestosterone (DHT), which would suggest that there are defects in the systemic processing of DHEA into more active sex steroids. We also hypothesized that in addition to the decreased salivary levels of DHEA, its active metabolites are also abnormally low in SS. This hypothesis was tested by measuring salivary DHEA, testosterone, and DHT levels in patients with SS and comparing these levels to healthy age- and sex-matched controls. Serum concentrations of testosterone, DHT, and 17- $\beta$ -estradiol were also measured.

Another aspect of the present work, besides studying systemic and local sex steroid concentrations, is to assess if the DHEA steroid pro-hormone is locally converted to other sex steroids in salivary glands and if this local conversion of DHEA to other sex steroids is perhaps disturbed in SS. DHEA is a weak androgen, actually a pro-hormone, which can be converted either to estrogen, such as 17- $\beta$ -estradiol in the breasts and uterus in women, or to androgens, such as testosterone and DHT in the prostate in men<sup>10</sup>. This local production of active sex steroids from a pro-hormone according to local tissue needs accounts for a significant proportion of all sex steroids produced in humans. Production of pro-hormone DHEA is a feature unique to primates and does not occur in rodents. Therefore, rats and mice cannot be readily used to study some of the fundamental questions related to SS, such as the intracrine DHEA metabolism, which may be related to the female dominance, late age of onset, and exocrine gland involvement in SS.

We examined the local processing of DHEA *in vitro* and *in vivo* using HSG cells and serum and saliva samples, respectively. Intracrine processing of DHEA in salivary glands was studied *in vitro* in HSG cells by stimulating the

cells with DHEA and DHT and with or without the 5- $\alpha$ -reductase inhibitor dutasteride, and studying the effect of stimulation on the expression of androgen-regulated CRISP-3. Saliva samples were used to assess the effectiveness of intracrine DHEA conversion in salivary glands *in vivo* in patients with SS compared to healthy controls. In addition, the ratios of DHEA/testosterone and testosterone/DHT were used to assess the effectiveness of the local intracrine conversion of the DHEA pro-hormone to its functionally active metabolites in salivary glands.

We hypothesized that in addition to an overall low salivary androgen level, local intracrine disruption of the conversion of the pro-hormone DHEA to its active terminal testosterone and DHT metabolites may contribute to the local androgen deficiency in the salivary glands of patients with SS.

## MATERIALS AND METHODS

**Patients and samples.** Blood was collected from 43 patients with primary SS (all women, aged 33–78 yrs, mean 52.9) and 15 age- and sex-matched healthy controls (aged 32–73 yrs, mean 54.9). Patients fulfilled the revised classification criteria for SS described by Vitali, *et al*<sup>1</sup>. Patients were positive for at least 4 of the 6 classification criteria, 1 of them showing the autoimmune character of the disease (either histopathological finding of focal sialadenitis in salivary glands and/or the presence of antibodies to Ro/SSA or La/SSB antigens in serum). Patients with SS were excluded if they were taking glucocorticoids, immunosuppressive therapy, or disease modifying antirheumatic drugs.

Patients had mild or more severe sicca symptoms in the form of xerostomia (98%), keratoconjunctivitis sicca (98%), rhinitis sicca (88%), laryngitis sicca (93%), dry pharynx (79%), dry skin (70%), and vaginitis sicca (79%). These high percentages are probably due in part to the Finnish climate and way of life, as many patients spend most of the wintertime inside in dry air in heated houses without air humidification. The so-called Oxholm wheal was used as the reference for the classification of extraglandular disease manifestations<sup>11</sup>. Different systemic and visceral manifestations may represent part of the syndrome or just coincidental comorbidity. Thirty percent ( $n = 13$ ) of the patients had some type of lung disease, usually bronchial hyperreactivity or asthma ( $n = 9$ ), but 2 had chronic obstructive lung disease, 1 had fibrosing alveolitis, and 1 had tracheobronchial malacia. One patient had had a mild acute pancreatitis and 1 had chronic pancreatitis. Fifty-eight percent ( $n = 25$ ) of patients had some other gastrointestinal manifestations, usually gastroesophageal reflux disease and/or hiatal hernia ( $n = 12$ ), but also gastritis and/or ulcer ( $n = 8$ ) and celiac disease ( $n = 4$ ). Other manifestations included irritable bowel syndrome, duodenitis, and lactose intolerance; the sum for these is higher than 25 as some patients had more than 1 past or present gastrointestinal manifestation.

Urinary tract changes were seen in 30% ( $n = 13$ ), comprising 7 patients with recurrent lower (cystitis) and/or upper (pyelonephritis) urinary tract infections, 2 with interstitial cystitis, and 2 with type I renal tubular acidosis. In addition, 1 patient was a carrier of Alport's syndrome and had microscopic hematuria, and 1 patient had had an epidemic nephropathy. Seven patients had or had had some hepatobiliary changes: 3 gallstones, 2 fatty liver and elevated transaminases, 1 Gilbert syndrome, and 1 hemangiomas of the liver. One patient had had MALT lymphoma successfully treated with operation and radiation and 2 patients had lymphadenopathy. Twenty-eight percent ( $n = 12$ ) had thyroid manifestations, most commonly either medicated hypothyroidism ( $n = 5$ ) or thyroid cysts and/or struma ( $n = 5$ ). Two patients had an autoimmune thyroiditis confirmed by thyroid autoantibodies and a thin-needle biopsy, but they had not developed hypothyroidism according to the sensitive serum thyroid stimulating hormone test,

and were not receiving thyroxine substitution. Three patients had had fever as a sign of SS (no other reason had been diagnosed) and almost all, 95% (n = 41), in our study suffered from fatigue.

Mild hematological manifestations (past or present, manifest after SS had been diagnosed, 53%, n = 23) were seen as follows: anemia (n = 14), leukocytopenia (n = 9), and thrombocytopenia (n = 1). The sum of the manifestations is 24 because 1 of these patients had had anemia and had slight and symptomless leukocytopenia. Thirteen patients had Raynaud's phenomenon and 3 had had serositis, 2 pleuritis, and 1 pericarditis. Thirty-five percent (n = 15) had some neurological manifestations, usually a peripheral mixed sensory-motor neuropathy (n = 11), but 2 had had a lymphocytic meningitis, 2 had restless legs, and there was 1 case of each of the following: trigeminal neuralgia, epilepsy, migraine, cluster headache, and a past stroke (from which the patient had recovered); 5 patients had 2 neurological manifestations. As the patients were seen by a rheumatologist, almost all had myalgia, arthralgia, and occasionally slight synovitis (91%, n = 39). Finally, 35% (n = 15) of the patients had had some SS-related skin changes, usually vasculitic urticaria (n = 5) or purpura (n = 4), other skin changes being sun rash (n = 2), vitiligo (n = 2), dermatitis herpetiformis (n = 1), and acne in the back (n = 1).

Immediately after collection, blood samples were allowed to stand for 45 min, and centrifuged for 10 min (1500 × g) before the serum was collected and stored at -20°C. Resting saliva was collected from 43 patients with SS (all women, aged 40–58 yrs) and 15 age- and sex-matched controls (aged 40–55 yrs) as described<sup>12</sup>. Unstimulated whole saliva was collected for 15 min and stimulated saliva for 5 min when chewing paraffin capsules. Saliva samples were centrifuged (10 min, 100 ×g), a proteinase inhibitor (Complete, Roche, Basel, Switzerland) was added, and the samples were stored at -20°C.

Labial salivary gland tissue samples were received from 2 controls who were treated for mucocele (1 man, aged 17 yrs and 1 woman, age 17 yrs).

The controls did not have any signs or symptoms of SS or xerostomia. Our study was approved by the ethical committee of the Hospital District of Helsinki and Uusimaa.

**Measurement of serum sex steroids.** The levels of DHEA-S (the sulfated transport form of DHEA) in serum samples were measured with a radioimmunoassay (Thermo, Waltham, MA, USA). Free testosterone levels, DHT levels, and 17-β-estradiol levels were measured with ELISA kits (IBL, Hamburg, Germany).

**Measurement of salivary sex steroids.** Salivary DHEA, testosterone, and 17-β-estradiol were measured with salivary enzyme immunoassays from Salimetrics (State College, PA, USA). Salivary DHT was measured with DHT ELISA kits (IBL). Before measurement, the frozen samples were melted on ice and centrifuged (15 min, 200 ×g). Only the clear supernatant was used for the analysis.

**Cell and tissue culture and stimulation.** HSG cell line is a line of human submandibular gland cells, which in their naive state express ductal epithelial phenotype and differentiate to acinar cells upon culture on a basement membrane-like laminin-α1 chain containing Matrigel (BD Biosciences, San Jose, CA, USA). The cell line serves as a useful model of salivary gland epithelial progenitor cell to acinar cell transdifferentiation<sup>13</sup>. HSG cells were cultured on Matrigel as described<sup>9</sup>. The stimulations were performed in serum-free media (not containing, e.g., sex steroid binding globulins). Cells were stimulated with 100 μM DHEA (Fluka, St. Louis, MO, USA) or 100 nM DHT (Fluka) (diluted in absolute ethanol) for 72 h. Finasteride (type II selective 5-α-reductase inhibitor) or dutasteride (inhibits both type I and type II 5-α-reductase; type I 5-α-reductase 100 times more effectively than finasteride) was added at the same time as the hormones and incubated for the same 72 h.

Labial salivary gland explants were cultured as described<sup>9</sup>.

**RNA isolation and cDNA synthesis.** Total RNA was isolated from HSG cells with the Trizol protocol (Invitrogen, Carlsbad, CA, USA) followed by mRNA extraction with the Dynabeads mRNA Purification Kit (Dyna, Oslo, Norway). Tissue RNA was isolated with the High Pure RNA Tissue

kit (Roche, Basel, Switzerland). Complementary DNA (cDNA) synthesis was done with SuperScript First-strand cDNA Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions.

**Quantitative polymerase chain reaction (PCR).** Quantitative PCR was performed using 0.2 μg first-strand cDNA, 0.5 mM primers in LightCycler™ PCR mix and a LightCycler PCR instrument (Roche).

For the housekeeping gene β-actin, the primers used were 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' and 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3' and for porphobilinogen deaminase (PBGD) 5'-ACA TGC CCT GGA GAA GAA TG-3' and 5'-AGA TGC GGG AAC TTT CTC TG-3'. The primers used for CRISP-3 were 5'-TTC ACC CTT CTT CCC AAT CA-3' and 5'-CCT CCT TGA GTG GAG GGT ATT-3', for 5-α-reductase type I 5'-ATG TTC CTC GTC CAC TAC GG-3' and 5'-AGC CAC ACC ACT CCA TGA TT-3' and for 5-α-reductase type II 5'-ACG GTA CTT CTG GGC CTC TT-3' and 5'-ACA AGC CAC CTT GTG GAA TC-3'. The copy numbers of CRISP-3 and 5-α-reductase type I and II genes in each sample were normalized against β-actin or PBGD copy numbers (in labial salivary glands and in HSG cells, respectively).

**Western blotting.** Cell culture media was collected and concentrated using 10 kDa Centricon centrifugal filter device tubes (Millipore, Billerica, MA, USA). Samples were boiled for 5 min in sodium dodecyl sulfate-gel loading buffer and equal amounts of total protein (8.5–30 μg) were loaded onto each lane. After electrophoresis the samples were blotted into Immobilon-P transfer membranes (Millipore), which were blocked overnight. Membranes were incubated in 3 μg/ml rabbit anti-human CRISP-3 antibody<sup>14</sup> followed by alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch, Suffolk, UK). The detection was accomplished using Western Lightning CDP-Star Chemiluminescence Reagent (Perkin Elmer, Waltham, MA, USA), followed by imaging of the membranes with ProXpress 2D Imaging System (Perkin Elmer). The quantification of the results was completed with Phoretix software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

**Statistical analysis.** Data were analyzed with SPSS, version 13.0 (SPSS, Chicago, IL, USA). Baseline demographics and disease characteristics were assessed using descriptive statistics. Differences in sex steroid levels between patients with SS and controls were tested for significance with the t-test or Mann-Whitney U-test (for normally and non-normally distributed data, respectively). All results are given as mean ± standard error of the mean and median. Correlations were analyzed using Spearman's test. The significance level was set at p < 0.05. Two-tailed levels of significance were used throughout.

## RESULTS

**Concentrations of sex steroids in serum.** Concentrations of systemic DHEA-S and DHT were decreased in patients with SS compared to controls (mean 2.3 μM ± 0.2, median 2.1 vs 3.4 μM ± 0.3, median 3.1, p = 0.005, and 779.1 ± 72.8 pM, median 692.6 vs 1155.8 ± 141.8 pM, median 1030.6, p = 0.003). Although the systemic concentrations of testosterone were decreased in patients with SS, they did not differ significantly (2.79 ± 0.41 pM, median 2.1 vs 4.3 ± 1.3 pM, median 2.9, p = 0.238). In contrast to decreased concentrations of androgens, the serum concentrations of 17-β-estradiol were increased in SS (325.6 ± 57.3 pM, median 254.7 vs 123 ± 41.9 pM, median 75.7, p = 0.008).

**Concentrations of sex steroids in saliva.** All measured androgens, DHEA, testosterone, and DHT, were relatively low in patients with SS (n = 43) compared to controls (n = 15). Concentrations of DHEA differed significantly [244.3 ± 40.8 pM, median 178.0 vs 340.0 ± 38.5 pM, median 337.8



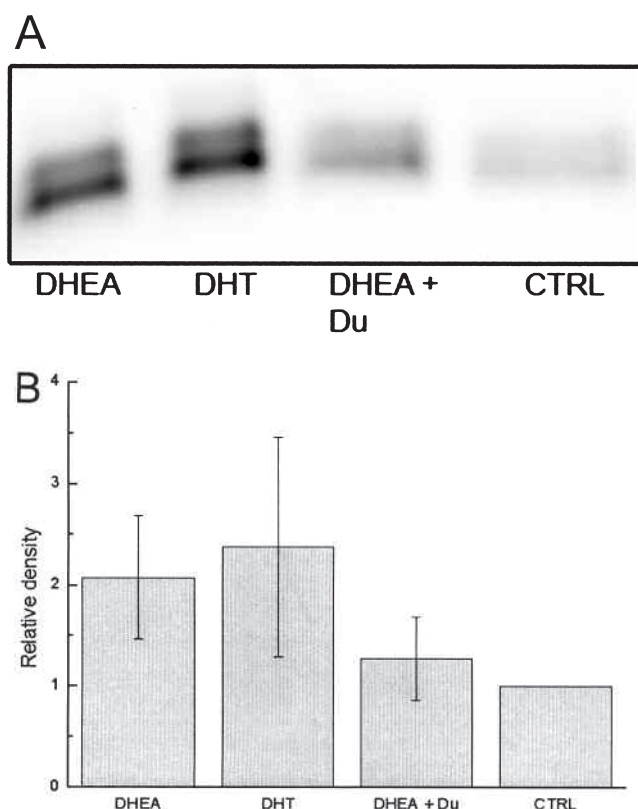
( $p = 0.021$ )). Concentrations of testosterone were  $126.3 \pm 11.4$  pM, median 107.6 versus  $151.2 \pm 13.1$  pM, median 169.5 ( $p = 0.088$ ) and DHT  $113.9 \pm 28.0$  pM, median 74.4 versus  $221.2 \pm 73.8$  pM, median 113.1 ( $p = 0.160$ ), respectively. The concentrations of 17- $\beta$ -estradiol in saliva in patients with SS versus controls were  $22.6 \pm 7.5$  pM, median 24.1 versus  $21.0 \pm 1.7$  pM, median 11.5, respectively ( $p = 0.033$ ). The amount of sex steroids corrected for salivary protein (pmol/mg total protein) showed the same trend between patients with SS and healthy controls: DHEA  $329.9 \pm 53.5$ , median 235.6 versus  $553.6 \pm 81.1$ , median 561.5 ( $p = 0.01$ ); testosterone  $185.4 \pm 27.8$ , median 150.2 versus  $245 \pm 39.6$ , median 204.6 ( $p = 0.056$ ); DHT  $155.6 \pm 41.4$ , median 85.3 versus  $207.1 \pm 51.9$ , median 151.6 ( $p = 0.323$ ); and 17- $\beta$ -estradiol  $25.2 \pm 6.7$ , median 15.4 versus  $33.9 \pm 4.8$ , median 29.2 ( $p = 0.007$ ). The same trend was also evident when the values were corrected for salivary volume (pmol hormone secreted in 5 min): DHEA  $0.98 \pm 0.23$ , median 0.57 versus  $2.83 \pm 0.83$ , median 2.35 ( $p = 0.015$ ); testosterone  $0.56 \pm 0.10$ , median 0.40 versus  $1.14 \pm 0.18$ , median 0.99 ( $p = 0.002$ ); DHT  $0.63 \pm 0.20$ , median 0.34 versus  $1.34 \pm 0.37$ , median 0.97 ( $p = 0.091$ ); and 17- $\beta$ -estradiol  $0.07 \pm 0.01$ , median 0.05 versus  $0.16 \pm 0.02$ , median 0.17 ( $p = 0.001$ ).

**Correlation between androgen levels and Multiple Fatigue Inventory (MFI)-20 scores.** Clinical and androgen levels of patients with SS showed no correlation between MFI-20 scores and serum and salivary androgen levels. P values were 0.526, 0.217, and 0.115 between serum DHEA-S, testosterone, and DHT and MFI-20 scores, respectively; and 0.770, 0.853, and 0.363 between salivary DHEA, testosterone, and DHT and MFI-20 scores, respectively.

**Expression of 5- $\alpha$ -reductase in salivary glands.** 5- $\alpha$ -reductase type I mRNA was expressed in labial salivary gland explants and in HSG cells (approximately 200 copies/10,000,000  $\beta$ -actin copies and 100,000 copies/100,000 porphobilinogen deaminase (PBGD) copies, respectively), whereas the expression of 5- $\alpha$ -reductase type II was very low (approximately 1 copy/10,000,000  $\beta$ -actin copies and 100 copies/100,000 PBGD copies, respectively).

**Effect of androgen stimulation with and without 5- $\alpha$ -reductase inhibitors on acinar cell CRISP-3.** DHEA and DHT stimulations increased the expression of androgen-regulated CRISP-3 in HSG cells cultured on Matrigel on both the mRNA and the protein level. The type I and II 5- $\alpha$ -reductase inhibitor dutasteride prevented the DHEA- but not the DHT-driven upregulation of CRISP-3 (Figure 1). The selective type II 5- $\alpha$ -reductase inhibitor finasteride did not have any effect in this respect (data not shown).

**Local processing of DHEA into more active sex steroids in salivary glands.** In healthy controls the correlation between DHEA and testosterone in saliva was low ( $r = 0.001$ ) and not significant ( $p = 0.911$ ), but the correlation between testos-

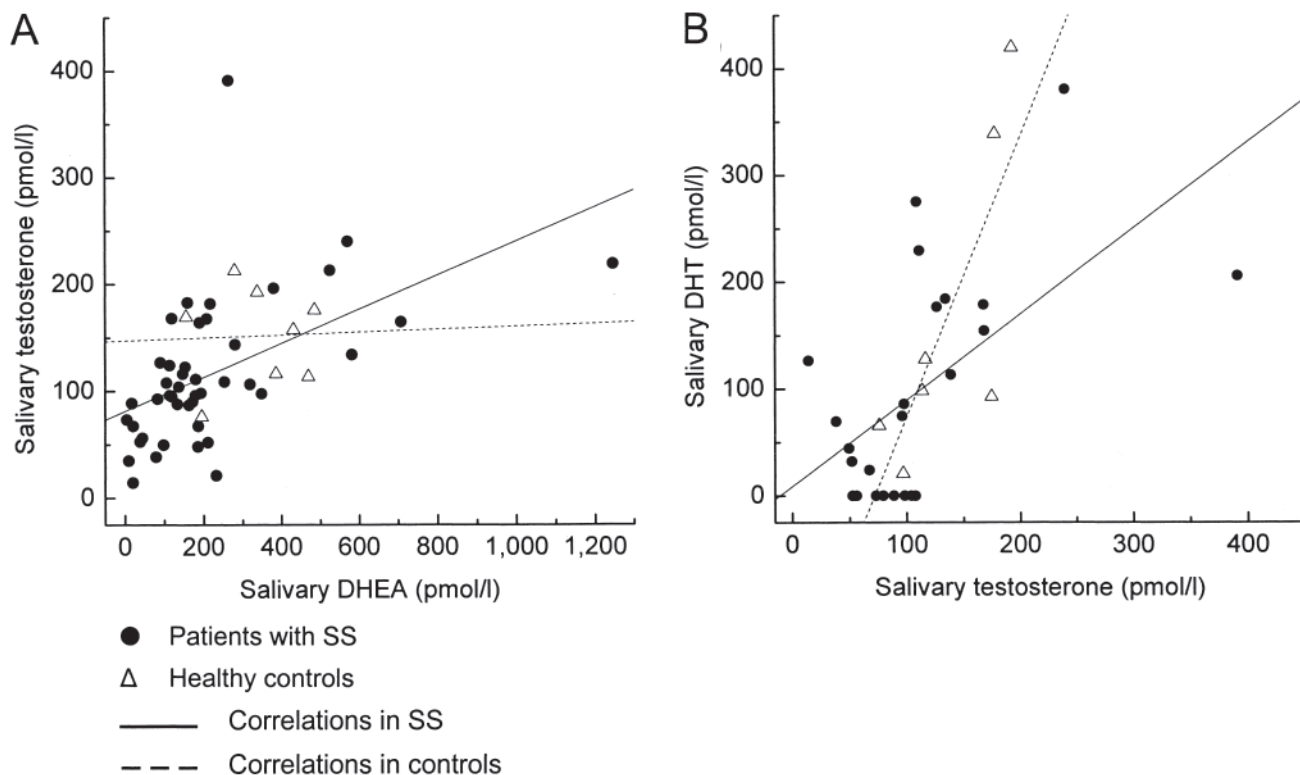


**Figure 1.** Effect of androgen stimulations on CRISP-3 protein expression of HSG cells cultured on Matrigel. DHEA and DHT stimulations increased the expression of CRISP-3 in Western blotting. Addition of the 5- $\alpha$ -reductase inhibitor dutasteride inhibited upregulation caused by DHEA, but had no effect on upregulation caused by DHT (data not shown). A. Western blot of stimulated HSG cell media. B. Quantification of Western blot: results given as relative density of the bands ( $n = 6$ ).

terone and DHT was high ( $r = 0.627$ ) and significant ( $p = 0.014$ ). In contrast, there was a significant correlation in SS between DHEA and testosterone ( $r = 0.26$ ,  $p < 0.001$ ), but the correlation between testosterone and DHT was lower than in controls ( $r = 0.35$ ,  $p = 0.002$ ; Figure 2). The correlations between the salivary concentration of DHEA and 17- $\beta$ -estradiol were low and did not differ significantly in patients with SS and in controls ( $r = 0.009$ ,  $p = 0.446$ , and  $r = 0.07$ ,  $p = 0.736$ , respectively, data not shown).

## DISCUSSION

SS is characterized by diminished lacrimal and salivary flow rates. As the acinar cell is responsible for the production of primary saliva, whereas the ductal epithelial cells only regulate its composition, it would seem that this diminished exocrine secretory function is due to some type of failure of the secretory acinar cells. We have recently described that the human submandibular gland HSG cells, which have the phenotype of intercalated duct epithelial cells, increase their CRISP-3 expression upon differentiation to terminally differentiated secretory acinar cells<sup>9</sup>. Such differentiation can



**Figure 2.** Correlations between salivary DHEA and testosterone (A) and testosterone and dihydrotestosterone (DHT; B) in patients with SS and controls. A. In SS patients the correlation between salivary DHEA and testosterone was highly significant ( $r = 0.267$ ,  $p < 0.001$ ,  $n = 42$ ); in controls no such correlation could be seen ( $r = 0.001$ ,  $p = 0.911$ ,  $n = 8$ ). B. In SS patients the correlation between testosterone and DHT was relatively low ( $r = 0.347$ ,  $p = 0.002$ ;  $n = 24$ ); in controls it was much higher ( $r = 0.627$ ,  $p = 0.014$ ,  $n = 7$ ).

be induced by the culture of HSG cells on a laminin- $\alpha$ 1 chain containing Matrigel substrate<sup>13</sup>, but CRISP-3 levels are much more enhanced if DHEA is also added to the culture medium. This suggests that DHEA, or some intracrine metabolic product of DHEA, supports this progenitor to acinar cell differentiation. Conversely, lack of such androgen drive could impair the progenitor-acinar cell transdifferentiation, which is necessary for the normal exocrine gland remodeling (maintenance of the acini). Based on earlier findings of decreased DHEA concentrations in serum and saliva of patients with SS<sup>9</sup> and the fact that SS is characterized by acinar cell atrophy and impaired secretory function, accompanied by a reciprocal ductal epithelial cell hyperplasia, our hypothesis was that in SS there is both a systemic and a local deficiency of androgens leading to diminished well-being of acinar cells in SS salivary glands. That androgens have been shown to contribute to the normal structure and function of submandibular salivary glands in BALB/c mice<sup>15</sup> further supports our theory of the effect of androgens on salivary glands in SS. Also, the predominance of women in SS and the reported low levels of salivary androgen-dependent marker (CRISP-3) encouraged us to test our hypothesis.

We decided to evaluate the androgen profile in the systemic (serum) and local (saliva) tissue compartments in

healthy controls and in patients with SS. We confirmed reports that serum DHEA-S levels are low in SS<sup>8,9</sup>, and extended these findings by demonstrating that serum DHT levels are also low in SS. The diminished systemic concentrations of the pro-hormone DHEA in SS, along with its functionally most active androgenic end-product DHT, indicate that exocrine glandular tissues, including acinar cells, are subjected to subnormal androgen levels in this syndrome. In contrast, serum 17- $\beta$ -estradiol was increased in SS, which can be a result of the altered balance of steroidogenic enzymes converting DHEA further into either estrogens or androgens. As a result, the serum androgen-estrogen ratio is low in patients with SS compared to healthy controls, which could contribute to an increased tendency to contract autoimmune diseases<sup>7</sup>.

DHEA is a systemic pro-hormone found in humans and other higher mammalian species and tailored in a tissue-specific way to either estrogens or androgen<sup>10</sup>. In the female breast and uterine tissue, it is predominantly converted to 17- $\beta$ -estradiol. In this intracellular or "intracrine" conversion, aromatase plays an important role. This has practical implications in aromatase inhibition, which is used in the treatment of estrogen-sensitive/driven breast cancer. In the male prostate, DHEA pro-hormone is converted to DHT<sup>16</sup> and plays a role in benign prostatic hyperplasia, which can

be treated by 5- $\alpha$ -reductase inhibition<sup>17</sup>. Also in SS it would seem to be more important to check the local tissue concentrations, conversions, and effects of DHEA rather than to assess their somewhat irrelevant systemic levels. Thus, in addition to studying systemic sex steroid concentrations in SS, we wanted to see if the local concentrations in the target tissue were also altered.

Previously, it was not known what the local target site (salivary gland) DHEA, testosterone, and DHT levels were, and if and how the salivary glands “tailor” the DHEA pro-hormone available to them from the systemic circulation<sup>18</sup>. We found that overall, the DHEA, testosterone, and DHT androgen levels were relatively low in saliva in SS. As there is no active transport system of sex steroids to saliva<sup>19</sup>, their concentrations in saliva reflect both systemic concentrations (passive transfer) and local intracrine processes (local metabolism). There were no correlations between MFI-20 scores and serum and salivary androgen levels. These sex steroid values vary notably between individuals, perhaps explaining why such correlations were not seen. It might be better to check correlations between various clinical measures and the actual effects of androgen using, e.g., serum CRISP-3 as a biomarker for the effect of androgen.

DHEA, testosterone, and DHT are not independent of each other. They form components in a metabolic intracrine chain in which DHEA is first converted to androstenedione, but then further to testosterone and DHT by 2 different enzymes. According to our hypothesis, this intracrine chain is broken in SS salivary glands, further decreasing the already low androgen concentrations in SS salivary glands. We first studied if there is local production of sex steroids from DHEA in salivary glands. We used acinar HSG cells cultured on Matrigel, which we had previously shown to upregulate their CRISP-3 production in response of DHEA stimulation. We showed that DHEA and DHT increase CRISP-3 expression in acinar cells. The upregulation caused by DHEA, but not that by DHT, can be prevented by inhibiting the synthesis of DHT with a 5- $\alpha$ -reductase inhibitor.

Next, we studied the possible defects of DHEA processing in SS. The efficacy of the conversion of DHEA into more active sex steroids can be assessed using the substrate to end-product ratio. If this ratio is low, the conversion is effective. In SS, the DHEA/testosterone ratio was 1.93 compared to 2.42 in healthy controls, indicating that intracrine conversion of DHEA to testosterone is relatively effective. This is also reflected in their correlation, which was low ( $r = 0.267$ ) but highly significant ( $p < 0.001$ ) in SS, whereas such a dependency did not exist between DHEA and testosterone in healthy controls ( $r = 0.001$ ,  $p = 0.911$ ). This suggests that in SS, but not in healthy controls, increase in salivary DHEA could lead to increased testosterone concentration. Further conversion of testosterone to DHT is catalyzed by 5- $\alpha$ -reductase, which according to mRNA expression levels is of type I in salivary glands. The apparent testos-

terone to DHT conversion seems to be ineffective in patients with SS compared to healthy controls, as the respective testosterone/DHT ratios were 1.18 and 0.53. In patients with SS the correlation between testosterone and DHT was low ( $r = 0.347$ ) but significant ( $p = 0.002$ ), whereas in controls this correlation was higher ( $r = 0.627$ ,  $p = 0.014$ ). Consequently, the profile of salivary sex steroids in controls clearly differed from that observed in SS. Men might be protected against this testosterone to DHT conversion failure due to systemic input of testosterone, which raises the substrate concentration.

Ours is the first study in which such a clear and consequent diminution of androgen levels is reported in a disease target tissue in a female-dominant autoimmune disease. This explains our recently published observation that the androgen-dependent biomarker CRISP-3 is, at both the mRNA and protein level, very low in saliva and salivary glands in patients with SS compared to healthy controls<sup>9</sup>. Our findings about the low expression of CRISP-3 in SS are opposite to observations by Tapinos, *et al*<sup>20</sup>, who reported that CRISP-3 would be expressed only in salivary glands of patients with SS but not in healthy controls. However, closer examination of the CRISP-3 primers used by Tapinos, *et al* revealed that they do not recognize human CRISP-3<sup>9</sup> but other, as yet unknown molecular species.

Based on our results, DHEA replacement therapy could be considered as a possible therapeutic application in the prevention and repair of the acinar atrophy in salivary glands in SS. However, taking into account the tissue and cell-specific intracrine processing of DHEA by different steroidogenic enzymes and the possible defects of them in SS, DHEA may not work in salivary glands in SS. On the other hand, in DHT replacement therapy the adverse effects of the hormone might override the benefits of the treatment.

One possibility for a therapeutic application might be the combination of DHEA and enzyme inductors, which could induce the steroidogenic DHEA processing enzymes in salivary glands in SS.

Our study shows a prominent and consistent androgen deficiency in salivary glands in SS. It was shown that in salivary glands the pro-hormone DHEA is subjected to active intracrine metabolism, and that this processing is different in healthy controls and in patients with SS. These defects in the local production of active androgens in SS could further worsen the androgen deficiency in the target tissue of the disease. Relatively low salivary DHT concentrations and the consequently low androgen biomarker (i.e., CRISP-3) concentrations<sup>9</sup> in SS seen in the target tissue of SS thus probably result from a multifactorial desulfation and intracrine conversion defects in this female-dominant autoimmune exocrinopathy. This observation has escaped attention in earlier studies as DHEA is produced (in adrenal glands) and locally tailored (in salivary glands) only in humans, but not in the widely used rodent SS models.

## REFERENCES

1. Vitali CS, Bombardieri R, Jonsson R, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002;61:554-8.
2. Thomas E, Hay EM, Hajeer A, Silman AJ. Sjögren's syndrome: a community-based study of prevalence and impact. *Br J Rheumatol* 1998;37:1069-76.
3. Birlik M, Akar S, Gurler O, et al. Prevalence of primary Sjögren's syndrome in Turkey: a population-based epidemiological study. *Int J Clin Pract* 2008; Apr 16 [Epub ahead of print].
4. Novljan MP, Rozman B, Jerse M, et al. Comparison of the different classification criteria sets for primary Sjögren's syndrome. *Scand J Rheumatol* 2006;35:463-7.
5. Talal N. Overview of Sjögren's syndrome. *J Dent Res* 1987;66:672-4.
6. McMurray RW, May W. Sex hormones and systemic lupus erythematosus. *Arthritis Rheum* 2003;48:2100-10.
7. Beeson PB. Age and sex associations of 40 autoimmune diseases. *Am J Med* 1994;96:457-62.
8. Valtysdottir ST, Wide L, Hällgren R. Low serum dehydroepiandrosterone sulfate in women with primary Sjögren's syndrome as an isolated sign of impaired HPA axis function. *J Rheumatol* 2001;28:1259-65.
9. Laine M, Porola P, Udby L, et al. Low salivary DHEA and androgen-regulated CRISP-3 in Sjögren's syndrome. *Arthritis Rheum* 2007;56:2575-84.
10. Labrie F, Bélanger A, Simard J, Van Luu-The, Labrie C. DHEA and peripheral androgen and estrogen formation: intracrinology. *Ann NY Acad Sci* 1995;774:16-28.
11. Oxholm P, Asmussen K. Primary Sjögren's syndrome: the challenge for classification of disease manifestations. *J Intern Med* 1996;239:467-74.
12. Konttinen YT, Platts LA, Tuominen S, et al. Role of nitric oxide in Sjögren's syndrome. *Arthritis Rheum* 1997;40:875-83.
13. Royce LS, Kibbey MC, Mertz P, Kleinman HK, Baum BJ. Human neoplastic submandibular intercalated duct cells express an acinar phenotype when cultured on a basement membrane matrix. *Differentiation* 1993;52:247-55.
14. Udby L, Cowland JB, Johnsen AH, Sorensen OE, Borregaard N, Kjeldsen L. An ELISA for SGP28/CRISP-3, a cysteine-rich secretory protein in human neutrophils, plasma, and exocrine secretions. *J Immunol Methods* 2002;263:43-55.
15. Treister NS, Richards SM, Suzuki T, Jensen RV, Sullivan DA. Influence of androgens on gene expression in the BALB/c mouse submandibular gland. *J Dent Res* 2005;84:1187-92.
16. Suzuki K, Nishiyama T, Hara N, Yamana K, Takahashi K, Labrie F. Importance of the intracrine metabolism of adrenal androgens in androgen-dependent prostate cancer. *Prostate Cancer Prostatic Dis* 2007;10:301-6.
17. Marks LS. 5 $\alpha$ -reductase: history and clinical importance. *Rev Urol* 2004;6 Suppl:S11-21.
18. Pinkstaff CA. Salivary gland sexual dimorphism: a brief review. *Eur J Morphol* 1998;36 Suppl:31-4.
19. Lewis JG. Steroid analysis in saliva: An overview. *Clin Biochem Rev* 2006;27:139-46.
20. Tapinos NI, Polihronis M, Tryphronitis G, Moutsopoulos HM. Characterization of the cysteine-rich secretory protein 3 gene as an early-transcribed gene with a putative role in the pathophysiology of Sjögren's syndrome. *Arthritis Rheum* 2002;46:215-22.