

Immunohistochemical Evaluation of Sex Steroid Hormones in Sicca Syndrome Patients

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ABSTRACT

JAMES ROKOS: Immunohistochemical Evaluation of Sex Steroid Hormones in Sicca Syndrome Patients

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Sjögren syndrome (SS) is a chronic autoimmune disease affecting mainly climacteric females. The coincidence of sex hormones decline and higher prevalence of SS in this population implies that such hormones participate in its pathogenesis. Their exact role in the pathogenesis of SS is still controversial. This study investigates the presence and location of sex hormone receptors within minor labial salivary glands (MLSG) in a cohort of sicca syndrome patients being evaluated for SS. An immunohistochemical comparison of the ratio of estrogen receptors (ER) to androgen receptors (AR) expression was correlated to the patients' focus score (FS). A greater intensity of androgen receptor expression in SS patients, especially in the nucleus of acinar cells was found when compared to the other receptors ($p < 0.001$). However, no statistically significant difference was found in the ratio of ER to AR as a function of FS in our group of patients.

DEDICATION

To my sons, Connor and Nicholas, I hope that the pursuit of my dream to be an Oral Pathologist will inspire you to always follow your dreams; and be assured that no matter how old you get, any dream worth dreaming can be realized if you persevere and trust in yourself.

To Dr. John Jacoway: a teacher; a friend; a Renaissance man who first ignited my intellectual passion for Pathology; and a man to whom I will always try to emulate. I can merely hope to achieve a fraction of your expertise and knowledge.

To my wife, Laura, for her encouragement, companionship, and emotional support throughout this journey. Thank you.

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“No man is an island...” (John Donne, “Meditation: XVII”, 1623), and I am no exception.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

5-diol- 5-androstene-3 β ,17 β -diol

3 α -diol-G-Androstane-3 α ,17 β -diol-G

17 β -estradiol- Estradiol

ADT-G- Androsterone glucuronide

AIB1- Amplified in breast cancer-1

ANA- Anti-nuclear antibodies

ANDRO- Androstenedione

AR- Androgen receptor

ARE- Androgen response element

Bx- Biopsy

bFGF- Basic FGF

CMC- Carolina's Medical Center

CG- Control group

CRISP 3- cysteine-rich secretory protein 3

DES- Diethylstilbestrol

DHEA- Dehydroepiandrosterone

DHEA-S- Dehydroepiandrosterone sulfate

DHT- 5-dihydrotestosterone

DIM-3,3-diindolymethane

DLBC-Diffuse large B cell lymphoma

DNA- De-oxy ribonucleic acid

Dx- Diagnosis, diagnostic

EGF-epidermal growth factor

EGFR- EGF receptor

ER- Estrogen receptor

ERE- Estrogen response element

ESE2-Epithelium-specific ETS factor 2

ESTR- Estrone

ETS-E-twenty-six

FFPE- Formalin-fixed paraffin embedded

FGF- Fibroblast growth factor

G6P-AT- Glucose 6 phosphatase

GIST- Gastrointestinal stromal tumor

HRT- Hormone replacement therapy

HF- Hydroxyflutamide

IGF- Insulin growth factor

IGFBP- IGF binding protein

IGFIR-IGF-I receptor

IQR- Inter-quartile range

KCS- Keratoconjunctiva sicca

LBA- Ligand binding assay

LBD- Ligand binding domain

LG-Lacrimal gland

M- Mean

MALT- Mucosal associated lymphoid tissue

Mdn- Median

MG-Meibomian Gland

MLSG- Minor labial salivary gland

MOD- Mean optical density

mRNA- Messenger ribonucleic acid

NHL-Non-Hodgkin lymphoma

NMT55- Nuclear matrix protein, 55 kDa

PCR-Polymerase chain reaction

PDEF-Prostate-derived ETS factor

PI3K-Phosphatidylinositol 3-kinase

PKA- Protein kinase A

PR- Progesterone receptor

PSA-Prostate-specific antigen

PTEN- Phosphatase and tensin homolog

PYK2- Proline-rich tyrosine kinase 2

RA- Rheumatoid arthritis

Rb-Retinoblastoma susceptibility gene

RF- Rheumatoid factor

rt-PCR- Real time polymerase chain reaction

RT-PCR-Reverse transcriptase polymerase chain reaction

RNA- Ribonucleic acid

SD- standard Deviation

SG-Salivary Gland

SH2- SRC homology 2

Sicca patients- Patients with complaints of dry eyes and dry mouth but have not met the other diagnostic criteria for Sjögren syndrome.

SIR-Standardized Incidence Ratio

SLE-Systemic lupus erythematosis

SMG-Submandibular gland

SMRT-Silencing mediator of retinoid and thyroid hormone receptor

SRC-Steroid receptor co-activator

SS: Sjögren's syndrome

SS+: FS \geq 1

SS-: FS $<$ 1

STAT-Signal transducer and activator of transcription

TFM-Testicular feminized

TIF-Transcriptional intermediary factor

TPL- Translational Pathology Laboratory

UNC-CH- University of North Carolina at Chapel Hill

INTRODUCTION

Sjögren Syndrome (SS) is an autoimmune disease with debilitating oral sequelae. It is the second most common rheumatic disease in the US, affecting between 2 and 4 million people, and it typically afflicts climacteric females nine times more often than men. (1); 292 Fox, R.I. 2005}} It has been hypothesized that the coincidence of the hormonal decline and the rise in prevalence in Sjögren Syndrome implies that sex hormones play a role in the pathogenesis of the disease. (2,3)(4) Research has shown that both estrogens and androgens are involved in the development, differentiation, and normal functioning of the salivary glands. In addition, withdrawal of these sex hormones, in both human and animal models leads to glandular dysfunction. (5,6) In other autoimmune processes, androgenic influence curtails the severity of the inflammatory response, and estrogenic participation enhances it. (7) Yet, in SS, the typical age of onset is during a time when both androgens and estrogens are declining.

Despite extensive research to explain the role of sex hormones in the pathogenesis of SS, most of the answers still elude explanation. Conflicting theories abound as to whether the disease is caused by an androgenic deficiency, an estrogenic deficiency, or a combination of the two. (8-14) Contributing to this lack of knowledge

is the fact that very little is known about the normal expression and distribution of receptors for the active hormones in the affected glands.

It is the intention of this study, to contribute to the knowledge and understanding concerning the role of sex hormones in SS. Through immunohistochemical staining of sex hormone receptors, we will investigate if there is a difference between the expression of androgen (AR), estrogen receptors (both ER- α and ER- β), and progesterone receptors (PR) in the minor labial salivary gland (MLSG) tissue of a cohort of patients who have some, but not all the diagnostic criteria of primary SS. In addition, a sample of age-matched female patients, who have had lower labial biopsies for other reasons that incidentally include MLSG tissue will be used for observation and comparison of the normal receptor expression. The ratio of ER to AR will be compared between the groups in its relationship to focus scores (FS) (i.e. FS ≥ 1 vs. FS < 1 vs. control). We hypothesize that the ratio of ER to AR will increase as FS increases.

LITERATURE REVIEW

Sjögren's Syndrome is a common autoimmune disease with serious oral sequelae and an increased risk of malignancy

Sjögren's Syndrome (SS) is an autoimmune exocrinopathy first described by Mikulicz in 1892 and then further characterized by the Swedish ophthalmologist, Henrik Sjögren, in 1933. (15) At the time of its recognition by Mikulicz, the patient presented with bilateral enlargement of the parotid glands which, upon histologic examination, were focally infiltrated with lymphocytes. Sjögren went on to describe a cohort of women who had clinical symptoms of dry eyes, dry mouth, and probable rheumatoid arthritis in addition to similar histologic findings as Mikulicz's patient. Keratoconjunctivitis sicca was the term Sjögren introduced for this constellation of signs and symptoms. It was later proven by Morgan and Castleman that both Mikulicz's and Sjögren's patients had identical histologic features. In 1956, Bloch et al outlined the features common to what we now know as Sjögren's syndrome (SS).(16,17)

Sjögren syndrome is the second most common autoimmune rheumatic disease in the US, affecting 2-4 million people. (1,18) Primary SS is characterized by a lymphocytic infiltrate of the exocrine glands, most significantly the lacrimal, meibomian, and salivary leading to a characteristic keratoconjunctivitis and

xerostomia. When seen unaccompanied by other connective tissue diseases, such as rheumatoid arthritis, systemic lupus erythematosus, or scleroderma it is considered primary SS. When seen in combination with these other diseases it is considered secondary SS. The incidence of primary SS equals that of secondary SS. (18)

The defining qualities of the exocrine involvement of the disease have many quality of life ramifications. The keratoconjunctivitis sicca caused by profound aqueous tear deficiency and decreased lipid content, can lead to corneal abrasions and secondary bacterial and fungal infections. Of greatest concern is the development of sterile or microbial corneal ulcerations and subsequent blindness. (16)(19) Direct effects of xerostomia include: atrophy of the mucosa and associated discomfort; difficulties in swallowing, chewing, and speaking; diminished taste; and decrease nutrition. In addition, further complications due to xerostomia can include increased risk of dental caries, and increased frequency of bacterial and fungal infections. These, in turn, can cause secondary stomatodynia (18)

Despite the quality of life issues associated with the glandular sequelae of SS, extraglandular involvement is also significant. Generalized xerosis, affecting other mucosal surfaces besides the ocular and oral, are frequently reported: dryness of the nose; throat; skin; and vagina are common. Systemic manifestations of SS can involve peripheral neuropathies, myalgias, arthralgias, thyroid disorders like autoimmune thyroiditis, and pulmonary and renal pathosis. (18) (20)

One of the most serious complications of SS is the increased relative risk for malignant lymphoma seen in this patient population. Originally it was reported that primary SS patients had an increased relative risk of developing a lymphoma as high as 44.4 times normal, mainly of the MALT type (low grade).(21) This was based on 7 cases of lymphoma in 142 patients over a 29 year period of time. In a meta-analysis of autoimmune associated lymphoma risk, Zintzaras showed that non-Hodgkin lymphoma (NHL) is more common in patients with autoimmune diseases, and SLE and primary SS showed the greatest association. However the standardized incidence ratios (SIR) from the various studies occurred over quite a broad range (8.7-44.4).(22) More recently the upper limit of this range has come under scrutiny. Theander, et al re-evaluated the relative risk to be more realistically 16 times higher than the general population. Moreover, the type of lymphoma that is more likely to develop is a diffuse large B-cell type (DLBC) and a CD4+ T cell lymphocytopenia was found to be a strong risk factor for lymphoma development. (23)

Diagnosis of SS is based on a combination of signs and symptoms not unlike other rheumatic diseases. Since no single diagnostic test to indicate or exclude the diagnosis of SS exists, the current diagnostic criteria include a combination of 6 subjective and objective findings the patient must possess. These include: subjective ocular symptoms; subjective oral symptoms; objective ocular signs such as a positive Shirmer's test or Rose Bengal score; histopathological evidence of disease such as a minor labial salivary gland (MLSG) biopsy with a focus score ≥ 1 ; objective evidence of salivary gland involvement by an assessment of unstimulated

salivary flow, salivary scintigraphy, or parotid sialography; and laboratory abnormalities as evidenced by the presence of autoantibodies, SS-A (Ro) ,SS-B (La), or rheumatoid factor. (24) None of these criteria by themselves is specific to SS and several are common to several autoimmune diseases. This has led to numerous criticisms of the American-European classification system, and other diagnostic classification schemes being proposed. (25,26)(27) This lack of agreement makes the diagnosis of SS somewhat problematic and inconsistent. By extension, this complicates collaboration between care providers utilizing different diagnostic classifications, and thwarts efforts to develop treatment strategies, and study their effectiveness.

SS affects women more frequently than men

Although men and children can present with SS, the disease primarily affects middle-aged women nine times more often than men. (28)(29)(30) It is this female dominance and late age of onset that leads investigators to question the role of sex hormones in the pathoetiology of the disease.

Sjögren syndrome affects women 9 times more often than men.(31)(18) It is not alone in this gender differential. Other autoimmune disorders that preferentially affect women include rheumatoid arthritis (2-4:1), systemic lupus erythematosus (5-13:1), scleroderma (3:1), and Graves' disease (4-8:1).(32-34)(31) As gender appears to be a risk factor in all of these diseases, sex steroid hormones, especially estrogens, may be mediators of the process. Sex hormones influence both humoral

and cell mediated immune responses.(7)(35) Furthermore, estrogens have been shown to have a perpetuating effect on inflammatory conditions. (36,37)(38) (14) By contrast, testosterone has been shown to ameliorate autoimmune inflammation in systemic lupus erythematosus (SLE) models and in the target organs affected by SS.(39)(40)(41)(42)(43)

SS mainly affects perimenopausal women, why then?

Epidemiological data indicates that fertile age women are more affected by rheumatic autoimmune diseases than men, probably owing to a higher concentration of pro-inflammatory estrogens.(44) This would seem likely given the dramatic decrease in incidence in other autoimmune diseases like SLE after menopause.(45) So then why would an autoimmune disease, such as SS, have its genesis in the perimenopausal female?

During menopause, serum concentrations of sex-steroid hormone are declining in women, due to a regression of the gonadal production of testosterone and estrogen.(46) However, there are two sources for androgens and estrogens in the body. One is the well known gonadal source, but additionally there are sex steroid precursors, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S), produced by the adrenal cortex and converted peripherally in target tissues to either androgens or estrogens depending on the tissue need or local conditions. This peripheral production is not organ specific, nor even tissue specific,

but can be different for each individual cell. When introduced, this new sector of endocrinology, called intracrinology highlighted the subtle, cellular control peripheral tissues have over the formation of sex hormones.(47). In adult human males, 50 % of all androgens are derived from adrenal precursors. In women, 75% of estrogens are produced peripherally prior to menopause, and 100% after menopause. (47) Moreover, women produce 66% as much androgens as men from DHEA.(48) Therefore, serum measurement of either androgens, estrogens, or their adrenal precursors may not reflect a true profile of the patient's hormonal status.

A similar phenomenon to menopause, called adrenopause, occurs synchronously. It is characterized by a functional decline in the secretion of DHEA and DHEA-S from the adrenal cortex.(49)(48).

Is it this decline in both gonadal and adrenal hormones the cause for SS being seen most likely in climacteric females? Sullivan demonstrated that postmenopausal females are not just estrogen deficient; they are also androgen deficient as well.(2). His study showed a serum deficiency in DHEA, 5-androstene-3 β , 17 β -diol (5-diol), DHT, androsterone glucuronide (ADT-G), and androstane-3 α ,17 β -diol-G (3 α -diol-G) in SS patients compared with age-matched controls, but it did not find decreased serum concentrations of androstenedione, testosterone, estrone, or 17 β -estradiol. Like Sullivan, Valtysdottir, et al showed that SS patients had lower serum concentrations of the adrenally produced pro-hormones compared with controls. (50)

Taiym, et al showed significantly higher serum levels of prolactin, and higher ratios of prolactin/progesterone and estrogen/progesterone between SS patients and healthy age-, sex-, race-matched controls. No significant differences were detected in the levels of estrogen or progesterone alone.(51)

Sullivan's group reported that androgen deficiencies may play a critical role in autoimmune dry eye syndromes such as SS.(8) Furthermore, it was shown that estrogen and progesterone promoted the development of dry eye symptoms in women, especially those who were taking hormone replacement therapy or who were pregnant.(9,10,13) In addition, Nagler, et al presented 2 cases of previously healthy young women who developed SS following in vitro fertilization therapy.(12) Conversely, Ishimaru, et. al., using animal models, suggested that it is an estrogen deficiency that leads to the exocrinopathy typical of SS.(11,52) Hayashi, et al proposed that an estrogen deficiency promoted the production of auto antigens within the gland parenchyma.(53)

Yet serum may not be an accurate assessment of the sex hormone status of SS patients. More recently, Porola, et al has demonstrated not only low serum concentrations of DHEA and DHT in SS patients, but went to show that salivary concentrations of the pro-hormone and its end –product (DHT) were also low.(54) This reflects a more accurate picture of the intracrine processing and the sex hormone levels within one of the main target organs of SS.

Why are the exocrine glands preferential targets of SS?

The main exocrine glands involved in SS are the lacrimal (LG), the meibomian (MG), and the salivary glands (SG). In various mammalian species, including humans, there is evidence highlighting the gender differences in these structures. These differences include morphologic, physiologic, and even immunologic differences. For example, in the lacrimal gland of both mice and rats, the size and shape of the lacrimal acini are larger and more irregular in the males and smaller and more regular in the females. In hamsters, males have higher number and affinities of β -adrenergic binding sites compared to females, and females have greater amounts of melatonin and N-acetyltransferase compared to the males. Immunologically, male rats show a higher synthesis of secretory component, greater production of IgA, and an increased number of IgA-containing cells compared to females. Most significant of all is the greater incidence of autoimmune diseases in the lacrimal glands of mouse and human females.(55) Moreover, these types of sex-related differences extend to the salivary glands of various species as well. (56,57)

Sex-related differences can be appreciated at the genomic level as well. Señorale-Pose, et al showed that the Vcs2 gene is differentially expressed in the acini epithelium of the submandibular gland of mice according to gender. No Vcs2 transcripts were detected in the female submandibular gland. Moreover, transcripts were lost upon orchietomy of male mice and induced in females by androgen administration.(58)

In 2006, Richards et al showed significant differences in gene expression in over 490 different genes when comparing male and female mouse lacrimal glands using microarray and rt-PCR assays.(59) In addition, Treister et al demonstrated tissue-specific sex-related differences in gene expression in the BALB/c mouse major salivary glands.(5)

Androgen target: a theory for sex-related differences

Considering that sex related differences in species is under the influence of sex-steroid hormones, research was undertaken to determine whether androgens or estrogens predominated in the development, maintenance, and normal functioning of these glands. Through manipulation of androgenic influences, studies have shown that the LG, MG, and SG are androgen target organs. If mice, rats, hamsters, guinea pigs and/or rabbits are castrated or are treated with an androgen receptor antagonist degenerative changes ensue in the LG. (55). Decreased growth and functional activity, disappearance of glandular elements, and attenuation of acinar cell size are but a few of the changes observed. Moreover, if androgens are exogenously replaced following orchiectomy, LG structure and function can be restored and, in some cases, even lead to acinar cell hyperactivity.

In the submandibular glands of male and female mice, there was an observed 10 fold increase in the activity of glucose-6-phosphate amino transferase (G6P-AT) in the females. If the mice were ovariectomized, no change in activity was observed. If,

however, testosterone was injected into the female mice, the activity of the G6P-AT was reduced to the normal male level. Conversely, if male mice were given 17 β -estradiol no change in their G6P-At activity was noted, but, upon castration, the level rose to that of the normal female glands.(60) This would suggest that the influence of estrogens is secondary to that of androgens in the glands. It was also shown that ovariectomized female mice subsequently treated with testosterone differentially expressed 500 genes compared to controls. Interestingly, 214 of these genes were also expressed by androgen treated male mice, but many were unique to the females.(6)

On a molecular level, Richards, et al showed that 2000 genes were regulated by testosterone in the mouse LG. He used orchietomized or testicularly feminized mice that were given testosterone implants.(61) Similar results were observed by Treister, et al when looking at the magnitude of the effect androgens have on submandibular gland of the BALB/c mouse. Orchietomized male mice were implanted with a subcutaneous pellet containing either slow-release testosterone in a vehicle or just the vehicle. It was shown that androgens significantly influenced the expression of over 1300 genes, 366 of them had been previously shown to be differentially expressed by males.(5,62)

Similar evidence of androgenic influence on the meibomian gland has also been discovered. Given the fact that the MG is a large sebaceous gland, and elsewhere in the skin, sebaceous glands fall under androgen control, it is not remarkable that this

ocular gland would also be an androgen target. Sullivan, et al, using immunohistochemistry (IHC) found androgen receptor (AR) protein in the nuclei of both male and female rat meibomian acinar epithelium. Also, when examining rabbit MG following castration, the application of topical ocular 19-nortestosterone or systemic 19-nortestosterone lead to significant differences in the lipid content profile of the glandular acini without affecting gross morphology.(63)

In 2005, Schirra, et al went on to show that in castrated, male, mouse MG, testosterone administration significantly influenced the expression of 1590 genes compared to control treated mice.(64)

The presence of AR in human ocular tissues was demonstrated by Rocha, et al. The AR was located in the epithelial cell nuclei in the LG and in acinar epithelial nuclei in the MG. In addition 5 α -reductase mRNA was also identified. This enzyme catalyzes the conversion of dehydroepiandrosterone (DHEA) to the potent active metabolite, dihydrotestosterone (DHT).(65) (66) Moreover, Wickham, et al demonstrated estrogen receptor (ER), progesterone receptor (PR) and AR mRNAs in various ocular tissues in various mammalian species including humans using rt-PCR. Their results confirmed the presence of AR, ER, and/or PR in tissue from the LG and MG of rats, rabbits, and humans.(67)

Li, et al demonstrated AR localization, using IHC, in the serous acini and ductal epithelium of the submaxillary glands of male rats independent of whether the

animal had been castrated or not. AR mRNA was localized utilizing in situ hybridization to the epithelial cells of the convoluted, the secretory, and the excretory ducts in the control groups only. In the castrated males the AR mRNA was confined to the convoluted and secretory ducts only. An overall reduction in the quantity of AR in the gland was observed following castration. (68) Laine, et al showed AR in the serous, mucous, and ductal epithelial nuclei of normal human salivary gland.(69)

Is SS mediated by an androgen deficiency, an estrogen deficiency, or an interaction of the two?

Numerous studies have addressed why the glandular effects of SS may be mediated by sex hormones. It has been demonstrated in the MRL/lpr mouse model of SS that the lacrimal lymphocytic lesions can be suppressed with androgen treatment.(70) Sullivan demonstrated that anti-inflammatory benefits of androgen treatment in this animal model, and also an enhanced functional output as measured by total amount of IgA secretion in the saliva.(43) Conversely, if androgens are deficient one would expect an increase in lacrimal inflammatory lesions, and a decrease in glandular function. This was tested using testicular feminized mice, castrated rats, rabbits, guinea pigs, and human males receiving androgen antagonists. No correlation between androgen receptor dysfunction and increased lacrimal gland or submandibular salivary gland inflammation was observed in all the animal models. Moreover, no decrease in tear volume was noted in the mice o(71)r in the human males.(71)

In a similar assessment of the androgenic influence in the meibomian glands of the eye, Sullivan, et al found striking evidence of meibomian dysfunction and SS-like evaporative dry symptoms in humans receiving androgen antagonists. These symptoms included: greater frequency of tear film debris; abnormal tear menisci; metaplasia of meibomian gland orifices; reduction in the quality of secretions; and an increase in blurred vision, light sensitivity, and painful eyes.(72)

Estrogen receptors (ER) were identified in the rat submandibular and parotid glands in ovariectomized females.(73) There was found to be a differential expression of ER between the two glands with the submandibular gland containing a significantly higher concentration of ER. Camacho-Arroyo showed that ER and PR were located mainly in the acinar nuclei of female and male rabbits. Timing during estrous, whether the animal had been castrated, or castrated and estrogen treated determined the concentration of both ER and PR with a decreasing trend, respectively. Male rabbits showed lower concentrations of the receptors than females.(74)

Estrogens also have been shown to have a target tissue in the oral cavity, most notably, the epithelia of the surface mucosa and salivary glands.(75)(76) This becomes evident when levels of estrogen fall and the mucosa atrophies and salivary function decreases as is seen during menopause. (77,78) Furthermore, these symptoms can be ameliorated by the re-establishment of normal estrogen blood levels with hormone replacement therapy (HRT).

Interestingly, in 2000, Esmaeli, et al showed that the conjunctiva and accessory lacrimal glands were devoid of either AR, ER, or PR. In the MG sampled, he gave evidence for ER in the human MG (22 of 22), but, was able to demonstrate AR and PR in only 1 of 22 specimens.(79) This is in distinct contrast to other sebaceous glands of the skin and scalp.

ER- α and ER- β mRNA was also found in human LG.(80) By contrast, ER- α was not detected in oral buccal or gingival epithelium or in SG. The other isoform, ER- β was widely expressed in all oral tissues especially keratinocytes and acinar and ductal SG epithelium. (75)

Obviously, a controversy exists between the interplay of androgens and estrogens in the pathogenesis of SS. There is most likely a delicate balance between androgenic and estrogenic influences leading to the exocrinopathy indicative of SS that remains to be uncovered. Furthermore this delicate balance appears not at the system or organ level, but peripherally, at the tissue and cellular level. Peripheral production of these hormones may hold the key to this controversy. Porola, et al have shown that within the milieu of a systemic androgen/DHEA deficiency, there is also a suspected defect in the intracrine enzymatic machinery causing a lack of androgens to be produced at the cellular level and an over production of estrogens.(54) Furthermore, the enzymes which catalyze the conversion of DHEA to DHT or estradiol, 5 α -reductase and aromatase, respectively, were found to be distributed differently within the cytosol of SS patients when compared to healthy controls.(81)

Local factors, such as the presence of cytokines and circulating cortisol may push the equilibrium towards an androgenic environment or to one that is more estrogenic. (82) Cutolo showed that the cytokines IL-1, IL-6, and TNF- α stimulated aromatase activity in RA patients synovium.(44) Rocha had previously shown that of these three cytokines, testosterone would cause a decrease in mRNA of IL-1 and TNF- α in the lacrimal glands of a mouse model of SS.(42)

Previous studies evaluating sex hormone influence on glands of SS mainly are molecular in nature

Studies looking at serum levels of hormones, RT-PCR analysis of hormone receptor mRNA, and DNA- microarray analysis abound,(50,83-88) but very little research has been directed toward the IHC analysis of MSLG biopsies of SS patients. The earliest mention of using IHC to evaluate sex hormone influence in salivary gland tissue of SS patients was by Kumagami and Onitsuka in 1993. They stained for the hormones estradiol, testosterone, and dihydrotestosterone in only (9) patients with secondary Sjögren's syndrome. Compared to controls, the SS patients showed more reactivity for the androgenic hormones. Unfortunately the controls were neither age nor sex matched.(89) In 1998, Onodera, and colleagues used IHC to analyze the estrogenic enzyme, aromatase in MSLG biopsies of SS patients of both sexes and varying ages. His study showed more aromatase activity in the SS patients compared to patients with other salivary gland pathoses.(90) More recently, however, Spaan, et al localized the intracrine enzymes within the cytoplasm in SS patients and healthy controls.(81)

No previous IHC evaluation of the ER/AR ratio in sicca syndrome patients' MLSG biopsies

Several studies have mentioned the possibility of an interaction or a delicate balance between the seemingly opposing influences of androgens and estrogens in the exocrinopathy of SS. (54,71,81,91) To date no study utilizing an IHC evaluation of ER to AR ratio in a population of sicca syndrome patients has been reported. A comparison between these sex steroid hormone receptors to an age/sex matched control population is also lacking. The advantage of immunohistochemical (IHC) evaluation of biomarkers is that it allows for the anatomic localization of the marker at the tissue level (glandular) as well as at the cellular level within that tissue. With sex hormone receptors for example, IHC allows for the evaluation of where, within the gland, the cells that house the receptors are located, i.e. acini vs. ducts, as well as where, within the cells, the receptors are found, i.e. cytoplasm vs. nucleus. The intracrine formation of sex hormones at the cellular level, mentioned previously, makes this type of evaluation essential since each cell can potentially have its own level of sex hormone activity completely independent from the rest of the tissue. Additionally, a technique which uses homogenized tissue may miss circulating levels of pro-hormones that are within normal limits but are inappropriately processed at the target cell as has been suggested(54).

In SS, IHC has been used to detect the hormones themselves, the receptors for the hormones, the steroidogenic enzymes involved in converting the precursor to the active hormone, and other downstream, surrogate, biomarkers which are influenced

by the hormones. By example, the use of a surrogate biomarker for androgen activity (CRISP-3) was expertly demonstrated in the 2007 study by Laine, Porola, and colleagues who combined both in vitro and in vivo techniques and utilized a combination of IHC, in situ hybridization, microarray analysis, ELISA and RT-PCR to convincingly show how SS is characterized by a “insufficient androgen effect” at the salivary gland level.(88) Recently, Spaan, et al compared the distribution of steroidogenic intracrine enzymes in the MLSGs of both SS patients and healthy controls using immunofluorescent IHC.(81)

Limited information about IHC evaluation of normal expression of sex hormone receptors in normal human salivary glands

There is very little baseline IHC data that demonstrates the expression of AR and/or ER in normal human salivary glands. Most of the existing data comes from studies evaluating the presence of these receptors in salivary gland neoplasms.(92-95) In 1993, Laine, demonstrated the presence of AR in salivary tissue using IHC.(69) Valimaa, has shown that, although there are (2) isoforms of the estrogen receptor (α and β) the beta isoform predominates in the oral mucosa and salivary glands.(75) Yet there is no study, to date, which has used IHC to characterize the expression of ER to AR receptors in climacteric females.

In the LG, MG, and SG androgens exert their effect through high-affinity, saturable, steroid-specific androgen receptors (AR) found within epithelial cells. Once activated, the monomeric hormone-receptor complex translocates to the nucleus,

and associates with the promoter region of an “androgen response element” (ARE) of specific target genes. Here it effects the transcription of various androgen-dependent mRNA species.(55)

An alternative signaling pathway for steroid receptors is being defined in which rapid cellular responses can be effected by plasma membrane bound AR, ER, and PR. (96,97)(96-98)

Novel measurement of immunoreactivity in MSLG biopsies: The Allred Score

In 1998, Allred, et al proposed an alternative method to the then, current “gold standard” of ligand binding assays (LBAs) used to assess the IHC ER status of breast cancer patients. The evaluation was essential to better predict which patients would benefit from adjuvant endocrine therapy.(99,100)The LBAs were technique sensitive, labor intensive, as well as expensive. IHC assessment of the receptor status proved to easier and had similar specificity and sensitivity. The “Allred Score” as it became known was validated, and has become widely accepted as a way to semi-quantitatively assessment of ER of a breast carcinoma. (101) It looks at the percentage of cells that are immunoreactive and assigns a “proportion score” (PS) on a scale of 0-5 (see figure). It then looks at the average intensity of the staining in those cells and assigns an “intensity score” (IS) on a scale of 0-3. The PS and IS are then added together to obtain the “total score” (TS), the Allred score. An Allred score of <3 is considered negative by medical pathologists. When introduced the intra- and

inter-reproducibility of the scoring was seen to be >90% in Allred's laboratory.(99)

There are no published studies in which the Allred score was used to assess sex hormone receptor in MSLG biopsies. This will be the first.

METHODS

Institutional Review Board approval (**Study #:** 08-0989) to conduct studies on archived pathology specimens was obtained for both the University of North Carolina, Chapel Hill (UNC-CH), and Carolina's medical Center, Charlotte (CMC). A search of the Oral & Maxillofacial Pathology Laboratory's database for the last 3 years yielded a potential cohort of patients whose tissue was submitted for histomorphometric analysis as part of the diagnostic work-up for primary Sjögren's syndrome (SS) (N=47).

Patient selection

Sicca Specimens

Tissue used in this study consisted of excess archival minor labial salivary gland (MLSG) tissue submitted from a single institution (CMC). Pertinent medical information about each specimen was obtained through chart review of medical records at CMC. Information gathered included: patient age; race; focus score (FS) from MLSG biopsies; smoking history; subjective ocular symptoms; Shirmer's test results; objective oral dryness; hormone replacement therapy (HRT) history; history of systemic steroid use; duration of symptoms; other medical conditions; previous treatments rendered; and treatment response. The "sicca" patients were further

divided into two groups: those with a FS ≥ 1 (group A); and those with a FS < 1 (group B).

Control Specimens

Control specimen (group C) tissue was obtained from the tissue archives of the Oral & Maxillofacial Pathology Laboratory at UNC- CH. The control specimens were tissue samples previously submitted from peri- and post-menopausal females who had MLSG tissue incidentally included in their specimens, and had no reported history of xerostomia. A database with the patients' information was created.

Subsequently all paraffin blocks were de-identified regarding accession numbers from UNC-CH and assigned a study number that corresponded to the one in the database and was different from the accession number. The data base is kept in a password-protected secure server behind a firewall at UNC School of Dentistry's Research server. Only two of the investigators have access to the database.

Inclusion Criteria

SS Specimens

All specimens were minor labial salivary gland biopsies (MLSG) of peri- and post-menopausal females. Only specimens from patients who exhibited 3 of the 5 diagnostic criteria for primary SS established by the joint American European consensus of 1996, and revised in 2001 were included (24). All specimens came from patients who had complaints of dry eyes and dry mouth, and a negative blood test for anti- nuclear antibodies (ANA), SS A/Ro and SS B/La. (N= 28)

Control specimens

All specimens were from patients who were peri - or post-menopausal females who had incidental findings of MLSG tissue within their specimens submitted for other reasons besides salivary gland pathosis. At least 2 mm² of gland parenchyma had to be included in the specimen. (N=8)

Exclusion Criteria

Sicca Specimens

Only specimens from the clinic at CMC were included in these groups. Any specimen from a patient who had evidence of anti-nuclear antibody test (ANA) in their serum was excluded. Patients who had secondary SS were not included in the study. Specimens obtained from any other intra-oral location besides the lower lip were excluded as well.

Control Specimens

Submitted MLSG tissue could not contain neoplasms, either benign or malignant, amyloidosis, or granulomatous inflammation. If the area of the gland parenchyma in the specimen measured less than 2 mm² the specimen was excluded. Any specimens from intraoral locations besides the lower lip were likewise excluded.

Immunohistochemical (IHC) Staining

Tissue specimens were formalin-fixed paraffin-embedded (FFPE) MSLG biopsies. Seven micrometer sections were cut and mounted on plus-coated glass slides. IHC was performed at the APTCL (Anatomical Pathology Translational Core Lab) at UNC-CH according to the following protocol.

Antibodies:

Rabbit polyclonal anti-Androgen Receptor PG21 antibody (Cat# 06-680) was purchased from Chemicon® International Company/Millipore Corporation, Temecula CA 92590. Mouse monoclonal anti-human Estrogen receptor α antibody (M7047), clone 1D5 was from Dako North America, Inc. Carpinteria, CA 93013. Rabbit polyclonal anti-Estrogen Receptor-beta Ready to use antibody (Cat#AR385-5R) was purchased from Biogenex 4600 Norris Canyon Rd. San Ramon, CA Mouse monoclonal anti-Human Progesterone receptor (NCL-PGR-312), clone 16 from (Leica Microsystems Inc. Norwell MA 02061)

Automated detection:

Estrogen Receptor (ER- α) and progesterone Receptor (PR) stains were carried out in the Bond Autostainer (Leica Microsystems Inc. Norwell MA 02061) according to the manufacturer's IHC protocol. Briefly, slides were de-waxed in Bond Dewax solution (AR9222) and hydrated in Bond Wash solution (AR9590). Antigen retrieval was performed for 30 min at 100°C in Bond-Epitope Retrieval solution 1 (pH-6.0) or solution 2 (pH-9.0) for PR and ER respectively. Slides were incubated with the appropriately diluted primary antibodies for 2h (EGFR). Antibody detection was

performed using the Bond Polymer Refine Detection System (DS9800). Stained slides were dehydrated and cover slipped.

Estrogen receptor- β (ER- β) was carried out in the DAKO Autostainer according to the Dako Auto-Envision IHC protocol. Steam Antigen retrieval was performed for 30 min in DAKO Target Retrieval Solution pH-6.0 (Cat.#S2369) 30 min.

Antibody detection was performed using the DAKO Envision Labelled Polymer HRP Rabbit (Cat.#K4003). Chromogen 2 min. Innovex DAB (Cat.# NB314SBD).

Counterstained stained slides in Hematoxylin DAKO 30 sec.(Cat.# S3309). Bluing 30 secs. Stained slides were dehydrated and cover slipped.

Manual detection:

Androgen receptor (AR) stain was conducted manually using Vectastain ABC Rabbit kit Mouse detection system according to manufacturer's instruction (Vector laboratories, INC Burlingame, CA94010). Heat induced antigen retrieval was carried at pH -6.0 using Dako Cytomation Target Retrieval Solution(S1699) for 30 min. Slides were incubated overnight at 4°C with the primary antibody.

Positive and Negative Controls

Appropriate positive controls were utilized for each of the antibodies reacted. Human prostate tissue was used for the AR antibody, and human breast tissue was used for the ERs and PR antibodies. Sections of MSLG tissue from each of the three groups were incubated with the appropriate serum that did not contain the primary antibody for the negative controls.

Histomorphometry

Focus scores (FS) were determined prior to the start of the study by two, Board-Certified Oral & Maxillofacial Pathologists (VM,RP) experienced in histomorphometric analysis. MLSG specimens were sectioned, mounted on glass slides, stained with hematoxylin & eosin, and visualized with standard light microscopy using an Olympus BX41 stereoscopic microscope (Olympus America Inc.,Center Valley, PA).The total area of glandular parenchyma was measured using a reticule within the ocular of the microscope. Lymphocytic “foci” were then counted within that area. A focus of lymphocytes is determined to be an aggregation of 50 or more lymphocytes adjacent to viable parenchyma of salivary gland. The “focus score” was then calculated according to the following formula:

$$\text{Focus Score} = (\# \text{ of Foci} \times 4) / \text{Area}$$

Analysis of IHC

All slides were examined using standard light microscopy using an Olympus BX41 stereoscopic microscope (Olympus America Inc.,Center Valley, PA). The immunohistochemical signal was scored using a modified version of the “Allred Score”(99), which is a semi-quantitative tool originally developed to evaluate hormone receptor immunoreactivity in breast cancer specimens. It is a two-part assessment that looks at the number of cells that are staining, and also the intensity

of the staining in those cells. In the original Allred scoring system, a “Proportion score” in a scale of 0-5, corresponding to increasing percentages of cells reacting is added to the “intensity score” in a scale of 0-3 which assesses the intensity of the immunoreactivity of the cells. The resulting sum total of proportion and intensity scores gives the final “Allred Score” of 0-8 (excluding 1). See Table 1A and B and Figure 1

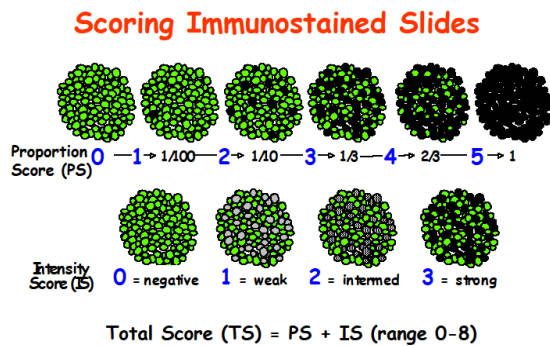
Table 1A: Table showing the Allred Score proportion score and its corresponding percentage of cells.

Proportion Score	Percentage of cells
0	0
1	<1
2	1-10
3	11-33
4	34-66
5	67-100

Table 1B: Table showing the Allred Score intensity score and its corresponding reactivity.

Intensity Score	Reactivity
0	None
1	Weak
2	Moderate
3	Strong

Figure 1 : Illustration of the Allred score, depicting visually, the immunoreactivity of tissue. (99)



A “Modified Allred score” was created in the current study (Figure 2). It consists of the same intensity scale, but the proportion scale is reduced from 0-5 to 1-4, yielding a total score in the range of 1-7. This allows for a more equal distribution of the percentages within each proportion level and for more consistent scoring of specimens, especially at the lower end of the scale, where subtle differences tended to be more common.

Table 2: Table showing a modified version of the Allred Score with compression of all values less than 10% of the proportion score being represented by a score of 1. The same intensity score is used.

Proportion Score	Percentage of cells
1	<10
2	11-33
3	34-66
4	67-100

Intensity Score	Reactivity
0	None
1	Weak
2	Moderate
3	Strong

Statistical Analysis

All data was collected and entered into Excel spreadsheets. Statistical analysis of the data was performed. Comparisons between the three groups' ages were performed using the Kruskal-Wallis test. Comparisons of the ratios ER- α and ER- β to AR in each of the three groups were performed using the Kruskal-Wallis test (SPSS v.16 GP 2007; SPSS Inc., Chicago, IL). Due to sample size of group B and C, statistical testing of the reactivity patterns of each receptor antibody was only performed within group A. The intensity and proportion stains were modeled separately using a proportional odds model with cumulative logits and a general estimating equation method for ordinal categorical data.

Photomicrography

Photomicrographs of the specimens were captured using an Olympus DP 70 digital camera mounted atop a Olympus BX41 stereoscopic microscope (Olympus America Inc., Center Valley, PA).

RESULTS

Patient Demographics

The focus scores, age and race of the patients are summarized in table 1. Patients were divided into three groups based on FS: group A had $FS \geq 1$ (n=20) ; group B $FS < 1$ (n=8); and group C were controls (n=8). The ranges of FS were: A= 1.0-9.9; B=0.30-0.80.

The age ranges of each group were: A= 34-79yrs; SS-= 46-69yrs; and CG= 42-81.

The mean age for each group was: SS+= 60.3yrs (SD=14.1); SS-= 53.9yrs (SD=8.8); and CG=56.6 (SD= 13.8). The unpaired t-Test was utilized to show there was no statistically significant difference in the average ages of the groups (p=0.34).

Of the sicca patients: two were African American (A=1, B=1), all others were Caucasians; four were current smokers, two from groups A and B, respectively. See Table 3

Table 3: Demographic data showing age, race and focus score for the three experimental groups. Group A has FS ≥ 1 , group B has FS < 1 , and group C is the control group. ($p=0.34$)

Group A (FS ≥ 1)	Age	FS				Group C	Age
1	77	3.56					
2	77	1.5					
3	34	1.68					
4	56	1.71					
5	34	1.98					
6	79	1.68					
7	53	1.9					
8	76	2.2					
9	64	2.24					
10	48	1.38					
11	76	9.9					
12	53	1.28					
13	62	1.51					
14	50	1.06					
15	65	1					
16	76	1					
17	48	1.04					
18	69	1.16					
33	53	2.35					
36	55	1.2					
Group B (FS < 1)	Age	FS					
19	50	0.41				27	42
20	66	0.51				28	69
21	48	0.3				29	48
22	46	0.43				30	81
23	52	0.82				31	50
24	47	0.5				32	48
25	69	0.74				34	67
26	53	0.62				37	48

Hormone Receptor Immunoreactivity

The results for the statistical analysis of the receptor immunoreactivity are summarized in Table 4. Based on the intensity of immunohistochemical reactivity of MSLG of patients from group A, and controlling for subcellular location, a proportional odds model with cumulative logits using a general estimating equation method for ordinal categorical data showed that the likelihood of having greater intensity of AR reactivity is 37.3 times higher than reactivity to ER- α , and 7.71 times higher than reactivity to ER- β (i.e. AR $>$ ER- β $>$ ER- α) ($p < 0.001$). See Table 4A

Table 4A: Intensity of immunoreactivity in the SS group (group A) as a function of receptor type. When controlling for subcellular location, AR is 37.7 times more likely to be more intense than ER- α , and 7.7 times more likely than ER- β .

Contrast	Predicted OR	P-Value
AR vs. ER	37.33	< 0.0001
AR vs. ERB	7.71	< 0.0001
ERB vs. ER	4.84	< 0.0001

By the same method of assessing antibody reactivity, the, likelihood of having a higher proportion of cells that react to AR is 92.5 times higher than ER- α , and 187 times higher for ER- β when controlling for subcellular location (i.e. AR > ER- α > ER- β) ($p < 0.001$), and there is a 4.8 times higher likelihood of having a greater intensity of cells that react with ER- β compared to ER ($p < 0.01$) and a twice the likelihood of having a higher proportion of cells that react to ER- α than to ER- β ($p = 0.02$). See Table 4B

Table 4B: Proportion of cells immunoreacting in the SS group (group A) as a function of receptor type. When controlling for subcellular location, AR is 92.49 times more likely to have a greater proportion of cells immunoreacting compared to ER- α , and 186.77 times more likely than ER- β ; ER- α is 2.02 times more likely to have a greater proportion than ER- β .

Contrast	Predicted OR	P-Value
AR vs. ER	92.49	< 0.0001
AR vs. ERB	186.77	< 0.0001
ER vs. ERB	2.02	0.0167

Hormone Receptor Distribution

Androgen receptors

The most intense AR immunoreactivity score was observed in both the nuclei of the ductal cells (Mdn=7, IQR= 0) and the nuclei of the acinar cells (Mdn=7, IQR= 0). See Figure 2A and B.

Figure 2A: Androgen receptor immunoreactivity in the ductal nuclei (arrow)

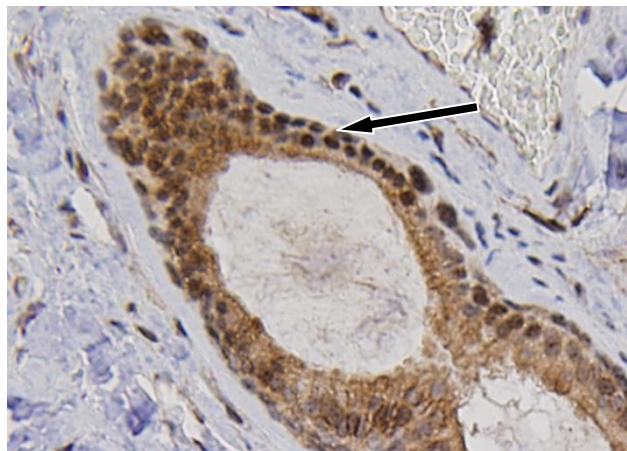
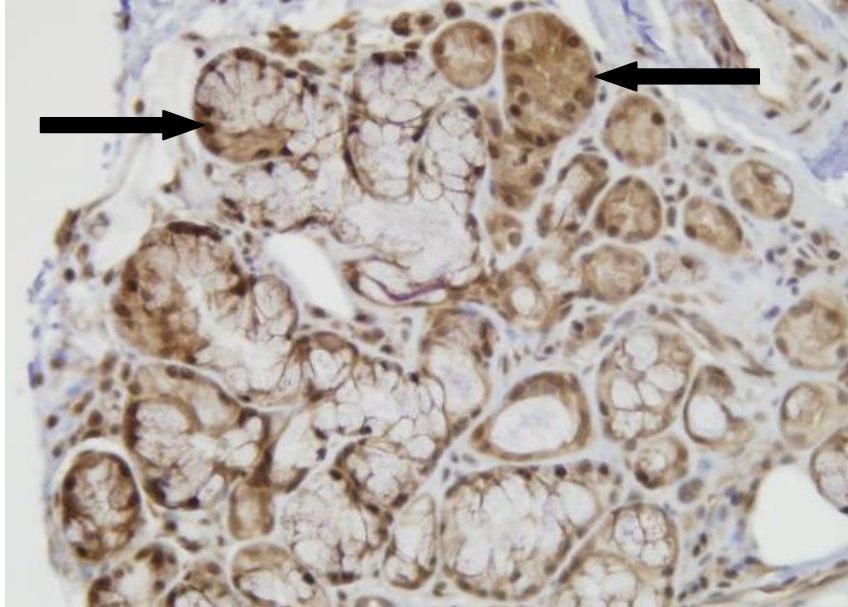


Figure 2B: Androgen receptor immunoreactivity in the acinar nuclei (arrows).

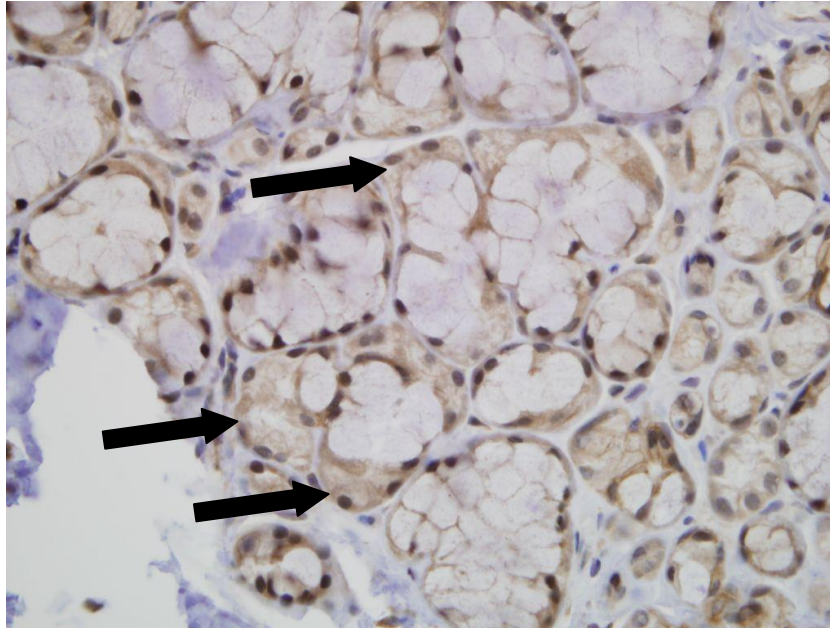


The ductal cell cytoplasm, although noticeably reactive, was less intense with a median score of 6 (IQR= 0), and the acinar cytoplasm was the least immunoreactive with a median score of 4 (IQR= 0). See Results Figure 2A and B. This distribution of reactivity was independent of the FS. The cells whose nuclei stained most intensely were at the periphery of the acini and mostly abluminal to the ducts. In these locations all of the cells were immunoreactive.

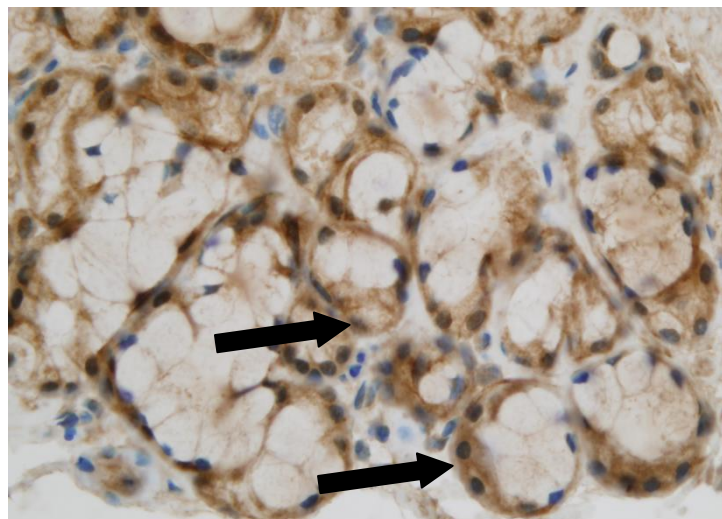
The cytoplasm of serous demilunes surrounding mucous acini was more immunoreactive for ER- α than for AR. See Figure 3A and B.

Figure 3: Immunoreactivity of receptor antibodies within serous demilunes of the MLSG.

A: Weak AR immunoreactivity in the acinar cytoplasm except in the serous demilunes where moderate reactivity was seen (arrows).



B: Stronger ER- α immunoreactivity in the serous demilunes and, as with AR, a similar lack of reactivity in the other acinar cytoplasm (arrows).

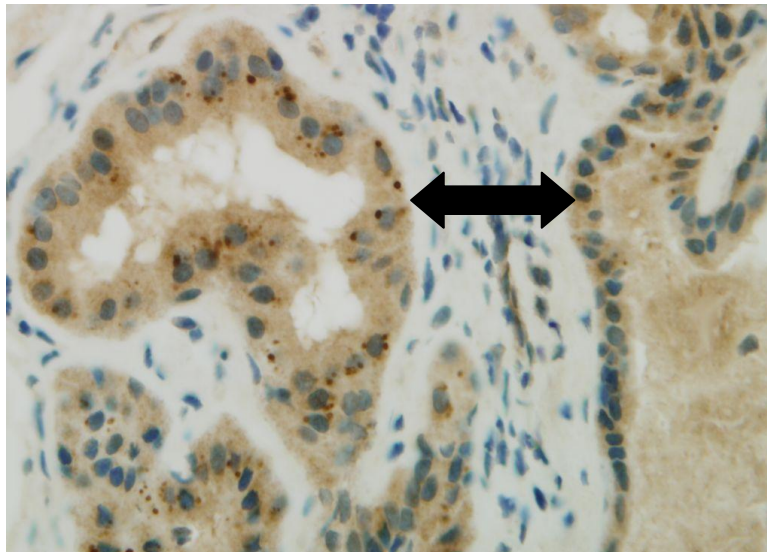


Estrogen- α Receptors

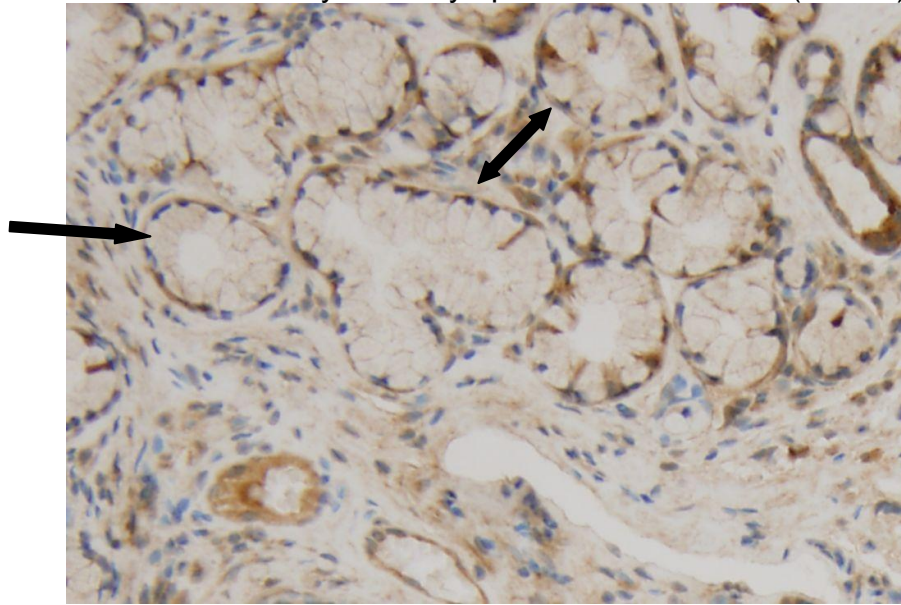
All groups showed weak reactivity to ER- α antibody in all but the cytoplasm of the ductal cells, median score of 5 with an IQR of 1. See Figure 4 A and B.

Figure 4: ER- α immunoreactivity in cytoplasm of the ductal and acinar cells

A: Moderate ER- α immunoreactivity in the cytoplasm of ductal cells (arrows)

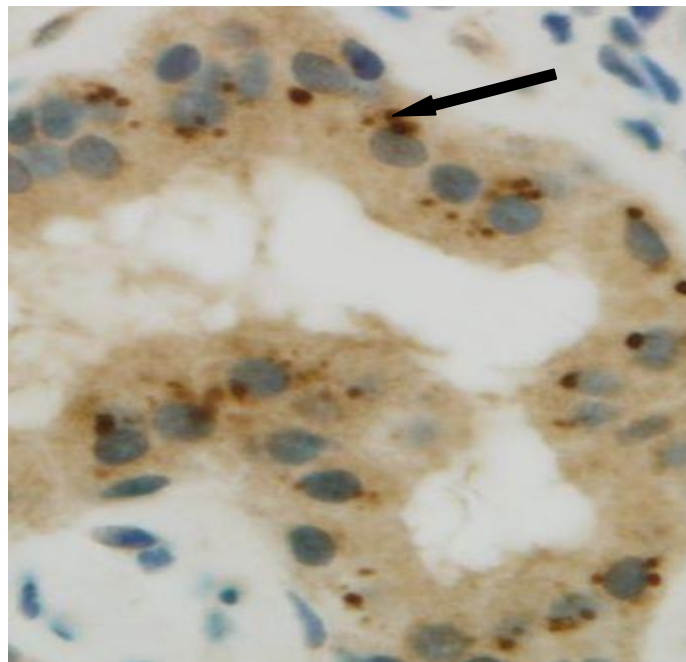


B: Weak ER- α immunoreactivity in the cytoplasm of acinar cells (arrows).



As can be seen in figure 4, we observed intense globular areas of cytoplasmic immunoreactivity adjacent to the nuclei in both acinar and ductal cells in all three groups.

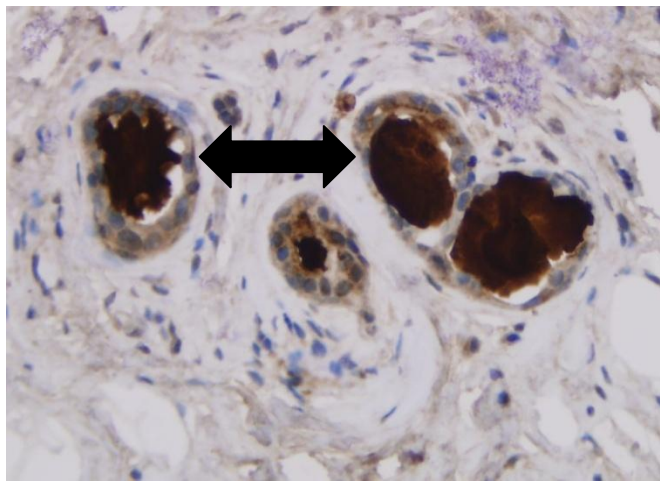
Figure 5: High power (40x) view of image seen in figure 4A showing globular areas of cytoplasmic immunoreactivity (arrow).



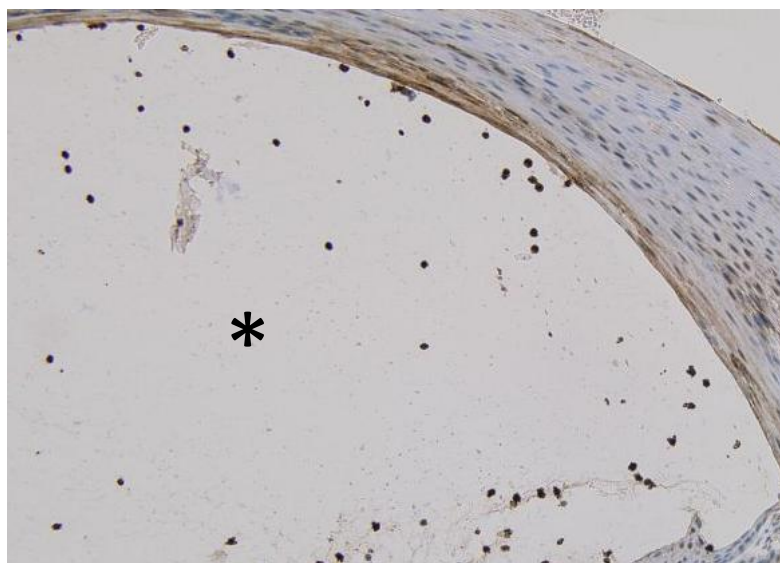
Inspissated pools of saliva were highly immunoreactive to ER- α compared to the other receptors in groups A and B. This phenomenon was not observed in the mucoceles from control patients. See Results Figure 6 A-D.

Figure 6: ER- α immunoreactivity in mucous pools within the SS group (group A) compared to extravasated mucus in the control group (group C), and lack of immunoreactivity with the other receptor antibodies.

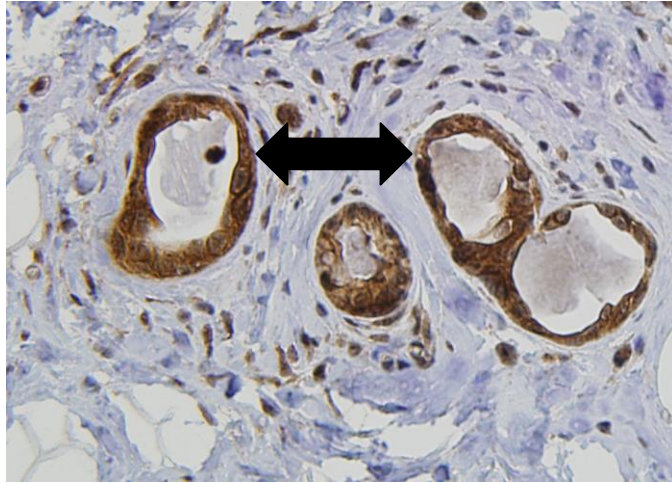
6A: Strong ER- α immunoreactivity in the inspissated mucus (arrows).



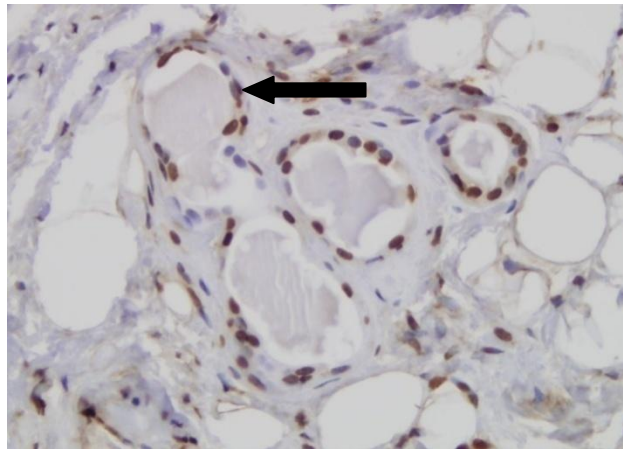
6B: Lack of ER- α immunoreactivity in the extravasated mucus of a mucocele (asterisk).



6C: Lack of AR immunoreactivity in the inspissated mucus (arrows).



6D: Lack of ER- β immunoreactivity in the inspissated mucus (arrow).



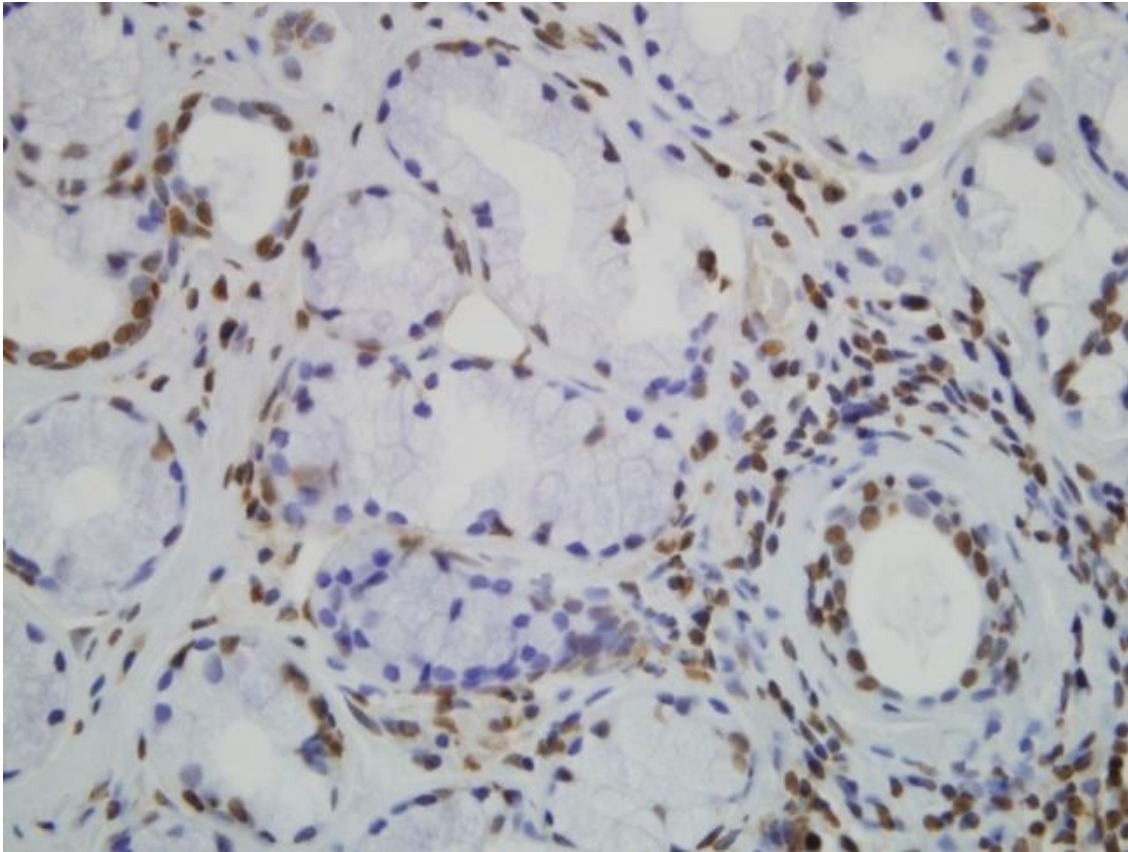
Estrogen- β Receptors

All groups revealed intense ER- β immunoreactivity. The ductal cells' nuclei had a median score of 6 with an IQR of 0, and in the nuclei of the acinar cells had a median score of 5, with an IQR of 1. Only weak antibody staining was observed in the cytoplasm of the acinar and ductal cells. Intense nuclear staining was also seen

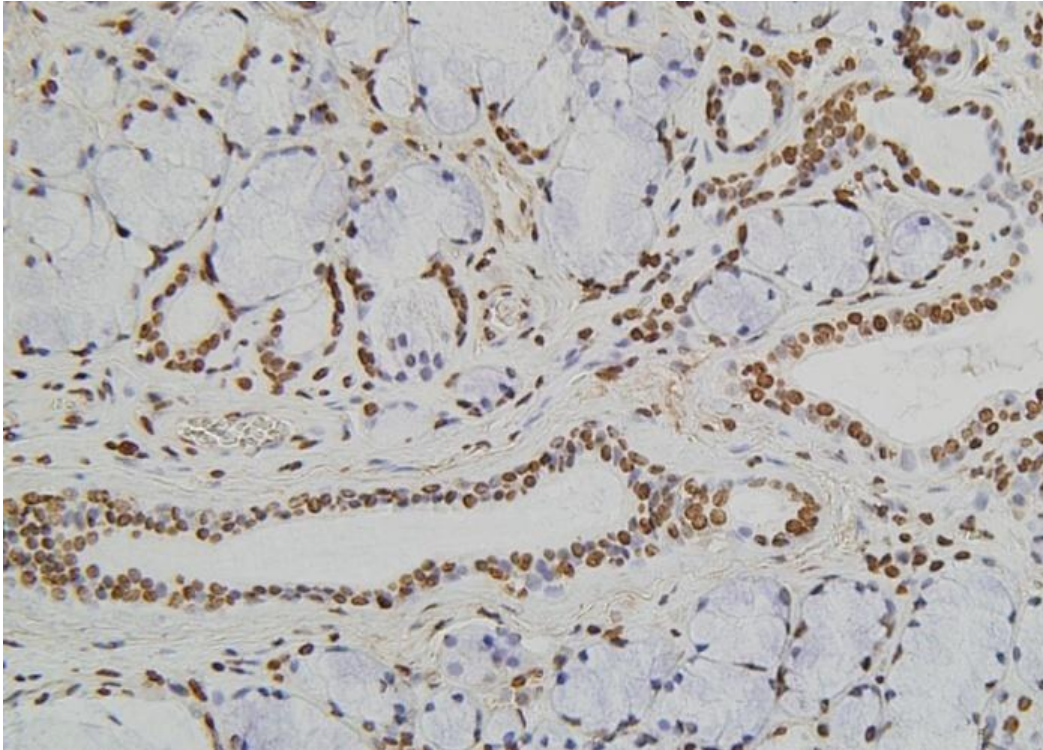
diffusely throughout the parenchyma of the gland and in a subset of cells at the periphery of acini and abluminally in ducts. See Figure 7.

Figure 7: ER- β immunoreactivity in the different experimental groups showing viable intensity and number of cells reacting, but consistently nuclear in its location.

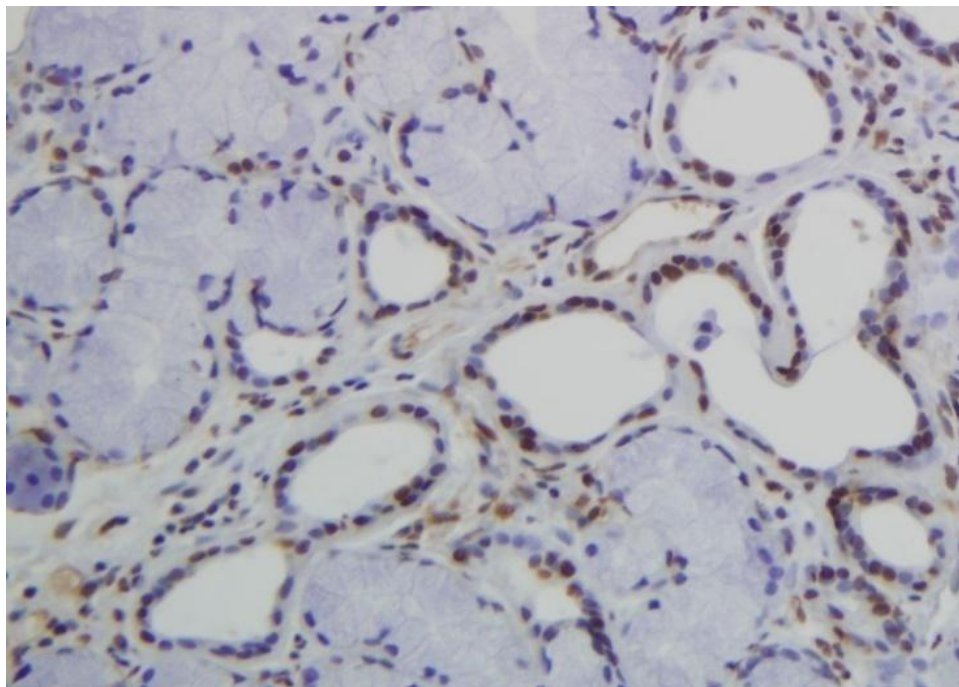
A: Group A showing strong but inconsistent nuclear reactivity



B: Group B showing moderately intense immunoreactivity in subsets of cells. The ductal nuclei appear to be in the majority of cells reacting. Reactivity is moderately intense



C: Group C showing moderately intense but more variable proportion of cells reacting. Still, strictly a nuclear pattern.

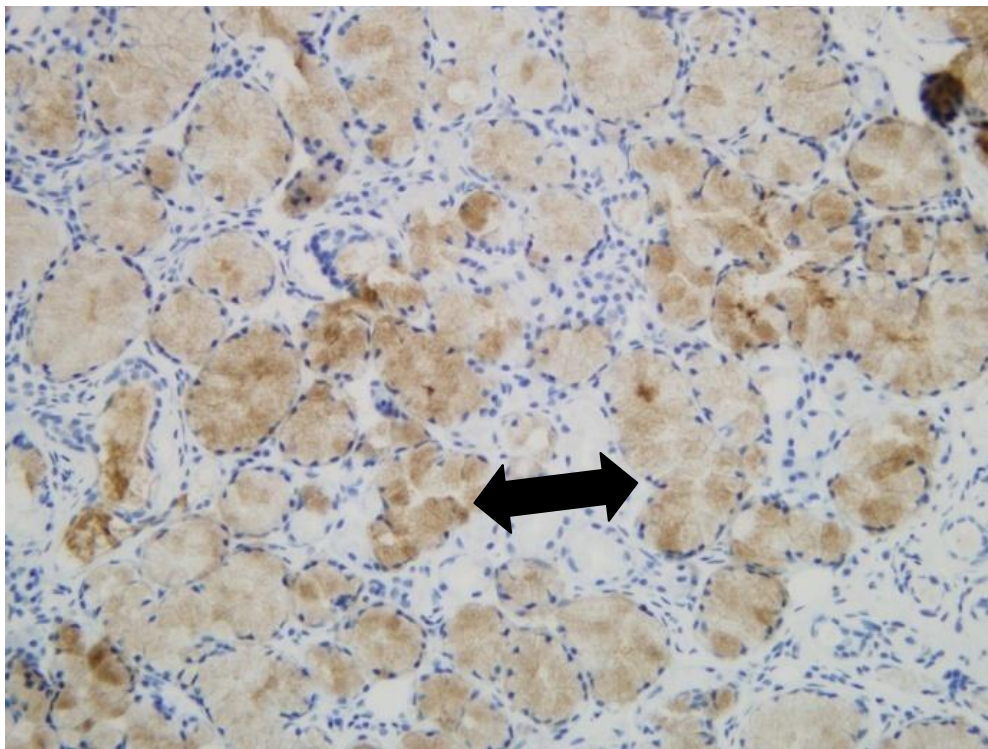


Progesterone receptors

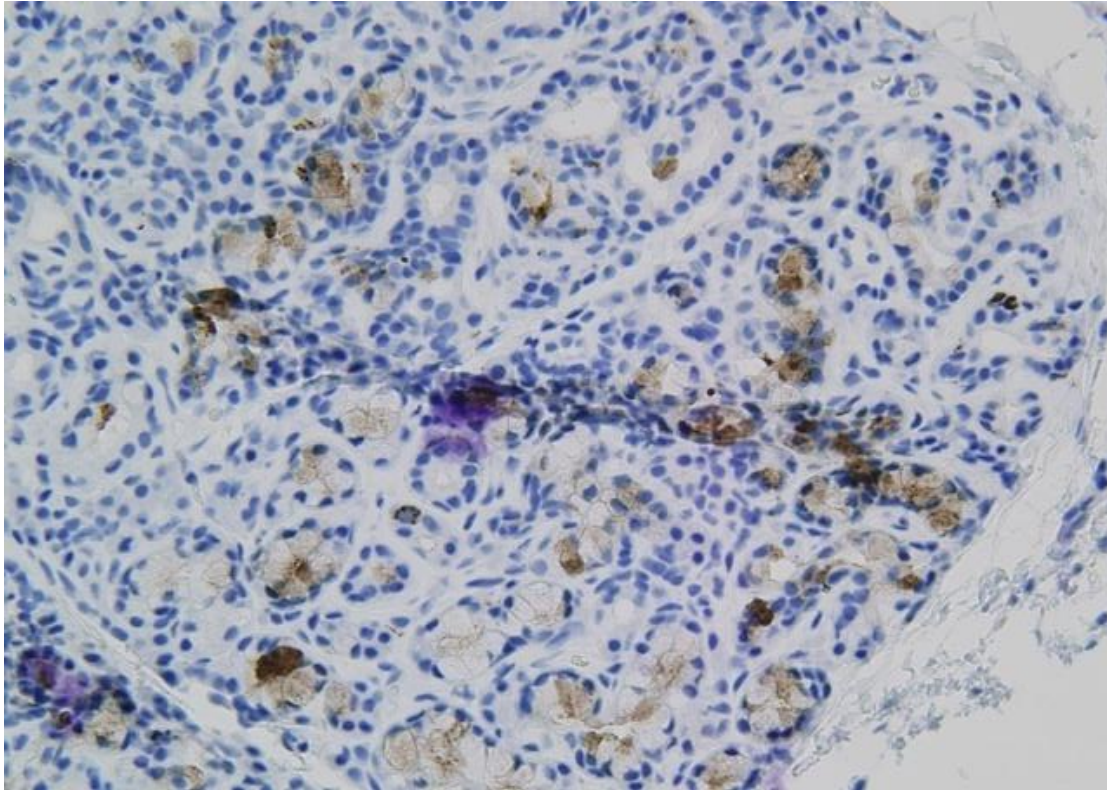
PR immunoreactivity was observed in only two cases: one in group A; and one in group C. In both instances, reactivity was noted only in the acinar cytoplasm. See Figure 8.

Figure 8: Isolated cases of PR immunoreactivity.

8A: In Group A showing weak, diffuse acinar cytoplasmic reactivity (arrows).



8B: In Group C showing very weak variable immunoreactivity in a subset of cells.



Comparison of ratios of ER to AR by group (Group A vs. Group B vs. Group C)

When the ratio of ER to AR is compared, there is no statistically significant difference noted between the groups. No difference in this trend is noted whether ER- α or ER- β is used in the ratio. Although differences do exist, none reach statistical significance. See Table 5A and B.

Table 5A: Comparison of ER- α /AR as a function of FS. No statistically significant comparisons were identified

	ER- α /AR NA	ER- α /AR CA	ER- α /AR DN	ER- α /AR DC
Group A	0.250	0.439	0.458	0.793
Group B	0.226	0.477	0.415	0.810
Group C	0.220	0.523	0.635	0.818
P-Value*	0.9958	0.2965	0.2050	0.9073

*Kruskal-Wallis test

5B: Comparison of ER- β /AR as a function of FS. No statistically significant comparisons were identified

	ER- β /AR NA	ER- β /AR CA	ER- β /AR DN	ER- β /AR DC
Group A	0.639	0.297	0.877	0.314
Group B	0.726	0.310	0.882	0.262
Group C	0.709	0.221	0.929	0.191
P-Value*	0.5040	0.1039	0.3646	0.0522

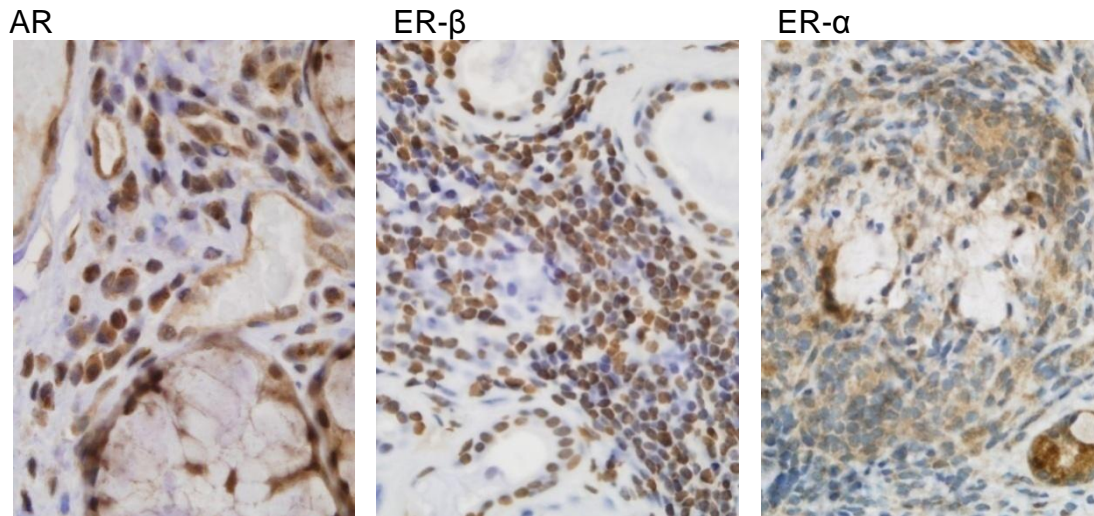
*Kruskal-Wallis test

Other areas of staining within the glands

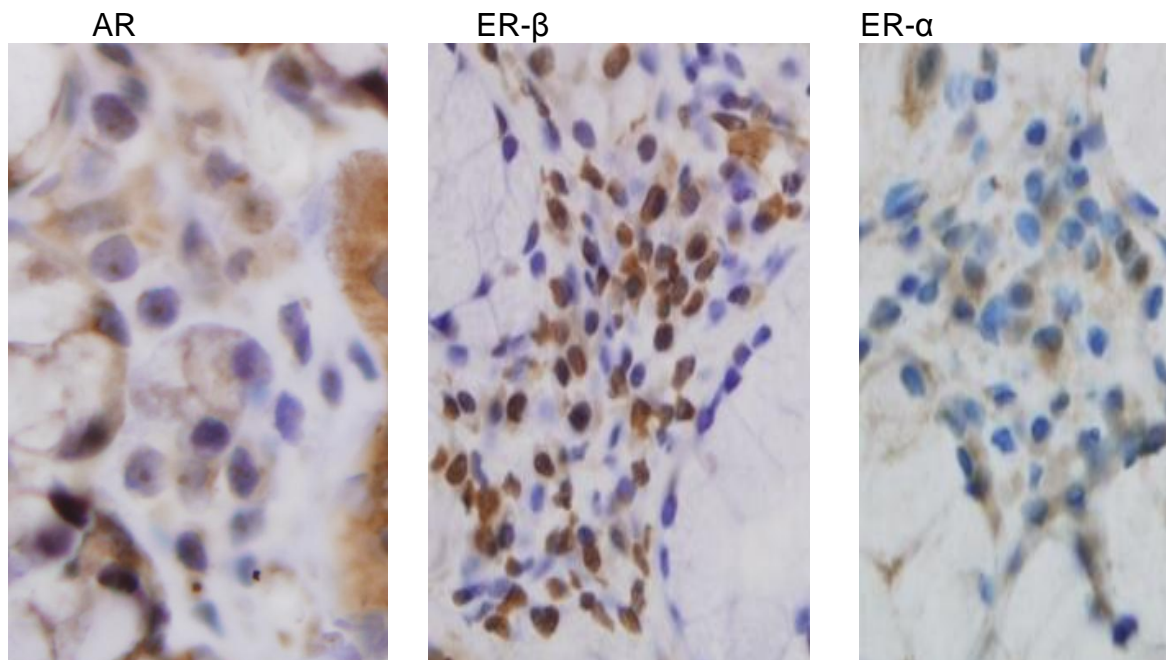
The lymphoplasmacytic foci within the glands of both group A and group B reacted with AR, ER- β , and to a lesser extent, ER- α . Within these foci, the plasma cells were the more immunoreactive cell type, and were mainly stained by AR and to a lesser extent ER- β . In control patients, who also had lymphoplasmacytic infiltrates, were observed to be overall less intensely reactive to the receptor antibodies compared to the sicca patients. See Figure 9.

Figure 9: Differences in immunoreactivity of hormone receptor antibodies in inflammatory infiltrates of sicca patients compared to controls

9A: Comparison of AR, ER- α , and ER- β in sicca patient specimens' lymphoplasmacytic infiltrates. Note the relatively intense reactivity of all the stains with AR > ER- β > ER- α .



9B: Comparison of AR, ER- α , and ER- β in control patient specimens inflammatory cell infiltrates. Note the reduction in intensity of all the antibodies with ER- β > AR > ER- α .



DISCUSSION

In this pilot study we analyzed described a cohort of perimenopausal women who were being evaluated for SS. All of the women met many of the diagnostic criteria for SS except for serologic evidence of SS-A or SS-B in their blood analysis. A MLSG biopsy was indicated to make the final diagnosis. Twenty of the women had a FS \geq 1 in their MLSG biopsy (group A) and therefore were confirmed to have primary SS. Eight other women in this group had a FS $<$ 1 negating the diagnosis of SS (group B), despite meeting many of the other diagnostic criteria. We examined the expression of the sex steroid hormones receptors for differences between these two groups. In addition, we compared the receptor expression in MLSGs of eight sex- age-matched patients who were not being evaluated for SS-like symptoms (group C).

Estrogens are pro-inflammatory while androgens ameliorate inflammation.(35,102,103) We hypothesized that SS is not mediated only by a decrease in systemic and local androgenic influence as some authors have proposed(2); nor is it precipitated by a local, intracrine overproduction of estrogen. We hypothesized also that the balance between the sex steroid hormones' influence is altered, contributing to a pro-inflammatory environment in genetically susceptible individuals. We propose that group A will exhibit a differentially increased expression of ER compared to group B, who should have less of a difference in their ratio of ER to AR. Finally, group C should have relatively more AR

expression compared to the other two groups. By comparing the ratio of estrogen receptor expression to the androgen receptor expression (ER/AR) between the groups, we anticipate finding an increasing trend in this ratio as FS increases.

This hypothesis has its basis in the fact that AR and ER are auto-regulated. (55,104)

This means that the presence of their ligand causes an upregulation of the receptor protein itself in addition to other response proteins. By using the receptor protein expression as our biomarker rather than the presence or absence of the hormones themselves, prevents the erroneous interpretation that measuring hormone level reactivity may foster. The presence of hormone does not necessarily mean it is effecting a change in the cellular functions. Furthermore evaluating “hormone response biomarkers” (CRISP-3, LIV-1) does not account for the possibility of ligand independent stimulation of receptors leading to receptor response protein expression. Additionally, receptor protein expression is pivotal in reflecting the milieu of the cells’ intracrine status. For example, if there is an estrogenic influence enhancing aromatase activity, there will be a consequent increase in the expression of ERs and PRs (90). On the other hand, if there is an androgenic environment, AR protein expression will occur.

While our results show that antibodies for AR, ER- α , ER- β all showed appreciable amounts of immunoreactivity in the glandular parenchyma as well as variable immunostaining in adjacent structures, the three comparison groups did not show any statistically significant difference in their ratio of ER to AR. Furthermore, PR immunoreactivity was seen to be virtually non-existent.

In the SS group, however, a significant trend became apparent: AR had the greatest odds of being reactive regardless of location in the gland or subcellular compartment. For decades it has been proposed that SS is driven by an overall androgen deficiency. (2,8,71,91) More recently, however, tissue-level androgen and DHEA deficiencies have been proposed as a mechanism for the glandular destruction seen in SS.(54,81,88) Results of the present study demonstrate that the predominant presence of AR over the other steroid hormone receptors in SS patients indicates that androgens are present, at least at the tissue and cellular level, in adequate concentrations in these patients as well.

There are several reasons why no statistically significant difference was seen in the reactivity patterns between the groups. Most importantly, sample size was a significant limiting factor in this study, raising the possibility of introducing a type II statistical error (false negative). Our database only yielded twenty-eight patients in whom the inclusion criteria were met. Achieving statistical significance was not possible considering the sample sizes and the number of comparisons attempted. Due to these statistical limitations, we were only able to analyze the SS group for trends, and, even within this group, there were insufficient numbers of patients to be able to analyze each subcellular location for trends involving each receptor protein.

The data did not support the hypothesis as stated, and this could have been caused by several factors. Our study groups were shown to be similar in terms of age as proven by a p value= 0.34. However, the groups may not have differed significantly

in terms of the following clinical parameters: salivary flow, HRT history, corticosteroid use. With a larger sample size these factors could be controlled for.

The main differentiation among the sicca patients was based on FS. It might prove useful in the future to test the inter-observer reliability of the Oral Pathologists (VM and RP) to determine if FS values are reproducible.

Furthermore, the control group sample size was small and was based on a convenience sampling from the tissue archives. The former was driven by the fact that the population age of our study very few MLSG biopsies are performed that yield glandular tissue that could not be excluded for other reasons, such as significant morbidities associated with them. For the purposes of this study, we did not have enough preliminary data to merit enrolling age-, sex-matched control subjects with stricter inclusionary and exclusionary criteria. Therefore, the present control group, being represented by mainly traumatically motivated specimen collection may not be optimal for comparison with our sicca patient sample. Both have inflammatory cell infiltrates: one is autoimmune induced; the other reactive in nature.

Our choice of sex steroid receptors could have been specious; although at the genesis of this project it did not seem so. Controversy exists as to whether the autoimmune inflammatory infiltrates in the main exocrine glands affected by SS were mediated by androgens or estrogens. Studies investigating serum levels of gonadal

and adrenal hormones and prohormones, respectively, showed that SS was a hormone mediated disease, (3,8,51,66,71,105). However, it was Labrie, et al who agreed that SS was hormone mediated, but its pathogenesis may lie at the cellular level of the affected glands.(47,106) Several studies had previously shown that the sex hormones themselves could be demonstrated in the salivary and ocular glands.(89,93) Furthermore, intracrine, steroidogenic enzymes have been demonstrated in both salivary and ocular tissues. (65,90)Laine, et al proved that the effects of androgens' influence were being rendered via androgen response proteins, like CRISP-3, although at lower levels, hypothesizing that an androgen deficiency might be the inciting factor.(88) In summary, past studies have shown that, despite a general systemic deficiency of androgens and estrogens: 1)the receptor ligand was proven to be present; 2)the intracrine machinery to produce this ligand was demonstrated; and 3) the effects of ligand binding to the receptor were shown. However, no studies addressed the expression or localization of the receptor.

Although all of these receptors have been shown to be expressed in the human salivary gland, no study ever established what the normal expression, distribution, or subcellular localization of these receptors was in formalin fixed, paraffin embedded archival tissue. Moreover, the studies that exist use various assays to evaluate the presence of the receptor proteins and do not give consistent results.(69,93-95,107-110) Other studies discussed receptor protein expression in the context of salivary

gland neoplasms but did not compare this expression pattern to normal expression patterns.(92,111,112)

Another reason may be that both AR and ER, in the presence of their ligand at physiologic concentrations, reach a steady state between their activated form in the nucleus and their inactive cytoplasmic form. Once that threshold is reached only minor variations may occur unless the ligand is completely withdrawn (113,114)Even though innumerable copies of the mRNA can be made by a transcription factor (i.e. activated receptor), there are only a finite number of estrogen response elements (EREs) and androgen response elements (AREs) in the DNA with which the receptors can directly interact.

Another explanation for our results may be methodological. Immunohistochemistry has many benefits over ligand binding assays of the past and molecular assays used in more recent studies. (113,115)Most importantly, the preservation of the tissue and cellular architecture allows more accurate assessment of receptor distribution within the cells of interest. However, the IHC technique also has technical pitfalls that may falsely mask significant trends. (113,116)Although the antibodies used are all commercially available and had been previously optimized by our laboratory for use on formalin-fixed paraffin embedded (FFPE) tissue, there can be a progressive loss of antigenicity of ERs in the tissue as time elapses after sectioning.(116-118) This technical detail was not known at the time that multiple levels of the archival specimens were cut in preparation for this and future studies. In

the process, much of the sample tissue was depleted in the paraffin blocks. If all of the receptors were equally affected by this decrease in immunoreactivity, the ratios between them would be intact, albeit less intense. If the ERs are preferentially affected, then a very significant negative bias is could potentially be introduced. Furthermore, the subcellular location of the receptors can preferentially make them more or less susceptible to degradation with tissue processing. (119) Time from excision to fixation, type of fixative, duration of fixation, and section thickness can all impact the immunoreactivity and interpretation of the receptor protein.(116) Estrogen receptors, being housed in the cytoplasm, are particularly vulnerable to tissue processing artifacts.

The methodology for assessing the reactivity can introduce another variable and potential bias. In this study we attempted to adapt the “Allred Score” as a semi-quantitative evaluation of an otherwise ordinal grading scale. The “Allred score” is one of several visual assessment tools used to evaluate immunoreactivity. Others include: the subjective “Gestalt” method where one visually assigns a value of 0 (non-reactive), 1+ (weak), 2+(moderate), 3+ (strong); the “H score”, which includes measures of the percentage of positive nuclei with intensity of the staining; and video image analysis using mean optical density (MOD) of the staining.(113,120,121) All of these methods have unique strengths and weaknesses depending on the situations they are applied. The “Allred score” is specifically used to determine ER/PR immunoreactivity in breast carcinomas to assess the estrogen sensitivity of the tumor and its responsiveness to endocrine therapy (Tamoxifen™).

In this method, a “proportion score” (PS), which is a survey of the percentage of positively staining cells, is combined with a score for the intensity of the immunostaining (intensity score (IS)). It yields a range of possible scores of 0-8, with the exclusion of a score of 1. (99,100) Although the “Allred score” is designed to appreciate the heterogeneity of the reactivity patterns in breast cancer specimens, it begins to lose its sensitivity when attempting to assess subtle variations in immunoreactivity, especially when the proportion score and the intensity diverge equally. For example, if a specimen has 34% of the cells reacting (PS=3), and that reactivity is strong (IS=3), the resultant Allred Score= 6. Conversely, in a different specimen has 100% of the cells reacting (PS=5), but the reactivity is only weak (IS=1), this specimen also has an Allred score =6 even though the immunoreactive patterns of the two specimens differ greatly. Furthermore, in surgical pathology, any “Allred score” <3 is considered to be negative. It was not designed for “shades of gray”, but more for accurate assessment of grossly positive vs. negative.

Upon its introduction, the Allred score was shown to be reproducible when utilized by different observers. In the current study, only one observer (JR) scored the IHC immunoreactivity. At the time of the experiment, no previous studies provided insight into the trends that might emerge. Because of this, the data collected was assumed to be reliable. A possible source of error in the data may have been a lack of intra-observer reliability. In the future, it would be prudent to perform an intra-observer reliability assessment.

The use of a ratio of hormone receptors to explore the delicate balance that may exist between the pro-inflammatory influences of the estrogens and the anti-inflammatory influences of the androgens. It was hoped that the previously proven androgen deficiency combined with the increasing estrogenic environment of the glands would easily become apparent. Unfortunately, the use of ratios may mask significant trends. This would be particularly true if there were significant, but proportional changes in both the numerator and the denominator.

The patterns of the receptor proteins' in our study are intriguing. As discussed above, AR reactivity was significantly more common than any of the other receptor proteins. We observed intense AR immunoreactivity in most of the nuclei of the acinar cells and good reactivity, but less reactivity in the cytoplasm of the acini compared to the nuclei. The cytoplasmic reactivity highlighted the serous demilunes. As seen in figure 3B.

This result is consistent with a previous study of AR localization in the salivary glands of humans by Laine, et al.(69) Alternatively, our results differ with a later study by the same group. In 2007, Laine's group found that an androgen response protein, crisp-3, was polarized to the area of serous demilunes in healthy controls, but lost this polarization in SS patients.(88) In contrast to this finding, our study showed that despite the presence of SS, AR reactivity seemed more intense in the cytoplasm of the serous demilunes. In the ductal cells, a similar pattern emerged,

although the ductal cell cytoplasm and nuclei were both quite immunoreactive. As seen in figure 2A.

All of the sex steroid hormones are ligand-binding transcription factors that are produced in the cytoplasm and then either reside in the cytosol in an inactive state, as is the case with AR, or are shuttled into the nucleus prior to interacting with their hormone ligand, as seen with ER and PR. (122) If there is cytoplasmic AR reactivity, this is evidence of the presence of the receptor, however it is unbound, inactive, and not resulting in DNA transcription (119,123,123). In the present study, the nuclei of acinar cells exhibited the greatest AR reactivity, indicating effective transcription. In the ductal cells, however, it appears that either no hormone is available or that it is not causing active transcription via the classic receptor pathway.

The reaction pattern of ER- β revealed an almost exclusive nuclear reactivity which decorated the periphery of ducts and to a lesser degree, acini. Architecturally it was difficult to determine if the acinar reactivity was in mucocytes, myoepithelial cells, or a combination of both. As seen in figure 7A. This pattern was seen throughout the entire specimen, although, in areas of immunoreactivity, not all of the cells expressed ER- β positivity. Very minimal cytoplasmic reactivity was noted, whether in proportion of cells reacting or in intensity. The almost exclusive nuclear reactivity is consistent with the findings of Valimaa, et al in 2004.(75) In the present study, consistent immunoreactivity of nuclei localized to the periphery of the acini and ducts, in the position often occupied by myoepithelial cells, might indicate reactivity

of these cells. Further studies, including possible double labeling of these cells may answer that question.

When ER- α staining is observed, there is a distinct reduction in intensity of the tissue reactivity. ER- α was found diffusely staining the cytoplasm of the ductal cells, and to lesser extent acinar cells. An interesting observation, unique to ER- α , was that, within the cytoplasm immediately adjacent to the nucleus, were noted numerous focally intense globular areas of immunoreactivity (Figure 5). This phenomenon was seen in a number of specimens in both acinar and ductal cells, and in all groups compared. No mention of this type of staining could be found in the literature, but it is reminiscent of perinuclear, "Golgi dot" pattern seen with CD30 reactivity in Hodgkin lymphoma and the CD117 (C-kit) reactivity in gastrointestinal stromal tumors (GISTs). This pattern may indicate a derangement in the post translational maturation and subsequent sequestration within the Golgi apparatus of the ER- α receptor protein. (124)

Another interesting finding is the intense immunoreactivity of ER- α in inspissated mucous. This was not seen with the other receptor antibodies. It was not seen in the extravasated mucous of the mucocele patients in the control group. Spaan, et al recently demonstrated that there is an intracellular architecture to the intracrine enzymes involved in processing DHEA-S to the active hormones, in which aromatase was preferentially located near the apical pole of the acinar cells.(81) Aromatase is the enzyme needed to catalyze the conversion of testosterone to

estradiol. Porola also has shown that SS patients have slightly higher concentrations of salivary estrogen.(54) One could conceive of a process where aromatase catalyzes the conversion of testosterone to estradiol and then the estradiol is secreted with the salivary mucins. Moreover, if there is nascent estradiol in excess, like in a SS patient, this may bind the cytoplasmic ER and both could then be found in the saliva as well. There has been no mention in the literature of transport of hormone receptor protein out of the cell, so the reason and significance of this finding remains to be explained.

Our study reveals immunoreactivity with ER- α contrary to previous studies. In 1992, Ojanotko-Harri, et al found only AR in sections of gingival and buccal mucosa.(108)In 2000, Leimola-Virtanen, et al demonstrated ER expression in mucosa and salivary gland only by RT-PCR but was not able to demonstrate its expression by IHC. It is assumed that both of these studies would have used antibodies for ER- α protein. The 1992 study was prior to the discovery of ER- β in 1996, and Leimola-Virtanen did not state which ER antibody they used. By 2000, however, even though ER- β had been discovered, there may not have been an antibody for it commercially available. Moreover, Valimaa, in 2004 stated definitively that ER- β was the only ER found in human oral buccal and gingival epithelium or in human salivary glands. Could it be that our ER- α reactivity is artifactual? Evidence in support of this possibly lies in the lack of immunoreactivity to PR in all but two of our specimens. In both cases, however, higher cytoplasmic reactivity of ER- α was seen when compared to other specimens.

In the breast, it is well documented that PR is under estrogenic control.

(114,125,126) Progesterone receptor expression indicates that the stimulation of ER by its hormone ligand has resulted in transcription of the DNA to induce PR expression. In the salivary glands, only Ozono showed evidence for PR expression and stated that it was exclusively found in cells that also contained estrogen and progesterone hormones in their cytoplasm despite the variable numbers of PR + cells. (93) In breast cancer patients ER/PR expression is a useful prognostic indicator. (127) ER+/PR+ tumors convey a favorable prognosis, and tumors that express neither receptor do not. Ironically, a tumor can express PR in the absence of ER and this still confers a favorable prognosis due to the inter dependence of PR on ER. (126) Considering this, it is conceivable that a cell can be ER+ and yet PR- . This may explain our results showing a variable and inconsistent PR immunoreactivity.

Sjögren syndrome is characterized by lympho-plasmacytic infiltrates into the parenchyma of salivary and ocular glands.(128) The inflammatory cell infiltrates in the specimens in this study were commonly found to be immunoreactive for AR, ER- β , and ER- α in a decreasing order of intensity. There were variable patterns depending on the receptor, the cell type reacting, as well as the source of the inflammation. Androgen receptor reactivity was the most intense, and highlighted numerous plasma cells. This reactivity was seen mainly in the cytoplasm, but this may be due to the fact that plasma cells have abundant visible cytoplasm on tissue sections compared to lymphocytes. This finding is supported by others who have

demonstrated hormone receptors being expressed in immune cells, and ER- β reactivity in the lymphocytic infiltrates has also been seen in breast tissue. (129-131) What is interesting is that in 2007, Pernis, et al showed that CD4+ T-lymphocytes expressed ER- α and B- lymphocytes expressed ER- β . (132) Considering that the lymphocytic infiltrates of SS comprise mainly T- lymphocytes, it is unusual that ER- α should be the least intense in our specimens. Furthermore, in 1995, Ono, et al, using a mouse model of SS was unable to demonstrate AR expression in the lymphocytic infiltrates within the lacrimal glands.(104)Future studies exploring the immunophenotyping of the inflammatory cell infiltrates of both AI-induced and traumatically induced inflammation would be valuable.

CONCLUSIONS

The data presented does not support the hypothesis as stated. Therefore we conclude that:

No difference in the ratios of ER- α or ER- β to AR exists in MLSG biopsy specimens of patients with sicca syndrome regardless of focus score.

However, the data has revealed other significant trends such as:

1. In MLSG biopsies of SS patients, the likelihood of finding sex hormone receptor immunoreactivity is higher in the nucleus of the acinar cells regardless of which hormone is being evaluated.
2. The likelihood of observing intense AR immunoreactivity in MLSG of Sjögren syndrome patients is 37.7 times greater than ER- α and 7.7 times greater than ER- β regardless of subcellular location.
3. Mucous secretions within the glands of patients with sicca are immunoreactive with ER- α while they are not reactive in patients from the control group. All other hormone receptors were not found in inspissated mucous secretions from any of the groups.

4. Lymphoplasmacytic foci within MSLG specimens were immunoreactive to ER- β , AR, and ER- α ; and qualitatively more intense in the sicca patients than in the control group.

Future Direction

- 1) Repeat the study with a larger sample size allowing for more elegant statistical analysis and reduction in the potential for type II statistical error.
- 2) Based on the current results, reanalyzing the overall ER expression versus the overall AR without designation of the glandular and subcellular compartments. This can be accomplished utilizing raw data scores as opposed to ratios of ER to AR.
- 3) Intentional enrollment of age-, sex-matched control patients who have no clinical evidence of inflammation in the MLSGs.
- 4) Prospective study where potential participants are identified and enrolled prior to MLSG biopsy so as to better control how specimens are handled prior to processing. This allows for comparison of reactivity of both fresh frozen as well as FFPE tissue.
- 5) Compare results to clinical measurements of disease, clinical outcomes, response to therapies, etc.
- 6) Attempt double-labeling of cells for better localization of hormone receptor expression within glands and within inflammatory cell infiltrates.
 - a) Myoepithelial markers to highlight myoepithelial cells from acinar and luminal ductal cells

- b) B- and T-cell markers to distinguish which cells are more intensely staining with the hormone receptor antibodies
- 7) Utilize laser capture micro-dissection to enable molecular studies on hormone receptor expression of acini vs. ducts vs. lymphoplasmacytic infiltrates.
- 8) Improved methodology:
 - (a) Intra-observer testing to validate reproducibility of the IHC scoring
 - (b) Inter-observer testing to validate consistency in the FS of the sicca patients.
 - (c) Utilize several analysis methods (manual and automated) for comparison of reproducibility and sensitivity.
 - (d) Utilize digital cell counters to facilitate standard protocol for better quantification of numbers of cells reacting.
 - (e) Section specimens at a thinner dimension to reduce overlap of cells within the glandular parenchyma.

REFERENCES

1. Kassan SS, Moutsopoulos HM. Clinical manifestations and early diagnosis of Sjogren syndrome. *Arch Intern Med* 2004;164:1275-84.
2. Sullivan DA, Belanger A, Cermak JM, et al. Are women with Sjogren's syndrome androgen-deficient? *J Rheumatol* 2003;30:2413-9.
3. Sullivan DA. Sex hormones and Sjogren's syndrome. *J Rheumatol Suppl* 1997;50:17-32.
4. Fox RI. Sjogren's syndrome. *Lancet* 2005;366:321-31.
5. Treister NS, Richards SM, Lombardi MJ, Rowley P, Jensen RV, Sullivan DA. Sex-related differences in gene expression in salivary glands of BALB/c mice. *J Dent Res* 2005;84:160-5.
6. Treister NS, Richards SM, Rowley P, Jensen RV, Sullivan DA. Influence of testosterone on gene expression in the ovariectomized mouse submandibular gland. *Eur J Oral Sci* 2006;114:328-36.
7. Cutolo M, Capellino S, Sulli A, et al. Estrogens and autoimmune diseases. *Ann N Y Acad Sci* 2006;1089:538-47.
8. Sullivan DA. Androgen deficiency & dry eye syndromes. *Arch Soc Esp Oftalmol* 2004;79:49-50.
9. Suzuki T, Schaumberg DA, Sullivan BD, et al. Do estrogen and progesterone play a role in the dry eye of Sjogren's syndrome? *Ann N Y Acad Sci* 2002;966:223-5.
10. Schaumberg DA, Buring JE, Sullivan DA, Dana MR. Hormone replacement therapy and dry eye syndrome. *JAMA* 2001;286:2114-9.

11. Ishimaru N, Saegusa K, Yanagi K, Haneji N, Saito I, Hayashi Y. Estrogen deficiency accelerates autoimmune exocrinopathy in murine Sjogren's syndrome through fas-mediated apoptosis. *Am J Pathol* 1999;155:173-81.
12. Nagler RM, Pollack S. Sjogren's syndrome induced by estrogen therapy. *Semin Arthritis Rheum* 2000;30:209-14.
13. Stern ME, Gao J, Siemasko KF, Beuerman RW, Pflugfelder SC. The role of the lacrimal functional unit in the pathophysiology of dry eye. *Exp Eye Res* 2004;78:409-416.
14. Ahmed SA, Aufdemorte TB, Chen JR, Montoya AI, Olive D, Talal N. Estrogen induces the development of autoantibodies and promotes salivary gland lymphoid infiltrates in normal mice. *J Autoimmun* 1989;2:543-52.
15. Sjogren H. Zur kenntnis der keratocconjunctivitis sicca (keratitis follofornis bei hypofunktion der tranendrusen). *Acta Ophthamol (Copenh)* 1933;11:1,2-151.
16. Srinivasan S, Slomovic AR. Sjogren syndrome. *Compr Ophthalmol Update* 2007;8:205-12.
17. Bloch KJ, Buchanan WW, Wolh MJ, Bunim JJ. Sjoegren's Syndrome. a Clinical, Pathological, and Serological Study of Sixty-Two Cases. *Medicine (Baltimore)* 1965;44:187-231.
18. Fox PC. Autoimmune diseases and Sjogren's syndrome: an autoimmune exocrinopathy. *Ann N Y Acad Sci* 2007;1098:15-21.
19. Baudouin C. The pathology of dry eye. *Surv Ophthalmol* 2001;45 Suppl 2:S211-220.
20. Connolly MK. Sjogern's syndrome. *Semin Cutan Med Surg* 2001;20:46-52.
21. Kassan SS, Thomas TL, Moutsopoulos HM, et al. Increased risk of lymphoma in sicca syndrome. *Ann Intern Med* 1978;89:888-92.

22. Zintzaras E, Voulgarelis M, Moutsopoulos HM. The risk of lymphoma development in autoimmune diseases: a meta-analysis. *Arch Intern Med* 2005;165:2337-44.
23. Theander E, Henriksson G, Ljungberg O, Mandl T, Manthorpe R, Jacobsson LT. Lymphoma and other malignancies in primary Sjogren's syndrome: a cohort study on cancer incidence and lymphoma predictors. *Ann Rheum Dis* 2006;65:796-803.
24. Vitali C, Bombardieri S, Jonsson R, et al. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002;61:554-8.
25. Manthorpe R, Oxholm P, Prause JU, Schiodt M. The Copenhagen criteria for Sjogren's syndrome. *Scand J Rheumatol Suppl* 1986;61:19-21.
26. Manthorpe R. Sjogren's syndrome criteria. *Ann Rheum Dis* 2002;61:482-4.
27. Homma M, Tojo T, Akizuki M, Yamagata H. Criteria for Sjogren's syndrome in Japan. *Scand J Rheumatol Suppl* 1986;61:26-7.
28. Anaya JM, Liu GT, D'Souza E, Ogawa N, Luan X, Talal N. Primary Sjogren's syndrome in men. *Ann Rheum Dis* 1995;54:748-51.
29. Horvath IF, Szodoray P, Zeher M. Primary Sjogren's syndrome in men: clinical and immunological characteristic based on a large cohort of Hungarian patients. *Clin Rheumatol* 2008;27:1479-83.
30. Fox RI. Sjogren's syndrome. *Curr Opin Rheumatol* 1995;7:409-16.
31. Cooper GS, Stroehla BC. The epidemiology of autoimmune diseases. *Autoimmun Rev* 2003;2:119-25.
32. Da Silva JA. Sex hormones, glucocorticoids and autoimmunity: facts and hypotheses. *Ann Rheum Dis* 1995;54:6-16.

33. Ansar Ahmed S, Penhale WJ, Talal N. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol* 1985;121:531-51.
34. Ansar Ahmed S, Talal N. Sex hormones and autoimmune rheumatic disorders. *Scand J Rheumatol* 1989;18:69-76.
35. Grossman C. Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. *J Steroid Biochem* 1989;34:241-51.
36. Cutolo M, Lahita RG. Estrogens and arthritis. *Rheum Dis Clin North Am* 2005;31:19,27, vii.
37. Lahita RG. Sex hormones and systemic lupus erythematosus. *Rheum Dis Clin North Am* 2000;26:951-68.
38. Siiteri PK, Jones LA, Roubinian J, Talal N. Sex steroids and the immune system-I. Sex difference in autoimmune disease in NZB/NZW hybrid mice. *J Steroid Biochem* 1980;12:425-32.
39. Steinberg AD. Role of sex hormones in autoimmune disease: comment on the article by Nelson. *Arthritis Rheum* 1996;39:1766-7.
40. Roubinian J, Talal N, Siiteri PK, Sadakian JA. Sex hormone modulation of autoimmunity in NZB/NZW mice. *Arthritis Rheum* 1979;22:1162-9.
41. Michalski JP, McCombs CC, Roubinian JR, Talal N. Effect of androgen therapy on survival and suppressor cell activity in aged NZB/NZW F1 hybrid mice. *Clin Exp Immunol* 1983;52:229-33.
42. Rocha EM, Wickham LA, Huang Z, et al. Presence and testosterone influence on the levels of anti- and pro-inflammatory cytokines in lacrimal tissues of a mouse model of Sjogren's syndrome. *Adv Exp Med Biol* 1998;438:485-91.
43. Sullivan DA, Edwards JA. Androgen stimulation of lacrimal gland function in mouse models of Sjogren's syndrome. *J Steroid Biochem Mol Biol* 1997;60:237-45.

44. Cutolo M, Sulli A, Capellino S, et al. Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity. *Lupus* 2004;13:635-8.
45. Ackerman LS. Sex hormones and the genesis of autoimmunity. *Arch Dermatol* 2006;142:371-6.
46. Burger H, Woods NF, Dennerstein L, Alexander JL, Kotz K, Richardson G. Nomenclature and endocrinology of menopause and perimenopause. *Expert Rev Neurother* 2007;7:S35-43.
47. Labrie F, Belanger A, Simard J, Van L, Labrie C. DHEA and peripheral androgen and estrogen formation: intracrinology. *Ann N Y Acad Sci* 1995;774:16-28.
48. Labrie F, Belanger A, Cusan L, Gomez JL, Candas B. Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. *J Clin Endocrinol Metab* 1997;82:2396-402.
49. Gonzales GF, Gonez C, Villena A. Adrenopause or decline of serum adrenal androgens with age in women living at sea level or at high altitude. *J Endocrinol* 2002;173:95-101.
50. Valtysdottir ST, Wide L, Hallgren R. Low serum dehydroepiandrosterone sulfate in women with primary Sjogren's syndrome as an isolated sign of impaired HPA axis function. *J Rheumatol* 2001;28:1259-65.
51. Taiym S, Haghghat N, Al-Hashimi I. A comparison of the hormone levels in patients with Sjogren's syndrome and healthy controls. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2004;97:579-83.
52. Ishimaru N, Arakaki R, Watanabe M, Kobayashi M, Miyazaki K, Hayashi Y. Development of autoimmune exocrinopathy resembling Sjogren's syndrome in estrogen-deficient mice of healthy background. *Am J Pathol* 2003;163:1481-90.
53. Hayashi Y, Arakaki R, Ishimaru N. Apoptosis and estrogen deficiency in primary Sjogren syndrome. *Curr Opin Rheumatol* 2004;16:522-6.

54. Porola P, Virkki L, Przybyla BD, et al. Androgen deficiency and defective intracrine processing of dehydroepiandrosterone in salivary glands in Sjogren's syndrome. *J Rheumatol* 2008;35:2229-35.
55. Sullivan DA, Wickham LA, Rocha EM, Kelleher RS, da Silveira LA, Toda I. Influence of gender, sex steroid hormones, and the hypothalamic-pituitary axis on the structure and function of the lacrimal gland. *Adv Exp Med Biol* 1998;438:11-42.
56. Murai S, Saito H, Masuda Y, Itoh T, Kawaguchi T. Sex-dependent differences in the concentrations of the principal neurotransmitters, noradrenaline and acetylcholine, in the three major salivary glands of mice. *Arch Oral Biol* 1998;43:9-14.
57. Denny PC, Denny PA, Chai Y, Klauser DK. DNA synthesis and development strategies with possible consequences on sexual dimorphism in adult mouse submandibular glands. *Crit Rev Oral Biol Med* 1993;4:511-6.
58. Senorale-Pose M, Jacqueson A, Rougeon F, Rosinski-Chupin I. Acinar cells are target cells for androgens in mouse submandibular glands. *J Histochem Cytochem* 1998;46:669-78.
59. Richards SM, Jensen RV, Liu M, et al. Influence of sex on gene expression in the mouse lacrimal gland. *Exp Eye Res* 2006;82:13-23.
60. Hosoi K, Kobayashi S, Ueha T. Sex difference in L-glutamine D-fructose-6-phosphate aminotransferase activity of mouse submandibular gland. *Biochim Biophys Acta* 1978;543:283-92.
61. Richards SM, Liu M, Jensen RV, et al. Androgen regulation of gene expression in the mouse lacrimal gland. *J Steroid Biochem Mol Biol* 2005;96:401-13.
62. 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.
63. Sullivan DA, Sullivan BD, Ullman MD, et al. Androgen influence on the meibomian gland. *Invest Ophthalmol Vis Sci* 2000;41:3732-42.

64. Schirra F, Suzuki T, Richards SM, et al. Androgen control of gene expression in the mouse meibomian gland. *Invest Ophthalmol Vis Sci* 2005;46:3666-75.
65. Rocha EM, Wickham LA, da Silveira LA, et al. Identification of androgen receptor protein and 5alpha-reductase mRNA in human ocular tissues. *Br J Ophthalmol* 2000;84:76-84.
66. Sullivan DA, Schaumberg DA, Suzuki T, et al. Sex steroids, meibomian gland dysfunction and evaporative dry eye in Sjogren's syndrome. *Lupus* 2002;11:667.
67. Wickham LA, Gao J, Toda I, Rocha EM, Ono M, Sullivan DA. Identification of androgen, estrogen and progesterone receptor mRNAs in the eye. *Acta Ophthalmol Scand* 2000;78:146-53.
68. Li HJ, Yao B, Liang W, Wang YM, Xu QL, Huang YF. Localization and potential function of androgen receptor in rat salivary gland. *Asian J Androl* 2005;7:295-301.
69. Laine M, Blauer M, Ylikomi T, et al. Immunohistochemical demonstration of androgen receptors in human salivary glands. *Arch Oral Biol* 1993;38:299-302.
70. Sato EH, Ariga H, Sullivan DA. Impact of androgen therapy in Sjogren's syndrome: hormonal influence on lymphocyte populations and Ia expression in lacrimal glands of MRL/Mp-lpr/lpr mice. *Invest Ophthalmol Vis Sci* 1992;33:2537-45.
71. Sullivan DA, Krenzer KL, Sullivan BD, Tolls DB, Toda I, Dana MR. Does androgen insufficiency cause lacrimal gland inflammation and aqueous tear deficiency? *Invest Ophthalmol Vis Sci* 1999;40:1261-5.
72. Sullivan DA, Sullivan BD, Evans JE, et al. Androgen deficiency, Meibomian gland dysfunction, and evaporative dry eye. *Ann N Y Acad Sci* 2002;966:211-22.
73. Campbell PS, Ben-Aryeh H, Swanson KA. Differential distribution of an estrogen receptor in the submandibular and parotid salivary glands of female rats. *Endocr Res* 1990;16:333-45.

74. Camacho-Arroyo I, Cerbon MA, Gamboa-Dominguez A, Gonzalez-Aguero G, Gonzalez-Mariscal G. Immunocytochemical detection of estrogen and progesterone receptors in the rabbit submandibular gland. *Comp Biochem Physiol A Mol Integr Physiol* 1999;123:179-86.
75. Valimaa H, Savolainen S, Soukka T, et al. Estrogen receptor-beta is the predominant estrogen receptor subtype in human oral epithelium and salivary glands. *J Endocrinol* 2004;180:55-62.
76. Kassi E, Moutsatsou P, Sekeris CE, Moutsopoulos HM, Manoussakis MN. Oestrogen receptors in cultured epithelial cells from salivary glands of Sjogren's syndrome patients. *Rheumatology (Oxford)* 2003;42:1120-2.
77. Wardrop RW, Hailes J, Burger H, Reade PC. Oral discomfort at menopause. *Oral Surg Oral Med Oral Pathol* 1989;67:535-40.
78. Forabosco A, Criscuolo M, Coukos G, et al. Efficacy of hormone replacement therapy in postmenopausal women with oral discomfort. *Oral Surg Oral Med Oral Pathol* 1992;73:570-4.
79. Esmaeli B, Harvey JT, Hewlett B. Immunohistochemical evidence for estrogen receptors in meibomian glands. *Ophthalmology* 2000;107:180-4.
80. Spelsberg H, Klueppel M, Reinhard T, et al. Detection of oestrogen receptors (ER) alpha and beta in conjunctiva, lacrimal gland, and tarsal plates. *Eye* 2004;18:729-33.
81. Spaan M, Porola P, Laine M, Rozman B, Azuma M, Konttinen YT. Healthy human salivary glands contain a DHEA-sulphate processing intracrine machinery, which is deranged in primary Sjogren's syndrome. *J Cell Mol Med* 2009;13:1261-70.
82. Labrie F. Extragonadal synthesis of sex steroids: intracrinology. *Ann Endocrinol (Paris)* 2003;64:95-107.
83. Wakamatsu E, Nakamura Y, Matsumoto I, et al. DNA microarray analysis of labial salivary glands of patients with Sjogren's syndrome. *Ann Rheum Dis* 2007;66:844-5.

84. Hu S, Wang J, Meijer J, et al. Salivary proteomic and genomic biomarkers for primary Sjogren's syndrome. *Arthritis Rheum* 2007;56:3588-600.
85. Hjelmervik TO, Petersen K, Jonassen I, Jonsson R, Bolstad AI. Gene expression profiling of minor salivary glands clearly distinguishes primary Sjogren's syndrome patients from healthy control subjects. *Arthritis Rheum* 2005;52:1534-44.
86. Brennan MT, Fox PC. Sex differences in primary Sjogren's syndrome. *J Rheumatol* 1999;26:2373-6.
87. Brennan MT, Sankar V, Leakan RA, et al. Sex steroid hormones in primary Sjogren's syndrome. *J Rheumatol* 2003;30:1267-71.
88. Laine M, Porola P, Udby L, et al. Low salivary dehydroepiandrosterone and androgen-regulated cysteine-rich secretory protein 3 levels in Sjogren's syndrome. *Arthritis Rheum* 2007;56:2575-84.
89. Kumagami H, Onitsuka T. Estradiol and testosterone in minor salivary glands of Sjogren's syndrome. *Auris Nasus Larynx* 1993;20:137-43.
90. Onodera K, Sasano H, Ichinohasama R, Ooya K. Immunolocalization of aromatase in human minor salivary glands of the lower lip with primary Sjogren's syndrome. *Pathol Int* 1998;48:786-90.
91. Sullivan DA, Wickham LA, Rocha EM, et al. Androgens and dry eye in Sjogren's syndrome. *Ann N Y Acad Sci* 1999;876:312-24.
92. Williams MD, Roberts D, Blumenschein GR, Jr, et al. Differential expression of hormonal and growth factor receptors in salivary duct carcinomas: biologic significance and potential role in therapeutic stratification of patients. *Am J Surg Pathol* 2007;31:1645-52.
93. Ozono S, Onozuka M, Sato K, Ito Y. Immunohistochemical localization of estradiol, progesterone, and progesterone receptor in human salivary glands and salivary adenoid cystic carcinomas. *Cell Struct Funct* 1992;17:169-75.

94. Pires FR, da Cruz Perez DE, de Almeida OP, Kowalski LP. Estrogen receptor expression in salivary gland mucoepidermoid carcinoma and adenoid cystic carcinoma. *Pathol Oncol Res* 2004;10:166-8.
95. Dimery IW, Jones LA, Verjan RP, Raymond AK, Goepfert H, Hong WK. Estrogen receptors in normal salivary gland and salivary gland carcinoma. *Arch Otolaryngol Head Neck Surg* 1987;113:1082-5.
96. Levin ER. Rapid signaling by steroid receptors. *Am J Physiol Regul Integr Comp Physiol* 2008;295:R1425-30.
97. Levin ER. Plasma membrane estrogen receptors. *Trends Endocrinol Metab* 2009;20:477-82.
98. Lemp MA. Evaluation and differential diagnosis of keratoconjunctivitis sicca. *J Rheumatol Suppl* 2000;61:11-4.
99. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998;11:155-68.
100. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* 1999;17:1474-81.
101. Shousha S. Oestrogen receptor status of breast carcinoma: Allred/H score conversion table. *Histopathology* 2008;53:346-7.
102. Cutolo M, Capellino S, Straub RH. Oestrogens in rheumatic diseases: friend or foe? *Rheumatology (Oxford)* 2008;47 Suppl 3:iii2-5.
103. Craft RM. Modulation of pain by estrogens. *Pain* 2007;132 Suppl 1:S3-12.
104. Ono M, Rocha FJ, Sullivan DA. Immunocytochemical location and hormonal control of androgen receptors in lacrimal tissues of the female MRL/Mp-lpr/lpr mouse model of Sjogren's syndrome. *Exp Eye Res* 1995;61:659-66.

105. Porola P, Laine M, Virkki L, Poduval P, Konttinen YT. The influence of sex steroids on Sjogren's syndrome. *Ann N Y Acad Sci* 2007;1108:426-32.
106. Labrie F, Belanger A, Luu-The V, et al. DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging. *Steroids* 1998;63:322-8.
107. Lamey PJ, Leake RE, Cowan SK, Soutar DS, McGregor IA, McGregor FM. Steroid hormone receptors in human salivary gland tumours. *J Clin Pathol* 1987;40:532-4.
108. Ojanotko-Harri A, Forssell H, Laine M, Hurttia H, Blauer M, Tuohimaa P. Immunohistochemical detection of androgen receptors in human oral mucosa. *Arch Oral Biol* 1992;37:511-4.
109. Shick PC, Riordan GP, Foss RD. Estrogen and progesterone receptors in salivary gland adenoid cystic carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1995;80:440-4.
110. Leimola-Virtanen R, Salo T, Toikkanen S, Pulkkinen J, Syrjanen S. Expression of estrogen receptor (ER) in oral mucosa and salivary glands. *Maturitas* 2000;36:131-7.
111. Nasser SM, Faquin WC, Dayal Y. Expression of androgen, estrogen, and progesterone receptors in salivary gland tumors. Frequent expression of androgen receptor in a subset of malignant salivary gland tumors. *Am J Clin Pathol* 2003;119:801-6.
112. Dori S, Trougouboff P, David R, Buchner A. Immunohistochemical evaluation of estrogen and progesterone receptors in adenoid cystic carcinoma of salivary gland origin. *Oral Oncol* 2000;36:450-3.
113. Mohler JL. A role for the androgen-receptor in clinically localized and advanced prostate cancer. *Best Pract Res Clin Endocrinol Metab* 2008;22:357-72.
114. Horwitz KB, McGuire WL. Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. *J Biol Chem* 1978;253:2223-8.

115. Kim D, Gregory CW, Smith GJ, Mohler JL. Immunohistochemical quantitation of androgen receptor expression using color video image analysis. *Cytometry* 1999;35:2-10.
116. Poulsen HS, Ozzello L, King WJ, Greene GL. The use of monoclonal antibodies to estrogen receptors (ER) for immunoperoxidase detection of ER in paraffin sections of human breast cancer tissue. *J Histochem Cytochem* 1985;33:87-92.
117. Fergenbaum JH, Garcia-Closas M, Hewitt SM, Lissowska J, Sakoda LC, Sherman ME. Loss of antigenicity in stored sections of breast cancer tissue microarrays. *Cancer Epidemiol Biomarkers Prev* 2004;13:667-72.
118. DiVito KA, Charette LA, Rimm DL, Camp RL. Long-term preservation of antigenicity on tissue microarrays. *Lab Invest* 2004;84:1071-8.
119. Nguyen MM, Dincer Z, Wade JR, et al. Cytoplasmic localization of the androgen receptor is independent of calreticulin. *Mol Cell Endocrinol* 2009;302:65-72.
120. Dabbs DJ. *Diagnostic Immunohistochemistry*. 2006;1:828.
121. McCarty KS, Jr, Miller LS, Cox EB, Konrath J, McCarty KS S. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. *Arch Pathol Lab Med* 1985;109:716-21.
122. Dahlman-Wright K, Cavailles V, Fuqua SA, et al. International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacol Rev* 2006;58:773-81.
123. Bai Z, Gust R. Breast cancer, estrogen receptor and ligands. *Arch Pharm (Weinheim)* 2009;342:133-49.
124. Emile JF, Stock N, Corless CL, et al. Dotlike or Golgi-like KIT and PDGFRA Staining in GISTs. *Am J Surg Pathol* 2009;33:157,8; author reply 157-9.

125. McGuire WL, Zava DT, Horwitz KB, Chamness GC. Steroid receptors in breast tumors--current status. *Curr Top Exp Endocrinol* 1978;3:93-129.

126. Rosa FE, Caldeira JR, Felipes J, et al. Evaluation of estrogen receptor alpha and beta and progesterone receptor expression and correlation with clinicopathologic factors and proliferative marker Ki-67 in breast cancers. *Hum Pathol* 2008;39:720-30.

127. Scarpin KM, Graham JD, Mote PA, Clarke CL. Progesterone action in human tissues: regulation by progesterone receptor (PR) isoform expression, nuclear positioning and coregulator expression. *Nucl Recept Signal* 2009;7:e009.

128. Margaix-Munoz M, Bagan JV, Poveda R, Jimenez Y, Sarrion G. Sjogren's syndrome of the oral cavity. Review and update. *Med Oral Patol Oral Cir Bucal* 2009;14:E325-30.

129. Benten WP, Becker A, Schmitt-Wrede HP, Wunderlich F. Developmental regulation of intracellular and surface androgen receptors in T cells. *Steroids* 2002;67:925-31.

130. Benten WP, Stephan C, Wunderlich F. B cells express intracellular but not surface receptors for testosterone and estradiol. *Steroids* 2002;67:647-54.

131. Li S, Han B, Liu G, et al. Immunocytochemical Localization of Sex Steroid Hormone Receptors in Normal Human Mammary Gland. *J Histochem Cytochem* 2009;.

132. Pernis AB. Estrogen and CD4+ T cells. *Curr Opin Rheumatol* 2007;19:414-20.