Saliva as a Monitoring Fluid for Hormonal Activity in Systemic *Lupus Erythematosus*

IULIA IOANA STANESCU[1,2], BOGDAN CALENIC[1]*, ALINA DIMA[3], DANIELA MIRICESCU[1], DANIELA GABRIELA BADITA[2], CRISTIAN BAICUS[3,4], PAUL BALANESCU[3], EUGENIA BALANESCU[4], PAULA PERLEA[5], MARIA GREABU[1]

[1]University of Medicine and Pharmacy Carol Davila, Faculty of Dental Medicine, Department of Biochemistry, 8 Eroii Sanitari Blvd, 050474, Bucharest, Romania

[2]University of Medicine and Pharmacy Carol Davila, Faculty of Dental Medicine, Department of Physiology, 8 Eroii Sanitari Blvd, 050474, Bucharest, Romania

[3]University of Medicine and Pharmacy Carol Davila, Faculty of Medicine, Department of Internal Medicine, 19-21 Stefan cel Mare Blvd., 020125, Bucharest, Romania

[4]Colentina Clinical Hospital, Laboratory of Clinical Immunology, CDPC, 19-21 Stefan cel Mare Blvd., Bucharest, 020125, Romania

[5]University of Medicine and Pharmacy Carol Davila, Faculty of Dental Medicine, Department of Endodontics, 19 Calea Plevnei, 010221, Bucharest, Romania

Saliva is a remarkable diagnostic fluid being used to monitor a wide range of local and systemic diseases. Autoimmune diseases represent a major health threat to women worldwide with sexual hormones acting as co-factors for these pathologies and interfering with the normal immune response. The main aim of our study was to evaluate salivary levels of testosterone (T2) and 17-β estradiol (E2) in several autoimmune diseases with a special focus on systemic lupus erythematosus (SLE). The study included 45 SLE patients, 15 patients with other autoimmune diseases and 10 healthy subjects. Salivary T2 and E2 levels were determined using ELISA assays. Salivary E2 levels in female SLE patients were significantly increased versus vasculitis female patients. Positive correlations have been found between salivary E2 levels and important clinical parameters such as age at inclusion and duration of corticosteroid treatment. E2 salivary level was also identified as an independent predictor of lupus renal nephritis. Regarding T2, salivary levels were found to be significantly lower in SLE female patients compared to respective controls. Furthermore, positive statistical correlations were found between T2 and E2 salivary levels. Collectively, these results promote the use of saliva as a monitoring fluid for hormonal activity in SLE.

Keywords: saliva, autoimmune diseases, 17-β estradiol, testosterone

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease with miscellaneous clinical features, affecting multiple organs and systems [1]. Although the precise etiology of this rather rare affliction remains indeterminate, it is already established that environmental factors associated with genetic mechanisms contribute to the SLE pathogenesis [2,3]. Moreover, the endocrine system has been incriminated as a component that contributes to the SLE development [2].

Estrogens and androgens have been found to have effects not only on the reproductive system, but also to interfere in numerous other functions such as the normal immune response, often being co-factors to the installation of autoimmune diseases [4]. Similarly to other autoimmune disease, SLE affects mainly women [5,6]. This predilection for the female gender could be explained by the differences induced by the sex hormones. Thus, the female to male ratio of 9:1 together with the higher incidence of the disease in the reproductive years and in women using hormone replacement therapy post-menopause advocate for the implication of estrogen in the pathogenic mechanisms of SLE [7]. Theories regarding the involvement of estrogen in SLE etiology suggest that this steroid hormone promotes an imperfect T cells apoptosis that allows the endurance of autoreactive T cells [5]. Other studies indicate the disturbing effects that estrogen has on B cells, the altered molecular pathways conducting to an overproduction of autoantibodies [8]. 17-β estradiol (E2), the prevalent estrogen found in serum was reported by numerous studies to have increased levels in SLE patients compared to healthy subjects [8,9]. On the other hand, testosterone (T2) and other androgens were found to have protective effects and various researches reported lower serum concentrations of androgens in SLE patients [10].

Due to highly variable manifestations, with a unique clinical presentation for each patient, SLE diagnosis represents a challenge even today. Without having a specific test for this disease, blood and urine tests combined guide clinicians towards the diagnosis. However, medical research is still on a quest for a precise identification tool for this autoimmune disease. Recently, more and more studies have focused on saliva's potential as a diagnostic fluid not only for oral pathologies, but also for systemic diseases. Along with other advantages, the major benefit for the usage of this fluid is represented by the non-invasive collection procedures that contribute to a better collaboration with the patient who is normally more hesitant to taking blood tests [11].

Our research team has previously demonstrated that saliva can be a reliable diagnosis fluid in diseases with an inflammatory component, such as periodontitis [12,13], as well as a remarkable diagnosis fluid in autoimmune diseases, such as oral lichen planus [14-16]. Hence, the hypothesis of the present study is that saliva can be used as a dependable fluid for monitoring hormonal levels in SLE patients. To the best of our knowledge this is the first work to focus on salivary hormonal changes in patients with different types of autoimmune diseases.

Taking all this information into consideration, the main objective of our study was to measure and compare

* email: bcalenic@yahoo.co.uk; Phone: +40755044047

All authors contributed equally to the present work

salivary levels of testosterone and 17-β estradiol in patients with SLE, rheumatoid arthritis, systemic sclerosis and vasculitis.

Experimental part

Patient selection

The present research included 45 SLE patients (40 women and 5 men) that fulfilled the Systemic *Lupus* Collaborating Clinics (SLICC) SLE's criteria. Patients with another autoimmune disease overlapping were excluded. The control group consisted of 10 healthy subjects (5 females and 5 males), with no autoimmune diseases. A second control group comprised of patients with other autoimmune diseases, including 5 patients with rheumatoid arthritis, 5 patients with systemic sclerosis and 5 vasculitis patients (all women). For each subject included in our research, clinical data such as gender, age, disease duration, as well as information regarding current treatments and numerous biochemical parameters have been documented. The present study was approved by the ethics board of Colentina Clinical Hospital, Bucharest, Romania. Informed consent was obtained from each participant who agreed to participate voluntarily.

Samples collection

Salivary samples were collected at the moment of inclusion for all the participants in the study. 2 mL of unstimulated whole saliva was collected for each participant, around 8 AM before eating, drinking or smoking in all subjects and after a single mouth rinse with 5 mL of distilled water. The subjects were asked to sit down and be relaxed, as well as not to swallow during the collecting procedure. Communication with others was also prohibited. After the collection, the samples were kept on ice and transported in an isotherm box and subsequently they were centrifuged at 4000 rpm for 15 min. Samples were aliquoted and stored at -70°C until further determinations.

Hormone levels determination

Salivary testosterone and 17-β estradiol levels were determined using an Enzyme-linked immunosorbent assay (ELISA) (IBL International GMBH, Hamburg, Germany) kit. The assays employed a quantitative sandwich enzyme immunoassay technique for both hormones. The working procedures strictly followed the manufacturer's instructions. Briefly standards, controls and samples were pipetted into wells precoated with a monoclonal antibodies specific for each hormone. In a subsequent step the wells were washed with PBS and antibodies specific for each marker were added to all wells. Following another washing step, a substrate solution was added to wells. The color developed in proportion to hormonal levels found in each sample. When color development was stopped, the optical density was determined using a microplate reader set to 450 nm with a wavelength correction set to 540 nm.

Statistical analysis

The statistical analysis was performed using SPSS software. The characteristics were expressed as median (quartile 1; 3). Statistically significant correlations were found using the Mann-Whitney test (a two-sided p-value less than 0.05 was noted as statistically significant). A Spearman test was used to evaluate possible bivariate correlations between salivary testosterone and 17-β estradiol levels, as well as for other significant bivariate correlates identified for the 17-beta estradiol salivary levels (p-value< 0.05 was considered statistically significant). Regression logistic models (by enter method) have been used to assess the employment of salivary 17-β estradiol and testosterone as predictors for renal involvement in SLE.

Results and discussions

Clinical data

Clinical parameters such as age at inclusion, disease duration as well as body mass index (BMI) have been acquired for all of the 70 subjects included in the present study (table 1).

General characteristics of the SLE female patients, such as Complement 3, Complement 4, cutaneous involvement, renal or hematological involvement or the European Clinics Activity Measure (ECLAM) score were also noted (table 2).

17-β estradiol monitoring

Salivary levels of 17-beta estradiol (E2) in female SLE patients (7.0 (4.3-32.3) pg/mL) have been found to be significantly increased only comparing to vasculitis female patients (3.9 (3.3 - 4.6) pg/mL, p=0.04). The E2 levels were higher in women with SLE than in all the other groups, except the female controls, but no statistical significance has been found regarding any of the other group included in the study (table 3).

When age was taken into consideration, as expected, lower E2 salivary levels were determined in SLE females older than 40 years when compared with the respective control group: 4.5 (4.2 -14.8) pg/ mL vs. 51.0 (18.0 - 77.2) pg/ mL, p=0.001 (Mann-Whitney test). Also, results

	Cases, n	Gender, F/ M	Inclusion age, Years	Disease duration, years	BMI, kg/ m^2
All subjects	70	60/ 10	51.0 (37.5 – 60.5)	8.0 (4.0 – 14.0)	25.5 (21.8 – 29.9)
SLE	40	40/ 0	50.0 (38.0 – 59.0)	10.0 (5.0 – 15.0)	25.1 (21.5 – 30.2)
	5	0/ 5	51.0 (31.5 – 58.0)	6.0 (4.0 – 10.0)	25.6 (24.9 – 34.7)
Control	5	5/ 0	39.0 (30.0 – 50.5)	-	21.9 (19.5 – 22.6)
	5	0/ 5	60.0 (40.0 – 68.5)	-	29.4 (23.7 – 29.9)
SS	5	5/ 0	54.0 (42.0 – 60.0)	2.0 (0.5 – 9.0)	25.9 (22.4 – 30.9)
RA	5	5/ 0	64.0 (47.0 – 66.0)	6.0 (3.0 – 14.0)	26.0 (22.7 – 32.6)
Vasculitis	5	5/ 0	47.0 (42.0 – 70.0)	8.0 (2.0 – 14.0)	25.8 (24.8 – 35.1)

Table 1
GENERAL CHARACTERISTICS OF THE SUBJECTS INCLUDED

BMI – body mass index; F – female; M- male; kg – kilogram; m – meter; SLE – systemic lupus erythematosus; SS – systemic sclerosis; RA –rheumatoid arthritis

Characteristic	N=40
ESR, mm/ h	21.0 (11.0 – 31.5)
CRP, mg/ Dl	3.7 (2.1 – 6.9)
Complement C3,	1.1 (0.9 – 1.2)
Complement C4,	0.2 (0.1 – 0.2)
Leucocytes, no/ uL	6110 (5290 – 8595)
Lymphocytes, no/ uL	1610 (1255 – 2425)
Hemoglobin, mg/ dL	12.1 (11.8 – 13.2)
Cutaneous involvement, n (%)	27/ 40 (67.5)
Renal involvement, n (%)	20/ 40 (50.0)
Neurological involvement, n (%)	12/ 40 (30.0)
Hematological involvement, n (%)	31/ 40 (77.5)
ECLAM score, points	10.0 (5.0 – 20.0)
Corticosteroids – duration, years	6.5 (4.0 – 14.0)
Corticosteroids – daily dose, mg Prednison	6.5 (4.0 – 14.0)

CRP – C-reactive protein; ESR – erythrocyte sedimentation rate; ECLAM – European Clinics Activity Measure

Data are expressed as median (quartile 1; 3)

Table 2
GENERAL CHARACTERISTICS OF THE SYSTEMIC LUPUS ERYTHEMATOSUS FEMALE PATIENTS

Subjects	Salivary 17-beta estradiol, pg/ mL	p-value*
All subjects	4.8 (4.2 – 21.9)	--
SLE female patients	7.0 (4.3 – 32.3)	--
SLE male patients	4.3 (1.8 – 5.6)	0.08
Control female patients	8.9 (5.6 – 40.4)	0.45
Control male patients	4.9 (4.6 – 13.4)	0.77
SS female patients	4.6 (4.4 – 36.2)	0.68
RA female patients	4.1 (3.7 – 7.1)	0.07
Vasculitis female patients	3.9 (3.3 – 4.6)	**0.04**

Mann- Whitney test in respect with the salivary testosterone levels in SLE female patients

BMI – body mass index; F - female; M- male; kg - kilogram; m – meter; SLE-systemic lupus erythematosus; SS -systemic sclerosis; RA -rheumatoid arthritis

Table 3
SALIVARY 17-BETA ESTRADIOL LEVELS IN THE GROUPS EVALUATED

showed significant higher E2 salivary levels in patients with higher ECLAM score at inclusion: 31.2 (21.8 - 75.2) pg/ mL vs. 4.7 (4.2 - 19.4) pg/ mL, p=0.02 (Mann-Whitney test).

Moreover, in regression logistic model (by enter method), adjusted for the patients' characteristics (age at inclusion and BMI), we identified the E2 salivary level as independent predictor of the lupus renal nephritis, OR (95% CI): 10.3 (1.02 – 104.2).

Positive correlations have been found between salivary levels of the 17-β estradiol and other clinical parameters such as age at inclusion, duration of corticosteroid treatment, systolic blood pressure or pulse pressure (table 4).

Testosterone monitoring

Salivary testosterone levels were found to be significantly lower in SLE female patients (18.1 (11.5 – 72.8) pg/mL) compared to both control female patients (78.3 (40.9 - 230.4) pg/mL, p=0.05) and control male patients (218.4 (68.5 -269.0), p=0.004). As a matter of fact, salivary T2 levels were found to be lower in SLE female patients compared to all the other groups included in the study with

	Inclusion age, years	Corticosteroids duration, years	Systolic blood pressure, mmHg	Pulse pressure, mmHg
17-beta estradiol, pg/mL	p=0.026 rs=-0.357	p=0.042 rs=-0.331	p=0.042 rs=-0.345	p=0.020 rs=-0.393

p=p-value; rs= Spearman's rho coefficient

Table 4
SIGNIFICANT BIVARIATE CORRELATIONS IDENTIFIED FOR SALIVARY 17-β ESTRADIOL LEVELS

Subjects	Salivary testosterone, pg/ mL	p-value*
All subjects	28.7 (12.9 – 83.9)	--
SLE female patients	18.1 (11.5 – 72.8)	--
SLE male patients	67.1 (9.4 – 182.1)	0.33
Control female patients	78.3 (40.9 – 230.4)	**0.05**
Control male patients	218.4 (68.5 – 269.0)	**0.004**
SS female patients	27.7 (4.9 – 316.5)	0.86
RA female patients	11.8 (8.7 – 29.1)	0.18
Vasculitis female patients	38.5 (26.9 – 71.3)	0.28

* Mann- Whitney test in respect with the salivary testosterone levels in SLE female patients

BMI - body mass index; F - female; M- male; kg - kilogram; m - meter; SLE - systemic lupus erythematosus; SS - systemic sclerosis; RA -rheumatoid arthritis

Table 5
SALIVARY TESTOSTERONE LEVELS IN PATIENTS GROUPS EVALUATED

the exception of the RA patients, but held no statistical relevance (table 5).

Salivary T2 levels proved to be significant lower in SLE female patients with lupus nephritis anytime than in those without renal involvement: 13.3 (8.6-54.2) pg/mL *vs.* 21.0 (14.7-99.6) pg/mL, p=0.02 (Mann-Whitney test). However, in regression logistic model, adjusted for the patients' characteristics (age at inclusion and BMI), we did not identify the salivary T2 level as an independent predictor of the lupus renal nephritis.

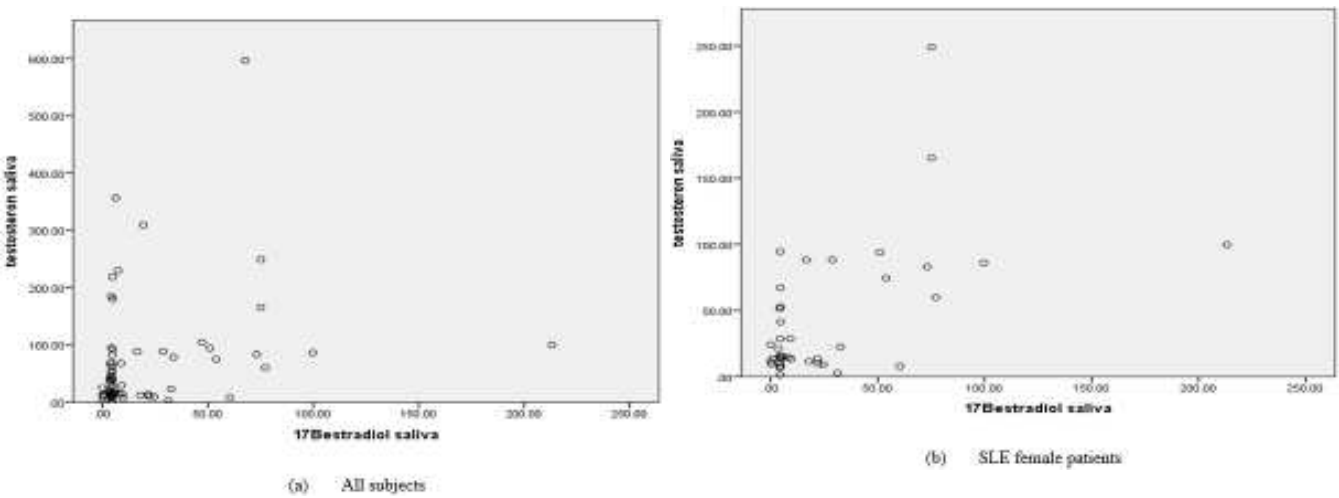
Also, our results show that salivary T2 and E2 levels correlate both when we analyzed hormonal levels for all subjects (p=0.004; rs=0.341), as well as for when only SLE female patients are taken into consideration (p=0.02; rs=0.376) (fig. 1).

Although the exact etiology of SLE remains unclear, numerous studies have focused on the role played by gonadal hormones in the pathogenetic mechanisms of this complex, acute and chronic inflammatory affliction that shows an upsurge of incidence and prevalence [17-19].

On the other hand, saliva has become a key diagnosis fluid in recent years, proving to be an exceptional alternative to classic diagnosis mediums (such as serum, urine or cefaloraquideous liquid) and acting as a genuine *mirror of the body* [20-21]. Moreover, biomarkers found in this remarkable amalgam of organic and inorganic components have shown to correlate with those from the blood. Among the many features that promote the use of saliva as a diagnosis fluid for systemic afflictions we can include good sensitivity, simple collection procedures and the requirement of limited quantities for determinations [22].

Regardless of the many features that recommend saliva as an exceptional diagnosis fluid, there hasn't been enough investigation is this field focusing on sexual hormones and their connection with SLE. To the best of our knowledge, there are only several other studies exploring the use of salivary testosterone levels in order to monitor SLE [23,24]. In this context, our research team investigated the prospect of saliva as a monitoring environment for sexosteroid hormones levels and also as a diagnosis fluid in SLE patients.

While SLE's various clinical traits could be explained by the environmental and genetic influences, its heterogeneity could also be owed to the alterations displayed in the cytokine modulation processes [2,25-27]. Even though the precise means through which estrogen influences cytokine production remains unknown, it has been found that this hormone can affect both nuclear (by activating intracellular receptor) and extranuclear structures, a very important pathway involved in the pathogenesis of this autoimmune disease being represented by a dysfunction of the immune



(a) All subjects (b) SLE female patients

Fig. 1. Testosterone levels correlate with the 17-beta estradiol levels (p = p-value; rs= Spearman's rho coefficient; (a) all subjects (n=70): p=0.004; rs=0.341; (b) SLE female patients (n=40): p=0.02; rs=0.376)

cell's mitochondria [28]. Thus, 17-β estradiol could explain the peak SLE rate during childbearing years [28,29].

Our results showed that there was no statistically significant increase in salivary 17-β estradiol levels in SLE female patients compared to the healthy women included in the study as controls. Similar data has been displayed by previous research performed on serum that found estrogen not significantly increased in SLE female patients [30,31]. A meta-analysis performed on 8 studies taking into consideration only female SLE patients revealed that only 2 of those investigations reported higher serum levels for 17-β estradiol, while 5 of them found no statistical dissimilarity compared to the respective control groups [30]. At the same time, salivary levels for 17-β estradiol levels in SLE female patients included in our research have been found statistically increased only *vs* women with vasculitis when women with other autoimmune diseases have been used as control groups. E2 salivary levels were higher also *versus* women with systemic sclerosis or rheumatoid arthritis, however no statistical significance could be found.

Nevertheless, in regard with lupus disease activity, we identified significant higher E2 salivary levels in patients with a higher ECLAM score at inclusion, these findings suggesting that there could be a correlation between E2 activity and SLE development and progression. In light of the hormonal changes related to the aging process, our research team also took into consideration age as a factor in the analysis of the salivary hormonal levels. The median age at inclusion for the SLE patients was of 50 years for the women and 51 years for the men, while the control group had a median age of inclusion of 39 years for the women and of 60 years for the men. Median age at inclusion for the 5 women with systemic sclerosis included in the study was of 54 years. Meanwhile, the 5 patients with rheumatoid arthritis (all of feminine sex) had a median age at inclusion of 64 years and the last group that participated to the study, consisting of 5 female patients with vasculitis had a median age at inclusion of 47 years (table 1). In this regard, the results we obtained displayed lower E2 salivary levels is SLE females older than 40 years when compared with the respective control group, as it was expected.

Moreover, our results also showed significant positive correlations between salivary 17-β estradiol levels and systolic blood pressure and pulse pressure, supporting previous findings advocating for the association of SLE with hypertension, one of the most important determinants for cardiovascular disease. Furthermore, the E2 salivary level was found to be an independent predictor of the lupus renal nephritis. Previous studies show that up to 50% of SLE patients present renal manifestations of the disease, with nephritis also being one of the causes of SLE hypertension [29-32].

In contrast to estrogen, testosterone has been found to exert immunosuppressive effects [30]. The mechanisms through which testosterone has an anti-inflammatory impact include a decreased production of pro-inflammatory cytokines such as IL-6 and stimulation of a higher secretion rate for IL-10, one of the most important anti-inflammatory interleukins [33]. Moreover, research has shown that salivary testosterone levels are an accurate reflection of serum free-circulating testosterone concentration [23]. The significantly decreased salivary levels of testosterone found in SLE female patients compared to both female and male control subjects included in our research are in concordance with the results reported by previous studies performed both on serum [30] and when saliva samples were analyzed [23]. Additionally, salivary T2 levels were lower in female SLE patients *versus* women with other autoimmune diseases, such as systemic sclerosis or vasculitis, but no statistical significance could be found.

Furthermore, while evaluating the presence of renal impairment at any time, we found that the salivary T2 levels proved to be significant lower in SLE female patients with lupus nephritis anytime than in those without renal involvement, suggesting a protective role played by testosterone against renal damage.

Conclusions

In conclusion, although salivary levels of 17-β estradiol were not significantly increased compared to healthy subjects, the positive correlations between this hormone's concentrations in saliva and age at inclusion, duration of corticosteroid treatment, systolic blood pressure and renal impairment, as well as the positive correlation between higher E2 levels and ECLAM score suggest that the salivary level of this estrogen could be used to monitor SLE activity. In addition, our results showed that salivary testosterone were lower in SLE female patients *versus* the healthy subjects and displayed a positive correlation between E2 and T2 salivary levels, hence promoting the use of saliva as a monitoring fluid for hormonal activity in systemic lupus erythematosus. Nonetheless, further research is needed.

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