# Bioactivity of prolactin in systemic sclerosis

G. La Montagna, R. Meli[1], T. Criscuolo, S. D'Angelo, G. Valentini

*Dipartimento Scientifico di Internistica Clinica e Sperimentale "F. Magrassi & A. Lanzara", Seconda Università di Napoli;*
*[1]Dipartimento di Farmacologia Sperimentale, Università Federico II, Naples, Italy*

## Abstract

### Objectives

*To evaluate basal serum prolactin (PRL) levels in systemic sclerosis (SSc) patients with different degrees of skin involvement, and investigate its relationship with some of the clinical and serological parameters of the disease.*

### Methods

*Basal serum PRL was measured in 44 SSc patients (38 F, 6 M) using a rat NB2 lymphoma line cell proliferation assay. Other parameters measured were: serum aminoterminal propeptide of type III procollagen (PIIINP) by RIA; soluble α interleukin-2 receptor (IL-2 sRα), serum intercellular adesion molecole-1 (ICAM-1), von Willebrand factor (vWF) by ELISA; the erythrocyte sedimentation rate (ESR); and C-reactive protein (CRP). Skin and organ/system involvement were assessed according to Medsger* et al.*'s organ/system severity scale, and global disease activity index according to Valentini* et al.

### Results

*The serum PRL concentration in the SSc patients was 13.8 ng/ml (95%CI from 3.2 to 49.1 ng/ml), similar than that in control subjects (12.8 ng/ml; 95%CI 3.0 to 18.4 ng/ml). Hyperprolactinemia, defined as a level > 20 ng/ml (mean 30.9 ng/ml, median 29.3) was found in a total of 6 cases (13.6%; 95% CI 5.8 to 28%) cases: in 1 out of 6 men (16.7%; 95%CI –26% to 59%) and similarly in 5/38 women (13.2%; 95%CI 1.9% to 24.4%). No correlation was found between PRL levels and SSc subgroup (lcSSc, icSSc, dcSSc), serologial parameters, or the level of disease activity. Finally, no significant correlations were found with clinical or serological variables.*

### Conclusions

*The findings confirm that mild hyperprolactinemia occurs in a subgroup of SSc patients. However, prospective studies are needed to better define the relationship between PRL and disease activity in scleroderma.*

**Key words**
Bioactive prolactin, systemic sclerosis, disease severity, disease activity.

*Giovanni La Montagna, MD, Assistant Professor of Rheumatology; Rosaria Meli, MD, Associate Professor of Pharmacology; Tullio Criscuolo, MD, Assistant Professor of Endocrinology; Salvatore D'Angelo, MD, Investigator in Rheumatology; Gabriele Valentini, MD, Professor of Rheumatology and Chief of Rheumatology Unit.*

*Please address correspondence and reprint requests to: Giovanni La Montagna, MD, Unità Operativa di Reumatologia, Policlinico-Seconda Università di Napoli, Via S. Pansini no. 5, 80131 Napoli, Italy. E-mail: giovanni.lamontagna@unina2.it*

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## Introduction

Recent data have shown that prolactin (PRL) plays a role as a cellular and humoral immunomodulator *in vivo* (1, 2). In particular, experimental studies in the autoimmune female B/W mouse model of systemic lupus erythematosus (SLE) have demonstrated that PRL induces disease activation (3). Clinical studies have shown that hyperprolactinemia (HPRL) occurs in autoimmune diseases such as SLE and systemic sclerosis (SSc) (4-8). However, there are conflicting results on the relationship between PRL levels and disease activity in SLE, as well as in SSc (5, 6, 8, 9).

It is well known that various factors, such as physiological conditions, the occurrence of comorbidity, a stress event, or the intake of some drugs (10, 11), can affect serum levels of PRL. Moreover, the differing biological activity of various molecular forms of immunoreactive human PRL, such as the occurrence of anti-PRL antibodies (12-14), could be relevant factors conditioning the interpretation of PRL behavior under different clinical conditions (15, 16).

We evaluated the basal serum PRL in SSc patients as detected by a bioactive method and investigated the relationship with clinical and serological features of the disease.

## Patient and methods

*Study groups*

Forty-four consecutive patients (38 women and 6 men; aged from 15 to 72 years, median 49.5 years with a disease duration lasting from 1 to 34 years, median 9.5 years) admitted to the Rheumatology Unit of the 2nd University of Naples from 1998 to 2000, all of whom fulfilled the American College of Rheumatology preliminary criteria for the classification of systemic sclerosis (previously, ARA), were studied. The patients were categorized into 1 of 4 SSc subsets (normal skin, limited, intermediate, diffuse) based on the extent of sclerodermatous skin involvement (17) and then were grouped into three clinical subsets according to Giordano *et al.* (18) [i.e., limited cutaneous systemic sclerosis (lcSSc), including SSc sine scleroderma, intermediate cutaneous systemic sclerosis (icSSc), and diffuse cutaneous systemic sclerosis (dcSSc)]. Moreover, they were divided into three serological subsets as defined by assessing antinuclear antibodies (ANA) on HEp-2 cells (cut-off level 1:40), and anti DNA topoisomerase I antibodies (anti Scl-70) by ELISA (cut-off level, 20 EU/ml), i.e. anticentromere antibody (ACA) positive, anti-Scl-70 antibody positive, and ANA positive with undetectable ACA and/or anti-Scl-70.

The patients were receiving low-dose glucocorticoids (prednisone equivalent ≤ 10 mg/day) (n = 23), D-penicillamine (n = 5), hydroxychloroquine (n = 2), cyclophosphamide (n = 13). In addition, they were taking other supportive drugs such as prostacyclin agonists and other vasoactive drugs, ACE-inhibitors, anti H2-receptors or omeprazole, prokinetics) when appropriate.

All patients had normal TSH levels; 4 among them had thyroid nodular hyperplasia, and 2 were using substitutive thyroxin for hypothyroidism. No patient suffered from other conditions that are associated with hyperprolactinemia (19).

Twenty healthy subjects (2 men and 18 women) aged from 20 to 60 years comprised the control group. All participants were enrolled after they had given their informed consent.

## Methods

Sera from SSc patients were collected under fasting conditions from 8 to 9 am, after a washout period of at least 4 days, and were immediately frozen at –20°C until the hormonal assay was performed. Basal serum PRL levels were determined by a biological method (direct measurement of a cellular response) using proliferation of a rat Nb2 lymphoma line cell, according to Pacilio *et al.* (9). This method, which was developed by Tanaka *et al.* (20), is comparable in terms of sensitivty and specificity to the radioimmunological assay (15). Briefly, 50 µl of varying sera dilutions or standard or medium (controls) were added in triplicate to wells of a microtest plate containing 200 µl of a suspension of rat Nb2 lym-

phoma cells (2 x $10^5$ cells) in phenol red-free RPMI 1640 with 10% horse serum after 24 h of starvation, and incubated for 3 days at 37°C in 5% $CO_2$ with 95% humidity. To exclude the possible interference of serum growth hormone on the bioassay, a rabbit polyclonal antibody against hGH (anti-hGH-IC3, NIDDK) was added to each sample. The PRL levels in the serum samples were calculated by measuring the hormone concentrations from the standard curve. In our laboratory, intra-assay and inter-assay variation coefficients were 1.3% to 2.9%, and 3.7% to 6.2%, respectively. As is generally accepted, HPRL was defined as a level higher than 20 ng/ml.

A clinical evaluation was also carried out. Skin involvement was measured according to the modified Rodnan total skin thickness score (mTSS) (21). Internal organ/system involvement was assessed using the following examinations: electrocardiogram, Doppler echocardiography, capillaroscopy, pulmonary function tests and diffusing capacity for carbon monoxide (DLCo), high resolution computed thomography of the chest; esophageal and/or gastrointestinal barum study and oral xylose absorption test, blood creatinine, urinalysis and arterial blood pressure evaluation. The severity of both skin and single internal organ/system involvement were graded from 1 to 4 using the preliminary organ/system severity scale according to Medsger *et al.* (22).

Disease activity was evaluated using a 10-point validated index (23); a value of 3 has been found to have the best discriminant capacity for active to very active disease.

In addition to PRL, we investigated, on sera frozen at –20°C immediately after collection, parameters of endothelial activation/damage (von Willebrand factor, vWF), fibroblast function (amino-terminal propeptide of type III procollagen, PIIINP), lymphocyte activation (soluble serum interleukin-2 receptor alpha, IL-2 sRα, soluble intercellular adhesion molecole-1 (ICAM-1), and acute phase reactants [erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)]. ESR was measured by the Westergren method and C-reactive protein (CRP) by immunonephelometry (using a Behring nephelometer). Soluble interleukin-2 receptor alpha (IL-2sRα) was measured using a solid phase enzyme immunoassay (ELISA) (Quantikine R&D Systems, USA) with a detection limit < 6 pg/ml. Aminoterminal propeptide of type III procollagen (PIIINP) was measured by radioimmunoassay (RIA) according to the manufacturer's instructions (ORION Diagnostica radioimmunoassay) with a detection limit less than 0.2 mcg/l. Von Willebrand factor (vWF) was detected according to the manufacturer's procedure (Shield Diagnostics ELISA kit) with a detection limit < 1.6% of the activity. Intercellular adesion molecule-1 (ICAM-1) was measured using a commercial ELISA kit (Quantikine R&D Systems, USA) according to manufacturer's instructions. The sensitivity of the kit was less than 3.3 ng/ml.

These serological parameters were not investigated in control subjects, for whom we relied on our previous studies devoted to each marker (unpublished data).

*Statistical analysis*

The statistical analysis of the results was carried out using the statistical Package for Social Science (SPSS) for Windows (release 6.1). Data were expressed as the median and range or mean ±standard deviation (SD) with the confidence interval (95%CI), where appropriate. Differences between groups were analyzed using non-parametric methods. The linear correlation between continuous variables was evaluated after log transformation of the data because their distribution was not normal, using Spearman's rho coefficient. The significance was set at a p value < 0.05 using two-tailed tests.

## Results

*Clinical assessment*

The patients were divided into 3 clinical subsets: 15 cases with lcSSc, 19 with icSSc and 10 with dcSSc. They were divided into 3 serological subsets: ACA positivity in 13 cases (29.5%), anti Scl-70 antibody positivity in 27 cases (61.4%), and ANA positivity with ACAor anti Scl-70 negativity in 4 cases (9.1%).

The mTSS ranged from 0 to 43 (mean 14.7 ±10.9; median 13).

The assessment of organ/system involvement showed: general health in 23 cases (score 1: 40.9%; score 2: 11.4%); peripheral vascular in 38 (score 1: 15.9%; score 2: 36.4%; score 3: 34.1%); heart in 20 (score 1: 11.4%; score 2: 31.8%; score4: 2.3%); lung in 42 (score 1: 31.8%; score 2: 31.8%; score 3: 31.8%); gastrointestinal tract in 43 (score 1: 86.4%; score 2: 11.4%); joint/tendon in 13 (score 1: 6.8%; score 2: 15.9%; score 3: 2.3%; score 4: 4.5%); kidney in 5 (score 1: 2.3%; score 2: 2.3%; score 3: 4.5%; score 4: 2.3%); muscle in 1 (score 1: 2.3%). On the whole, the organ system involvement was mild (1) or moderate (2) in our series.

The disease activity score ranged from 0 to 6, median 3.

The basal serological parameters of inflammation or cellular activation/damage as median and ranges are shown in Table I.

*Basal serum prolactin*

Table II shows the basal levels of serum prolactin in SSc patients divided by sex and menopausal status, and in healthy controls. In all SSc patients serum PRL levels were similar to those in controls as indicated by the inclusion of the mean of the values detected in SSc patients within the confidence interval of the control group, in which all the subjects had normal serum levels (< 20 ng/ml) of PRL. The mean value detected in male patients was significantly lower than that in females. No differences were seen between the PRL levels in women of child-bearing age with respect to those in menopause. Hyperprolactinemia was found in 6 SSc patients (13.6%; mean PRL value 30.9 ng/ml, median 29.3). The frequency in men and women was similar: 1 out of 6 men (PRL value, 21 ng/ml; 16.7%, 95%CI –26% to 59%) and 5 out of 38 women (13.2%, 95%CI from 1.9% to 24.4%). In one female patient renal failure was found that could account for the increased PRL level. We did not investigate the underlying cause of hyperprolactinemia in the only male SSc patient with this condition.

**Table I.** Basal laboratory parameters for our SSc patients.

| Parameter | Median | (range) | Percentage of abnormal values |
|---|---|---|---|
| ESR (mm/h) | 16 | (2 – 80) | 21% |
| CRP(mg/dl) | 0.79 | (0.3 – 7.5) | 44% |
| VWF (% activity) | 104.7 | (63.2 – 253) | 14% |
| PIIINP(mcg/ml) | 3.33 | (1.89 – 5.29) | 50% |
| IL-2 sRα (pg/ml) | 1124.8 | (147 – 9608) | 21% |
| ICAM-1 (ng/ml) | 324.4 | (209.5 – 695.3) | 38% |

**Table II.** Basal serum prolactin levels (PRL) (mean and 95% CI) and hyperprolactinemia (HPRL) prevalence in SSc patients and control group

| Study groups | No. of cases | Basal PRLserum levels | 95% CI | HPRL* (No. cases) |
|---|---|---|---|---|
| SSc | 44 | 13.8 ng/ml (3.2 – 49.1) | 11.1 – 16.5 ng/ml | 6 |
| Women | 38 | 14.3 ng/ml (4.6 – 49.1) | 11.3 – 17.3 ng/ml | 5 |
| Child-bearing age | 11 | 15.3 ng/ml (4.9 – 49.1) | 10.7 – 17.1 ng/ml | 3 |
| Menopausal | 27 | 13.9 ng/ml (4.6 – 37.6) | 7.4 – 23.1 ng/ml | 2 |
| Men | 6 | 10.7 ng/ml (3.2 – 21.0) | 3.6 – 17.7 ng/ml | 1 |
| Controls | 20 | 12.8 ng/ml (3.2 – 18.4) | 11.6 – 14.0 ng/ml | 0 |

The range is indicated between parentheses. *Cut-off point = 20 ng/ml.

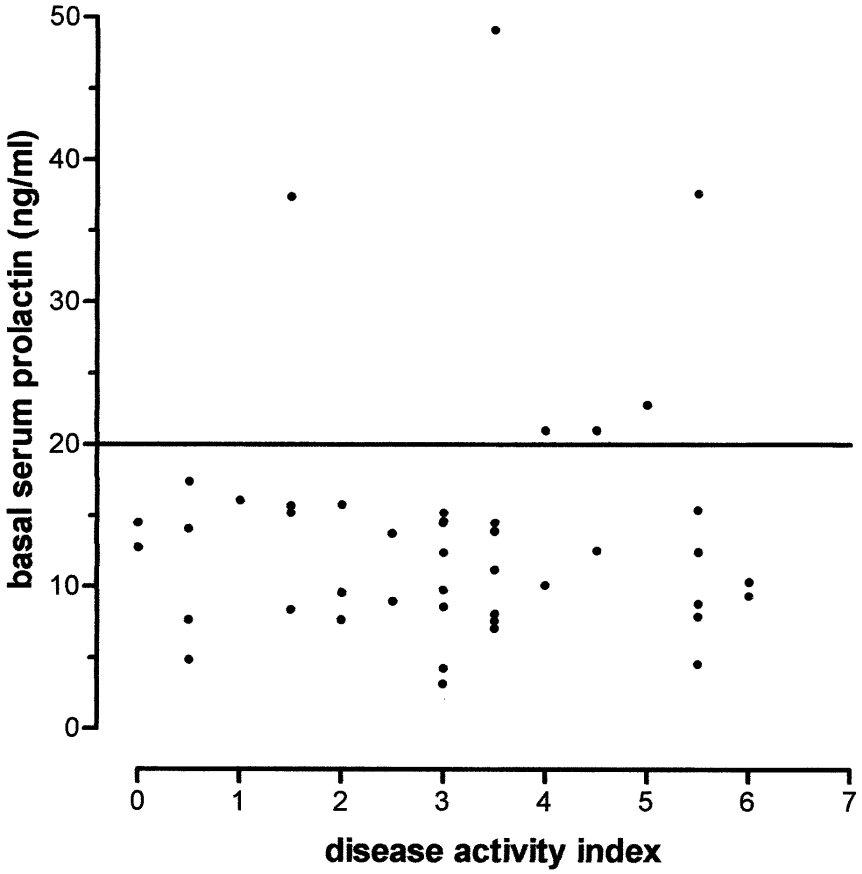


**Fig. 1.** Distribution of basal serum prolactin according to the disease activity index in 44 SSc patients. The horizontal line indicates the prolactin cut-off value.

Nevertheless, we did not found any evidence of pituitary enlargement.

We found no significant difference in the mean serum PRL concentration among patients belonging to the three SSc clinical and serological subgroups (data not shown).

Figure 1 shows the relationship between basal serum PRL levels and the global disease activity index. Dividing the patients into two groups according to the cut off value of the preliminay disease activity score, we identified 27 patients with active to very active disease, i.e. with an index > 3. Hyperprolactinemia occurred in 5 out of these patients (18.5%; 95%IC from 2.8% to 34%), and in one out of the 17 patients with a value of disease activity score £ 3 (5.9%, 95%CI from –6.6 to 18.3%). However, the difference between two groups was not significant.

Univariate analysis showed no statistical significant correlations among log serum PRL levels and age, disease duration, disease activity index, whereas a significant correlation was found between log serum PRL levels and joint involvement according to Medsger *et al.* ($r_s$ = 0.369; p = 0.014), the significance of which persisted after the exclusion of confounding variables such as age (p = 0.048). No correlations were found among serum PRL values and serological variables (ESR, CRP, vWF, IL-2 sRα, ICAM-1, and PIIINP concentrations) (data not shown).

When the patients were divided into two groups according to the current therapeutic regimen, no differences emerged in the comparison of the PRL levels of patients who had previously received cyclophosphamide or glucocorticoids and those who had not (data not shown). The relationship between PRL levels and hydroxychloroquine or D-penicillamine was not useful because only a few patients were taking these drugs.

## Discussion

This is the first report evaluating the basal serum PRLconcentrations in SSc patients using a biological assay. Mild hyperprolactinemia was seen in 13.6% (1 male and 5 females), confirming that in a subgroup of SSc patients hyperpro-

lactinemia can occur (5,6,13). The prevalence of hyperprolactinemia in our series was lower than that recently reported in two studies evaluating serum PRL levels by RIA or ELISA in SSc (7, 13). Moreover, we did not find any difference between SSc patients and control subjects. This result is in contrast with our previous study in which significant differences were found using the IRMA method (6). On the whole, we hypothesize that these results probably mainly depend on the different clinical and serological characteristics of the SSc patients enrolled, and/or the methods of hormonal testing.

In accordance with previous reports using RIA or ELISA (6, 13), on the whole we could not demonstrate any association between basal serum PRL levels and clinical characteristics of the disease such as cutaneous or single internal organ/system involvement, apart from a weak correlation found with joint involvement. Our data therefore confirm those reported by some authors (6,13) but not are in agreement with one study which reported a relationship between high serum prolactin and the severity of systemic sclerosis (5). However, in that study disease severity was not assessed using the Medsger scale. Finally, a significant relationship was not found between hyperprolactinemia and the preliminary global disease activity index.

It is difficult to explain why hyperprolactinemia occurs in SSc. It is generally thought that there is a relationship between the neuroendocrine peptide prolactin and the immune system (24). This hypothesis has been raised in relation to some autoimmune diseases, such as SLE, as several reports have demonstrated a relationship between hyperprolactinemia and disease activation (7, 25, 26). Few studies of SSc patients have focused on this issue and no univocal conclusions have been reached. Recently, it has been shown that an elevated serum level of PRL in SSc patients may be due to a sustained increase over 24 h and a shift in the diurnal rhythm (13). This aspect has not been addressed in our study.

Anti-PRL autoantibodies in SLE patients have been reported (27) to make hyperprolactinemia devoid of any functional consequence, since the complexes PRL-antiPRL change *in vivo* into biological activity of PRLbecause, probably, their high molecular weight prevent them from crossing the capillary's barrier (12). It is not quite clear whether these autoantibodies have a role in other connective tissue diseases such as SSc. This aspect was not addressed in our study and therefore we are unable to rule out the possibility that the bioactivity of prolactin may be influenced by the presence of PRL autoantibodies, as has been observed in SLE patients (12). However, no relationship was found between PRL autoantibody levels and serum PRL concentrations in SSc patients or control subjects in another study (13). Moreover, the time interval between low dose glucocorticoid withdrawal and blood sampling could have been too short to exclude a possible interference on Nb2 cell proliferation through the antiproliferative effect of glucocorticoids, even if it has been pointed out that this effect is reversed in the presence of prolactin (28). Nevertheless, no relationship was found in our SSc patients between glucocorticoid users and non users. In addition, despite the fact that this study was not designed to elucidate the influence of therapy on PRL levels, we were unable to demonstrate a significant influence of cyclophosphamide on hormonal concentrations. Hyperprolactinemia was not related to therapy. In fact, abnormal levels of PRL have not been found in patients using chloroquine (4) or D-penicillamine (29).

Our study has some limitations that could have partially influenced the results. The main ones are the small size of the patient series, the cross-sectional design of the study, and the high prevalence of anti DNA-topoisomerase I, that was, however, a well-established feature of our series (18, 30) and may relate to unestablished ethnic or environmental factors. Finally, the possibility that prolactin autoantibodies may have the effect of reducing bioactivity in SSc patients with mild hyperprolactinemia was not evaluated in our study and remains open.

A number of reports have been already focused on HPRL in SLE. Since conflicting results have been reported, the conclusions of our study, particularly those concerning the lack of any association with clinical or epidemiological features, must be confirmed in other studies.

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