Absence of Circadian Salivary Cortisol Rhythm in Women with Anorexia Nervosa

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Abstract. Study Objective: To compare the cortisol levels and 24 hour salivary cortisol rhythm in patients with anorexia nervosa (AN) and normal controls.

Design: Prospective transversal controlled study.

Setting: Tertiary-referral University Hospital.

Participants: Twenty-five patients aged 15 to 35 years, 13 of them with regular ovulatory cycles, and 12 with diagnosis of AN.

Interventions: Salivary and blood collection for cortisol 24-hour rhythm determination.

Main Outcome: Salivary cortisol was determined at 9 AM, 5 PM, and 11 PM. Seric follicle-stimulating hormone, luteinizing hormone (LH), prolactin, estradiol (E2), progesterone, dehydroepiandrosterone-S (DHEA-S), and cortisol were sampled together with the 9 AM salivary sample.

Results: LH, E2, and DHEA-S levels were reduced in patients with AN. A correlation between salivary and serum cortisol levels was observed in the 9 AM sample only in controls (r = 0.67, P = 0.01; AN: r = 0.48, P = 0.12). Cortisol rhythm was present in all control subjects, whereas it was absent in one third of AN patients. The area under the curve for the AN group with preserved rhythm was significantly higher than for the control group (Me = 6811 ng/dl/24h vs 3708 ng/dl/24h; P = 0.034).

Conclusion: Patients with AN have higher salivary cortisol levels when compared to normal women and some of them do not present circadian rhythm.

Key Words. Anorexia nervosa—Salivary cortisol—Biological rhythm

Introduction

Anorexia nervosa (AN) is a psychoneuroendocrine disease associated with psychological stress whose effects on the reproductive system are changes in gonadotrophin pulses and consequent hypoestrogenism. The inhibitory effects of stress on reproductive function are mediated by changes in the hypothalamus-pituitary-adrenal (HHA) axis. Corticotropin-releasing hormone (CRH) is increased, but adrenocorticotropic hormone (ACTH) levels are normal and the response of the hormone to CRH stimulation is reduced. However, the adrenal glands of anorectic women respond more than those of normal women to ACTH stimulation in the production of cortisol.

It has been reported that the circadian rhythm of plasma cortisol may be preserved in these patients, even in the presence of these changes in the HHA axis. According to some investigators, the rhythm of plasma cortisol can be considered to be a stable marker of the circadian cycle because of its reproducibility over time both in separate individuals and in groups. On the other hand, because the major component of plasma cortisol is linked to carrier proteins, plasma cortisol may not reflect the fluctuations of free cortisol, which is actually responsible for the biological actions of the hormone. Thus, the determination of free cortisol in saliva may be a more reliable marker of the real functional status of the HHA axis.

Although several recent reports have demonstrated changes or loss of salivary cortisol rhythm associated with different diseases, few data are available about free salivary cortisol rhythm in AN. Putignano et al. demonstrated that salivary cortisol also maintains a circadian rhythm in women with AN, although with a flattening of the cortisol rhythm curve compared to normal women. Thus, it is possible that...
subtle individual and minor unexplored changes in free salivary cortisol rhythm may be present in patients with AN.

On this basis, the objective of the present study was to determine salivary cortisol levels and the presence of circadian rhythm in patients with AN analyzed as a group and individually and to compare the results to those obtained for ovulatory women of normal weight.

Materials and Methods

After being duly informed about the voluntary nature of their participation and about all the procedures involved in the study, the patients included in this investigation signed an informed consent form. When the patient was a minor the person legally responsible for her signed the consent form. The study was approved by the Research Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo (HCFMRP-USP).

Study Groups

The participants were divided into two groups:

Group I was a control group consisting of 13 normal and ovulatory volunteers. The inclusion criteria were age between 15 and 35 years, absence of clinical complaints, regular menstrual cycles, progesterone levels of 5 ng/ml or more in a blood sample obtained during the second phase of the menstrual cycle, absence of galactorrhea and hirsutism, and body mass index (BMI) between 18 and 25 kg/m².

Group II consisted of 12 patients with AN seen at the Outpatient Clinic for Disorders of Eating Behavior of the Department of Internal Medicine, FMRP-USP. Ten of these patients were of the restrictive type and two of the bulimic type. All patients were amenorrheic. Inclusion criteria according to the American Psychiatric Association were age between 15 and 35 years, onset of symptoms before 25 years of age, loss of 25% or more of initial body weight, BMI below 18, distorted attitudes with respect to self-image, weight and eating habits, absence of other psychiatric diseases, absence of organic disease, and other clinical manifestations such as bradycardia, lanugo, periods of hyperactivity, periods of bulimia, and vomiting (sometimes induced).

An exclusion criterion for both study groups was the use of any hormonal medication over a period of less than 3 months before the beginning of the study.

Hormonal Measurements in Saliva

Cortisol was determined in the saliva of all women, collected during the first phase of the cycle, between the 4th and 8th day of the menstrual cycle, from the controls and at any time from women with AN. The material was collected into plastic flasks with screw-on caps at 9 AM in the hospital and at 5 PM and 11 PM of the same day in the patient’s residence. Patients were instructed to first rinse their mouth with water twice and then to deposit the saliva directly into the container 15 to 20 minutes later. The collected saliva was centrifuged at 2000 rpm for 10 minutes and the supernatant was stored at −20°C until the day of determination. Salivary cortisol was determined in the Laboratory of the Discipline of Endocrinology and Metabolology, HCFMRP-USP.

Saliva samples (25 µl) were used for radioimmunoassay without previous extraction or dilution. Serum albumin-conjugated rabbit anti-bovine cortisol-3-carboxymethyloxime (Prof. José Gilberto H. Vieira, Escola Paulista de Medicina, São Paulo) was used as antibody. This antibody is highly specific for cortisol (100%), with little cross-reaction with cortisone (15%), corticosterone (3%) and 11-deoxycorticisol (2%) and does not react significantly with other steroids.14 Hydrocortisone [1,2-3H (N)] (New England Nuclear Products) was used as the labeled hormone. The limit of assay sensitivity was 62.4 ng/dl salivary cortisol and the intra-assay error for salivary cortisol was 7.3%.

Blood

Blood samples were collected from all patients at 9 AM together with the first saliva sample for luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), estradiol (E2), dehydroepiandrosterone-S (DHEA-S), progesterone (P4), and cortisol determinations. The purpose of the plasma cortisol measurement was to determine the correlation of the hormone with the 9 AM salivary cortisol level. In the control group, a second blood sample was collected during the second phase of the cycle for the determination of progesterone in order to confirm the occurrence of ovulation. Serum cortisol was determined by radioimmunoassay (RIA) in the Laboratory of the Discipline of Endocrinology and Metabolology, HCFMRP-USP. Serum albumin-conjugated rabbit anti-bovine cortisol-3-carboxymethyloxime was used as antibody. Hydrocortisone [1,2-3H (N)] (New England Nuclear Products) was used as the labeled hormone. The limit of assay sensitivity was 1.2 µg/dl serum cortisol and the intra-assay error was 7.3%.

DHEA-S was determined by RIA without previous extraction. Anti-DHEA-S antibody was produced in rabbits against the dehydroepiandrosterone-3-hemisuccinate-bovine albumin conjugate (Prof. José
sidered to be present when the 5PM and 11 PM values
were less than 78.1% of the morning value (9 AM),
which was considered to be 100%. 15

For statistical analysis of hormonal data, values be-
low the detection limit of the assays were considered
to be the minimum detection level.

The areas under the curves were compared by the
paired t-test and the 9 AM and 11 PM ratios were com-
pared by the Mann-Whitney test. Correlations were
calculated by the Pearson correlation test.

The level of significance was set at $P < 0.05$ in all
analyses. The Graph Pad Prism version 3.0 and the
GMC Biological Research, version 8.4 programs were
used for the statistical calculations.

**Results**

Median age was 21.0 years ($Q_1 = 19.5; Q_3 = 28.0$)
for the control subjects and 19.5 ($Q_1 = 16.5; Q_3 =
25.0$) for the AN group ($P = 0.25$). BMI was 21.4
($Q_1 = 20.6; Q_3 = 22.9$) and 15.6 ($Q_1 = 13.7; Q_3 =
16.6$) kg/m$^2$ for the control and AN groups, respec-
tively ($P = 0.0001$).

Plasma LH, FSH, PRL, E2, DHEA-S, and P4 levels
are listed in Table 1. There was a reduction in the
levels of all hormones in patients with AN, with sig-
nificant differences for LH, E2, and DHEAS. The
levels of P4 confirm the ovulatory cycle in the control
group. The individual concentrations of plasma (9 AM)
and salivary cortisol (9 AM, 5 PM, and 11 PM) for
the control and AN groups are presented in Tables 2 and
3, respectively.

There was a significant positive correlation ($r =
0.56; P = 0.035)$ between serum and salivary cortisol
levels in the samples collected at 9 AM when consider-
ing both groups together ($r = 0.45; P = 0.01$)
(Fig. 1A). However, when the groups were analyzed
separately, this correlation was significant for the con-
trol group ($r = 0.67; P = 0.01$) (Fig. 1B), but not for
the AN group ($r = 0.48, P = 0.12$) (Fig. 1C).

Individual analysis of the curves showed that, ac-
cording to the adopted criteria, the cortisol rhythm
was present in all control patients, but was absent in
one third of the patients with AN.

The median cortisol levels of AN patients were higher
at all time points considered (Tables 2 and 3). The
median salivary cortisol levels were 560, 180, and
108 ng/dl for 9 AM, 5 PM, and 11 PM, respectively, for
the control group and 690, 278 and 132 ng/dl, respec-
tively, for AN patients. When patients with circadian
rhythm were analyzed separately, the median values
were 940, 278, and 180 ng/dl for 9 AM, 5 PM, and 11
PM, respectively. The area under the curve was signifi-
cantly higher in the patients with preserved rhythm than
in the control group (Me = 6811 ng/dl/24h vs 3708 ng/
dl/24h; $P = 0.034$). See Fig. 2.

Between 9 AM and 11 PM, the ratio of cortisol levels
was 5.42 in the control group versus 3.42 in the AN
group, indicating a flattening of the cortisol rhythm
curve in AN. However, when the AN group was sub-
divided into patients with and without preserved

<table>
<thead>
<tr>
<th>Groups</th>
<th>LH mIU/ml</th>
<th>FSH mIU/ml</th>
<th>PRL ng/ml</th>
<th>E2 pg/ml</th>
<th>DHEA-S µg/dl</th>
<th>Progesterone ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control median</td>
<td>3.1*</td>
<td>2.9</td>
<td>8.8</td>
<td>47.10**</td>
<td>175.0t</td>
<td>10.1</td>
</tr>
<tr>
<td>Q1</td>
<td>2.6</td>
<td>2.5</td>
<td>7.5</td>
<td>36.7</td>
<td>150.0</td>
<td>8.1</td>
</tr>
<tr>
<td>Q3</td>
<td>3.8</td>
<td>4.4</td>
<td>13.3</td>
<td>66.6</td>
<td>270.0</td>
<td>13.9</td>
</tr>
<tr>
<td>AN median</td>
<td>0.7*</td>
<td>1.95</td>
<td>6.5</td>
<td>26.9**</td>
<td>110.0t</td>
<td>NA</td>
</tr>
<tr>
<td>Q1</td>
<td>0.7</td>
<td>1.12</td>
<td>5.1</td>
<td>20.0</td>
<td>66.0</td>
<td>NA</td>
</tr>
<tr>
<td>Q3</td>
<td>3.6</td>
<td>3.83</td>
<td>8.0</td>
<td>37.0</td>
<td>142.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

Q1 and Q3 = first and third quartiles. *$P = 0.0098$; **$P = 0.0065$; $tP = 0.0045$; NA = not applicable
Reference values. Follicular phase: (a) LH: 1.4—7.7 mIU/ml; (b) FSH: 3.4—10.0 mIU/ml; (c) PRL: 3.0—20.0 ng/ml; (d) E2: ≤102 pg/ml; (e) DHEA-S: 80.0 to 300.0 µg/dl; (f) cortisol: 5.0—20.0 µg/dl

**Data Analysis**

For the calculation of the circadian rhythm of salivary
cortisol we multiplied the intra-assay error (7.3%) by
3, with a resulting value of 21.9%. A rhythm was con-

The sensitivity limits for LH, FSH, PRL, E2 and P4
determinations were per-
formed in the Laboratory of Obstetrical Physiology
and Pharmacology of HCFMRP—USP. The method
used was chemiluminescence based on the double an-
tibody technique according to the protocols of the Di-
agnostic Products Corporation kits (DPC, Los
Angeles, CA) using an Immulite automatic analyzer.

The sensitivity of the method was 5 µg/dl DHEA-S
and the intra-assay error was 5.5%.

LH, FSH, PRL, E2, and P4 determinations were per-
formed in the Laboratory of Obstetrical Physiology
and Pharmacology of HCFMRP—USP. The method
used was chemiluminescence based on the double an-
tibody technique according to the protocols of the Di-
agnostic Products Corporation kits (DPC, Los
Angeles, CA) using an Immulite automatic analyzer.

The sensitivity limits for LH, FSH, PRL, E2 and P4
determinations were per-
formed in the Laboratory of Obstetrical Physiology
and Pharmacology of HCFMRP—USP. The method
used was chemiluminescence based on the double an-
tibody technique according to the protocols of the Di-
agnostic Products Corporation kits (DPC, Los
Angeles, CA) using an Immulite automatic analyzer.
rhythm the ratio was found to be 3.78 for the group with rhythm ($P > 0.05$) and 1.95 for the group without rhythm ($P = 0.029$).

**Discussion**

Although most reports in the literature show high cortisol levels in groups of patients with AN, the measurement of serum cortisol has not been useful for the diagnosis of the disease because individual cortisol values often overlap with those of normal subjects. This fact is observed in the present study, and, in our experience, the measurement of plasma cortisol has not been a good diagnostic indicator in patients evaluated on an individual basis. Similar data were reported by Couzinet et al.\(^\text{16}\) for women with nutritionally caused functional hypothalamic amenorrhea. A possible explanation is the wide variability of plasma cortisol values or the fact that total serum cortisol may not reflect the fluctuations of free cortisol.

It should be kept in mind that, when an axis activated by stress is assessed, hospitalization, medical procedures, or even a medical visit can induce further stress in most patients, thus possibly interfering with the results. For this reason, saliva collection at home seems to be adequate for this type of study, considering the low stress produced by this procedure. The measurement of free cortisol in saliva has been recommended as a noninvasive method of easy access, with the material being collected and stored at home by the patient, and the result obtained shows an accurate correlation with free plasma cortisol.\(^\text{1,17}\) However, although salivary cortisol determination involves simple and low cost techniques, it has not been routinely used and few reports are available about its use to assess patients with AN.

In the present study, a blood sample was collected in parallel to the 9 AM saliva sample in order to confirm the previously reported correlation between plasma and salivary cortisol levels.\(^\text{5,18}\) The determination of this correlation is important to validate the fact that free salivary cortisol levels reflect plasma cortisol levels. The data showed a significant correlation between serum and saliva levels in the samples considered as a whole (control and AN). However, when the groups were analyzed separately, the patients with AN did not show a significant correlation. According to some authors, when cortisol levels are above the corticosteroid-binding globulin (CBG) saturation point, a fact commonly occurring in patients with AN, there is a loss of this correlation.\(^\text{5,18}\) Also Casper et al.\(^\text{19}\) had already demonstrated loss of the affinity constant for CBG in patients with AN.

The fact is that patients with AN showed higher salivary cortisol levels, as observed both in the samples obtained at the different time points and in the measurements of the areas under the curve of circadian rhythm.

The increase in cortisol in AN has been attributed by most investigators to dysfunctions in the hypothalamic mechanisms that control ACTH secretion, but the mechanism involved has not been fully clarified. Hypercortisolism is not accompanied by an increase in other ACTH-dependent adrenal hormones, as is the case for DHEA-S. Sirinathsinghji et al.\(^\text{20}\) have demonstrated that in AN patients hypercortisolism is accompanied by loss of DHEA-S rhythm, with a reduction in its levels, a fact that cannot be explained.

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**Table 2.** Plasma and salivary cortisol levels of normal ovulatory women at 9 AM, 5 PM, and 23 PM

<table>
<thead>
<tr>
<th>Patients</th>
<th>Plasma Cortisol (ng/dl)</th>
<th>Salivary Cortisol (ng/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 AM</td>
<td>5 PM</td>
</tr>
<tr>
<td>EGC</td>
<td>19.2</td>
<td>540.0*</td>
</tr>
<tr>
<td>MCP</td>
<td>11.2</td>
<td>360.0*</td>
</tr>
<tr>
<td>EEU</td>
<td>22.4</td>
<td>308.0*</td>
</tr>
<tr>
<td>MAR</td>
<td>7.2</td>
<td>188.0*</td>
</tr>
<tr>
<td>LOS</td>
<td>24.8</td>
<td>820.0*</td>
</tr>
<tr>
<td>RPR</td>
<td>19.2</td>
<td>800.0*</td>
</tr>
<tr>
<td>LCS</td>
<td>27.2</td>
<td>920.0*</td>
</tr>
<tr>
<td>MAGF</td>
<td>16.8</td>
<td>360.0*</td>
</tr>
<tr>
<td>ACBS</td>
<td>13.6</td>
<td>560.0*</td>
</tr>
<tr>
<td>DPV</td>
<td>12.8</td>
<td>920.0*</td>
</tr>
<tr>
<td>ILC</td>
<td>11.6</td>
<td>200.0*</td>
</tr>
<tr>
<td>LFRM</td>
<td>22.4</td>
<td>1000.0*</td>
</tr>
<tr>
<td>KN</td>
<td>25.6</td>
<td>1040.0*</td>
</tr>
<tr>
<td>Median</td>
<td>19.2</td>
<td>560.0*</td>
</tr>
<tr>
<td>Q1</td>
<td>12.8</td>
<td>334.0</td>
</tr>
<tr>
<td>Q3</td>
<td>22.4</td>
<td>920.0</td>
</tr>
</tbody>
</table>

* Circadian cortisol rhythm; \(^*\) P < 0.05 compared to 5 PM and 11 PM

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**Table 3.** Plasma and salivary cortisol levels of patients with anorexia nervosa at 9 AM, 5 PM, and 11 PM

<table>
<thead>
<tr>
<th>Patients</th>
<th>Plasma Cortisol (ng/dl)</th>
<th>Salivary Cortisol (ng/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 AM</td>
<td>5 PM</td>
</tr>
<tr>
<td>RAB</td>
<td>5.9</td>
<td>920.0*</td>
</tr>
<tr>
<td>ASM</td>
<td>20.0</td>
<td>960.0*</td>
</tr>
<tr>
<td>ACFSGB</td>
<td>19.2</td>
<td>1,120.0*</td>
</tr>
<tr>
<td>CCZ</td>
<td>13.6</td>
<td>296.0</td>
</tr>
<tr>
<td>NAGN</td>
<td>17.6</td>
<td>556.0*</td>
</tr>
<tr>
<td>MRRSP</td>
<td>22.4</td>
<td>480.0*</td>
</tr>
<tr>
<td>JC</td>
<td>32.0</td>
<td>1,240.0*</td>
</tr>
<tr>
<td>JRM</td>
<td>24.0</td>
<td>580.0</td>
</tr>
<tr>
<td>PAI</td>
<td>28.8</td>
<td>800.0*</td>
</tr>
<tr>
<td>VAS</td>
<td>10.0</td>
<td>176.0</td>
</tr>
<tr>
<td>MFGM</td>
<td>12.8</td>
<td>240.0</td>
</tr>
<tr>
<td>MLGC</td>
<td>19.2</td>
<td>1,000.0*</td>
</tr>
<tr>
<td>Median</td>
<td>19.2</td>
<td>690.0</td>
</tr>
<tr>
<td>Q1</td>
<td>13.4</td>
<td>388.0</td>
</tr>
<tr>
<td>Q3</td>
<td>22.8</td>
<td>960.0</td>
</tr>
</tbody>
</table>

* Circadian cortisol rhythm; \(^*\) P < 0.05 compared to 5 PM and 11 PM
by the same mechanisms of cortisol increase. Reduction of DHEA-S also was observed in our data.

The interpretation of changes in cortisol rhythm, however, has some limitations. Although some literature reports indicate that the circadian profile of cortisol is highly consistent, changes in the circadian rhythm of cortisol have been reported even during normal menstrual cycles or in normal adult individuals and in young and elderly subjects, with individual variations from day to day, suggesting that the loss or variation of cortisol rhythm is not so infrequent.

In recent years, many reports have indicated changes in cortisol rhythm in different diseases known to be associated with psychic disorders and stress, such as colorectal cancer, fibromyalgia, chronic pain, Alzheimer disease, chronic fatigue syndrome, bronchial asthma, exhausting work, and depression. Individual changes in the circadian rhythm of salivary cortisol have been reported in these publications, such as disappearance of the rhythm or an inconsistent rhythm over successive days. Particularly in depressive patients changes in cortisol rhythm have been reported with recovery after treatment and although a certain rhythmicity persisted, increased diurnal cortisol secretion was observed in these patients. Thus, in situations of psychic disorders, considering that the temporal sequence of cortisol secretion is under the control of the central nervous system, it would not be surprising if some patients with AN presented subtle changes in the circadian rhythm of cortisol, reflected by a reduction of the 9 AM to 11 PM ratio or even a full loss, as was the case for one third of the patients studied here. These alterations, however, have not been described for AN. Most studies have reported maintenance of the cortisol rhythm in the plasma of patients with AN. As far as we know, only the study by Putgnano et al investigated the rhythm of free salivary cortisol in addition to the rhythm of plasma cortisol. The authors pointed out the maintenance of a circadian rhythm both in blood and saliva, although they reported a "flattening" of the curve of the cortisol cycle in patients with AN compared to control. However, they did not perform a more detailed individual analysis of this behavior of the cortisol rhythm. They analyzed the patients as a whole and therefore the mean (or median) values obeyed a circadian pattern of rhythm and a loss of rhythm in any patient was not described. Indeed, individual analysis may reveal different and interesting results.

The flattening reported by Putgnano et al was observed by analyzing the reduction of the mean values of the 8 AM to midnight ratio of salivary cortisol samples taken at these two times. Thus we noted that, as expected, the flattening markedly occurred in the patients with no rhythm. AN patients with preserved rhythm, even with a more elevated salivary cortisol, tended to show a flattening of the curve, with a reduction of the ratio, although in a statistically nonsignificant manner. Thus, we suppose that the flattening of cortisol rhythm described by the cited authors may reflect alterations of the circadian rhythm in some of the patients included in the analysis and that this detail may have disappeared when they were analyzed as a group.

Also, we thought that it would not be adequate to compare the control patients, all of whom presented the rhythm, to the AN patients as a whole, because 33% of the AN patients did not present the rhythm. This might have led to a bias in the interpretation of

![Fig. 1. Correlation between salivary and plasma cortisol: (A) all patients, (B) control patients and (C) anorexic patients.](image1.png)

![Fig. 2. Salivary cortisol levels (Me) in patients with anorexia nervosa and preserved circadian rhythm compared to control women at the different time points studied. Areas under the curve from AN vs Control with $P = 0.038$.](image2.png)
the results and for this reason we divided the patients into groups with and without the rhythm for the purposes of comparison.

The mechanisms involved in the increase in free cortisol and the absence of cortisol rhythm detected in some patients with AN still need to be elucidated. Besides the effects of the CNS on the HHA axis, other factors such as the reduction in CBG levels due to the low estrogen levels provoked by GnRH inhibition by CRH\(^{28}\) and/or to the malnourished status of these patients may be involved in the process. In addition, if food ingestion can influence the salivary cortisol rhythm\(^{29}\) it would be quite natural for patients with AN to be likely to present these modifications in view of their well-known eating disorder.

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References