

## Evaluation of levels of cortisol in saliva using electro-chemical luminescence in low-risk and high-risk pregnancies

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## Avaliação do cortisol salivar por electroquimioluminescência em gestantes de baixo e de alto risco

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### Abstract

*Objectives: to compare the levels of cortisol (cortisolemia refers to the level of cortisol in blood) in women with a high-risk pregnancy compared with those with a low-risk pregnancy, by way of evaluation of levels of cortisol in saliva, using the electrochemical luminescence technique (ECL).*

*Methods: 38 women aged between 17 and 40 years in the third trimester of pregnancy were divided in two groups: 20 low-risk pregnancies and 18 high-risk ones. Cortisol in saliva was collected at midnight and measured using ECL. The mean levels of cortisol in saliva in the two groups were compared using the Kruskal-Wallis test.*

*Results: the mean systolic and diastolic pressure was normal in both groups. The levels of cortisol in the saliva of women with high-risk pregnancies was significantly higher than those for the low-risk pregnancy group (20.2 ( $\pm$ 21,1) nmol/L vs 11.4( $\pm$ 16.2) nmol/L;  $p=0.007$ ).*

*Conclusions: a high risk pregnancy involves higher levels of cortisol than a low-risk one. The levels of cortisol in saliva, as measured using ECL, can be used to identify hypercortisolism in pregnancy.*

**Key words** Cortisol, Pregnancy, Pregnancy, high-risk

### Resumo

*Objetivos: comparar os níveis de cortisol em mulheres com gravidez de alto risco em relação às gestantes de baixo risco, por meio da avaliação do cortisol salivar utilizando a técnica da electroquimioluminescência (EQL).*

*Métodos: foram estudadas 38 mulheres de 17 a 40 anos de idade, no terceiro trimestre de gestação, divididas em dois grupos: 20 gestantes de baixo risco e 18 gestantes de alto risco. O cortisol salivar foi coletado à meia-noite e medido através da EQL. As médias do cortisol salivar foram comparadas entre os dois grupos de gestantes através do teste de Kruskal-Wallis.*

*Resultados: a média das pressões sistólica e diastólica foi normal nos dois grupos. O cortisol salivar das gestantes de alto risco foi significativamente mais elevado do que das gestantes baixo risco: (20,2 ( $\pm$ 21,1) nmol/L vs 11,4( $\pm$ 16,2) nmol/L;  $p=0,007$ ).*

*Conclusões: a gestação de alto risco cursa com níveis mais elevados de cortisol quando comparada à gestação de baixo risco. O cortisol salivar, medido pelo EQL mostrou-se promissor para identificar o hiper-cortisolismo na gestação.*

**Palavras-chave** Cortisol, Gravidez, Gravidez de alto risco

## Introduction

Human gestation brings about changes in the activity of most endocrine systems in a woman, including the hypothalamic-pituitary-adrenal axis (HPA).<sup>1</sup> These alterations in the HPA axis are important for maintaining an adequate environment for growth and development of the fetus, since an excess or lack of cortisol results in disruption of maternal-fetal homeostasis.<sup>2-4</sup>

Excess maternal corticoid during pregnancy has been being observed for forty years now, with elevated levels beginning to be detected at around the 12<sup>th</sup> week of pregnancy.<sup>5</sup> The difficulty studying alterations in the HPA axis in a pregnant woman is that of establishing a reliable biological marker and a practical diagnostic method which is not influenced by the physiological alterations arising from pregnancy itself.<sup>5,6</sup>

The neuroendocrine changes that occur in every pregnancy alter the parameters used by laboratory techniques for dealing with hypercortisolism.<sup>1</sup> Increased levels of estrogen stimulate the hepatic production of glycoprotein carrying globulin (GCG).<sup>1</sup> This increase in hepatic GCG, which continues until the 12<sup>th</sup> day after birth, causes a rise in circulating levels of cortisol linked to the protein and occasions a temporary fall in levels of free cortisol, which, in turn, leads to a reduction in negative feedback to the HPA axis.<sup>7</sup> Consequently, levels of the adrenocorticotrophic hormone (ACTH) rise and this stimulates the production of cortisol. The levels of free cortisol are initially normal and then rise during pregnancy and reach maximum levels at the end of the second and third trimesters.<sup>5</sup> Total cortisol and free plasmatic cortisol can reach values that are two or three times higher compared with women who are not pregnant. These high levels of plasmatic cortisol observed in pregnant women are equivalent to those found in Cushing's syndrome.<sup>1</sup> The increase in levels of free cortisol during pregnancy also leads to restriction of the action of cortisol during this period. Despite the increase in serum cortisol during pregnancy, the circadian rhythm of the system is preserved,<sup>8</sup> but it is not known what variation in levels of cortisol is responsible for complications occurring during high-risk pregnancies. Evaluating the levels in normal pregnant women as well as in the high risk group may provide information on these questions.

Studies evaluating hypercortisolism using dexamethasone are difficult to interpret during pregnancy, owing to the alterations that normally occur during pregnancy itself.<sup>1</sup> Studies have shown a

smaller reduction in levels of plasmatic and urinary cortisol in pregnant women, after a suppression test with 1 mg of dexamethasone, compared to women who are not pregnant.<sup>1</sup> In the post-partum period, this abnormality may persist for two or three weeks in a significant proportion of women.<sup>1-2</sup> This reduction in the suppressive action of dexamethasone contributes to the GCG effect on cortisol and restriction of the action of cortisol,<sup>1,5</sup> or to the possible antiglycorticoid effect of progesterone on tissue.<sup>5</sup> It is thus necessary to establish laboratory techniques and paradigms for pregnancy in order to identify variations in levels of hormones that truly differ from the values considered to be physiological.<sup>7</sup>

It is common to measure the levels of free urinary cortisol when diagnosing hypercortisolism in pregnancy.<sup>1-4</sup> The principle underlying this method is the detection of the free fraction of cortisol, thereby diminishing the influence of pregnancy on concentrations of cortisol in serum. However, this diagnostic method has limitations regarding elaboration and interpretation, as collection is a laborious process and the laboratory technique used is radioimmunoassay (RIA).<sup>8</sup> At present, the "gold standard" test for measuring free urinary cortisol involves structural assays, such as mass spectroscopy, which are more accurate than methodologies using assays based on antibodies.<sup>5</sup>

Measurement of cortisol in saliva at midnight has been used as a tracking test to identify hypercortisolism in non-pregnant women.<sup>9-13</sup> This method measures the free fraction of cortisol in serum and has sensitivity and specificity similar to that of other established methods for tracking increases in cortisol levels.<sup>14</sup> It also has the advantage that it is a non-invasive method and samples can easily be collected in the patient's own home.<sup>15</sup> The concentration of cortisol in saliva reflects the levels of free fraction cortisol in plasma and is not affected by the amount of saliva produced or by variations in the concentration of glycoprotein carrying globulins caused by the use of oral contraceptives and pregnancy.<sup>12</sup> Measurement of cortisol in pregnancy to evaluate hormone response to psychological stress has been used as a predictor of adversities in pregnancy.<sup>13-14</sup>

The aim of this study is to compare levels of cortisol in high-risk pregnancies by evaluating cortisol in saliva using electrochemical luminescence (ECL) to study the HPA axis during pregnancy.

## Methods

A cross-sectional type study was carried out among

women with high and low risk pregnancies after the 24<sup>th</sup> week of gestation, measured from the last date of menstruation or using ultrasound parameters. The participants were recruited at the pre-natal outpatients clinic of the Agamenon Magalhães Hospital, which attends women with high- and low-risk pregnancies referred by the Brazilian National Health System in the city of Recife and its surrounding metropolitan region, in the State of Pernambuco, Brazil, between August and October 2006. Levels of cortisol were analyzed in samples of saliva from the two groups of women with high- and low-risk pregnancies.

Thirty-eight pregnant women were selected, 20 with low-risk pregnancies and 18 with high-risk. The inclusion criteria were: a good level of understanding on being invited, not having systemic arterial hypertension, *diabetes mellitus* or heart disease either prior to pregnancy or related to it. The pregnant women were selected at the outpatients clinic and from the wards of the above mentioned hospital and the criteria were: being in the third term of pregnancy, being aged between 18 and 35 years, not using corticoid and not presenting any psychiatric disorders.

After selection the participants signed a free and informed consent form, answered the questionnaire to provide information on the woman and her pregnancy, and were told how to collect the saliva samples at home from the free flow of saliva, without expectoration, i.e. being careful to expel only salivary secretion and to avoid mixing it with tracheal secretion. The saliva was collected in a dry cylindrical plastic tube (8 mL), the amount of saliva collected was around 2 mL, always at midnight, the same time established for the investigation of non-pregnant women and men with Cushing's, since salivary cortisol is low at this time and alterations in cortisol production are thus easier to identify. The patients were informed that they could not eat, sleep or engage in strenuous exercise for at least three hours before collection. This advice was given to avoid any interference of these activities in the levels of cortisol in both groups. After collection, the women were told to store the sample in a refrigerator or a polystyrene container with ice in their own home (in the case of patients in hospital, the samples were kept in the high-risk ward refrigerator, at an average temperature of 4°C. The samples collected at home were brought to the hospital by the patient and sent in a polystyrene container to the laboratory researcher, within at most 10 hours after collection.

The technique used measure levels of cortisol involved electro-chemical luminescence immuno-

assay (EQL). EQL is a very precise method for measuring hormone levels. The test used for the study, Elecsys Cortisol, is based on the competition test principle using a polyclonal antibody specifically directed against cortisol. The use of saliva for measurement of cortisol levels facilitates the technique, as there is no need to separate the hormone from its protein carrier. This is a very simple technique, as it requires no catalyst, the reagents used for the reaction are highly stable, and have maximum sensitivity. The COBAS apparatus with the Elecsys 1010/2010 analyser and Roche modular analytics E170<sup>®</sup> were used. The saliva collected was centrifuged and incubated directly without treatment. The sensitivity of the test was <0.5 nmol/L. The intra-assay variation coefficient of the samples used to normalize the technique ranged from 1.5% to 6.1% and the corresponding interassay variation coefficients ranged from 4.1% to 37.1%.

The results were expressed as the medians of their respective interquartile intervals. The statistical comparisons for the quantitative variables describing the clinical characteristics of the groups were carried out using Student's *t* test. Evaluation of the level of salivary cortisol in the two groups of women was conducted using the Kruskal-Wallis test. In all the analyses, a *p* value equal to or lower than 0.05 was considered statistically significant.

The project received prior approval from the Agamenom Magalhães Hospital's Ethics Committee, protocol FR-072669.

## Results

In the low-risk pregnancy group, 22 of the 30 women originally selected returned with the sample, two of whom (9%) were excluded for not having provided a large enough sample. For this reason, only 20 low-risk pregnant women were finally included in the study. Of the 25 high-risk pregnant women, 20 collected the sample. Two of these had to be excluded for not providing a large enough sample (10%) resulting in a final study sample of 18 in this group.

Of the 18 pregnant women in the high-risk group, six (33.3%) were in hospital with pre-term labor, five (27.7%) had chronic systemic arterial hypertension, five (27.7%) had pre-eclampsia, one (5.5%) chronic hypertension and diabetes and one (5.5%) heart disease (heart failure following valvulopathy). Three of the women with chronic hypertension were using methyl dopa. No pregnant woman from either group presented clinical signs of hypercortisolism.

The main clinical characteristics of the two groups are shown in Table 1. The women in the high-risk group were, on average, younger ( $p<0.001$ ) and had higher systolic ( $p=0.003$ ) or diastolic ( $p=0.007$ ) arterial pressure than those in the low-risk group.

Table 2 shows the levels of salivary cortisol in the two groups. The levels of cortisol in high-risk pregnant women were significantly higher than those

in women with low-risk pregnancies ( $p=0.007$ ). There was an asymmetrical distribution of salivary cortisol in each group. Two extreme values were identified, one in each group, and, even when these were disregarded, the asymmetry persisted, as can be seen in Figure 1.

**Table 1**

Clinical characteristics of the population under study.

Parameters	High-risk pregnancies (G1)	Low-risk pregnancies (G2)	p
	(N=18)	(N=20)	
	$\bar{X} \pm DP$	$\bar{X} \pm DP$	
Age (years)	25,0 $\pm$ 7,0	29,6 $\pm$ 3,3	<0,001
Gestational age (weeks)	31,8 $\pm$ 4,1	31,9 $\pm$ 5,1	0,940
Weight at 3 <sup>rd</sup> trimester (Kg)	68,8 $\pm$ 14,0	68,8 $\pm$ 8,2	0,997
Weight at 1 <sup>st</sup> trimester (Kg)	62,8 $\pm$ 12,1	60,5 $\pm$ 7,2	0,496
Weight variation	6,0 $\pm$ 4,3	8,3 $\pm$ 7,2	0,127
SAP* (mmHg)	122,0 $\pm$ 17,0	107,5 $\pm$ 11,6	0,003
DAP** (mmHg)	75,5 $\pm$ 11,9	66,0 $\pm$ 8,8	0,007

\* SAP=systolic arterial pressure; \*\* DAP=diastolic arterial pressure.

**Table 2**

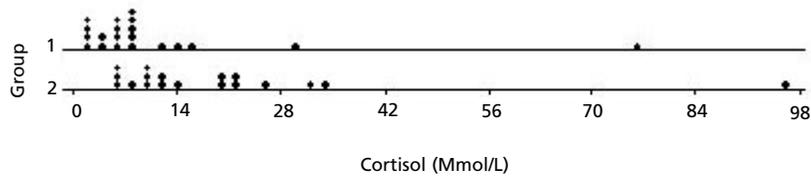
Levels of cortisol in the saliva of 38 pregnant women, as measured using electro-chemical luminescence.

	High-risk pregnancies	Low-risk pregnancies	p
	(N=18)	(N=20)	
Mean ( $\pm$ SD)	20,23 (21,10)	11,45 (16,23)	0,007
Median	12,64	6,533	0,007
25% percentile	8,757	3,892	
75% percentile	21,960	8,896	
Interquartile difference	13,205	5,004	

Cortisol level in nmol/L.

**Figure 1**

Distribution of levels of cortisol in saliva, by group.



Group 1 = Low-risk; Group 2 = High-risk. Each point represents an individual.

## Discussion

The results of the present study suggest that salivary cortisol, measured using electrochemical luminescence (ECL), is found at significantly higher levels in women with high-risk pregnancies compared to the low-risk group. The women with low risk pregnancies had mean cortisol levels in their saliva of 11.450 nmol/L, almost four times higher than standard laboratory reference value for cortisol at midnight, in individuals without hypercortisolism, which is 3.058 nmol/L.<sup>15</sup>

The use of ECL to measure cortisol in saliva in pregnant women has not been the subject of much study<sup>4,15</sup> and those that use this technique have not collected their samples at midnight.<sup>15</sup> In pregnancy, the evaluation of the neuro-endocrine system should take into account the alternations that are normal in women in this condition. Physiological hypercortisolism in pregnant women affects the measurements and interpretation of values obtained for salivary cortisol. As circadian rhythms remain the same, it is important that the sample be collected at midnight to reduce the interference of high morning levels of cortisol on the results.<sup>15</sup>

Many factors influence the level of cortisol in saliva. The figures produced by this study demonstrate an asymmetrical distribution. The data were collected in an attempt to establish general parameters for the health of pregnant women, although the concepts of well-being, stress-levels and family stability were not clearly defined. The literature reports that these factors affect the dynamic of the neuro-endocrine system during pregnancy.<sup>16</sup>

The women who attend prenatal clinics in public hospitals are predominantly from the low-income sector.<sup>17</sup> In view of this, many of these women may

be undernourished, with vitamin deficiencies and anemia, have an insalubrious home environment and low levels of education.<sup>17</sup> These factors may have interfered in the measurement of cortisol in both groups of pregnant women. Another point worth making is the fact that many of the women with high-risk pregnancies had already been hospitalized, which may have affected the levels of cortisol. This, along with the number of participants, makes it difficult to extrapolate references for pregnant women in general from these figures.

Cortisol levels were measured only once for each participant. One of the methodological limitations of this study was the difficulty in showing pregnancy events in chronological order. There were no laboratory data on the women's cortisol levels prior to pregnancy or in the first trimester. Nevertheless, the women did not have clinical manifestations of hypercortisolism on the initial evaluation, which supports the idea that the increase in cortisol levels was a result of pregnancy. It should be pointed out that the clinical symptoms of Cushing's syndrome worsen during pregnancy and that the two cases of extreme levels of cortisol need to be evaluated subsequent to pregnancy to rule out the presence of a neuro-endocrine disorder. The pregnant woman in the low-risk group who had a high level of cortisol was 24 weeks pregnant, had experienced greater than average weight-gain (4 kg) and did not display any of the clinical manifestations of Cushing's syndrome. These figures may be distorted by the influence of extraneous factors that were not clearly identified in the study, such as, for example, the degree of stress experienced in everyday life. Other factors that might have affected the results, such as contamination by bleeding gums, are less likely, since an oral cavity examination was carried out on

each of the women before the saliva was collected.

This study found that the cortisol levels in the high-risk pregnancy group were on average higher than they would be during a risk-free pregnancy, which raises a number of questions regarding the influence of this hormone in the course of gestation. Cortisol plays an important part in the development of the fetus, although excessive levels are related to negative metabolic changes. Given the limitations of the study, it was not possible to establish a prospective relationship between these events. Identification

of reference values for cortisol in pregnancy or variations in physiological hypercortisolism could lead the way to the development of new tools for health professionals managing pregnancy. Hitherto there have been no studies that have analyzed and compared cortisol levels in the saliva of women with low- and high-risk pregnancies. Further research needs to be carried out to clear up the outstanding questions.

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