Assessment of salivary amylase as a stress biomarker in pregnant patients.
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Running title: Salivary alpha-amylase: a stress biomarker in pregnant patients

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<table>
<thead>
<tr>
<th>MeSH key words</th>
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<tr>
<td>Stress, Psychological</td>
</tr>
<tr>
<td>Saliva</td>
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<td>Salivary alpha-Amylases</td>
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<td>Caesarean Section</td>
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**Abstract (words number: 266)**

**Background:** Chronic stress during pregnancy has been associated with worsened maternal, obstetric or foetal outcomes and poor pain relief during labor. Acute stress immediately before spinal anesthesia for caesarean section may contribute to the occurrence of hypotension. Objective measures of the level of acute stress may therefore help identify pregnant patients at risk for adverse outcomes. Salivary alpha-amylase (SAA) is a stress biomarker poorly investigated during pregnancy. The goals of this study were therefore to determine if (i) SAA increases in pregnant patients submitted to the stress of the transfer to the operating room (OR), (ii) this increase is significant by calculating the reference change value (RCV) in healthy volunteers. RCV is the critical difference that must be exceeded between two sequential results for a change to be considered clinically relevant.

**Methods:** In 15 pregnant patients undergoing planned caesarean section under spinal anesthesia, SAA, systolic blood pressure, heart rate, and immediate anxiety were measured on the morning of surgery and in the OR. RCV was calculated in 18 healthy volunteers.

**Results:** A median 220 % increase in SAA activity ($P = 0.0015$) and a median 17 % increase in systolic blood pressure ($P = 0.0006$) were observed between the ward and the OR in pregnant patients. No changes of immediate anxiety or heart rate were observed. RCV was $\pm 76 \%$ in volunteers and 13 out of the 15 pregnant patients had a SAA increase greater than the RCV.

**Conclusion:** Under stressful conditions, a clinically and statistically increase in SAA is observed in pregnant patients. Further studies are required to define the clinical utility of SAA.
Introduction

Chronic stress during pregnancy has been associated with worsened maternal, obstetric, foetal or neonatal outcome and poor pain relief during labor.\textsuperscript{1-3} Moreover, acute stress immediately before spinal anesthesia for planned caesarean section may contribute to the occurrence of hypotension.\textsuperscript{1, 4-6} More specifically, as demonstrated by Hanss et al, increased sympathetic activity before spinal anesthesia evidenced by an increased low-to-high frequency ratio with off-line heart-rate variability analysis is associated with hypotension and a prophylactic treatment of hypotension guided by this ratio is effective.\textsuperscript{4-6} Real-time, objective and non-invasive assessment of the level of acute stress may therefore help identify pregnant patients at risk for adverse outcomes (e.g. hypotension after spinal anesthesia for caesarean section). Unfortunately, non-invasive assessment of stress during pregnancy is challenging. Self-administered questionnaires like the Spielberger’s State-Trait Anxiety Inventory are of no help in uncooperative patients or in cases of language or comprehension barriers which represents up to 20\% of the patients followed in our Institution.\textsuperscript{7} Stress-induced hemodynamic changes i.e. increased heart rate may not indicate stress in pregnant patients owing to, firstly, the attenuation of the chronotropic response to endogenous catecholamines and beta-adrenergic agents observed during pregnancy and, secondly, patients’ chronic medications.\textsuperscript{8, 9}

In recent years, saliva components such as alpha-amylase (SAA) have gained interest as non-invasive indicators of body changes associated with stress. It is one of the principal salivary proteins secreted by the highly differentiated epithelial acinar cells of the exocrine salivary glands via activation of beta-adrenergic receptors. SAA is viewed as a measure of endogenous sympathetic activity and an association between salivary amylase activity and low-to-high frequency ratio was demonstrated.\textsuperscript{10,11} It has therefore been proposed as a stress biomarker in non-perioperative situations such as extreme sport activities or induced psychological stress.\textsuperscript{12,13-15} However, blunting of SAA increase to stress exposure has been reported in the second and third trimesters of pregnancy when compared with non-pregnant patients that may make SAA unsuitable to assess stress during late pregnancy.\textsuperscript{16} The first goal of this study was therefore to determine if SAA increases
in pregnant patients submitted to the stressful environment of the operating room for a planned caesarean section under spinal anesthesia.

To confer clinical relevance to changes in SAA activity observed in pregnant patients submitted to stress, the critical difference that must be exceeded between two sequential results that is above the biological and analytical "noise" must be determined. Reference change value (RCV) is proposed to compare two consecutive biological measurements. It takes into account the variability related to the measurement techniques of SAA activity (analytical variability measured by the inter-assay coefficient of variation) and the intra-individual variability of SAA activity in patients (biological variability measured by the coefficient of intra-individual variation).\textsuperscript{17, 18} Analytical and biological variability values used to calculate RCV are obtained from a normal, control population using a saliva collection technique, an amylase activity measurement technique and a delay between two saliva samples similar to those used in the population of interest, i.e. pregnant patients. The second goal of this study was therefore to calculate the RCV of SAA in healthy volunteers in order to determine if the change in SAA activity observed in pregnant patients exposed to the stress of the operating room is clinically significant (above the biological and analytical variability).
Materials and methods

The study was approved by the Ethics Committee of Cochin Hospital, Assistance Publique-Hôpitaux de Paris, France. Informed consent was obtained from each patient.

Pregnant patients

Fifteen ASA 1 or 2 female patients undergoing planned caesarean section under spinal anaesthesia were studied. Patients were fasting since midnight. Blood pressure, heart rate, anxiety and SAA activity were measured in the following temporal sequence on the ward and in the operating room.

Anxiety was measured with the Spielberger’s State-Trait Anxiety Inventory (STAI) form. This is a validated tool for self-reporting immediate anxiety. It comprises one set of 20 statements related to the immediate situation, the state anxiety. It includes statements like “I feel calm” or “I am worried”. To each statement, the patient is required to select one out of four responses: not at all, somewhat, moderately so or very much so. The score for the STAI-state changes as the context changes. It takes about 5 minutes to fill in the STAI-state form. The minimum and maximum values for the score are 20 and 80.

Non-invasive blood pressure and heart rate were measured after at least 15 minutes of bed rest with the patient lying supine with a 20° left tilt. The mean of at least three consecutive measurements was calculated.

Volunteer patients

Volunteers were recruited among members of the medical staff of the Anesthesia and Biochemistry Departments of Bichat Hospital. To avoid stress related to working conditions, volunteers came to hospital on a day off. Moreover, samplings were always performed in a quiet area of the Anesthesia Department after 10 minutes of resting. Volunteers were fasting since midnight.

Saliva collection
Subjects were required to abstain from smoking, eating or drinking water one hour and brushing teeth three hours before saliva sampling. No subject was on medication containing adrenergic agonists or antagonists. Saliva sampling used a saliva collection device (Salivette®, Sarstedt, France) over a 2-minute period. The collecting tube was centrifuged and the supernatant stored at -20°C until SAA assay.

In pregnant patients undergoing planned caesarean section, the first saliva sample was obtained on the day of surgery between 8 a.m. and 1 p.m., while the patient was waiting in her bedroom on the surgical ward. After this measurement, an 18-gauge intravenous catheter was inserted on the left hand. The second measurement was performed 3 hours later in the operating room immediately before performing spinal anesthesia and after the patients had been monitored with non-invasive blood pressure, five-lead electrocardiogram and pulse oxymetry.

In volunteers, two samples of saliva were obtained at 3-hour interval between 8 a.m. and 1 p.m.

The first and the second measurements were always performed in the sitting position, both in pregnant patients and in volunteers.

Measurement of salivary alpha-amylase activity

SAA activity was measured after 1/200 (vol:vol) dilution of saliva in saline, with a colorimetric assay using 4,6-ethyldene-(G7)p-nitrophenyl-(G1)-D-maltoheptaoside as substrate on the Modular P® analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Imprecision was determined using two samples of healthy controls (mean 54 UI/ml and 139 UI/ml). For each sample, SAA was measured 10 times from 10 different saliva dilutions. The inter-assay coefficient of variation (CVa) for SAA in volunteers was 4% and 3% at 54 and 139 UI/ml, respectively.

Statistical analysis

Results are expressed as median (extremes) or number of patients (%). Comparisons of quantitative variables used a Mann-Whitney U test or a Wilcoxon
test, as appropriate. Associations between quantitative variables were tested with the Spearman test.

Noto et al reported a salivary amylase activity (mean ± 1 SD) of 72 ± 48 UI/ml in 10 healthy female volunteers using a saliva collection method and amylase measurement technique similar to ours. Based on an expected 75% increase in salivary amylase activity in pregnant patients in the operating room compared with the surgical ward, a bilateral test, a power of 80% and an alpha risk of 0.05, 13 pregnant patients undergoing caesarean section needed to be included.

Coefficients of intra (CVi) and inter-individual (CVg) variation for volunteers were calculated with ANOVA for repeated measurements. RCV was calculated in volunteers as $2^{1/2} \times Z \times (CVa^2 + CVi^2)^{1/2}$ where $2^{1/2}$ is a constant that takes into account the 2 measurements, Z equals 1.96 for a 0.05 alpha risk and a bidirectional change in the measured activity and CVa the inter-assay CV. The index of individuality was calculated in volunteers as $(CVa^2 + CVi^2)^{1/2} / CVg$. 


Characteristics of the patients are presented in Table 1. Pregnant patients were significantly younger and had significantly higher BMI than volunteers.

In volunteers, median SAA activity did not differ between the first and the second salivary samples (121 (24-579) UI/mL and 167 (50-287) UI/mL, respectively; \( P = 0.279 \)). SAA activity on the first sample in volunteers did not differ from the one measured on the ward in pregnant patients. In volunteers, intra-individual variability was 27 % and inter-individual variability was 47 % leading to a RCV of ± 76 % (i.e. 1.76 times increase or decrease) and to an index of individuality of 0.58.

In pregnant patients, median SAA activity significantly increased from 72 (21-262) UI/mL on the surgical ward to 231 (86-586) UI/mL in the operating room (\( P = 0.0015 \); median increase + 220 %). Individual variations of SAA activity are presented in Figure 1. Thirteen of the 15 pregnant patients had an increase in SAA activity greater than 76 % i.e. greater than the RCV. A statistically significant increase in systolic blood pressure (median increase 17 %) was also observed between the ward and the operating room but no STAI-state or heart rate changes were observed (Table 2). In pregnant patients, no association was observed between the STAI-state and amylase activity, both on the morning of surgery (rho = - 0.238 and \( P = 0.37 \)) and in the operating room (rho = - 0.137 and \( P = 0.6094 \)).
We have observed a large increase in SAA activity in pregnant patients exposed to the stressful environment of the operating room. Moreover, this increase is significant because it exceeds the reference change value of SAA calculated in volunteers. Furthermore, the low individuality index demonstrates that the patient can be assessed with respect to her individual SAA level rather than a population-derived reference interval indicates that the analyte displays high individuality. The patient can therefore be assessed with respect to her individual SAA level rather than a population-derived reference interval.

The effect on the level of stress of transferring a patient from its comfortable room on the ward to the operating room has been little investigated. However, as we observed in the current study, it does not modify the level of self-reported anxiety – a causative factor of the stress response - assessed by patients with questionnaires such as the STAI-state form or by a numeric rating scale. This reinforces the limited interest of self-administered questionnaires in this situation and the value of an objective biomarker of stress (e.g., salivary alpha-amylase). A sympathetic stress response evidenced by an increase in plasma concentrations of epinephrine was observed between the ward and the operating room, although the factors responsible for this increase remain to be determined. It is worth mentioning that plasma epinephrine concentration values cannot be obtained immediately. Since, salivary amylase release by the highly differentiated epithelial acinar cells of the exocrine salivary glands depends on the activation of beta-adrenergic receptors, this sympathetic response may explain the increase in SAA activity we observed.

The occurrence of a measurable stress response in the operating room was further confirmed by the parallel increase in systolic blood pressure that is similar to the increase observed in previous studies. For instance, Gregg et al. reported a mean 18% increase in systolic blood pressure in female volunteers undergoing a mental arithmetic task which is considered as a “beta-adrenergic stress”, a stressful condition similar to the hostile environment of the operating room. Although the amplitude of the blood pressure response we observed is probably not clinically relevant, it indicates the occurrence of a stress response in the operating room. The non-statistically significant increase in heart rate is surprising since the hemodynamic
response to stress is mediated by epinephrine. Gregg et al reported a 13% increase in heart rate values in female volunteers undergoing a mental arithmetic task. Apart from a type one error, our results may be explained by the higher basal heart rate observed during the third trimester of pregnancy limiting therefore the potential range of heart rate increase. Another explanation could be the attenuation of the chronotropic response to beta-adrenergic agents observed during pregnancy.

In pregnant patients, no association was observed between the STAI-state and SAA activity, both on the morning of surgery and in the operating room. This result is surprising. It may be related to the fact that salivary amylase and the STAI-state do not explore the same aspect of the stress response. Indeed, salivary amylase is an objective assessment of the beta-adrenergic effector branch of the stress response while the STAI-state is a subjective assessment of stress experience.

An increase in SAA activity has been demonstrated in many stressful situations such as skydiving, mental arithmetic test, stressful video viewing or psychosocial stress. Salivary amylase activity quickly increases after an individual is submitted to stress. For instance, Takai et al. reported that salivary amylase activity increased as early as the third minutes after starting viewing a stressful video. Nater et al. reported an increase in salivary amylase activity 5 minutes after stating a mental stress consisting of a mental arithmetic task. The range of SAA activity increase in these situations was between 100 and 200%. Conversely, a decrease in SAA has been observed as soon as the third minute after exposure to soothing conditions. However, an increase in SAA has never been demonstrated in the preoperative period and the definition of what is a clinically relevant increase taking into account analytical and biological variability was never published. Although we did not take into account the gradual increase in SAA activity from morning to evening, the median 220% increase we observed in pregnant patients over a 3-hour period was higher than the 75% increase observed during the day.

RCV was proposed by Hariss and Yasaka for comparing two consecutive measurements. It is defined as the critical difference that must be exceeded between two sequential results for a significant (or true) change to occur. Analytical and biological variability values used to calculate the RCV are obtained from a normal, control population using a sampling technique, a measurement technique
and a delay between two samples similar to those used in the population of interest i.e. pregnant patients. The choice of non-pregnant patients as a control group can be criticised. A control group made of pregnant patients not submitted to the stress of the OR could have been more appropriate. We decided to choose non pregnant patients as the control group in order to have results that can be extended to various populations i.e. not limited to pregnancy. The choice of this population seems justified since salivary amylase activity did not differ between control patients and pregnant patients in non-stressful situation (“on the ward”). Presently, RCV is commonly used to assess consecutive measurements of many biomarkers such as N-terminal proBNP. Therefore, an increase of SAA higher than 76 % i.e. a signal to noise ratio of 1.76 may be suggested as biologically and clinically-relevant. In 87% of the pregnant patients SAA activity increase exceeded the RCV. The index of individuality lower than 0.6 indicates that the analyte displays high individuality. The patient can therefore be assessed with respect to her individual SAA level rather than a population-derived reference interval. The current measurement technique of SAA does not allow real-time results. However, portable hand-held monitors are now available and may be used as point-of-care.

In conclusion, this study demonstrates that a significant increase in SAA activity is observed in pregnant patients placed in the stressful environment of the operating room. Further studies are required to define the clinical utility of SAA as a stress biomarker in pregnant patients.
**Table 1:** Demographic characteristics of volunteers and pregnant patients. Results are expressed as median (extremes) or number of patients (%). Comparisons use the Mann-Whitney U test.

<table>
<thead>
<tr>
<th></th>
<th>Volunteers ( n = 18 )</th>
<th>Pregnant patients ( n = 15 )</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>48 (28-58)</td>
<td>34 (23-45)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165 (151-183)</td>
<td>163 (154-176)</td>
<td>0.2533</td>
</tr>
<tr>
<td>Weight (kg) (at delivery for caesarean section)</td>
<td>67 (51-94)</td>
<td>71 (59-99)</td>
<td>0.2127</td>
</tr>
<tr>
<td>Body mass index(kg/m²)</td>
<td>25.5 (18.7-33.7)</td>
<td>26.4 (23.0-34.0)</td>
<td>0.0344</td>
</tr>
<tr>
<td>Pregnancy term (weeks of amenorrhea)</td>
<td>__</td>
<td>39 (36-39)</td>
<td>__</td>
</tr>
<tr>
<td>Gravidity</td>
<td>__</td>
<td>2 (1-4)</td>
<td>__</td>
</tr>
<tr>
<td>Parity (including current pregnancy)</td>
<td>__</td>
<td>2 (1-3)</td>
<td>__</td>
</tr>
<tr>
<td>Indication for caesarean section</td>
<td>__</td>
<td></td>
<td>__</td>
</tr>
<tr>
<td>- previous caesarean section</td>
<td></td>
<td>6 (40 %)</td>
<td></td>
</tr>
<tr>
<td>- perineal protection</td>
<td></td>
<td>4 (27 %)</td>
<td></td>
</tr>
<tr>
<td>- breech presentation</td>
<td></td>
<td>3 (20 %)</td>
<td></td>
</tr>
<tr>
<td>- twin pregnancy</td>
<td></td>
<td>1 (6.5 %)</td>
<td></td>
</tr>
<tr>
<td>- abnormal placenta insertion</td>
<td></td>
<td>1 (6.5 %)</td>
<td></td>
</tr>
<tr>
<td>Time elapsed between the 2 saliva samples (hours)</td>
<td>3 (2-5)</td>
<td>3 (1-6)</td>
<td>0.3619</td>
</tr>
</tbody>
</table>
Table 2: Systolic blood pressure, heart rate and STAI-state changes between the surgical ward and the operating room in pregnant patients. Results are expressed as median (extremes) or number of patients (%). Comparisons use the Wilcoxon test.

<table>
<thead>
<tr>
<th></th>
<th>On the ward</th>
<th>In the operating room</th>
<th>P_value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>107 (80-136)</td>
<td>126 (105-149)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Heart rate (/min)</td>
<td>80 (54-91)</td>
<td>77 (71-109)</td>
<td>0.0502</td>
</tr>
<tr>
<td>STAI-state score</td>
<td>31 (20-66)</td>
<td>33 (24-69)</td>
<td>0.55</td>
</tr>
</tbody>
</table>
**Figure 1:** Individual variations of salivary alpha-amylase activity in 18 volunteers (top) and in 15 pregnant patients (bottom).


