## Improving the methodology for non-invasive autonomic nervous system recording and its implementation in behavioral research

van Lien, R.

2014

***document version***
Publisher's PDF, also known as Version of record

# CHAPTER 4

## Comparison of within and between subject variation in salivary alpha-amylase and the preejection period

René van Lien, Jos Bosch, Jennifer May, Stefan Wüst, and Eco J.C. de Geus

This chapter wil be merged with chapter 5 and submitted to PNEC.

**Abstract**

Recently, the secretion of the salivary enzyme alpha-amylase (sAA) has gained interest as a potential non-invasive biomarker for activity of the sympathetic nervous system (SNS). In this study we compared sAA to an established measure of SNS activity, the preejection period (PEP). Twenty-three students between 18 and 35 years old wore an ambulatory recording device (VU-AMS) during one night and one day. Values for PEP were extracted from the impedance- and electrocardiograms recorded during the 30 minutes before a saliva sample was obtained by Salivette cotton rolls to determine sAA. This was repeated 7 times during the daytime recording. Respiratory sinus arrhythmia (RSA) was used to test for a possible interactive effect of the SNS and parasympathetic nervous system (PNS) on sAA. Replicating prior research, a daytime pattern for sAA was found showing an increase in sAA during the day. No significant within or between participant correlation between PEP and sAA was found, even when taking concurrent RSA levels into account. Within and between participant differences in ambulatory measured sAA are not correlated to parallel differences in an established measure of cardiac sympathetic activity.

**Introduction**

Activity of the sympathetic nervous system (SNS) may be paramount to the detrimental effects of stress on cardiovascular health (Palatini et al., 2004; Schwartz et al., 1992). As a consequence, cardiovascular psychophysiologists need reliable and valid strategies to measure sympathetic nervous system activity in humans. Recently, the secretion of the salivary enzyme alpha-amylase (sAA) has gained interest as a potential non-invasive biomarker for activity of the SNS. An immediate advantage of sAA assessment in large scaled studies is that it can be combined with salivary cortisol assessment, which is an established biomarker of the activity of the hypothalamic-pituitary adrenocortical (HPA) axis. Hence, if sAA truthfully reflected SNS activity, repeated salivary sampling would allow parallel research on the two major stress systems in large-scale samples (Chatterton, Jr. et al., 1996) including those of the sizes needed for genetic epidemiology (Lander & Kruglyak, 1995; The Wellcome Trust Case Control Consortium, 2007). However, before engaging in such efforts, the validity of ambulatory sAA as an index of SNS activity needs to be established beyond reasonable doubt.

Alpha-amylase is synthesized and secreted by the acinar cells of the salivary glands, in particular those of the parotid gland, and makes up about 20% of the total protein in saliva. The theoretical idea that sAA might serve as a non-invasive and easily obtained surrogate marker of SNS activity has been based on the presence of adrenoceptors in the acinar cells of the saliva glands and the increases in the protein-to-fluid ratio of alpha-amylase in response to norepinephrine. Empirical support comes from previous studies that found elevations of sAA after being exposed to stressors known to increase SNS activity, like a stressful academic examination (Bosch et al., 1996), parachute jump (Chatterton, Jr., Vogelsong, Lu, & Hudgens, 1997), stressful computer games (Skosnik et al., 2000; Takai et al., 2004), watching a stressful video (Bosch, de Geus, Veerman, Hoogstraten, & Nieuw Amerongen, 2003a), mental arithmetic test (Noto et al., 2005), and the Trier Social Stress Test (TSST) (Nater et al., 2006; Nater et al., 2005; Rohleder, Wolf, Maldonado, & Kirschbaum, 2006). Reduced sAA was found after watching a relaxing movie (Takai et al., 2004; Takai et al., 2007) although others have reported an increase during relaxation (Morse, Schacterle, Furst, Esposito, & Zaydenburg, 1983). The stress-induced sAA increase can be blocked pharmacologically by the β-adrenergic blocker propanolol (Van Stegeren, Rohleder et al. 2006), and Ehlert et al. (Ehlert, Nater et al. 2005) found a significant increase in sAA after administration of yohimbine which blocks the inhibitory α1-adrenergic autoreceptor.

While the above findings show that, at the level of group comparisons, increases in SNS activity by pharmacological means or through mental and physical stress is indeed accompanied by increases in sAA, they do not show whether between-participant differences in the overall levels of SNS activity are reflected in between-participant differences in sAA or the extent to which changes in SNS activity within an individual are reliably reflected in parallel within-participant differences in sAA. A design is needed that compares between-participant differences and within-participant changes in an established index of SNS activity to parallel between-participant differences and within-participant changes in sAA. For between-participant differences, this comparison has been made using venous catecholamine levels. Chatterton (Chatterton, Jr. et al., 1996) found moderate to good correlations between sAA and plasma epinephrine and norepinephrine (r=.49 and r= .64 respectively) during exercise, but other studies using mental stressors reported no or weaker correlation between plasma catecholamines and sAA (Chatterton, Jr. et al., 1997; Milad, Klock, Moses, & Chatterton, 1998; Morrison, Haas, Shaffner, Garrett, & Fackler, 2003; Rohleder et al., 2004; Skosnik et al., 2000).

These discrepancies do not by necessity reflect poorly on the sAA, as the validity of plasma catecholamines is not perfect. Concerns have been raised about differences in intraneuronal vesicular storage and leakage, re-uptake, extraneuronal clearance that may (severely) distort the relation between actual SNS activity and plasma catecholamine concentrations (Eisenhofer et al., 2004; Esler et al., 1990; Goldstein, 1995; Goldstein, McCarty, Polinsky, & Kopin, 1983; Hjemdahl, Larsson, Johansson, Zetterlund, & Eklund, 1990). If plasma catecholamines are themselves unreliable markers of SNS activity, weak correlations to sAA cannot tell us much about the validity of sAA.

In this study we compare sAA to an alternative measure of SNS activity, the preejection period (PEP) which can be obtained non-invasively by thoracic impedance cardiography. Within-participant changes in PEP index changes in β-adrenergic inotropic drive to the left ventricle as shown in laboratory studies manipulating β-adrenergic tone by epinephrine infusion (Mezzacappa et al., 1999; Schachinger et al., 2001; Svedenhag et al., 1986; Svedenhag et al., 1991), amyl nitrite inhalation (Nelesen et al., 1999) and adrenoceptor blockade (Harris et al., 1967; Schachinger et al., 2001; Winzer et al., 1999). Within participants, the PEP decreases in a dose-dependent way to exercise (Krzeminski et al., 2000; Miyamoto et al., 1983b; Smith et al., 1989b), emotional stress (Berntson et al., 1994a; Newlin et al., 1979; Sherwood et al., 1986) and monetary reward (Richter et al., 2009). Between-participant differences in PEP level are stable over time (Goedhart et al., 2006; Vrijkotte et al., 2004), heritable (Kupper et al., 2006), correlate modestly but significantly to plasma catecholamines (McCubbin, Richardson, Langer, Kizer, & Obrist, 1983) and reliably reflect interindividual differences in cardiac sympathetic activity assessed by dual blockade (Cacioppo et al., 1994). Since the PEP can be obtained by thoracic impedance cardiography (Sherwood et al., 1990) a number of devices are now available for ambulatory recording of the PEP (Cybulski, 2000; Nakonezny et al., 2001; Sherwood et al., 1998; Willemsen et al., 1996). This allowed us to use the ambulatory 'salivary design' typically employed to assess HPA-axis activity, whilst simultaneously recording the PEP values in the period preceding the salivary measurements. To support the sAA as a measure of SNS activity it should show a negative correlation to the PEP such that salivary samples with high sAA should be accompanied by a shorter PEP.

This study also assessed parasympathetic nervous system (PNS) activity using respiratory sinus arrhythmia (RSA) as a proxy of cardiac vagal control. As reviewed in detail elsewhere PNS activity can also increase sAA, and activation of the SNS during co-activation of the PNS can drastically increase the effects of SNS activity on sAA (Garrett, 1987; Rohleder & Nater, 2009). To account for a possible interactive effect of SNS and PNS activity on sAA, we used the concept of autonomic space as detailed in Berntson et al. (Berntson et al., 2008). Specifically, we used PEP and RSA to define a measure that reflects co-activation (Co-AR) of the PNS and SNS. Correlation of the sAA to the RSA and Co-AR was additionally tested.

**Methods**

*Participants*

Thirty-three participants (10 males) between 18 and 35 years old (mean = 23 years, SD = 4.3) were recruited and included. All participants were free of any obvious somatic or psychiatric disease, did not use medication, had not travelled through time zones or attended shift work in the prior three weeks and did not smoke or drink excessively (defined by more than 5 drinks or cigarettes a day). Participants were recruited at the VU University Amsterdam campus via the Biological Psychology department's online sign-up system for student participation in experiments.

*Procedure*

All participants wore an ambulatory recording device (VU University Ambulatory Monitoring System, VU-AMS) for a period of about 30 hours. For twenty-three participants monitoring with the VU-AMS began at 13:00 on the first day and was continued on a second day up till 19:00. For ten participants monitoring started at 16.00 on the first day till 19.00 on the second day. During the second day sAA levels were measured at 7 common time points (see table 1). Participants were instructed to take the first sample immediately upon awakening. A prepaid text message phone service was used to communicate their wake up time to the experimenter. Awakening time was verified and, if appropriate, corrected after visual inspection of the ambulatory recording of heart rate and body movement according to the procedure outlined by Kupper et al. (2005).

Thirty minutes after awakening participants were reminded via text messages to take the second sample. Three samples were taken under the supervision of the researcher during two 1-hour visits to the department for neurocognitive testing, two samples at 10.00 and 18.00 during active testing and at 19:00 at the end of the experimental protocol. The two remaining samples were taken outside of the laboratory (at 12:00 and at 14:00) and for both samples participants were prompted by a text message. The VU-AMS device was removed after the last sample taken in the laboratory (19:00).

**Table 1.** *Seven common saliva sample moments.*

| Recording period | Time of Day | Saliva Sample |
|---|---|---|
| Awakening | 05:04 – 08:00 | Sample 1 |
| Awakening + 30 min | 05:34 – 08:30 | Sample 2 |
| Morning sample (supervised) | 10:00 | Sample 3 |
| Afternoon sample 1 | 12:00 | Sample 4 |
| Afternoon sample 2 | 14:00 | Sample 5 |
| Afternoon sample 3 (supervised) | 18:00 | Sample 6 |
| Evening sample (supervised) | 19:00 | Sample 7 |

*sAA*

Salivary samples were obtained by the standard protocol for salivary cortisol collection (Bartels, de Geus, Kirschbaum, Sluyter, & Boomsma, 2003; Bartels, van den, Sluyter, Boomsma, & de Geus, 2003; Kirschbaum & Hellhammer, 1994) where salivary is collected while participants gently hold a cotton roll (Salivette sampling device, Sarstedt, Nümbrecht, Germany) in their mouth for approximately one minute. Salivettes were stored by the participant in a cool and dark location until transport to the laboratory where they were immediately stored at -20$^{o}$C. Participants were

requested not to eat (breakfast) until after the second saliva sample and were asked to refrain from eating 30 minutes prior to each saliva sampling.

After thawing for biochemical analysis, samples were centrifuged at 2000 x g at 10°C for 10 min. The sAA assay utilizes the enzymatic action of sAA on the chromagenic molecule, 2-chloro-4-nitrophenyl -D maltotrioside (Fuitest Amyl CNPG3, Biocon, Vöhl-Marienhagen, Germany) that was used as the assay substrate to estimate amylase concentration (Lorentz, Gutschow, & Renner, 1999; Winn-Deen, David, Sigler, & Chavez, 1988). Enzymatic activity of alpha-amylase on this substrate yields 2-chloro-p-nitrophenol, which can be spectrophotometrically measured at 405 nm. The amount of alpha-amylase activity present in the sample is directly proportional to the increase in absorbance at 405 nm. Saliva samples were diluted 1:200 with ddH2O, and 8µl of the diluted saliva was pipetted in duplicates into a 96-well microtiter plate. After adding 320µl substrate solution (CNP-G3), the plate was incubated for 60 seconds at 37°C and mixed at 500rpm in a microtiter plate thermoshaker. After 60 seconds the plate was read kinetically in a plate reader at 405nm and incubated again for exactly 120 seconds at 37°C and 500rpm. After this second incubation the OD was read again at 405nm. The OD change was calculated by subtracting the OD of the first reading from that of the second reading and alpha-amylase activity was expressed in U/ml. (Lorentz et al., 1999; Winn-Deen et al., 1988).

*Ambulatory measurement*



The VU-AMS device uses a six-electrode configuration to record the ECG, thorax impedance (Z0), changes in thorax impedance (ΔZ) and the first derivative of these changes (dZ/dt) as described in detail elsewhere (Goedhart et al., 2006; Riese et al., 2003). From these signals, the preejection period (PEP), respiratory sinus arrhythmia (RSA), and the inter-beat intervals (IBI) can be extracted. Briefly, PEP was calculated using the R-wave-locked ensemble averaged dZ/dt signal, using all valid beats within 60-second periods. This averaging reduces noise caused by respiration and limb movement related changes in thorax impedance. In these 60-second averages, the onset of a rapid change in dZ (B-point) was manually scored with the VU-AMS interactive software program as described previously (Riese et al., 2003). PEP was computed as the distance between the R-wave and the B-point, and a fixed Q-onset to R-wave period was added (48msec). RSA was obtained through the peak-valley method (Bosch et al., 1996; Grossman, 1983; Katona et al., 1975). Breathing cycles that showed irregularities like gasps, breath holding, coughing etc., were not considered valid and were removed from further processing, as were the shortest and longest breaths that deviated more than 3SD. When no respiratory phase-related acceleration or deceleration was found or when the shortest beat in inspiration was longer than the longest beat during expiration, the breath was assigned an RSA value of zero. Mean respiration rate (RR) and RSA were computed across all valid breaths to a single mean RSA for each of the experimental conditions. The kinetics of SNS effects on cardiac innervation and sAA salivary protein secretion may be quite different. SNS effects on PEP are immediate but sAA might be viewed as a more integrative measure of SNS activity. For a fair comparison PEP levels should be aggregated for a period preceding the time of sAA sampling. The optimal time window for this is unknown so we computed mean PEP, RSA and IBI across various time windows before each of the eight saliva samples were taken. These include windows from 1) 30 minutes before sampling up to 20 minutes before sampling (-30/20), 2) 20 minutes before sampling up to 10 minutes before sampling (-20/10), 3) 10 minutes before sampling up to sampling (-10/0) and 4) sampling moment till 10 minutes after sampling. Within these periods we selected only the

fragments during which the participants were 'sitting with minimal physical activity' (or lying in case of the first pre-awakening sample). To detect physical activity we used the motility signal of the inbuilt vertical accelerometer in the VU-AMS device. Next we assessed the mean PEP during these selected periods. Likewise, a mean RSA and IBI was computed across all breaths falling in the pre-sample sitting/lying fragments during the 4 different periods used. As almost identical results were obtained for all four periods we report in detail only on the 10 minute pre-sample recording period (-10 / 0).

The concept of autonomic space was developed by Berntson et al. (Berntson et al., 2008) to account for a possible interaction between the sympathetic and parasympathetic branches of the autonomic nervous system. They derived two measures of autonomic control from RSA and PEP that reflected reciprocal activation/deactivation versus co-activation/co-inhibition of these branches. Cardiac autonomic regulation (Co-AR) was calculated as the sum of the normalized values of RSA and PEP (formula = zRSA + (-ZPEP)) for each of the 30 minute periods. Co-AR represents the dimension of co-activation/co-inhibition, with low values representing co-inhibition and high values co-activation of the cardiac SNS and PNS branches.

*Data analysis*

Mixed model analysis of variance was used to test a main effect of time of day on the pre-sample PEP, RSA, and Co-AR levels and the sAA at the time of sampling. We included sex and age as covariates. To examine the between-participant correlations we computed Pearson correlations between sAA and PEP, RSA, and Co-AR for the 7 of the samples. The distribution of the Spearman rank correlations across the 7 samples within each of the 33 participants were used to examine within-participant correlations. In view of multiple testing we adopted a significance level of 0.001, based on an experiment-wise alpha of 0.05 with a Bonferoni correction for the 40 most critical sAA/PEP correlations computed. sAA was mildly skewed so we repeated all analysis with the log-transformed form. Virtually identical results were obtained and we report the analyses on the observed values only.

**Results**

Table 2 displays the average duration of the sitting periods used to compute the pre-sample PEP, RSA, and Co-AR. The means and standard deviations are given per sample. Mixed model analysis revealed no significant effect of sample time on RSA, PEP, and Co-AR.

**Table 2.***Mean (±SD) for IBI, RSA, PEP, and Co-AR in the 10 minute pre-sample recording period .*

| **Saliva sample** | **Time of Day** | **IBI** (ms) | *SD* | **RSA** (ms) | *SD* | **PEP** (ms) | *SD* | **Co-AR** | *SD* |
|---|---|---|---|---|---|---|---|---|---|
| Awakening | 05:04 – 08:00 | 776.71 | 142.6 | 70.38 | 31.4 | 107.00 | 21.1 | -.06 | 1.2 |
| Awakening + 30 min | 05: 34 –08:30 | 727.00 | 172.8 | 57.96 | 27.9 | 101.20 | 22.5 | -.15 | 1.1 |
| Morning (supervised) | 10:00 | 804.23 | 96.9 | 77.25 | 30.9 | 101.00 | 26.4 | .13 | 1.5 |
| Afternoon 1 | 12:00 | 815.58 | 134.9 | 75.62 | 30.4 | 107.10 | 24.8 | .10 | 1.2 |
| Afternoon 2 | 14:00 | 792.40 | 137.5 | 66.03 | 23.9 | 102.84 | 23.5 | .01 | .9 |
| Afternoon 3 (supervised) | 18:00 | 672.32 | 117.1 | 54.29 | 23.4 | 93.13 | 21.3 | .11 | 1.1 |
| Evening (supervised) | 19:00 | 786.93 | 112.1 | 73.73 | 29.0 | 103.31 | 19.3 | .25 | 1.1 |



Table 3 and figure 1 display the mean sAA at the various sampling times. Mixed model analysis revealed a significant effect of sample time on sAA ($F(6, 32) = 3.32$, $p < .008$). Post hoc analysis revealed a gradual increase in sAA throughout the day.

**Table 3.** *Mean (±SD) Daytime variation in sAA.*

| **Saliva sample** | **Time of Day** | **sAA** (Unit/ml) | SD |
|---|---|---|---|
| Awakening | 05:04 – 08:00 | 28.85 | ±23.87 |
| Awakening + 30 min | 05: 34 –08:30 | 27.38 | ±35.31 |
| Morning sample (supervised) | 10:00 | 43.60 | ±40.40 |
| Afternoon sample 1 | 12:00 | 37.24 | ±39.75 |
| Afternoon sample 2 | 14:00 | 43.38 | ±45.82 |
| Afternoon sample 3 (supervised) | 18:00 | 68.58 | ±65.44 |
| Evening sample (supervised) | 19:00 | 62.85 | ±70.20 |

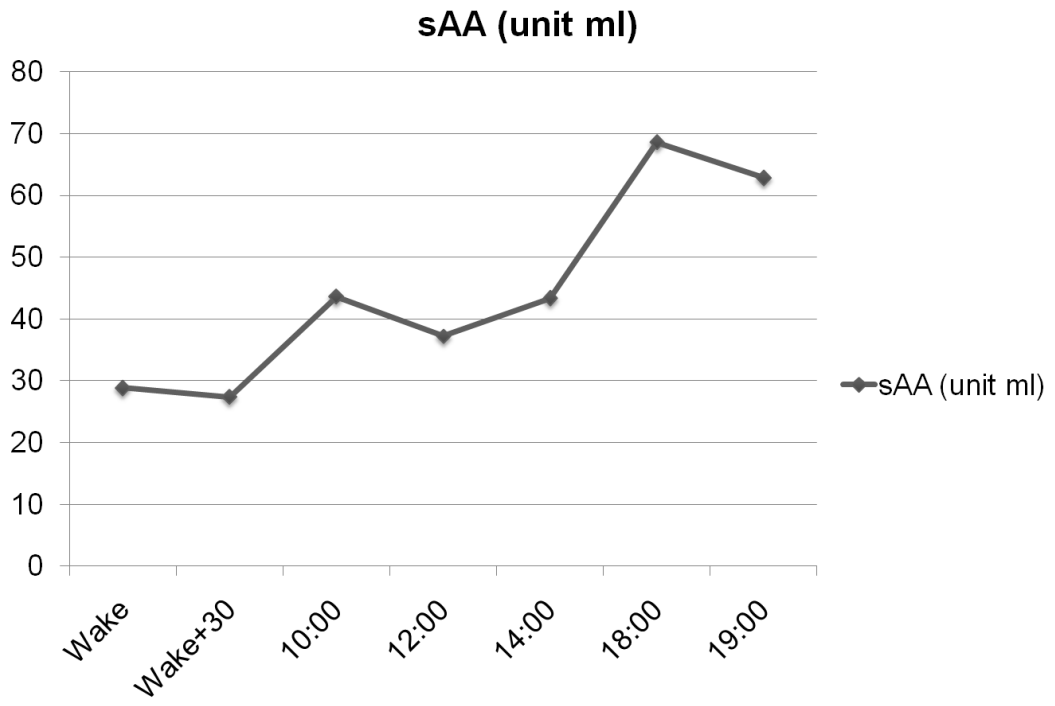


**Figure 1.** *Daytime increase in sAA (U/ml).*

*Correlational analysis*

The scatterplots between PEP and sAA and RSA and sAA are given in Figure 2. To examine the between-participant component of the covariance in our measures we first calculated Pearson correlations across the 33 participants, separately for each of the 7 samples (see table 4). No significant correlations between sAA and PEP were detected (range -.18 to .22), and sAA also did not correlate to RSA (range -.22 to .19) or Co-AR (range -.20 to .36).

**Table 4.** *Between-participant correlations of sAA with IBI, RSA, PEP, and Co-AR (10-minute pre-sample recording period).*

| **Recording period** | **Time of Day** | **IBI-sAA** | p | **RSA-sAA** | p | **PEP- sAA** | p | **CAR- sAA** | p |
|---|---|---|---|---|---|---|---|---|---|
| Awakening | 05:04 – 08:00 | 0.33 | .09 | 0.19 | .36 | -0.05 | .82 | 0.24 | .23 |
| Awakening + 30 min | 05: 34 –08:30 | -0.12 | .58 | -0.03 | .86 | 0.22 | .25 | -0.14 | .47 |
| Morning (supervised) | 10:00 | -0.16 | .39 | -0.22 | .25 | 0.03 | .88 | -0.20 | .27 |
| Afternoon 1 | 12:00 | 0.07 | .72 | 0.05 | .78 | -0.07 | .72 | -0.01 | .98 |
| Afternoon 2 | 14:00 | 0.16 | .38 | 0.09 | .65 | -0.18 | .33 | 0.04 | .83 |
| Afternoon 3 (supervised) | 18:00 | -0.19 | .32 | 0.03 | .87 | 0.05 | .78 | -0.12 | .53 |
| Evening (supervised) | 19:00 | -0.01 | .94 | 0.02 | .94 | 0.10 | .61 | 0.36 | .05 |

In addition, no significant within-participant relation between sAA and PEP was seen, and, as indicated, this did not reflect the choice of the time period around saliva sampling (see table 5). For the -10 /0 time period, mean Spearman rank correlation between PEP and sAA was -0.09. Only three individuals showed a significant PEP–sAA correlation in the expected direction.

**Table 5.** *Mean, maximum and minimum within-participant correlations of sAA with IBI, RSA, PEP and Co-AR for various time windows around sAA sampling.*

| | Timeframe | -30/-20 | -20/-10 | -10/0 | 0/10 |
|---|---|---|---|---|---|
| IBI-sAA | Mean | -.10 | -.06 | -.02 | -.01 |
| | Maximum | -.79 | -.73 | -.69 | -.83 |
| | Minimum | .67 | .81 | .71 | .75 |
| | # Sig. Negative* | 1 | 2 | 1 | 3 |
| RSA-sAA | Mean | -.09 | -.03 | .01 | .02 |
| | Maximum | 0.61 | .88 | .93 | .84 |
| | Minimum | -0.82 | -.58 | -.87 | -.86 |
| | # Sig. Negative* | 1 | 1 | 1 | 4 |
| PEP-sAA | Mean | -.05 | -.18 | -.04 | -.09 |
| | Maximum | -.91 | -.98 | -.88 | -.68 |
| | Minimum | .88 | .86 | .87 | .73 |
| | # Sig. Negative* | 2 | 5 | 3 | 0 |
| Co-AR-sAA | Mean | .00 | .10 | .02 | .00 |
| | Maximum | .57 | .69 | .86 | .68 |
| | Minimum | -.95 | -.66 | -.64 | -.81 |
| | # Sig. Negative* | 0 | 1 | 2 | 4 |

** =p < 0.05*

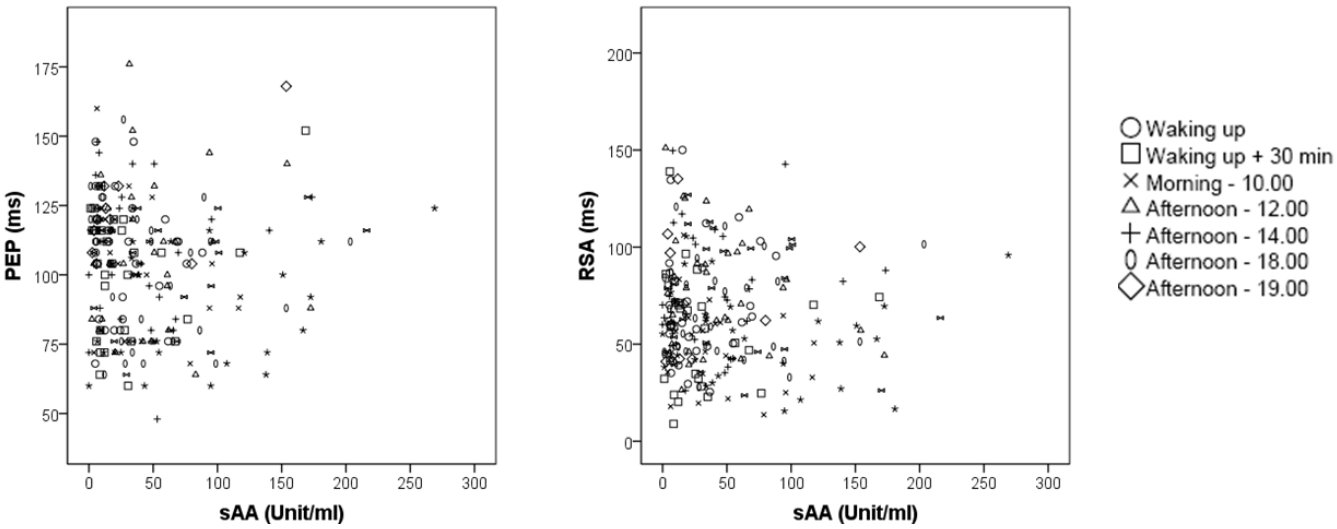


**Figure 2.** *Scatter plots of sAA with PEP and RSA.*

*Area under the curve*

In keeping with a recent study that showed the potential relevance of overall daytime sAA levels (Wolf, Nicholls, & Chen, 2008) we also computed the Area Under the Curve (AUC) and incremental AUC (AUCi) of the diurnal sAA curve using the trapezoid formula as described by Pruessner (Pruessner, Hellhammer, Pruessner, & Lupien, 2003). In parallel, we computed the overall daytime averages for PEP, RSA, IBI, and Co-AR. The sAA AUC variables are less dependent on between-participant differences in the basal values in sAA. Table 6 illustrates that no evidence was found for a relationship between sAA and PEP, RSA, or Co-AR.

**Table 6.** *Pearson correlations between the AUCi and AUCg of sAA with all-day PEP, IBI, RSA, and Co-AR .*

| | **IBI** | | **RSA** | | **PEP** | | **Co-AR** | |
|---|---|---|---|---|---|---|---|---|
| | r | p | r | p | r | p | r | p |
| AUCi | .10 | .58 | -.03 | .87 | -.02 | .89 | -.11 | .53 |
| AUCg | .05 | .79 | -.07 | .71 | -.08 | .67 | -.13 | .48 |

**Discussion**

Enzymatic AA activity in salivary samples obtained by the Salivette device has gained a lot of interest as a potential non-invasive biomarker for activity of the SNS (Nater et al., 2009; Rohleder et al., 2009). If sAA truthfully reflects SNS activity, repeated salivary sampling for determination of cortisol and sAA would allow parallel research on the two major stress systems, the HPA-axis and the SNS, in large-scaled epidemiological research. To support sAA as a measure of SNS activity we tested its correlation to the PEP, which is an established marker of cardiac sympathetic control (Cacioppo et al., 1994; Harris et al., 1967; Mezzacappa et al., 1999; Miyamoto et al., 1983b; Nelesen et al., 1999; Newlin et al., 1979; Schachinger et al., 2001; Sherwood et al., 1986; Smith et al., 1989b; Svedenhag et al., 1986; Winzer et al., 1999). Our results failed to show the expected negative correlation between PEP and the sAA, either within- or between-participants.

The absence of a significant relation between PEP and sAA might have reflected confounding effects of within- or between-participant differences in PNS activity. The PNS mainly controls fluid secretion by the salivary glands, whereas sympathetic activity mostly regulates salivary protein secretion, including secretion of sAA (Bosch, Ring, de Geus, Veerman, & Amerongen, 2002; Garrett, 1987). As reviewed in detail elsewhere (Bosch et al., 2002; Garrett, 1987; Rohleder et al., 2009) an additional effect of the PNS activity is that it synergistically increases SNS-mediated protein secretion. In keeping, we have previously shown that sAA steeply increases during a laboratory stressor that evokes sympathetic-parasympathetic co-activation (viewing a surgical video), whereas only a marginal increase was seen during a stressor that evoked sympathetic activation in conjunction with a vagal withdrawal (a time-paced memory test) (Bosch et al., 2003a). Similarly, El-Sheikh et al. (El-Sheikh, 2005) found that children with higher vagal tone, as indexed by RSA, during stressful mirror-star tracing had higher sAA.

To account for confounding by PNS activity we repeated our analyses using either RSA, a measure that reflects PNS activation, and Co-AR, a measure that reflects SNS-PNS co-activation,

which was adopted from Berntson et al (Berntson et al., 2008). Results from these additional analyses suggest that differences in PNS activity do not explain the absence of a PEP-sAA correlation.

The absence of a significant relation between PEP and sAA also cannot easily be attributed to the procedures specific to this study. We used a salivary sampling procedure that has become standard in sAA research (Nater et al., 2009; Rohleder et al., 2009), under which conditions we fully replicated the circadian rhythm in sAA reported previously, i.e. the sAA showed a significant and progressive increase across the test day (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007). Low levels of sAA upon awakening and an increase of sAA during the day has been shown in rats before (Bellavia, Sanz, Chiarenza, Sereno, & Vermouth, 1990; Dawes, 1974), and was later confirmed in humans (Artino et al., 1998; Jenzano, Brown, & Mauriello, 1987; Nater et al., 2007; Rantonen & Meurman, 2000; Wolf et al., 2008). If sAA reflects SNS activity this would suggest that SNS activity is low in the morning and increases towards the evening. This is opposite from what is found using other measures of SNS activity like PEP and plasma catecholamines that show high SNS activity in the morning and a decline during the day (Gold et al., 2005; Karas et al., 2005; Turton & Deegan, 1974); (Burgess, Trinder, Kim, & Luke, 1997; Holmes, Burgess, & Dawson, 2002; McCubbin et al., 1983; van Eekelen, Houtveen, & Kerkhof, 2004).

Clearly, the lack of an association between sAA and PEP in part reflects the fact that autonomic activity at the level of the heart does not fully mirror autonomic activity in other organs (Folkow, 2000). However, the diffuse and highly interconnected anatomy of the SNS is also not consistent with a complete organ response specificity and the correlation between the two measures should have captured some of this generalized SNS activity. In view of the substantial literature supporting PEP as a measure of SNS activity (Berntson et al., 2008; Harris et al., 1967; Krzeminski et al., 2000; Mezzacappa et al., 1999; Miyamoto et al., 1983b; Nelesen et al., 1999; Newlin et al., 1979; Schachinger et al., 2001; Sherwood et al., 1986; Smith et al., 1989b; Svedenhag et al., 1991; Winzer et al., 1999) we believe that sAA, as assessed by utilizing the current protocol, may have some shortcomings.

A first shortcoming is that salivary fluid secretion and protein secretion are partially independently regulated processes, so that sAA concentrations may vary due to between- and within-participant variation in fluid secretion. This study, as the vast majority of sAA studies, did not measure salivary fluid secretion. According to Rohleder et al. (Rohleder et al., 2006) this should not be a problem, but results by Bosch et al. (Bosch et al., 2003a) provide reasons for concern, showing that a substantial part of sAA can be attributed to flow rate changes. A second shortcoming is that saliva secretion may have been induced by mild mechanical stimulation (gentle moving the salivette around in the mouth and possibly chewing). Although this method of saliva collection is common practice in sAA research (Rohleder et al., 2009), it may be problematic because stimulation of mechano-receptors in the mouth, e.g. by chewing, induces local autonomic reflex activity that influences glandular function independent of central sympathetic regulation (Garrett, 1987).

A related issue is that saliva composition can change upon stimulation of saliva flow. Here the problem is not a diluting effect of oral fluid, discussed above, but rather the combination of the facts that saliva glands vary in their response to stimulation and contain differing concentrations of AA. For example, under passive conditions (i.e., without mechanical or gustatory stimulation), most saliva derives from the submandibular glands and only one-fifth derives from the parotid gland, which is very rich in AA (Humphrey et al., 2001; Schenkels, Veerman, & Nieuw Amerongen, 1995). However, during stimulation (e.g. chewing) the contributions of individual glands changes dramatically, whereby half of the total saliva derives from the parotid glands. Importantly, parotid

gland secretions have a 4- to 10-fold higher AA concentration than those of the submandibular glands (Veerman, van den Keybus, Vissink, & Nieuw Amerongen, 1996). In sum, the Salivette collection protocol, which was used in the current study, involves mechanical stimulation that may invoke localized autonomic reflex activity and variability in terms of glandular sources of sAA, which both may mask shared variance in cardiac and glandular SNS regulation.

The above shortcomings may compromise the validity of sAA as a biomarker of SNS activity and could explain the absence of a correlation between PEP-sAA. We should add, however, that the small number of saliva samples per participant (7) implied limited statistical power to detect significant within-participants associations. We deliberately choose this sampling scheme because it reflects a typical HPA-axis study and we aimed to determine if additional sAA assessments would be feasible under such a scenario. Less realistic, but more dense sampling would have yielded better power. Even so, inspection of the analyses presented in Figure 2 argues against anything but a very weak relation between PEP and sAA to begin with.

In conclusion, within- and between-participant differences in ambulatory-assessed sAA are not correlated to parallel differences in an established measure