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Anxiety Chronicity and Psychiatric Comorbidity: Influences on Salivary Alpha-Amylase in a Diagnostically Heterogeneous Sample of Outpatients with Anxiety Disorders

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Abstract

Anxiety disorders are the most prevalent class of mental disorders, often characterized by a chronic course and comorbid psychopathology. Reports of anxiety-cortisol relationships are inconsistent in the literature. Salivary alpha-amylase (sAA), a biomarker of autonomic nervous system activation, provides an opportunity to examine the stress response more fully. This study recruited a diagnostically heterogeneous outpatient sample attending a specialized anxiety treatment center to explore relationships between trait anxiety and salivary stress biomarkers and tested the influence of symptom chronicity and psychiatric comorbidity on this relationship. Multiple regression analyses were conducted to examine associations between psychosocial and physiological variables. Forty-four adults completed study procedures. Univariate associations were detected between chronicity and cortisol (r= -0. 348; p= 0. 028) and between comorbidity and sAA (r=0. 381; p= 0. 026). Although the statistical significance of these associations at α=0. 05 was lost when multiple regression was used to control for covariates, the relationship between comorbidity and sAA retained its strength of association (β=0. 341; p=0. 075). Chronicity moderated the anxiety-stress relationship such that greater chronicity significantly strengthened the relationship between trait anxiety and sAA; this interaction accounted for a significant proportion of the observed variance (ΔR^2=0. 469; p = 0. 001). This exploratory study supports the feasibility of sAA in anxiety-stress research using diagnostically heterogeneous samples. This work also suggests that the factors of symptom chronicity and psychiatric comorbidity may contribute variance to the anxiety-stress relationship in typically presenting anxiety disorder samples. Further research is needed to replicate the utility of alpha amylase in ecologically valid samples demonstrated here and understand in greater detail how these highly prevalent characteristics of anxiety may influence autonomic activation.

Keywords: Salivary alpha-amylase; Salivary A-amylase; Cortisol; Stress; Anxiety; Chronicity; Comorbidity

Abbreviations: SAA: Salivary Alpha-Amylase; ANS: Autonomic Nervous System; HPA Axis: Hypothalamic Pituitary Adrenal Axis; PTSD: Post-Traumatic Stress Disorder; STAI-T: State-Trait Anxiety Inventory-Trait Subscale; PANAS: Positive Affect-Negative Affect Scale; BDI-II: Beck Depression Inventory-II

Introduction

Anxiety disorders are the most prevalent class of mental disorder worldwide with the highest lifetime prevalence rates reported by the United States [1-3]. Anxiety disorders follow a clinical course that is typically chronic, recurrent [4] and characterized by comorbid psychopathology. The vast majority of individuals with a primary diagnosis of an anxiety disorder present with depression, additional anxiety disorders, and/or co-occurring substance abuse disorders [5]. Anxiety disorders are disorders of perceived threat with physiological symptoms figuring prominently in their assessment and diagnosis [6]. This physiological component may reflect activation of the stress response as anxiety and stress are two tightly interwoven phenomena that often co-occur [7]. The stress system is a neuroendocrine network cued to respond to perceived threat with the Hypothalamus Pituitary Adrenal (HPA) axis and sympathetic branch of the Autonomic Nervous System (ANS) mediating the stress response [8-13]. Stress biomarkers can be used to track stress response activation and may be measured through the collection of saliva, urine, or blood. Of these methods, salivary assay provides the least-invasive and most practical methodology for the physiological examination of the anxiety-stress relationship [14,15]. Researchers seeking to establish a relationship between anxiety and stress using salivary biomarkers have traditionally focused on the HPA axis and its end point effector, cortisol [16]. The inconsistent literature examining cortisol and anxiety relationships suggests that this may be only part of the story [17-20]. Research has supported use of salivary alpha-amylase (sAA) as a biomarker of Autonomic Nervous System (ANS) activation in response to stress [21-25]. The measurement of these two biomarkers in concert may afford the researcher a more complete picture of the stress response in anxiety disorders.

There is a still small literature exploring associations between anxiety and sAA. In a pilot study, 10 volunteers undergoing a 15-minute mental arithmetic test experienced statistically significant increases in sAA levels, heart rate, and self-report state anxiety. No corresponding associations with salivary cortisol were found [26]. Kang experimentally induced an anxious state in 16 healthy college students and reported statistically significant elevations of sAA and blood pressure compared to the control group [27]. Other studies have extended this research

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on the association between anxious arousal and sAA from community samples to specific clinical populations. Using a sample of untreated patients diagnosed with generalized social anxiety disorder, researchers noted significantly higher basal sAA levels in the diagnosed group than among matched controls without a comparable cross-group difference in cortisol [28]. Another research team observed sharp increases in waking sAA levels among patients with post-traumatic stress disorder (PTSD), suggesting an atypical diurnal profile of sAA in PTSD [29], while Tanaka and colleagues observed elevated sAA in a subgroup of their sample of panic disorder patients [30]. These promising studies of clinical samples have used well controlled samples of individuals recently diagnosed with a single Axis I disorder to examine anxiety-stress relationships and necessarily excluded patients with psychiatric comorbidity or who are chronic in their experience of anxiety. Since the existing anxiety-stress literature has yet to consider the factors of symptom chronicity or psychiatric comorbidity and given the high prevalence of chronicity and comorbidity within the anxiety disorders population, research is warranted that examines what effect, if any, these two factors may have on the stress response in an anxiety disorder sample.

In addition, there is accumulating evidence in support of dimensional approaches to the nosology of mental health disorders, rather than a categorical approach [31-33]. This is reflected in the new strategic plan of the United States' National Institute of Mental Health, which includes a goal to "develop, for research purposes, new ways of classifying mental disorders based on dimensions of observable behavior and neurobiological measures [34]". The existing research on alpha amylase in clinical samples has focused on examinations of individual anxiety disorder categories. The feasibility and utility of anxiety-stress research with alpha amylase in a dimensional context using a diagnostically heterogeneous sample is unsupported.

The present paper reports on an exploratory investigation of possible associations between trait anxiety and salivary biomarkers of the human stress response. This study aimed to explore these relationships in a sample of anxiety disorder outpatients as they typically present in the clinical setting (i.e., chronic in their experience of anxiety and carrying comorbid mental health diagnoses) and examine the possible impact of chronicity and psychiatric comorbidity on the anxiety-stress relationship. An additional aim of this research was to assess the feasibility of anxiety research using stress biomarkers in a diagnostically heterogeneous sample.

Methods

Participants

Participants were men and women experiencing anxiety symptoms presenting for evaluation at an outpatient anxiety disorders treatment center located on a medical center psychiatric campus. Eligible participants were 1) between 18-65 years of age, 2) able to provide legal, informed consent, 3) fluent in English, and 4) attending their initial assessment session at time of recruitment. We only excluded participants with conditions that compromised the ability of the participant to consent or complete study procedures (e. g., cognitive impairment). Participants were not otherwise excluded for the presence of comorbid psychopathology.

Procedures

Upon arrival for their initial assessment at the center, patients were informed of this study by center staff. Individuals who expressed interest met with a member of the research staff in a confidential setting to receive more information regarding the study. Those who agreed to participate underwent a written informed consent in which a staff member verbally assured the participants' understanding of all study procedures. After this, participants were asked to provide approximately 1. 5-ml of saliva via expectoration or passive drool into a 2-ml polyethylene vial designed for that purpose. Saliva samples were frozen directly after collection and maintained at -20°C until time of assay. Following provision of the saliva sample, participants completed a packet of self-report questionnaires and psychosocial measures. Study procedures required approximately 45 minutes to complete. Participants were also permitted to take the packet home for completion if necessary, to be returned prior to participants' third appointment at the center. The third appointment was set as the deadline for completion of study procedures because that is when interventions are typically implemented at the center. This deadline assured that data collection preceded and therefore avoided treatment effects. For analysis, all saliva samples were kept frozen and overnight shipped to Salimetrics, Inc. , a laboratory that specializes in biochemical salivary assay. Participants received no payment or other compensation for completion of study procedures.

Measurement

Participants completed the State-Trait Anxiety Inventory-Trait Subscale (STAI-T), a 20-item scale that assesses the stable tendency to experience anxiety [35]. Participants also completed the Positive and Negative Affect Schedule (PANAS): a 20-item scale designed to measure the current "here-and-now" experience of those two primary dimensions of mood [36]. Scores from the Beck Depression Inventory (BDI-II), a validated measure of depressive symptoms [37], were obtained via chart access. In addition, participants completed a study-specific saliva collection questionnaire with questions about medication, physical health, and health behaviors. This instrument was designed to identify the most common potential confounds of biomarker assay (e. g., caffeine use, smoking; see Data Analytic Plan below for a complete listing of assessed factors). Assays of cortisol and alpha amylase were reported in standard units of concentration (μg/dL and U/ml, respectively).

As part of treatment intake, all patients at the center where recruitment took place receive the Mini-International Neuropsychiatric Interview, a short structured diagnostic interview that has demonstrated convergent validity with longer instruments [38]. Participants' primary diagnosis and number of comorbid Axis I diagnoses were extracted from patient charts. For the purpose of analysis, these data were operationalized as a categorical variable with 3 levels: no Axis I comorbidity, 1 comorbid Axis I diagnosis, and 2 or more comorbid Axis I diagnoses. Self-report on the duration of patients' experience of anxiety is included on intake paperwork at this center where patients can select from one of the following possible responses:1 month or less, 1 to 6 months, 6 months to 1 year, 1 to 5 years, 5 to 10 years, or more than 10 years. Participant responses to this question operationalized anxiety chronicity as a categorical variable.

Data analysis

Analyses were conducted using the statistical software package SPSS version 20. Zero-order correlations examined the strength of relationships between psychosocial measures, potential confounds, and salivary biomarker levels in the sample. Next, we used linear regression to examine direct associations between psychosocial measures and stress biomarkers, controlling for potentially confounding variables where appropriate (see below). Moderator analyses examined interaction effects [39] wherein variables were centered and entered on

the first step of the regression and an interaction term between anxiety and the potential moderator being tested was calculated and entered on the second step of the model [40].

Many factors that influence concentrations of stress biomarkers in saliva have been described in the literature [20,41-45]. These range from demographic (e.g., age) to pharmacological (e.g., presence of selective serotonin reuptake inhibitors) to behavioral (e.g. , exercising or smoking prior to saliva collection). In order to account for the influence of these factors, any significantly associated($P \leq 0.05$) factors from the initial correlational analysis were entered as covariates in all subsequent analyses however if two variables were highly correlated ($r \geq 0.70$) only one was included to prevent multicollinearity among predictors. A full list of examined potential covariates is included in Table 3.

Salivary alpha amylase data typically exhibits a positive skew and transformation has been recommended in order to normalize the distribution and remain in keeping with analytic assumptions [44]. In the present study, sAA data did exhibit a positive skew and square root transformation normalized its distribution. Other continuous variables (e.g., STAI-T scores, cortisol levels) were distributed normally in this sample.

A statistical power analysis was conducted for this study using Length's power calculator [42] by setting the alpha coefficient at 0.05 and proposing a sample size of 35 participants. Given a multiple regression analysis with up to 4 regressors and/or interaction terms, this study was sufficiently powered (0.824) to detect a medium effect ($r = 0.3$) using the effect-size guidelines suggested by Cohen [46,47].

Results

Sample characteristics

Forty-nine clinic outpatients consented to participate in this study. Of these, one participant withdrew prior to providing a saliva sample and 4 participants were unable to return study specific packet in the timeframe prescribed by the protocol. The final sample consisted of 44 adults ranging in age from 18-65 years (mean=35.86; SD=14.12). This sample was 71.4% (n=30) female and 90.5% (n=38) of participants identified as non-Hispanic white (Table 1). Consistent with the study's aims, this sample was heterogeneous in terms of primary diagnosis (Table 2). This sample was also predominantly chronic in its experience of anxiety with the majority (73.1%) of participants experiencing their presenting problem for more than 5 years. Comorbidity was a similarly common feature, with 26 participants (59.1%) reporting 2 or more Axis I diagnoses total. Few participants reported having smoked (7.1%) or exercised (10.5%) on the same day of data collection, while a much larger proportion (46.9%) reported caffeine use.

	N (mean)	% (SD)
Age	(35.86)	(14.12)
Gender		
Female	31	70.4
Male	13	29.6
Ethnicity		
Black	0	0
White	40	90.9
Hispanic/Latino	2	4.5
Asian	1	2.3
Hawaiian/Pacific	1	2.3
Employment		
Full Time	13	29.5
Part Time	14	31.8
Not Working	7	15.9
Disability	3	6.8
Student	6	13.6
Retired	1	2.3
Education		
Graduate Degree	10	22.7
Bachelor's Degree	16	36.4
High School Diploma	17	38.6
Primary School	1	2.3
Income		
$70,001 or greater	22	50.0
$40,001 to $70,000	7	15.9
$20,001 to $40,000	8	18.2
Less than $20,000	4	9.1
Declined to report	3	6.8

Table 1: Sample descriptive statistics.

Primary Diagnosis	n
Generalized Anxiety Disorder	4
Hypochondriasis	4
Obsessive-Compulsive Disorder	13
Panic Disorder	7
Post-Traumatic Stress Disorder	3
Social Anxiety Disorder	5
Specific Phobia	6
Unknown at time of data collection	2

Table 2: Heterogeneity of primary diagnosis in sample.

Covariates	r_{sAA}	$r_{cortisol}$
Age	-0.032	-0.038
Gender	0.145	0.027
Body Mass Index	0.216	-0.022
Time Awake until Sampling	-0.247	-0.135
Prescribed Medications		
SSRIs	-0.195	-0.069
Beta-blockers	0.052	-0.285
Thyroid Replacement	0.243	0.045
Oral Contraceptive	-0.189	-0.032
Health Behaviors on Day of Sampling		
Exercising	0.072	-0.158
Alcohol Use (≤ 24 hours)	0.273	0.053
Food intake (≤ 2 hours)	-0.391*	-0.101
Smoking	-0.199	-0.178
Caffeine Use	-0.133	-0.329*
Psychological Variables		
STAI-T	0.042	-0.093
BDI-II	0.157	-0.052
PANAS(negative affect)	0.059	-0.076
PANAS(positive affect)	0.088	0.218
Chronicity	-0.022	0.348*
Comorbidity	0.394*	0.095

*$p \leq 0.05$

Table 3: Correlations among salivary biomarkers and potential covariates.

Univariate associations

The psychological variables of trait anxiety, current mood state, and depressive symptoms did not significantly correlate with cortisol or sAA. The variable of comorbidity was significantly associated with sAA levels (r=0.381;p=0.026) in this sample, while the variable of chronicity was associated with cortisol levels (r=-0.348; p=0.028). A complete listing of correlations between psychosocial variables and biomarkers is found in Table 3. Anxiety (STAI-T) and depression

(BDI-II) scores were highly correlated (r= 0. 833; p<0. 001) in the sample.

Of tested potential confounds to salivary assay, statistically significant correlations were observed between sAA and recent food intake (Spearmans *rho*= -0. 391; p=0. 03) and between cortisol and caffeine use on the day of sampling (Spearmans *rho*= -0. 329;p=0. 05). These covariates were added to subsequent regression analyses. All tested biomarker covariates and their associated correlations are shown in Table 3.

Multivariate analyses

In order to further examine the associations between comorbidity and sAA and between chronicity and cortisol, we used multiple linear regression of sAA on comorbidity while controlling for the covariate of recent food intake and multiple linear regression of cortisol on chronicity while controlling for the covariate of caffeine intake. Inspite of the fact that the statistical significance of the association was lost at the level of α =0. 05, the overall strength of the association between sAA and comorbidity (β=0. 341; p = 0. 075) was retained with the addition of the covariate to the regression model. In contrast, with the regression of cortisol on chronicity and caffeine consumption, the chronicity and cortisol association was considerably weakened (β= -0. 161; p=0. 395). Complete regression data can be found in Table 4.

Univariate analysis did not support a direct relationship between the stress response and trait anxiety in this sample. A moderating effect of chronicity on the trait anxiety/sAA relationship was observed such that greater chronicity of anxiety strengthened the association between trait anxiety and sAA in this sample (Figure 1). The regression model included the variables of STAI-T scores, chronicity, a STAI-T*chronicity interaction term, and the covariate of recent food intake. When the criterion of sAA value was regressed on this model, a

Cortisol on:	B	SE	Beta	t	p
Constant	0.245	0.100		2.457	0.020
Chronicity	-0.015	0.018	-0.161	-0.864	0.395
Caffeine Intake	0.001	0.001	-0.037	-0.198	0.844
sAA on:					
Constant	7.259	0.869		1.100	0.284
Comorbidity	5.390	2.879	0.341	1.872	0.075
Food Intake	-1.988	0.869	-0.417	-2.288	0.033

Table 4. Multiple regression of significant univariate associations with salivary biomarkers.

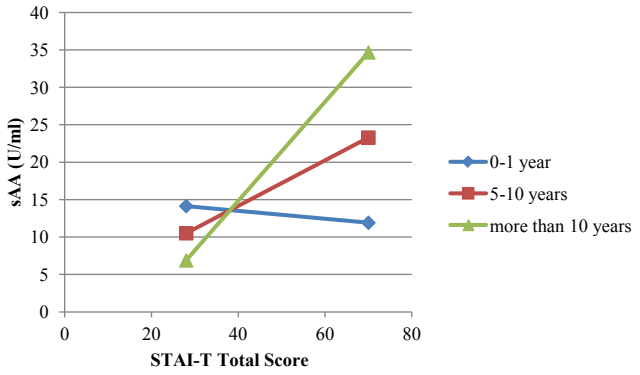

Figure 1: Chronicity moderates the relationship between trait anxiety and sAA.

sAA on:	B	SE	t	p
Constant	13.658	1.730	7.897	<0.0001
Food Intake	0.284	0.575	0.495	0.626
STAI-T	0.427	0.171	2.492	0.0211
Chronicity	10.776	2.433	4.429	0.0002
STAI-T*Chronicity	2.1758	0.456	4.776	0.0001

Table 5: Chronicity moderates the relationship between trait anxiety and sAA.

significant proportion of the variance was accounted for (R^2=0. 568; p = 0. 001) with a large proportion of that value attributable to the addition of the interaction term (ΔR^2=0. 469; p = 0. 001). Table 5 contains a full summary of this regression with the interaction term. In contrast, the model testing the interaction of chronicity on the trait anxiety/cortisol relationship accounted for a nominal proportion of the variance and was not statistically significant (R^2=0. 114; p= 0. 618). Tests of comorbidity as a moderator also did not produce statistically significant interactions with STAI-T/sAA(R^2=0. 245; P=0. 255) or STAI-T/cortisol (R^2=0. 087; p=0. 753) relationships.

Discussion

To our knowledge, this is the first study to explore possible associations between trait anxiety and salivary stress biomarkers while examining the influence of chronicity and psychiatric comorbidity on the anxiety-stress relationship. In addition, this is the first study to our knowledge that examines the feasibility and utility of anxiety-stress research using salivary biomarkers with a diagnostically heterogeneous sample.

Greater numbers of comorbid psychiatric diagnoses were associated with higher sAA levels in this sample. While this relationship lost statistical significance at α=0. 05 levels when tests controlled for covariates, the association retained its strength of effect. Because a statistic's associated p-value is a function of sample size, examination of effect sizes can be more useful in exploratory studies [48]. Considering the strength of the effect apart from probabilistic testing, these data suggest that the number of comorbid diagnoses (e.g., depression, anxiety, substance use) patients present with contributes to the basal state of autonomic arousal in addition to the contribution of anxiety symptoms. Additional phobias, intrusive thoughts, persistent apprehensions, or other clinical symptoms may be experienced as cumulative and contribute to the patient's overall psychopathological burden with a resultant heightened basal level of stress activation.

In this sample, chronicity of anxiety symptoms moderated the anxiety-stress relationship such that greater chronicity strengthened the association between anxiety scores and sAA levels. The finding that chronic anxiety is associated with basal sympathetic arousal has been demonstrated in past literature using measurement of heart rate [49,50]. Moreover, there is similarly long-standing evidence that symptoms of sympathetic arousal are robust and can be observed across anxiety disorder diagnoses [51]. More recent research has also suggested that different associations with anxiety may exist across the two biomarkers under examination in this study, as described earlier [26,28,30]. Our findings appear consistent with these prior observations, and to our knowledge, this study is the first to observe such findings in a diagnostically heterogeneous and comorbid outpatient sample.

A pertinent negative finding in our study was the lack of any significant association between trait anxiety and cortisol in our sample even when considering possible interaction effects of chronicity and comorbidity. Null associations between anxiety and cortisol are not unusual and are part of the mixed literature on this biomarker. A similar

finding was reported in a study of a non-clinical sample [18], while in the clinical realm a recent meta-analysis of 38 studies of cortisol levels in persons with PTSD reported that overall cortisol levels did not differ between PTSD and control groups [17]. A population based study saw a lower cortisol awakening response among older adults with chronic anxiety disorders compared to those without [52]. Our finding is consistent with these reports and also with the sAA and cortisol comparison studies reviewed above [26,28,30]. It is also consistent with the association between chronic stress and hypocortisolism in healthy humans and animal models [53,54]. In this way, our study supports the assertion that salivary cortisol alone appears insufficient to explain the relationship between psychological factors and stress activation [20].

Limitations and Future Directions

The authors wish to acknowledge the limitations of the work reported herein. This study was cross-sectional in design and as such no conclusions about causality or directionality in observed relationships can be made. This was an exploratory study using a small convenience sample and all findings should be considered tentative. While power analysis suggested that the sample used was sufficient for the detection of the medium effects observed, this study was still insufficiently powered to detect lower strength associations among the investigated variables and covariates. Further research is needed in order to test whether these findings might replicate with larger samples using more covariates. Distributions of gender, symptom chronicity, and extent of psychiatric comorbidity were all consistent with the demographics of the U. S. anxiety disorders population; however, the study sample was not ethnically or socio-economically diverse. We believe the study was limited in this regard by the sampling frame: clinic data indicate that less than 20% of the patients treated at the recruitment site belong to ethnic minority groups. This diminishes the generalizability of the study's results across ethnic groups and socio-economic strata. Finally, the cross-sectional study design necessitated use of a single time point for salivary assay. Best practices favor saliva collection at multiple time points as this provides more information on overall biomarker levels as well as their diurnal variations.

In conclusion, these findings support the use of the salivary biomarker sAA in addition to cortisol in anxiety and stress research. Further, they suggest that highly prevalent adjunctive clinical factors such as chronicity and comorbidity may be contributing variance to observed associations between anxiety and stress biomarkers. This may be useful to consider in further investigations of quantitative physiological markers as correlates for the psychological state of anxiety, but larger scale research studies are needed to contribute additional evidence. Finally, the utility and feasibility of sAA in anxiety-stress research in samples with diagnostic heterogeneity and broader inclusion criteria with respect to chronicity and comorbidity is tentatively supported. Further investigation using this approach serves the science as this particular area of research transitions from lab-based, internally valid methodologies to more ecologically valid designs.

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