Psychoneuroendocrinology 71 (2016) 110–118

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Salivary latent trait cortisol (LTC): Relation to lipids, blood pressure, and body composition in middle childhood

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ARTICLE INFO

Article history:
Received 11 January 2016
Received in revised form 13 May 2016
Accepted 13 May 2016

Keywords:
Latent trait cortisol (LTC)
Middle childhood
Lipids
Body composition
Blood pressure
Toxic stress

ABSTRACT

Adversity experienced early in life has the potential to influence physical health later in life. The stress-health relation may be partially explained by stress-related effects on cardiovascular risk factors. This study explored links between individual differences in trait-like variation in the activity of the hypothalamic-pituitary-adrenal (HPA) axis with cardiovascular risk factors in children. 474 children (*M* age = 9.22 years; 54% female; 83% Caucasian) were included in this study, in which cardiovascular risk was assessed using the following indices – triglycerides (TG), HDL-cholesterol (HDL-C), glucose (Glu); resting systolic and diastolic blood pressure, body mass index (BMI), waist-to-hip ratio, and % fat. Saliva samples were measured 3 times a day (waking, 30 min post-waking and bedtime) over 3 days (later assayed for cortisol). A latent trait cortisol (LTC) factor explained 43% of the variance in cortisol levels within and across days. Confirmatory factor analysis identified three cardiovascular risk factors: lipids (i.e., TG and HDL-C), blood pressure (i.e., systolic and diastolic), and body composition (i.e., BMI, Waist-to-hip ratio, and % fat). Lower salivary LTC was associated with higher lipids, higher blood pressure, and higher body composition. The findings further support the internal and external validity of the LTC construct, and may also advance our understanding of the link between interindividual differences in HPA axis activity and cardiovascular risk in middle childhood.

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1. Introduction

Throughout childhood acute and chronic adversity influences individual differences in the reactivity and regulation of the hypothalamic-pituitary-adrenal (HPA) axis (e.g., Gunnar and Donzella, 2002; Johnson et al., 2013). Despite advances in our understanding of the consequences of these processes for children's behavioral, psychosocial, and mental health outcomes (e.g., Doom et al., 2014), the depth of our knowledge relative to the effects on children's health remains remarkably shallow (e.g., Shonkoff, 2012). An exception is the accumulating literature suggesting that for chronically stressed individuals, both behavioral and neuroendocrine mechanisms promote central obesity, insulin resistance, and metabolic abnormalities (e.g., Pervanidou et al., 2013). It is widely held that for children in particular, chronic stress-related alterations in the activity of the HPA axis at this early life stage may increase risk of early-onset obesity, metabolic syndrome, and type 2 diabetes and may subsequently also affect the timing of puberty, final stature, and also body composition (e.g., Pervanidou and Chrousos, 2011, 2012). A recent review by Rodriguez and colleagues, however, suggests that inconsistencies between studies in methodology and results appear to be commonplace (e.g., see Rodriguez et al., 2015). Here we apply a multiple saliva sampling approach (within and between days) combined with advanced statistical methods, to further our understanding of the internal validity of modeling stable interindividual differences in children's

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http://dx.doi.org/10.1016/j.psyneuen.2016.05.013
0306-4530/Published by Elsevier Ltd.

HPA axis activity (i.e., cortisol). We have done so using a latent variable approach, and relate stable interindividual differences in salivary cortisol levels to lipids, blood pressure, and body composition in middle childhood.

1.1. Latent state-trait modeling of salivary cortisol

Developmental science was an "early adopter" of salivary cortisol measurement to study relationships between children's HPA axis reactivity and regulation relative to individual differences in behavioral, psychosocial, and mental health outcomes. In addition to substantive findings, studies consistently report that a significant portion of the variance in salivary cortisol levels relates to moment-to-moment and day-to-day fluctuations within individuals (e.g., Doane and Zeiders, 2014; Ross et al., 2014). Given this inherent "state" related variability in cortisol levels, some investigators have taken advantage of non-invasive collection techniques, asked participants to donate multiple saliva samples, and employed latent state-trait (LST) modeling (Steyer et al., 1989; Steyer et al., 2012) to estimate *stable trait-like* interindividual differences in HPA axis activity (e.g., Doane et al., 2015; Giesbrecht et al., 2015; Kirschbaum et al., 1990; Shirtcliff et al., 2005; Stroud et al., 2016). LST modeling assumes that a *"trait"* is a stable, consistent interindividual difference, in a construct across time and situations (Kenny and Zautra, 2001) and that such a construct can be assessed and statistically extracted by repeatedly sampling measurements collected across time and settings (Epstein and O'Brien, 1985). More specifically, any single measurement assessed at a given time point is considered to be influenced by person-specific and situation-specific factors, as well as their interaction (Eid et al., 1994). A commonly used method to assess stable interindividual differences is to simply aggregate (i.e., average) repeated measures collected over time. However, to reduce situation-specific influences and measurement error, simple aggregation requires a large number of repeated measures (Steyer and Schmitt, 1990). Moreover, aggregation does not allow the decomposition of the total variance into components accounted for by (1) person-specific factors, (2) situation-specific factors and person-situation interaction, or (3) measurement error. An advantage of LST modeling (Steyer et al., 1989; Steyer et al., 2012;) is that it permits the identification of a stable indicator of individual differences in cortisol levels. Specifically, LST modeling uses the correlations among different cortisol samples to create two types of factors: (1) a latent trait cortisol factor (LTC), which captures individual differences by drawing from the commonalities amongst cortisol samples in reference to the grand mean (or whole sample); and (2) a latent state cortisol factor, which captures state-specific situational or environmental influences (which may change from moment-to-moment or day-to-day) and random error variances. As an illustration Doane et al. (2015) recently documented the longitudinal stability of a LTC factor in adults in a three-wave (9-month) study. The LTC factor was distinct from the cortisol awakening response (CAR, Clow et al., 2010) and the diurnal slope. In contrast to these indices, LTC largely captured between-person variability in diurnal cortisol, rather than day-to-day and between-wave variability. Using a similar design, Stroud et al. (2016) reported similar findings with an adolescent sample (Stroud et al., 2016). Also, Giesbrecht et al. (2015) extended our understanding by revealing that LTC models were strongly correlated between trimesters and stable throughout pregnancy.

1.2. Latent trait cortisol: biobehavioral correlates and concomitants

To date, the literature reveals associations between individual differences in LTC and early adversity, recent chronic and acute stress, and problem behavior (e.g., Chen et al., 2015; Doane et al., 2015; Shirtcliff et al., 2005; Stroud et al., 2016). Given there are only a handful of studies, this area of inquiry is clearly in its infancy, and there are many knowledge gaps. Very little empirical attention has focused on the possibility of an association between LTC and children's health. The exception is Giesbrecht et al. (2015) who reported an inverse relationship between maternal LTC during pregnancy and infant birthweight. Epidemiologic studies consistently report that infant birth weight is amongst the strongest predictors of children's developmental trajectories and outcomes (Curhan et al., 1996).

Regarding the linkage between HPA axis activity and children's health, chronic activation of the HPA axis may lead to secretion of insulin, growth hormone, and sex hormones, leading to increased central adiposity and insulin resistance—the core pathophysiological alterations leading to the development of metabolic syndrome and type II diabetes. Biochemical alterations related to metabolic syndrome may lead to endothelial dysfunction and atherosclerosis in later life (Pervanidou and Chrousos, 2016). Furthermore, among healthy individuals, high levels of HPA activity, associated with elevated cortisol levels, may lead to increased appetite and caloric intake (Epel et al., 2004). The significance of these associations is underscored by epidemiological studies reporting an increase in the incidence and prevalence of overweight and obesity amongst youth (Kassi et al., 2011; Pervanidou et al., 2013; Weiss et al., 2004). Between 2005-2008 and 2009-2012 the obesity rate for children and adolescents increased by 5% to 16.9% (Centers for Disease Control and Prevention, 2014). The consequences of this epidemic have implications at the level of the individual, family, community (e.g., Koh, 2010), and society (e.g., Bhattacharya and Bundorf, 2009). Not surprisingly, the U.S. Department of Health and Human Services has placed a premium on research that aims to elucidate the underlying mechanisms in an effort to advance our understanding of this complex set of problems (e.g., Aungst, 2011).

It is noteworthy that one of the most recent reviews on the association between HPA axis activity and obesity/overweight during childhood concluded that inconsistencies between studies in methodology, sample characteristics, and results appear to be commonplace (e.g., see Rodriguez et al., 2015). It seems reasonable to assume that the application of a multiple saliva sampling approach to estimate stable intrinsic individual differences in children's cortisol levels may increase the probability of detecting a relationship between HPA axis activity and cardiovascular risk in childhood. In this study we attempt to address this knowledge gap.

In the health literature, higher levels of cardiovascular risk have been commonly represented by four components: insulin resistance, dyslipidemia, elevated blood pressure, and obesity/body composition (McCaffery et al., 2012). Each of these components has been indexed by various biochemical markers. Literature has not reached an agreement on which markers are the best for representing each component. The selection of markers is often based on criteria, such as (1) previous studies that have demonstrated markers that tend to be more important for representing each component, (2) allowance of budget, (3) feasibility related to data collection procedures, (4) participant burden, and (5) statistical power to detect an effect that is determined by the proportion between number of biomarkers included in the model and sample size. In most studies, insulin resistance was captured by insulin and glucose; dyslipidemia was indexed by triglycerides and high-density lipoprotein (HDL) cholesterol; blood pressure was represented by systolic and diastolic blood pressure; and body composition was indexed by body mass index (BMI) and waist-to-hip ratio (e.g., Gallo et al., 2012; Juster et al., 2010; Marsland et al., 2010; McCaffery et al., 2012). The focus of this study is on cardiovascular risk rather than metabolism. Thus, while on one hand we limited our measure of insulin resistance to glucose, on the other hand we expanded our measures of body composition by including percent

fat, which has been found to provide unique information on one's cardiovascular fitness independent of BMI and waist-to-hip ratio (Lobelo et al., 2009).

Different statistical strategies have been used for summarizing individual differences in cardiovascular risk. Among them, confirmatory factor analysis (CFA) was employed in this paper. CFA is a theory driven data reduction strategy (Bollen, 1989). Based on previous biochemical literature, measured markers that represent the same physiological system/component are loaded onto a latent factor. This factor captures the common variance of the measured markers, and measurement error of each marker is partialled out to enhance statistical power for model testing. Models established by confirmatory factor analysis and latent state-trait analysis are different types of measurement models based on the structural equation modeling (SEM) framework. According to prior literature, we suggest a model structure with glucose; triglycerides and high-density lipoprotein (HDL) cholesterol; systolic and diastolic blood pressure; body mass index, waist-to-hip ratio and percent fat loaded on insulin resistance, lipids, blood pressure, and body composition factors, respectively to represent the four physiological systems/components of cardiovascular risk factors (e.g., Lobelo et al., 2009; Marsland et al., 2010; McCaffery et al., 2012; Novak et al., 2003; Shen et al., 2006).

1.3. The present study

In the current study, 474 4th grade boys and girls completed standard assessments to evaluate cardiovascular risk: triglycerides (TG), HDL-cholesterol (HDL-C), and glucose (Glu) via fasting finger stick; resting systolic and diastolic blood pressure; body composition by BMI, waist-to-hip ratio, and % fat. Saliva samples were collected 3 times a day (waking, 30 min post-waking and bedtime) over 3 days and assayed for cortisol. We hypothesized that lower LTC would independently relate to higher lipids, blood pressure, and body composition.

2. Method

2.1. Participants

The Cardiovascular Health Intervention Program (CHIP) is an ongoing study of cardiovascular (CV) risk profiles in Southern Maine (e.g., Peterson et al., 2016). Two cohorts of 4th grade students from two elementary schools were invited to participate. A total of 474 students (M age = 9.22 years; 54% female; 83% Caucasian) participated in the CHIP saliva and cardiovascular risk assessment. The participation rate was 78%, which reflects the number of students with a complete data set that participated versus total number of 4th grade students. Possible reasons for a lack of participation was due to not returning a completed consent/assent form, being absent at the screening assessment day, or deciding not to complete any part of the assessment. The most common reason for not completing the assessment was the participant's lack of willingness to complete the lipid/glucose analysis, which required a finger prick. All 4th graders were given the opportunity to participate with no selection bias. The participants were recruited from two local schools where the superintendents gave their permission and were in a geographically close distance to the university. Fourth graders were chosen as they represented an age group in which researchers envisioned potential for maximum benefit with regard to comprehendive assessment results and making healthy lifestyle adjustments. The current sample size was based on the total number of 4th grade classes that could be successfully screened in the two schools chosen to participate during the semester. The study protocol was approved by the institutional review board at the University of New England (UNE), as well as the superintendent and principal at each participating school.

2.2. Procedures

Prior to the screening date at each participating school, a 15–20 min presentation was given to students to explain the importance of cardiovascular health and provide an overview of the program. A packet was given to each child, which contained a cover letter explaining the program, as well as a medical history form, a consent form, and an assent form. The packets were collected and data from the medical history were entered into a secured university website prior to the screening date. Students and teachers from 2 to 3 classes at a time came to the UNE on a field trip to participate in the assessment, which took approximately 3 h. When the children arrived at the university, they participated in stations to assess (1) blood lipids and glucose, (2) resting blood pressure (BP), and (3) an anthropometric assessment. Students were also given saliva collection devices and instructions to take home to their parents/guardians. The instructions for collecting saliva were also emailed directly to participants' parents/guardians.

2.3. Blood lipids and glucose collection

The night before screening, participants were asked to complete a 12 h fast. To verify fasting, children were asked if they had anything to eat or drink, other than water, in the past 12 h. If a child was in a non-fasting state, they were not included in this data set. A capillary tube of blood was obtained using the finger-stick method and analyzed using a calibrated Cholestech LDX cholesterol analyzer (Cholestech Corporation, Hayward CA). Calibration procedures were completed based on recommendations by Cholestech Corporation. The lipids/glucose panel included triglycerides, HDL-C, and blood glucose. The Cholestech LDX analyzer has been shown to be a valid instrument in comparison to venipuncture (Carey et al., 2006). Immediately following this measurement, the children were provided a juice drink and a granola bar. The missing data rate for all three biomarkers extracted from blood samples was 16%.

2.4. Resting blood pressure

An aneroid sphygmomanometer (Welch-Allyn Handheld Aneroid-Skaneateles Falls, NY) was used with appropriate sized cuffs for children to determine BP. Two BP measurements were taken two minutes apart, which were subsequently averaged, from the right arm of each child after sitting quietly for 3 min. Systolic BP was recorded at the first appearance of a clear repetitive sound (Korotkoff Phase I) and diastolic BP was taken at the disappearance of repetitive sounds (Korotoff Phase V). The missing data rate for blood pressure was 1%.

2.5. Anthropometrics

Height and weight were measured without shoes or bulky clothing. Height was measured to the nearest 0.5 cm using a portable stadiometer (Seca Mobile Height Stadiometer). Weight was recorded to the nearest 0.5 kg using an electronic scale (BWB-800-Tanita, Toyko Japan). Body mass index (BMI) was calculated as weight (in kg) divided by height (in meters) squared. Waist circumference (WC) was measured in (cm) and determined at the umbilicus, and hip was measured at the widest point of the trunk in triplicate with a Gulick measuring tape. FitnessGram software was used for assessing percent fat, which integrated the measurements of the child's right tricep, calf and gender in the model (Meredith

Table 1
Descriptive statistics of salivary biomarkers and cardiovascular risk indices.

Variable	Mean	Standard Deviation	Skewness	Kurtosis
Cortisol at bedtime on day 1 (μg/dl)	0.08	0.11	3.60	15.20
Cortisol at waking on day 1 (μg/dl)	0.30	0.23	2.45	10.26
Cortisol at 30-min post-waking on day 1 (μg/dl)	0.34	0.24	1.30	2.70
Cortisol at bedtime on day 2 (μg/dl)	0.08	0.12	4.53	26.47
Cortisol at waking on day 2 (μg/dl)	0.30	0.23	2.17	6.56
Cortisol at 30-min post-waking on day 2 (μg/dl)	0.35	0.21	0.93	1.10
Cortisol at bedtime on day 3 (μg/dl)	0.10	0.18	4.33	23.75
Cortisol at waking on day 3 (μg/dl)	0.30	0.19	1.58	3.94
Cortisol at 30-min post-waking on day 3 (μg/dl)	0.34	0.21	1.09	1.93
Triglycerides (mg/dl)	66.75	50.23	1.20	3.05
High-density lipoprotein (mg/dl)	51.93	12.40	0.31	0.22
Systolic blood pressure (mmHg)	105.43	12.61	1.17	6.73
Diastolic blood pressure (mmHg)	67.56	10.01	0.33	−0.35
Body mass index (kg/m^2)	19.48	4.29	1.56	4.44
Waist-to-hip ratio (cm)	0.87	0.07	0.47	−0.03
Percent fat (%)	28.68	10.09	0.68	0.38
Glucose (mg/dl)	89.58	9.42	0.70	25.30

and Welk, 2010). The missing data rates for BMI, waist-to-hip ratio, and percent fat were 3%, 2%, and 1%, respectively.

2.6. Determination of salivary analytes

During the university visit, participants were taught how to facilitate saliva collection three times per day (waking, 30 min post waking, and bedtime) on each of the three days. Participants were instructed to have saliva collection materials beside their bed so that they could reach them immediately when awakening and while still in bed, 30 min later with the least amount of activity, then again immediately before going to bed. In line with procedures delineated by Granger et al. (2012), participants were asked to avoid eating and brushing their teeth one hour prior to donating the sample, and rinse their mouth out with water 10 min prior to giving the sample. Participants were asked to place a 1 × 4 CM saliva collection swab (Salivabio, Carlsbad, CA) under their tongue for two minutes, then put the swab into a collection vial and place that vial in their home freezer. After all samples were collected, participants transported all vials to school, and samples were taken to the university and frozen at −20 °C. Determination of actual sampling times was not recorded. All samples remained frozen until the day of assay. All samples were assayed for cortisol in duplicate using a commercially available assay without modification to the manufacturers recommended protocol (Salimetrics, State College, PA). This immunoassay had a test volume of 25 μl, range of standards from 0.01 to 3 μg/dl, lower limit of sensitivity of 0.003 μg/dl, and average intra- and inter-assay coefficients of variation less than 5% and 10% respectively.

Missingness in the cortisol data is largely due to 58 participants who did not provide any samples on any day or time point. These 58 participants were not statistically different from the 416 participants who provided at least one cortisol sample on triglycerides (TG), HDL-cholesterol (HDL-C), glucose (Glu); resting systolic and diastolic blood pressure, body mass index (BMI), waist-to-hip ratio, and % fat, gender, age, race, SES, and medication use. For the participants who provided saliva samples, there was 3.9% missing cortisol data. We also set cortisol values as missing if the data fell under on of the following categories: cortisol values that were biologically implausible (>4.0 μg/dl) (0.4%); greater than 4 standard deviations above the sample mean (1.1%); samples that did not have sufficient volume for assay (5%); and cortisol values below the assay's lower limit of sensitivity (0.4% morning samples, 3.0% bedtime samples, 3.4% in total). In this study, we employed Full Information Maximum Likelihood (FIML) estimation in M*plus* version 7 for model testing (Muthén and Muthén, 1998-2012). This estimation routine performs the state-of-the-art, advance missing data handling technique. It generates parameter estimates and standard errors for the model as if missing data were to be "filled in" based on available data in the model (Enders, 2010; Enders and Bandalos, 2001; Graham, 2003; Schlomer et al., 2010). Thus, setting the unreliable cortisol values as missing and using FIML estimation allowed us to yield the least bias results amongst all available techniques to handle unreliable values in biomarker data.

2.7. Potential covariates

Covariates that might confound the relations between latent trait cortisol and cardiovascular risk variables included: gender, age, race, socioeconomic status, and medication use. Socioeconomic status was indexed by the father's and mother's levels of education, and the child's free lunch status. Medication use was coded as 0 = no use of any medication, or 1 = used any medications.

2.8. Data analytic strategy

The analytic strategy comprised four stages based on the structural equation modeling (SEM) framework. The four steps in this study were: (1) estimation of latent state-trait models for cortisol to identify a stable latent trait cortisol (LTC) factor; (2) using confirmatory factor analysis (CFA) to establish a multi-factor structure for representing the cardiovascular risk factors; (3) employing path analysis using the latent variables established in steps 1 and 2 to test hypotheses; and (4) adjusting for the potential confounding effects of gender, age, race, socioeconomic status (SES), and medication use. All models were estimated by Full Information Maximum Likelihood (FIML) (Muthén and Muthén, 1998-2012). Four global fit indices were used to evaluate model fit, criteria listed in parentheses indexed good fit: chi-square test (p-value > 0.05); Comparative Fit Index (CFI > 0.95); Standardized Root Mean Square Residual (SRMR < 0.05); and Root Mean Square Error of Approximation (RMSEA < 0.05, between 0.05 and 0.08, and > 0.08 indexed a good, fair, and poor fit, respectively) (Hu and Bentler, 1998). A deviance test (-2 log likelihood test) was used for model comparisons amongst nested models.

3. Results

3.1. Preliminary analyses

3.1.1. Descriptive statistics and correlations

Descriptive statistics are summarized in Table 1. Log-transformation (ln) was applied to cortisol, and BMI due to skewness. After transformation, normality was obtained for these

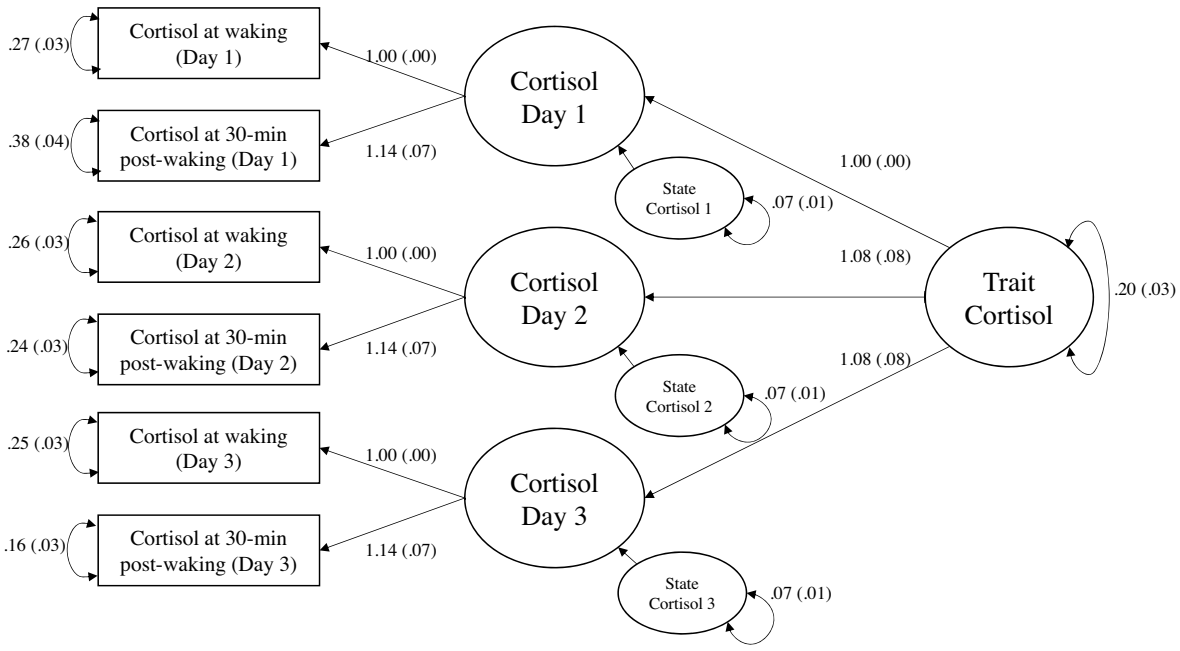

Fig. 1. Singletrait-multistate (STMS) model with salivary cortisol collected at wakening and 30-min post-wakening across three consecutive sampling days. All the loadings had *p*-value <0.001. Unstandardized parameter estimations and standard errors (in parentheses) were reported.

Table 2
Correlations among cardiovascular risk indices.

Metabolic Index	1	2	3	4	5	6	7	8
1. Triglycerides	1							
2. High-density lipoprotein	−0.40	1						
3. Systolic blood pressure	0.14	−0.08	1					
4. Diastolic blood pressure	0.21	−0.08	0.64	1				
5. Log-transformed body mass index	0.35	−0.33	0.38	0.34	1			
6. Waist-to-hip ratio	0.36	−0.29	0.18	0.14	0.59	1		
7. Percent fat	0.33	−0.27	0.40	0.39	0.84	0.51	1	
8. Glucose	0.06	−0.01	−0.03	0.01	0.03	0.02	0.00	1

variables. Across sampling days, the range of correlations for the log-transformed cortisol values was 0.35 – 0.48, 0.43 – 0.53, and 0.44 – 0.53 for samples collected at waking, 30-min post-waking, and bedtime, respectively. Averaging the cortisol values across days, correlations between the two morning samples was 0.65, waking and bedtime was 0.27, and 30-min post-waking and bedtime was 0.24. The intercorrelations amongst the cardiovascular indices (TG, HDL-C, systolic and diastolic blood pressure, log-transformed BMI, waist-to-hip ratio, and percent fat, and glucose) suggested that multiple latent factors representing different cardiovascular risk factors could be constructed through confirmatory factor analyses (Table 2).

3.1.2. Latent state-trait models for cortisol

Three Singletrait-Multistate (STMS) models were estimated, using waking, 30 min post-waking and bedtime cortisol data. In *Model 1*, the waking and 30 min post-waking samples from each day were used as indicators to form a latent state cortisol factor. Three state factors representing the three sampling days were subsequently loaded onto one latent trait cortisol factor. The fit indices yielded from this model were: χ^2 (11) = 41.018 (p < 0.001); CFI = 0.957; SRMR = 0.042; RMSEA = 0.081 (90% CI: 0.056-0.109); CFI and SRMR indicated a good fit, whereas RMSEA indicated a fair fit. *Model 2* was similar to Model 1, except that the bedtime sample from each day was included in each state factor. The fit indices yielded from this model were: χ^2 (24) = 243.438 (p < 0.001); CFI = 0.765; SRMR = 0.116; RMSEA = 0.148 (90% CI: 0.132-0.166); CFI, SRMR, and RMSEA indicated a poor fit. In *Model 3*, the waking samples across the three sampling days were used as indicators to form a waking latent state factor, and the 30 min post-waking and bedtime samples across days were loaded onto the 30 min post-waking and bedtime latent state factors, respectively. These three latent state factors were subsequently loaded onto one latent trait factor. The model did not reach convergence. The poor model fit in Model 2 and the non-convergent Model 3 were due to low correlations amongst the morning and bedtime samples.

Finally, before accepting Model 1 as the best fit, a more parsimonious, single-trait model was estimated. In *Model 4*, the six morning samples collected across days were loaded onto one latent trait factor. The fit indices yielded from this model were: χ^2 (9) = 76.159 (p < 0.001); CFI = 0.904; SRMR = 0.049; RMSEA = 0.135 (90% CI: 0.108-0.163); SRMR indicated a good fit. However, both CFI and RMSEA indicated a poor fit. After examining the collective findings, Model 1 was retained.

Model 1 was subjected to measurement invariance tests, which compared four nested models – baseline, weak (equated loadings from state factors to indicators), strong (weak plus equated loadings from trait factor to state factors), and strict (strong plus equated state factor variances) invariance models using deviance tests. The results of model comparison tests indicated that there were no significant differences across the four measurement models (Table 3). Thus, increasing the number of equality constraints imposed from the baseline to the strict invariance model was reasonable. To maximize the degrees of freedom, the strict invariance model was employed for subsequent analyses. The unstandardized solution illustrating the constraints for model identification and its strict invariance is depicted in Fig. 1. For Model 1 (strict invariance specifications), the consistency coefficients reflecting the proportion of stable/trait variance of the six manifested variables calculated by formula described in Steyer et al. (1989) ranged from 0.357 to 0.549 (Mean = 0.432), i.e., on average, the latent trait cortisol (LTC) factor explained 43% of the variance in cortisol levels within and across days.

Table 3
Measurement Invariance of STMS Model 1.

	a. Baseline	b. Weak Invariance	c. Strong Invariance	d. Strict Invariance
Number of Parameters	21	19	18	16
Log Likelihood	−2195.85	−2196.84	−2197.90	−2198.09
Deviance	4391.70	4393.67	4395.80	4396.17
Models Compared		a vs.b	b vs.c	c vs.d
Delta in Number of Parameters		2.00	1.00	2.00
Delta in Deviance		1.97	2.13	0.37
Chi Square Difference Test		0.37	0.14	0.83
Chi-Square Test of Model Fit	36.55	38.52	40.64	41.02
df	6	8	9	11
RMSEA	0.11	0.10	0.09	0.08
SRMR	0.03	0.04	0.04	0.04
CFI	0.96	0.96	0.96	0.96

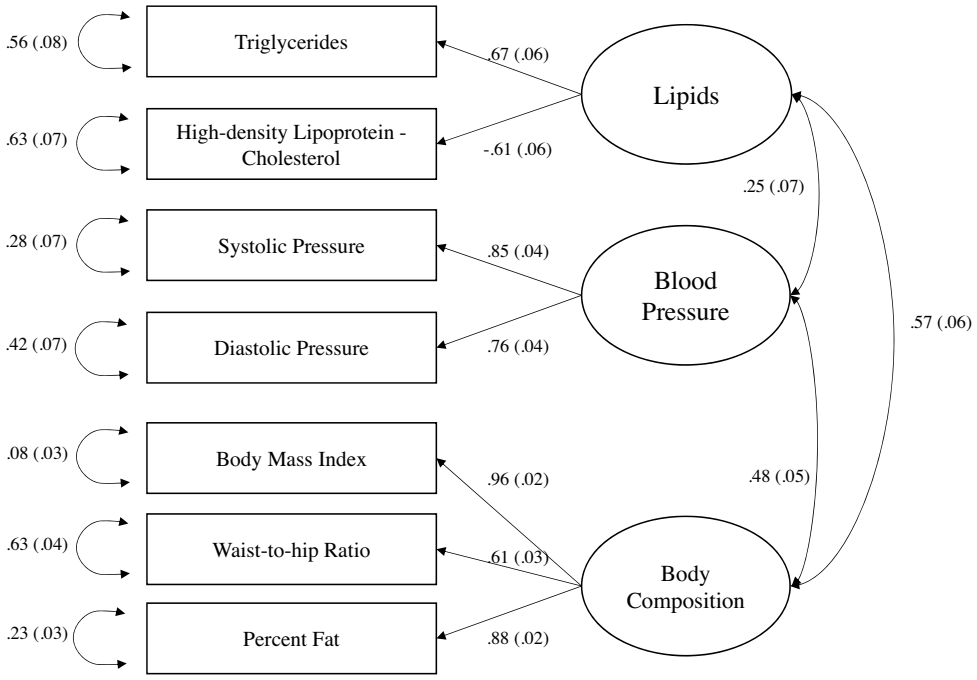

Fig 2. Confirmatory factor analysis model depicting factor structure of cardiovascular risk factors. All the loadings had *p*-value <0.001. Standardized parameter estimations and standard errors (in parentheses) were reported.

3.1.3. Measurement models for cardiovascular risk factors

Confirmatory factor analysis was conducted to test a four-factor model in which TG and HDL-C were loaded on a *Lipids* latent factor; systolic and diastolic blood pressure were loaded on a *Blood Pressure* latent factor; BMI, waist-to-hip ratio, and percent fat were loaded on a *Body Composition* latent factor; and glucose formed a one-indicator *Insulin Resistance* latent factor. This model failed to reach convergence due to the low correlations between glucose and other cardiovascular indicators. Subsequently, a *Three-Factor Model* with the elimination of Insulin Resistance factor from the four-factor model was estimated and evaluated (Fig. 2). Its fit indices suggested retaining this model for subsequent analyses: χ^2 (11) = 46.097 ($p < 0.001$); CFI = 0.973; SRMR = 0.040; and RMSEA = 0.082 (90% CI: 0.059-0.108). CFI and SRMR indicated a good fit, and RMSEA indicated a fair fit. The standardized solution illustrating the relative strength of the relations between the latent trait cortisol and their latent factors was depicted in Fig. 2.

3.2. Main analysis

3.2.1. Salivary biomarkers and cardiovascular risk factors

Using a SEM model (see Fig. 3), the latent trait cortisol (LTC) from the STMS model was specified to predict the Lipids, Blood Pressure, and Body Composition latent factors (Fig. 3). The fit indices were: χ^2 (61) = 131.184 ($p < 0.001$); CFI = 0.965; SRMR = 0.040; and RMSEA = 0.049 (90% CI: 0.038-0.061), indicating good fit. The trend indicated that lower LTC was associated with higher levels of Lipids. Lower LTC was also significantly associated with higher levels of Blood Pressure, as well as higher Body Composition. LTC explained 2.0%, 4.6%, and 4.0% of the variation in lipids, blood pressure, and body composition. The standardized solution illustrating the relative strength of the associations between the salivary biomarkers and cardiovascular risk factors is depicted in Fig. 3. The robustness of the conclusions were tested by adding gender, age, race, SES, and medication use, all commonly tested as covariates in the physiology literature, into the model. After including the influences of these covariates, the effects of LTC remained robust and the adjusted estimates and standard errors were reported in Fig. 3.

4. Discussion

A latent trait cortisol (LTC) factor was successfully modeled using multiple cortisol values from children's saliva samples collected within and across three days. The LTC factor explained 43% of the variance in cortisol levels. As expected, we observed that

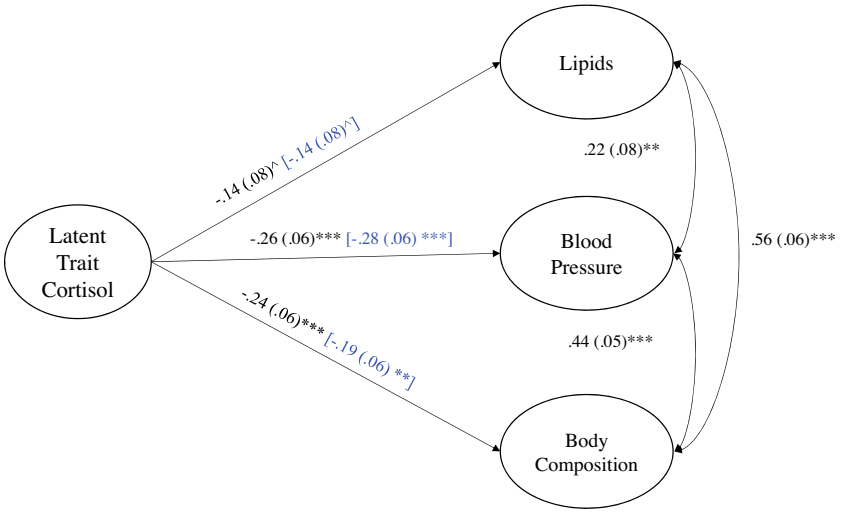

Fig. 3. Relations between salivary latent trait cortisol and cardiovascular risk factors. Chi-square = 131.184, df = 61, p < 0.001, CFI = 0.965, SRMR = 0.040, RMSEA = 0.049. All fit indices indicated the model was a good fit of the data. ^p < 0.10, *p < 0.05, **p < 0.01, and ***p < 0.001. Standardized parameter estimations and standard errors (in parentheses) were reported to illustrate the relative strength of the relations between the latent trait cortisol and their latent factors. Values in black represent parameters before adjusting for covariates. Values in blue enclosed by square brackets represent the parameters after adjusting for covariates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lower LTC scores were associated with higher lipid levels, higher blood pressure, and higher body composition. Importantly, LTC explained a significant portion of the variance in each of the three cardiovascular risk factors. Even after adjusting for gender, age, race, SES, and medication use, the effects of LTC on all cardiovascular factors remained. These findings suggest a strong relationship between stable individual differences in HPA axis activity and cardiovascular risk in middle childhood. Results further underscore the validity of operationalizing variation in HPA axis activity using LTC, and highlight the utility of applying LST-based analytical strategy when studying links between salivary cortisol and children's biobehavioral health.

Contemporary scientists posit that the regulation of the HPA axis (primary mediator) underlies the relationship between chronic stress and cardiovascular function. This is reflected in individual differences in the levels of triglycerides, HDL-C, blood pressure, and visceral fat deposits (secondary outcomes), which in turn, can lead to disorders and diseases such as CVD (tertiary outcomes) (e.g., Juster et al., 2010; McEwen, 1998). Some empirical evidence documents HPA axis dysregulation as associated with the disruption of multiple downstream physiological systems, including cardiovascular, metabolic, and immune systems in adolescents (e.g., Goodman et al., 2005), adults in midlife (e.g., Dowd and Goldman, 2006) and in old age (e.g., Seeman et al., 2002). Our findings suggest that a multiple sampling approach, enabling estimation of stable intrinsic individual differences in cortisol, can potentially enhance the probability of early detection of cardiovascular risk via the HPA axis link.

Given the high participant burden and high cost of repeated venipuncture, this multiple sampling approach is unlikely to be successful when used with traditional biospecimens. Fortunately, the salivary cortisol literature is now very mature – hundreds, if not thousands, of studies since the mid-1980s have sampled saliva repeatedly within and across days, and assayed cortisol. Most often, the measurement strategy employed in these studies has focused on acute reactivity to discrete events (e.g., Mrug et al., 2016), estimating the cortisol awakening response (e.g., Clow et al., 2010), the diurnal slope of cortisol production across the day (e.g., Lumeng et al., 2014), the total cortisol output across the day (e.g., Pruessner et al., 2003), or ecological momentary assessments (e.g., Adam, 2006). An important question is whether retrospective application of LST modeling to this literature has the potential to reveal relationships with individual differences in the reactivity and regulation of the HPA axis that, to date, have been overlooked.

One limitation of the study is that home saliva sampling was undertaken without a method of monitoring sample timing adherence (i.e., Stalder et al., 2016). As such, it is possible that time adherence plays a less significant role when cortisol is indexed by LTC, as LTC captures the stable, trait-like property of cortisol across repeated measures over discrete points in time (Eid et al., 1994; Stroud et al., 2016). Methodologically, structural equation modeling allowed errors in the measured variables, such as noncompliance, to be partitioned out from the latent variables (Steyer and Schmitt, 1990). Accordingly, in theory the implications of sample timing adherence for LTC on the results should be minimal. Given recent concerns by Stalder et al. (2016), LTC may be worth considering when investigators are studying research participants for whom sample timing adherence is likely to be problematic. A second limitation involves the degree of missingness in the biomarker data. As described earlier, the analyses were conducted in *M*plus 7 by using FIML for missing data handling. This allows for the reduction of biases in parameter estimates and standard errors by "borrowing" information from existing data to infer missing data, in order to provide robust findings in the face of missing data (Enders, 2010; Enders and Bandalos, 2001; Graham, 2003; Muthén and Muthén, 1998-2012; Schlomer et al., 2010).

Traditional measurement strategies for estimating cortisol values integrated over time include urine and hair cortisol. Typically, urine is collected over a 24 h period, and measured cortisol is normalized for creatinine, yielding a total cortisol output index. To the best of our knowledge, no available data addresses the consistency of 24 h urine cortisol across days, weeks, months, or years. Hence, the variance in each 24 h urine sample that captures stable individual differences in HPA axis activity is largely unknown. Recently, Meyer and Novak (2012) advanced the possibility of extrapolating measurement protocols designed for the assay of cortisol in the hair of non-human primates to human hair. Assuming hair grows at a constant rate, the length of harvested hair provides an estimate of the time interval over which cortisol was excreted in the body (e.g. weeks, months). The assay of cortisol from hair rests on the assumption that average rate of hair growth is 1 cm/month (Meyer and Novak, 2012). In reality, the variability of hair growth both across

and within individuals over time raises concerns. Comparing differences in the interpretation of cortisol sampled from urine and hair reveals that multiple repeated saliva samplings provides an avenue for indexing stable, trait-like components of cortisol over shorter (e.g. h) and longer (e.g. days, weeks, months) time periods. For instance, Shirtcliff and colleagues (2005) reported that consistency coefficients, which reflect the proportion of stable/trait variance on average across repeated cortisol measures, were 32.5% initially, and 26% at follow up one year later (Shirtcliff et al., 2005). Thus, the coefficients were relatively stable across years. Studies that compare salivary LTC, 24 h urine cortisol, and hair cortisol would seem like a worthwhile next step to provide insight into which measurement strategy best fits specific aims, budgets, and project-specific practical/logistical constraints.

In conclusion, when considered in tandem with recent findings by Chen et al. (2015); Doane et al. (2015); Giesbrecht et al. (2015), and most recently Stroud et al. (2016), the current results strengthen both the internal and external validity of the LTC concept. Links between LTC and cardiovascular risk factors in middle childhood reveal key questions about the origins of individual differences in LTC. One study raises interesting possibilities. Using objective contextual stress interviews with adolescents and their mothers to assess early adversity, Stroud et al. (2016) examined the cumulative impact of nine types of early adversity on adolescent girls' LTC. Greater early adversity was associated with a lower LTC level. It thus may be of more than passing interest that the direction of the effect in the current study was between *lower* LTC and higher lipids, blood pressure, and body composition. Estimating interindividual differences in HPA Axis activity by modeling LTC may provide an innovative opportunity to link the literature on toxic stress (Johnson et al., 2013; Shonkoff, 2012) to physiological changes in developmental trajectories that forecast cardiovascular risk.

Author contribution

H.G.-D., P.V. and E.H. contributed to the design of the study; R.P. and P.V. coordinated data collection; E.W.Y. conducted the statistical analyses; and all authors contributed to writing the manuscript. Some of the work on this project was completed at the Institute for Interdisciplinary Salivary Bioscience Research before it moved from Arizona State University to the University of California at Irvine in the spring of 2016.

Funding source

CHIP was supported by a subcontract from the Children's National Medical Center, Washington, DC that was funded from The Clark Charitable Foundation.

Conflict of interest

In the interest of full disclosure, DAG is Founder and Chief Scientific and Strategy Advisor at Salimetrics and SalivaBio and these relationships are managed by the policies of the committee's on conflict of interest at Johns Hopkins University School of Medicine and the Office of Research Adherence and Integrity at Arizona State University. DAG is in the process of transferring to the University of California, Irvine where similar oversight arrangements will be established. No other author has conflicts to disclose.

Acknowledgements

We thank the participating families and children, Autumn Breutzmann, Natashi Hui and Jimena Pelaez for assisting the completion of the reference list, and Jen Jewell and Judy Quang at Salimetrics for coordinating biotechnical support with salivary assays.

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