Longitudinal Associations between Overweight/Obesity and Stress Biology in Low-Income Children

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Running Title: Overweight/Obesity and Stress Biology

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Competing Interests statement: The authors have no competing financial interests in relation to this work.

Abstract

Background/Objectives: Associations between overweight and altered stress biology have been reported cross-sectionally during childhood, but it is unclear whether overweight precedes altered stress biology or if altered stress biology predicts greater likelihood of overweight over time. The current longitudinal study investigates associations between overweight/obesity, salivary alpha amylase and cortisol morning intercept, diurnal slope, and reactivity to social stress in a cohort of low-income children during preschool and middle childhood. Subjects/Methods: Children were recruited through Head Start and were observed and followed into middle childhood (N = 257; *M* = 8.0 years). Height and weight were measured at both time points. Saliva samples were collected across the day and in response to a social challenge at both ages for alpha amylase and cortisol determination. Results: Cross-lagged panel analyses indicated that overweight/obesity at preschool predicted lower morning alpha amylase (β = -0.18, 95% CI: -0.34, -0.03; p = .023), lower morning cortisol (β = -0.22, 95% CI: -0.38, -0.06; p = .006), lower sAA diurnal slope (β = -0.18, 95% CI: -0.34, -0.03; p = .021), and lower cortisol stress reactivity (β = -0.19, 95% CI: -0.35, -0.02; p = .031) in middle childhood. Lower alpha amylase reactivity at preschool was the only biological factor that predicted higher likelihood of overweight/obesity at middle childhood (β = -0.20, 95% CI: -0.38, -0.01; p = .035). Conclusions: These findings suggest that overweight/obesity may be driving changes in stress biology across early to middle childhood, particularly in down-regulation of morning levels of stress hormones, diurnal sAA slope, and cortisol reactivity to stress, rather than stress biology driving overweight/obesity.

Keywords: Early childhood, middle childhood, cortisol, alpha amylase, overweight, BMI

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Childhood and adolescent obesity rates have been increasing in the past three decades (1). In developed countries, over one in five children have overweight or obesity, and rates have also been increasing in developing countries (1). In the United States, children living in poverty are more likely to have overweight or obesity than children from higher socioeconomic groups (2). Overweight and obesity in childhood and adolescence are strong predictors of obesity in adulthood, so it is important to understand childhood factors that contribute to overweight and obesity in order to create early prevention and treatment interventions. It is likely that a combination of behavioral, biological, and environmental factors, and interactions between these factors, are involved in the increase in obesity over time, particularly for children living in poverty.

Associations between overweight/obesity and stress biology have been demonstrated in both children and adults, with overweight/obesity associated with disruptions in the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). Typical functioning of the HPA axis involves a cascade of sequential release of corticotropin-releasing hormone (CRH) from the hypothalamus, adrenocorticotropic hormone (ACTH) from the pituitary, and the glucocorticoid cortisol from the adrenal cortex. The HPA axis is involved in the regulation of metabolism, responses to challenge, and a host of other processes critical to homeostasis. Altered HPA axis regulation has been implicated in the development of overweight and obesity. In mammals, glucocorticoids maintain and enhance energy stores (3), and in humans, too, cortisol plays a central role in regulating food intake and metabolism (4). Obesity

also impacts cortisol regulation, as adipose tissue can generate cortisol, and there are interactions between adipose tissue and the HPA axis (5). Given this extensive cross-talk between systems coordinating stress responses, food intake, and metabolism (3), the dynamic patterns between cortisol regulation and obesity over time need to be investigated. Two functional measures of HPA biology, cortisol reactivity to stress and diurnal cortisol secretion, reflect the ability of the HPA axis to regulate responses to stress and to modulate circadian rhythm. These measures have been associated with overweight/obesity and will be assessed in the current study.

Associations between overweight/obesity and diurnal cortisol in children and adolescents have been mixed (6-13), and nearly all have been derived from cross-sectional studies, precluding an understanding of how associations between overweight and diurnal cortisol may be unfolding over time. One longitudinal study in adolescents provides evidence that a blunted diurnal cortisol pattern is associated with higher concurrent body mass index (BMI) and increasing BMI over time (14). In children with overweight and obesity, lower cortisol in the early and late morning and the evening have been observed compared to children with normal weight (8). Hypocortisolism has been associated with overweight for girls directly and mediated by reduced satiety responsiveness, and for boys, the association is mediated through emotional overeating (9). In girls aged 8-13 years, heightened cortisol reactivity is associated with higher BMI (15). Likewise, cross-sectional evidence suggests that for older children (8-9 years), higher cortisol reactivity is associated with higher BMI, but this association is not present in younger children (5-7 years) (16). However, there is also evidence in preschool children that a blunted cortisol response to stress is associated with a higher BMI (17).

Associations between body mass index and SNS activity have frequently been reported (18-21). The SNS promotes secretion of norephinephrine in response to stress, which leads to

increases in the enzyme salivary alpha amylase (sAA) (22); therefore, sAA has been used as a biomarker of SNS activity (23). sAA shows a diurnal pattern, with a rise across the day (24). There is evidence that chronic stress down-regulates the system, with children who have experienced chronic stress showing lower basal sAA patterns (25). Low SNS activity has been associated with low resting metabolic rate (18, 19), and medications that increase SNS activity have been demonstrated to reduce food intake (20). However, low SNS-obesity associations may be tissue-specific since high SNS activity may be more likely to promote pathogenesis in certain tissues such as the heart or blood vessels (e.g., hypertension) (26). These findings suggest that low SNS activity could be a risk factor for overweight and obesity, which could be exacerbated in children experiencing chronic stress. Alternatively, overweight could lead to greater SNS disruptions over time. For example, a higher BMI z-score at 2.5 years predicted lower cardiac reactivity to stress at age 5 years, showing a blunting of SNS reactivity over time (27). Overall, associations between basal SNS activity and overweight/obesity in children have been mixed (21, 28-31). In the studies using sAA as the marker of SNS activity, sAA output across the day was lower in school-aged girls with obesity than their normal weight counterparts (32). Lower morning sAA, a higher rise in sAA across the day, and blunted sAA reactivity were associated with increased BMI z-scores in low-income preschool-aged children (33). In one longitudinal study examining cortisol, sAA, and overweight/obesity in toddlers, lower morning sAA and higher sAA slope across the day at 27 months predicted a greater likelihood of overweight at 33 months for girls. For boys, overweight at 21 months predicted lower morning sAA at 27 months (34).

As most prior work has been cross-sectional, little is known about the directionality of associations between overweight and stress biology, particularly in children. Low socioeconomic

status and higher levels of stress are predictors of higher BMI; thus, investigating these associations longitudinally in low-income, highly stressed populations is a high priority for creating interventions that promote healthy weight and adaptive regulation of stress biology. The current study investigates longitudinal associations between overweight/obesity, cortisol, and sAA in low-income children, who are at higher risk for overweight/obesity, from preschool through middle childhood. Establishing whether overweight/obesity or stress biology—or both—drive changes in biology and weight status is essential for identifying developmental windows for prevention and intervention efforts that can address the child overweight and obesity epidemic. Our hypotheses were that cortisol and sAA that were lower in the morning and showed lower reactivity to stress would predict later overweight/obesity. We predicted that a blunted diurnal cortisol slope and a higher diurnal sAA slope would predict later overweight/obesity. These hypotheses were part of a secondary data analysis rather than primary hypotheses for the original data collection.

Methods

Participants

The current study uses data from the ABC Preschool and Kids cohort (9, 17, 33). Children and their parent(s) were recruited in preschool through Head Start, a federally-funded program for children from low-income backgrounds in the United States. A form was sent home to recruit children and their primary caregiver (92.6% mothers) for the study. Parents who returned the form and provided their contact information were compensated with $10. Parents were contacted to confirm eligibility and interest in participation. Exclusion criteria included: child or parent did not speak English; primary caregiver had a 4-year college degree or greater (to target a low-income sample); child was in foster care; child had medical problems, food

allergies, or perinatal complications; and gestational age < 35 weeks. Children were retained for the current analyses if they had valid data for cortisol and sAA reactivity or diurnal regulation at middle childhood. Informed consent was obtained, and the university's institutional review board approved this study.

Procedure

Children and parents participated in three assessments: two during preschool age (1st assessment age 2.9-5.2 years, N = 380; 2nd assessment age 3.2-7.1 years, N = 330) and one during middle childhood (age 7.0-10.2 years, N = 257). At the first preschool assessment, parents completed questionnaires on demographics and income, and children's height and weight were assessed. Diurnal salivary samples were collected from children at preschool for cortisol and alpha-amylase assessment 3 times per day for 3 days (morning, noon, late afternoon). At the second preschool assessment, five saliva samples were collected from the child in response to a social stressor for cortisol and alpha amylase, and children's height and weight were assessed. At the middle childhood assessment, parents completed questionnaires on demographics and income, and children's height and weight were assessed. Diurnal salivary samples were collected by parents at home 3 times per day for 3 days (morning, late afternoon, bedtime). MEMS caps were used for home data collection by parents to ensure timely home collection (92% accuracy within 15 minutes). Research assistants collected five saliva samples for sAA and cortisol determination in response to a social stressor. Details on saliva collection and stress tasks are in Supplement Sections 1.1-1.4. Trained research assistants measured child weight and height without shoes or heavy clothing at all three assessments according to standard protocols (35) (details in Supplement Section 1.5). Overweight/obesity was defined as ≥85th percentile for BMI

based on US Centers for Disease Control and Prevention growth charts for age and sex at each assessment (36).

Assays

Saliva samples were stored at −20° C until processing. Saliva samples were then thawed, vortexed, centrifuged for 15 minutes at 3000 rpm, separated from debris, and placed in Thermo Scientific Matrix Racks at -80° C. The same technician conducted all assays within each assessment using the same equipment following manufacturer's instructions. An Expanded Range High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (Catalog No. 1-3002, 96-Well Kit, Salimetrics LLC, PA, USA) with a detection limit of 0.007μg/dL was used to assay cortisol. At the first preschool assessment, the intra and inter-assay coefficients of variation (CV) were 7%. The sensitivity of the assays was 0.003 μg/dL. At the second preschool assessment, the intra and inter-assay CVs were 4.6% and 5.5%, respectively. At the middle childhood assessment, the average inter-assay CV was 4.0% and the intra-assay CVs were from 0.8-6.1%. Free cortisol is reported in μg/dL.

For alpha amylase, samples were assayed in duplicate with an alpha amylase kinetic reaction assay kit (Catalog No. 1-1902, 96-Well Kit, Salimetrics LLC, PA, USA). This assay uses a chromagenic substrate, 2-chloro-pnitrophenol linked with maltotriose, and the enzymatic action of alpha-amylase on this substrate produces 2-chloro-p-nitrophenol, which is measured spectrophotometrically 2 minutes after the reaction start time with a calibrated plate reader at 405 nm wavelength. The amount of alpha amylase activity is directly proportional to the increase in absorbance at 405 nm. Low, medium and high sAA controls were present in each assay. At the preschool assessments, intra-assay CVs were <6.5%, and the inter-assay CVs were <4.8%. At middle childhood, sAA intra-assay CVs averaged 4.8% and inter-assay CVs averaged 5.0%. The

sensitivity (0.01 units) is determined by the lower change in absorbance reading in each assay. Any sample below the low alpha amylase control was assayed again using a dilution recommended by the manufacturer to achieve a higher concentration and a greater absorbance reading. sAA is reported in enzyme units per milliliter (U/ml).

Data analytic plan

Cross-lagged panel analysis. Three cross-lagged models were fit using Mplus 7.1.4 to assess temporal associations between overweight/obesity, sAA, and cortisol at preschool and middle childhood assessments. All three models used overweight/obesity vs. normal weight (categorical variable) at both time points. The cortisol and sAA parameters used at both time points varied in each of the models. The first model used cortisol and sAA morning intercepts (standardized), the second used cortisol and sAA diurnal slopes (standardized), and the third used cortisol and sAA reactivity (AUCi). See Supplemental Information Section 2 for intercept, slope, and reactivity calculations. For the first and second models (diurnal cortisol and sAA), the preschool data was from the first preschool assessment. For the third model (cortisol and sAA reactivity), the preschool data was from the second preschool assessment. All of the variables in the model were observed. All pathways between Time 1 and Time 2 overweight/obesity, cortisol, and sAA were estimated simultaneously, which allows for more complex models than assessing multiple linear regressions. Covariates included age, sex, race/ethnicity, parent-reported birthweight, sleep quality, and medication use theorized to affect cortisol/sAA at preschool and middle childhood (see Supplement Sections 1.6-1.9). Pubertal development was included as a covariate for the middle childhood variables. Model fit was assessed using recommended guidelines in the field (37, 38), including the comparative fit index (CFI; > .90) and the root mean square error of approximation (RMSEA; .05 denotes good fit, .08 denotes adequate fit).

Results

Descriptive statistics. A total of 257 children had salivary cortisol and sAA data available at the preschool and middle childhood assessments (see Table 1 for demographics). Children in the current study did not differ from children who participated in earlier waves of the study or from those who did not provide cortisol at the middle childhood assessment as a function of the following T1 variables: age, sex, BMIz, income-to-needs ratio, primary caregiver education level, or race/ethnicity (all *p*s > .05). Due to missing data, 230 children were included in the diurnal cortisol/sAA analyses, and 219 were included in the cortisol/sAA reactivity analyses. Age was recorded at the first preschool assessment (M = 4.3 years, SD = 0.5), the second preschool assessment (M = 4.9 years, SD = 0.7), and the middle childhood assessment (M = 8.0 years, SD = 0.7). At the first preschool assessment, 39.7% of the sample was classified as having overweight/obesity, 40.9% as having overweight/obesity at the second preschool assessment, and 48.0% as having overweight/obesity in middle childhood. The income-to-needs ratio in preschool was 0.85 (SD = 0.68), indicating that children were generally living in low-income households.

Model fit indices. All three models demonstrated good fit according to the CFI. The intercept model had an RMSEA of 0.085, which demonstrates adequate fit, while the other two models demonstrated good fit with the RMSEA criteria.

Cortisol/sAA morning intercept and overweight/obesity. Overweight/obesity at preschool (first assessment) predicted a lower sAA morning intercept (β = -0.18, 95% CI: -0.34, -0.03; Table 2; Figure 1a), lower cortisol morning intercept (β = -0.22, 95% CI: -0.38, -0.06), and greater likelihood of overweight/obesity in middle childhood (β = 0.85, 95% CI: 0.75, 0.95). Higher sAA morning intercept at preschool predicted a higher sAA morning intercept at middle

childhood (β = 0.64, 95% CI: 0.57, 0.71). There was a significant within-time association between overweight/obesity and lower cortisol morning intercept in middle childhood (β = -0.25, 95% CI: -0.47, -0.03). The model explained 78.2% of the variance in overweight/obesity, 50.2% of the variance in sAA morning intercept, and 10.6% of the variance in cortisol morning intercept at middle childhood.

Cortisol/sAA diurnal slope and overweight/obesity. Overweight/obesity at preschool predicted a more blunted increase in sAA across the day (β = -0.18, 95% CI: -0.34, -0.03; Table 3; Figure 1b) and greater likelihood of overweight/obesity at middle childhood (β = 0.85, 95% CI: 0.75, 0.95). A blunted cortisol slope at preschool predicted a steeper cortisol slope at middle childhood (β = -0.21, 95% CI: -0.32, -0.10). Overweight/obesity at middle childhood predicted within-time associations with a blunted rise in sAA across the day (β = -0.32, 95% CI: -0.57, -0.06) and a more negative cortisol slope across the day (β = -0.40, 95% CI: -0.62, -0.17). The model explained 78.0% of the variance in overweight/obesity, 6.8% of the variance in sAA slope, and 13.8% of the variance in cortisol slope at middle childhood.

Cortisol/sAA reactivity and overweight/obesity. Overweight/obesity at preschool predicted more blunted cortisol reactivity (β = -0.19, 95% CI: -0.35, -0.02) and greater likelihood of overweight/obesity at middle childhood (β = 0.86, 95% CI: 0.77, 0.96; Table 4; Figure 1c). More blunted sAA reactivity at preschool predicted a greater likelihood of overweight/obesity in middle childhood (β = -0.20, 95% CI: -0.38, -0.01). The association between higher sAA reactivity at preschool and higher sAA reactivity in middle childhood was not statistically significant at p = 0.08 (β = 0.12, 95% CI: -0.01, 0.25). There were no within-time associations between overweight/obesity and sAA or cortisol reactivity at preschool or middle childhood. The

model explained 82.5% of the variance in overweight/obesity, 7.4% of the variance in sAA reactivity, and 13.2% of the variance in cortisol reactivity at middle childhood.

Sensitivity analyses. We conducted sensitivity analyses removing any participants who regularly take medications known to affect cortisol or sAA regulation and the results did not change (see supplement).

Discussion

The current study was the first to examine longitudinal associations between cortisol, sAA, and overweight/obesity across the preschool and middle childhood years, providing information about the directionality of observed associations between overweight/obesity and stress biology during childhood. Overall, analyses suggested that overweight/obesity predicted greater changes in stress biology over time, from early to middle childhood, rather than stress biology predicting increased likelihood of overweight/obesity over this time period. Specifically, overweight/obesity in preschool predicted future lower morning levels of cortisol and sAA, blunted cortisol reactivity, and a lower sAA slope across the day in middle childhood. However, the exception was that blunted sAA reactivity to stress in preschool predicted higher likelihood of overweight/obesity in middle childhood.

There are well-established associations between fat accumulation and cortisol regulation, which are consistent with findings in the current analyses. The literature supports that high cortisol levels and long-term HPA axis activation promote the accumulation of visceral fat over time (39). Elevated cortisol increases appetite and disrupts the regulation of energy balance (4). Greater cortisol secretion in adults with central obesity has been consistently noted (40). Conversely, a blunted diurnal cortisol pattern and low morning and evening cortisol levels have also been linked to higher BMI (8, 14). Thus, there are likely complex, bidirectional associations

that increase likelihood of fat accumulation and cortisol dysregulation over time. In the current study, overweight/obesity at preschool predicted lower morning cortisol at middle childhood, suggesting down-regulation of the HPA axis with excess adipose tissue. This finding is consistent with research in adults reporting abdominal fat is associated with lower morning cortisol, suggesting down-regulation of the HPA axis in response to the negative feedback resulting from high cortisol levels that can be secreted from fat tissue (41, 42). Chronically high levels of cortisol act on upstream mediators of the HPA axis (e.g., CRF, ACTH) to adaptively down-regulate the basal system to prevent the effects of chronic HPA activation (43), thus resulting in low morning cortisol levels. As heightened levels of morning cortisol are needed to mobilize energy resources, children with low morning cortisol levels may lack the resources necessary to behaviorally and biologically adapt to daily challenges (44). Low cortisol levels have been associated with overweight in children (8, 9) and the process leading to low cortisol could also increase vulnerability to certain health disorders (45).

In preschoolers, blunted cortisol reactivity has been observed in children with higher BMI z-scores (17); current findings suggest that overweight/obesity in preschool predicts a more blunted cortisol response to stress in middle childhood. Higher levels of adipose tissue could lead to or exacerbate metabolic problems that increase risk for cardiovascular disease and metabolic syndrome (42). Blunted cortisol reactivity may be a marker of risk in this low-income sample, particularly because blunted cortisol reactivity has been associated with social and emotional problems in high-risk children (46). Low morning cortisol levels and blunted cortisol reactivity could predispose vulnerable children to emotional or behavioral problems and may contribute to higher rates of these problems in children with overweight/obesity (47). There are individual differences in cortisol and sAA regulation due to genetics and epigenetics (48, 49) as well as

genetic and epigenetic differences in risk for overweight/obesity (50). Certain genetic or epigenetic profiles could have significant effects on pathways from stress biology to overweight/obesity or from overweight/obesity to stress biology. Although genetic or epigenetic factors were not examined in the current study, this is an important area for future research.

The current study provides additional evidence that low sAA activity predicts and is predicted by overweight/obesity in children. This finding addresses one of the most important avenues for research in SNS activation and overweight/obesity by assessing whether high or low sAA activity predicts greater likelihood of overweight/obesity over time (18). However, it must be noted that SNS activation may be higher or lower in individuals with overweight or obesity depending on the region of the body measured (18) and the type of measurement (e.g., hypertension in individuals with obesity). The finding of lower morning sAA and lower sAA slope across the day in middle childhood following overweight/obesity in preschool could reflect a down-regulation of the SNS as lower sAA levels have been associated with chronic stress (25), which could have implications for future behavior and physical health. Attenuated morning SNS activity and lower sAA diurnal slope could be due to down-regulation from chronically high levels of SNS activity, similar to down-regulation in the HPA axis following high HPA activity, which may reflect a failure to adequately prepare for daily challenges in the context of chronic stress, such as the stress of living in poverty for low-income children (51). This finding is consistent with a longitudinal study in toddlers finding that overweight at 21 months predicted lower morning sAA at 27 months, although that finding was specific to boys (34). Blunting of morning sAA levels in middle childhood following overweight/obesity in preschool, particularly in the context of poverty, could increase the likelihood of maladaptive biological and behavioral responses to stress in the future, which could influence future health through a number of

pathways. The finding that overweight/obesity predicted lower diurnal sAA slope was not consistent with evidence in young children that higher sAA slope is concurrently associated with and predictive of higher BMI (33, 34). Further research is needed to understand whether age is an important moderator of these associations.

The only biomarker that predicted greater likelihood of overweight/obesity in middle childhood was blunted sAA reactivity to stress in preschool. This is consistent with evidence that low SNS activity is associated with low resting metabolic rate (18, 19) and greater food intake (20). If children with blunted sAA reactivity have a lower resting metabolic rate and greater food intake over time, without offsetting this intake with physical activity, they may be more likely to develop overweight/obesity. Previous work has shown that higher sAA is associated with satiety (52), suggesting that lower sAA levels could be associated with greater hunger and less satiety, which could lead to excessive food intake. Low SNS activity has also been associated with obesity in children (30). However, the current findings are inconsistent with some work reporting heightened SNS activity in individuals with obesity (21, 28, 29, 53, 54). Most studies did not focus on SNS reactivity, however, and were conducted cross-sectionally with older children or adults. As this is the first study to examine these pathways longitudinally into middle childhood, and sAA is an indirect marker of SNS activity, this association needs to be replicated in other samples. These associations may be specific to certain individuals with a positive energy balance (18), so we need to understand whether there are genetic, psychobiological, or environmental factors that moderate the association between SNS activation and overweight/obesity in childhood. Our sample is socioeconomically high-risk, so greater experiences of psychosocial stress or exposure to obesogenic environments likely influenced overweight/obesity and stress biology compared to low-risk populations. These findings also may not generalize to

developmental periods outside early-to-middle childhood. It will be important to understand whether other aspects of SNS and HPA activity, such as chronic integrated cortisol measured in hair, are associated with overweight/obesity in a similar manner over time. Measures in different tissues address unique aspects of stress regulation and may show different associations with adiposity across development.

There were limitations to the current study. The stress reactivity task in preschool differed from the stress reactivity task in middle childhood. As preschool and middle childhood are very different developmental periods, the social stress tasks were designed to include a strong, developmentally appropriate social-evaluative component known to elicit stress responses at each age tested. Future work is needed to establish social stressors that are effective and similar across childhood. Timing of the diurnal saliva samples also differed between waves, with the preschool samples occurring between 8:30am and 4:30pm, and the middle childhood samples typically between 8am and 9pm. Our analytic strategy accounted for the timing of the samples when calculating the diurnal intercept and slope of sAA, but differences in methodology could still partially contribute to the results. Pubertal development was reported by parents, and thus may be biased or inaccurate compared to a medical exam. We did not measure physical activity as a potential covariate. We also used only BMI z-score as our measure of adiposity, and future research including additional measures of adipose tissue is needed. Finally, the study was limited to a low-income population in the rural Midwest, so it may not generalize to all children. We also did not include non-English speaking families in the study, so results will need to be replicated in non-English speaking populations. The current study did not adjust for multiple comparisons due to the nature of the pre-specified comparisons in the model (55), though future studies are needed to replicate the current findings.

Conclusions

The current study suggests that disruptions in stress biology, particularly down-regulation of morning levels of stress-mediating hormones, cortisol reactivity to stress, and lower diurnal sAA slope are more likely to follow overweight/obesity in children rather than precede overweight/obesity. A blunted sAA stress response at preschool was the only biological predictor of overweight/obesity in middle childhood. Importantly, these associations were reported in low-income children, a population with an outsized burden of the obesity epidemic. This prospective longitudinal study is the first to map associations between overweight/obesity and stress biology from preschool to middle childhood, providing insight into the directionality of observed associations and the course of overweight/obesity and disruptions in stress biology. Future research is needed to understand the mechanisms between these associations to improve prevention and intervention efforts that aim to enhance child health.

Acknowledgements: This research was supported by funding from F32HD088029 (PI: Doom), NICHD/NIDDK R01 DK095695 (PI: Miller and Lumeng), NIDDK R21DK090718 (PI: Miller and Lumeng), American Heart Association 10GRNT4460043 (PI: Miller), and NIDDK RC1DK086376 (PI: Lumeng).

Conflicts of Interest: The authors have no conflicts of interest to disclose.

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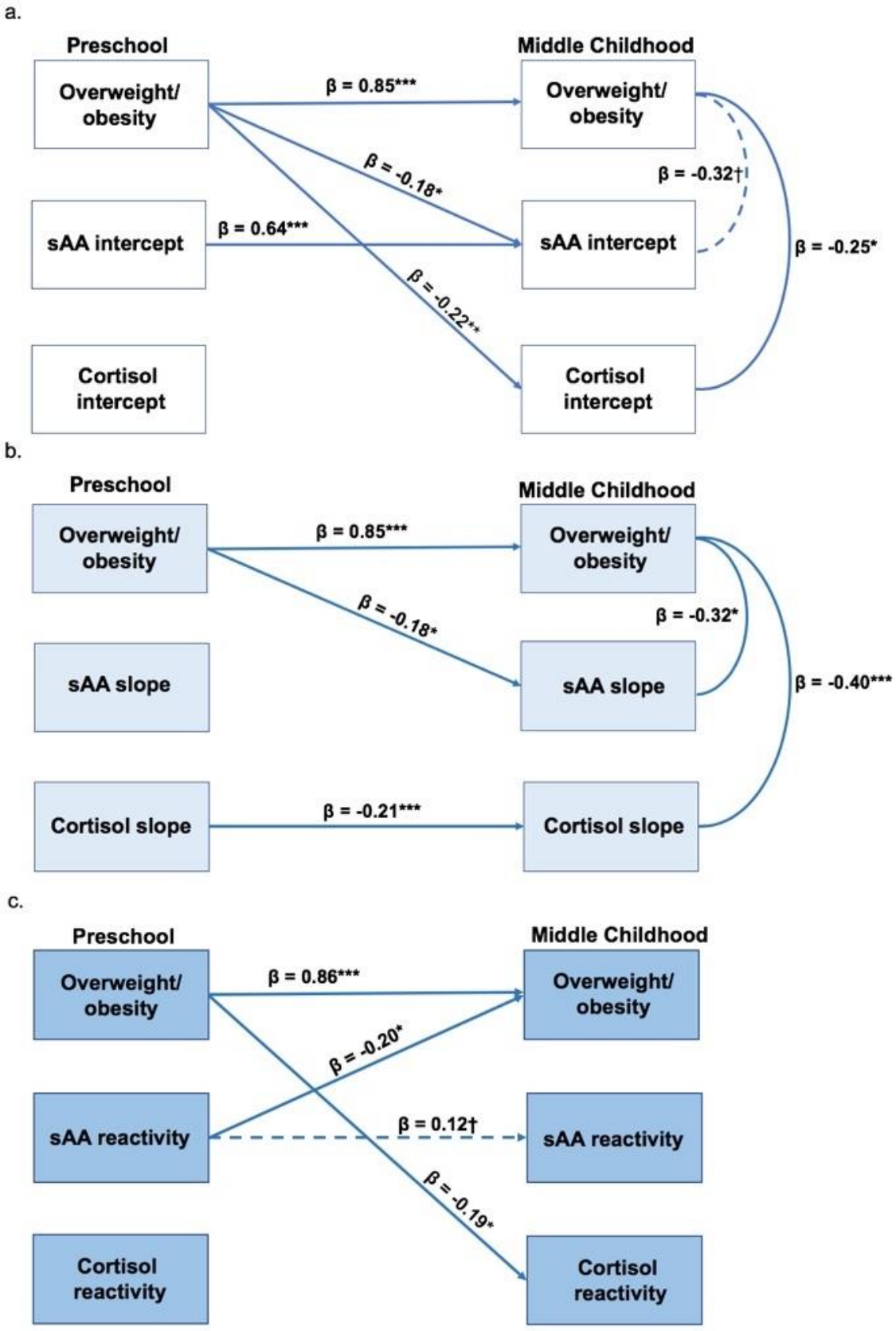
a.
Preschool
Middle Childhood
Overweight/ obesity
sAA intercept
Cortisol intercept
Overweight/ obesity
sAA intercept
Cortisol intercept
β = 0.85***
β = -0.18*
β = 0.64***
β = -0.22**
β = -0.32†
β = -0.25*
b.
Preschool
Middle Childhood
Overweight/ obesity
sAA slope
Cortisol slope
Overweight/ obesity
sAA slope
Cortisol slope
β = 0.85***
β = -0.18*
β = -0.21***
β = -0.32*
β = -0.40***
c.
Preschool
Middle Childhood
Overweight/ obesity
sAA reactivity
Cortisol reactivity
Overweight/ obesity
sAA reactivity
Cortisol reactivity
β = 0.86***
β = -0.20*
β = 0.12†
β = -0.19*

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Figure 1. Model tested for associations between overweight/obesity and 2a) sAA and cortisol intercept, 2b) sAA and cortisol diurnal slope, and 2c) sAA and cortisol reactivity. β values are standardized estimates. Statistical significance indicated by †$p < 0.10$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. During preschool, sAA and cortisol morning intercept and diurnal slope were measured at the first assessment, and sAA and cortisol reactivity were measured at the second assessment.

Table 1. Participant Characteristics.

	M	*SD*	%
Preschool: Age First Assessment (years)	4.25	0.52	
Preschool: Age Second Assessment (years)	4.88	0.70	
Middle Childhood: Age (years)	7.95	0.71	
Time Between Preschool Assessments (months)	7.46	5.35	
Time Between First Preschool and Middle Childhood Assessments (months)	44.40	7.89	
Female			49.0
Middle Childhood Pubertal Status	1.47	0.59	
Child Race/Ethnicity			
Non-Hispanic White			52.9
African American			16.7
Hispanic/Latino			10.1
American Indian			0.4
Asian/Pacific Islander			0.8
Multiracial			19.1
Parent Education			
Did Not Graduate High School			17.5
High School Degree or GED			30.0
Some College Courses			40.1
2-year College Degree			12.5
Preschool Income-to-Needs Ratio	0.85	0.68	
Middle Childhood Income-to-Needs Ratio	1.11	0.75	
BMIz at Preschool (First Assessment)	0.83	1.09	
Overweight/Obesity at Preschool (First Assessment)			39.7
BMIz at Preschool (Second Assessment)	0.86	1.09	
Overweight/Obesity at Preschool (Second Assessment)			40.9
BMIz at Middle Childhood	0.97	0.99	
Overweight/Obesity at Middle Childhood			47.9

Note. Means, standard deviations, and percentages of participants' demographic information and key variables. N = 257. T1 = preschool assessment, T2 = middle childhood assessment. GED = General Educational Development Test (high school equivalency test in the United States). Percentages are calculated for all participants with valid data on that measure.

Table 2. Overweight/obesity, sAA and cortisol morning intercept cross-lagged analysis.

	β	95% CI	*p*-value
Within-time paths			
T1 Overweight/obesity→T1 sAA intercept	-0.09	-0.27, 0.09	.31
T1 Overweight/obesity→T1 Cortisol intercept	-0.06	-0.23, 0.12	.53
T1 Cortisol intercept → T1 sAA intercept	0.08	-0.07, 0.23	.28
T2 Overweight/obesity→ T2 sAA intercept	-0.32	-0.67, 0.03	.069†
T2 Overweight/obesity→T2 Cortisol intercept	-0.25	-0.47, -0.03	.029*
T2 Cortisol intercept →T2 sAA intercept	0.08	-0.11, 0.27	.41
Autoregressive paths			
T1 Overweight/obesity→ T2 Overweight/obesity	0.85	0.75, 0.95	<.001***
T1 sAA intercept → T2 sAA intercept	0.64	0.57, 0.71	<.001***
T1 Cortisol intercept → T2 Cortisol intercept	-0.05	-0.19, 0.10	.53
Cross-lagged paths			
T1 Overweight/obesity→ T2 sAA intercept	-0.18	-0.34, -0.03	.023*
T1 Overweight/obesity→ T2 Cortisol intercept	-0.22	-0.38, -0.06	.006**
T1 sAA intercept → T2 Overweight/obesity	0.01	-0.11, 0.13	.88
T1 sAA intercept → T2 Cortisol intercept	-0.01	-0.14, 0.11	.83
T1 Cortisol intercept → T2 Overweight/obesity	0.06	-0.07, 0.19	.38
T1 Cortisol intercept → T2 sAA intercept	0.00	-0.11, 0.11	.99

Statistical significance indicated by †$p < 0.10$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. T1 = preschool (first assessment), T2 = middle childhood assessment.

Table 3. Overweight/obesity, sAA and cortisol slope cross-lagged analysis.

	β	95% CI	*p*-value
Within-time paths			
T1 Overweight/obesity→T1 sAA slope	0.09	-0.08, 0.27	.30
T1 Overweight/obesity→T1 Cortisol slope	-0.05	-0.21, 0.12	.57
T1 Cortisol slope → T1 sAA slope	0.05	-0.09, 0.18	.52
T2 Overweight/obesity→ T2 sAA slope	-0.32	-0.57, -0.06	.016*
T2 Overweight/obesity→T2 Cortisol slope	-0.40	-0.62, -0.17	<.001***
T2 Cortisol slope →T2 sAA slope	0.10	-0.02, 0.22	.10
Autoregressive paths			
T1 Overweight/obesity→ T2 Overweight/obesity	0.85	0.75, 0.95	<.001***
T1 sAA slope → T2 sAA slope	0.00	-0.12, 0.13	.97
T1 Cortisol slope → T2 Cortisol slope	-0.21	-0.32, -0.10	<.001***
Cross-lagged paths			
T1 Overweight/obesity→ T2 sAA slope	-0.18	-0.34, -0.03	.021*
T1 Overweight/obesity→ T2 Cortisol slope	0.00	-0.17, 0.17	>.99
T1 sAA slope → T2 Overweight/obesity	0.01	-0.11, 0.13	.86
T1 sAA slope → T2 Cortisol slope	0.02	-0.09, 0.13	.72
T1 Cortisol slope → T2 Overweight/obesity	0.04	-0.10, 0.17	.58
T1 Cortisol slope → T2 sAA slope	-0.03	-0.15, 0.09	.63

Statistical significance indicated by †$p < 0.10$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. T1 = preschool (first assessment), T2 = middle childhood assessment.

Table 4. Overweight/obesity, sAA and cortisol reactivity cross-lagged analysis.

	β	95% CI	*p*-value
Within-time paths			
T1 Overweight/obesity→T1 sAA reactivity	0.11	-0.05, 0.28	.18
T1 Overweight/obesity→T1 Cortisol reactivity	-0.06	-0.14, 0.02	.12
T1 Cortisol reactivity → T1 sAA reactivity	0.07	-0.06, 0.20	.27
T2 Overweight/obesity→ T2 sAA reactivity	-0.20	-0.70, 0.30	.43
T2 Overweight/obesity→T2 Cortisol reactivity	0.09	-0.31, 0.50	.65
T2 Cortisol reactivity →T2 sAA reactivity	0.06	-0.04, 0.16	.22
Autoregressive paths			
T1 Overweight/obesity→ T2 Overweight/obesity	0.86	0.77, 0.96	<.001***
T1 sAA reactivity → T2 sAA reactivity	0.12	-0.01, 0.25	.080†
T1 Cortisol reactivity → T2 Cortisol reactivity	0.02	-0.39, 0.42	.93
Cross-lagged paths			
T1 Overweight/obesity→ T2 sAA reactivity	0.16	-0.07, 0.38	.17
T1 Overweight/obesity→ T2 Cortisol reactivity	-0.19	-0.35, -0.02	.031*
T1 sAA reactivity → T2 Overweight/obesity	-0.20	-0.38, -0.01	.035*
T1 sAA reactivity → T2 Cortisol reactivity	0.11	-0.03, 0.24	.12
T1 Cortisol reactivity → T2 Overweight/obesity	0.03	-0.02, 0.09	.24
T1 Cortisol reactivity → T2 sAA reactivity	-0.06	-0.30, 0.18	.64

Statistical significance indicated by †$p < 0.10$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. T1 = preschool (second assessment), T2 = middle childhood assessment.

Supplementary Information Text

1. Methods

1.1. Diurnal saliva collection at preschool. RAs collected children's saliva at preschool 3 times per day on 3 consecutive days by having the child passively drool in a tube or chew on a piece of cotton. Samples were collected 1) upon arrival to preschool and before breakfast around 8:30am, 2) before lunch around 11:30am, 3) and at 4:30pm. Daily logs collected information about primary caregiver report of illness, medication use, unusually good or bad events, time of morning awakening and if it was the usual awakening time, eating or napping prior to the sample, and location during the afternoon sample. Children who did not provide enough saliva samples did not differ from those who did as a function of the following variables: sex, BMIz, income-to-needs ratio, primary caregiver education level, or race/ethnicity (all *p*s > .05). Those who did not provide saliva samples were younger than those who did (47.2 vs. 50.8 months), $t(378) = -2.13$, $p = .03$.

1.2. Diurnal saliva collection in middle childhood. Parents collected their child's saliva at home 3 times per day for 3 days. The morning sample was before breakfast and school around 8am, within 30 minutes of waking. The after-school sample was before snack or dinner at around 4pm. The bedtime sample was instructed to be around 8-9pm. The RA would call or text the parent at the scheduled sample time to confirm that the sample was collected and to answer any questions. MEMS caps were used as a check on parent report of saliva time. Parents were 92% accurate at reporting log times within 15 minutes of the actual cap-recorded time and 94% accurate at reporting times within 30 minutes of the cap-recorded time. Parents were told not to let the child eat in the 30-45 minutes prior to collecting the sample, to wait at least 3 hours between samples, and to not collect a sample if the child was sick. The parent was to have the

child rinse his or her mouth and then chew on dental cotton for 1-2 minutes. The parent was to place the sample in the correct color-coded tube, mark the time of sample and the child's last meal, report any medications or sickness, and place the tube in the home freezer. The RA would then pick up the samples, typically within a week of collection.

1.3. Stress reactivity protocol and saliva collection in preschool. Children and their primary caregivers attended a 1pm session for the stress reactivity protocol. There were no other research activities before the start of the stress reactivity protocol, and all sessions were done at the participants' Head Start location. First, the RA brought the child to a room that was separate from the parent and engaged in calming free play together for 20 min. Then the child participated in four challenge tasks. Tasks were designed to produce a mild to moderate stress level in young children, particularly by including a negative social evaluation component, a robust predictor of heightened cortisol reactivity (1, 2). Children rated six prizes ranging from a toy car or doll to a broken comb or deflated ball in order of preference. The RA told the child they could have the most preferred prize later as a gift and then took the prize out of the room.

The first challenge task, Perfect Circles (3), involved the RA asking the child to draw a "perfect circle." The RA would critique each circle the child drew for 3.5 minutes, saying that the circle was not perfect enough and they should keep trying. At the end, the RA told the child the final circle was "pretty good" before moving to the next task. During Puzzles, the second challenge task, the RA told the child to continue to solve a wooden puzzle that contained two incorrect pieces, which made it impossible to solve even though it was age-appropriate. After 3 minutes, the RA told the child, "We're out of time on that one," and removed the puzzle. Then the child was told to solve a puzzle designed for older children, which was not age appropriate because it was too difficult. The RA told the child that time was up after 4 minutes, and no child

correctly solved the puzzle. The RA did not provide help, reassurance, or encouragement, but at the end of the task acknowledged that the puzzles were "hard."

After these tasks, the RA told the child that he or she could have the preferred prize now, but that the RA needed to wrap it first. The third task, Gift Wrap/Wait (4), involved the RA pretending to wrap the gift by crinkling paper behind a screen for 1.5 minutes while the child waited. During the final task, Disappointing Gift (5), the RA presented the child with a box that was supposed to contain the selected gift. Instead, the box contained the child's least preferred prize. The child opened the box, and the RA remained unresponsive for 30 seconds while the child reacted to the gift. After 30 seconds, the RA "realized" the mistake and apologized. Then the RA retrieved the "correct" prize, which the child took home as a gift. The child was given the choice to engage in quiet free play with the RA or watch a children's movie for the next 40 minutes.

Cortisol and sAA reactivity were measured in saliva. Children provided saliva by passively drooling in a tube or chewing on a piece of cotton. Saliva was sampled five times during the protocol: (1) 20 minutes after entering the room, reflecting sAA/cortisol prior to the assessment; (2) 30 minutes after entering the room (10 min into the free play period) prior to beginning the challenge tasks; (3) 10 minutes after receiving the gift; (4) 20 minutes after receiving the gift; and (5) 40 minutes after receiving the gift. These multiple time points were samples to capture individual differences in sAA and cortisol reactivity and recovery (1, 6).

1.4. Stress reactivity protocol and saliva collection in middle childhood. Stress reactivity assessments were conducted in the afternoon, typically between 3-7pm and within a week of the diurnal saliva collection for the middle childhood assessment. The research assistant collected saliva sample #1 by instructing the child to chew on a piece of dental cotton for 1-2

minutes upon entry to a room where the child would have calming free play until 45 minutes post-snack. Saliva sample #2 was collected at 45 minutes post-snack and after calming free play. After sample #2, the child transitioned to the stress task. The stress reactivity task consisted of 10 minutes of academic testing with a strict teacher and 10 minutes of the Trier Social Stress Test for Children (TSST-C; (7). A female RA was the strict teacher, and she was instructed not to give any positive feedback to the child and to use a neutral, but not harsh, tone. The RA who introduced the teacher said that the teacher was very strict and proceeded to act nervous around the teacher. When the teacher walked in the room, she made several slight adjustments to the room setup in order to show that she was picky about rules. If the child did better than the other children tested, the child was told that he or she would earn a prize at the end.

The strict teacher conducted the oral word fluency task and the forward and backward digit span tasks from the Wechsler Individual Achievement Test-Third Edition (WIAT-III, 2009). The teacher then administered an adapted TSST-C story book task. The teacher instructed the child to tell a story about the pictures in the book for 30 seconds each (10 total pictures) and gave an example. The teacher used a timer to signal the beginning and end of the 30 seconds. After the 1st and 5th pictures, the teacher instructed the child to stop and then to speak into the microphone and say more about the next pictures. On the other pages, the teacher would proceed to the next picture after 30 seconds. If the child stopped, the teacher would say, “Keep going,” in a neutral voice for 3 times maximum per task.

After the pictures, the teacher continued to the math portion, reading grade-appropriate questions aloud from the WIAT-III. The child was given a paper and pencil to complete the problems. If the child provided 4 consecutive incorrect answers, the task was discontinued. In order to keep the task uncertain, the child was not given any feedback. If the child did not

717 respond for 30 seconds, he or she was prompted to answer. If the child requested help, the
718 teacher would tell the child that she could not help. Once the task was discontinued, the teacher
719 told the child she was leaving to score the child's answers to see if he or she had won the prize,
720 and then she left the room.

721 The RA re-entered the room two minutes after the teacher left and asked the child to
722 report his or her distress level. Saliva sample #3 was then immediately collected (20-25 minutes
723 after the beginning of the stress task). The RA and teacher debriefed the child, telling him or her
724 that the teacher was trying to practice being strict, and the teacher asked whether the child
725 thought she had done a good job of being strict. The child was given the prize and played calm
726 games with the RA while seated. Sample #4 was collected 15 minutes after the debriefing, and
727 Sample #5 was collected 35-40 minutes after the debriefing. Saliva was sampled several times
728 following the stress reactivity challenge tasks to capture individual differences in biological
729 reactivity and recovery (1, 6).

730 **1.5. Anthropometry.** At the first and second preschool assessments, child weight and
731 height were measured without heavy clothing or shoes in a Head Start private room by research
732 staff. Staff were trained by a pediatrician to reliably measure and weigh children using standard
733 protocols. Weight was measured with a ±0.1 kg Detecto calibrated scale (Detecto Physician's
734 Scale Model DR550). Height was measured with a ±0.1 cm calibrated Seca stadiometer (Seca
735 213/217). Measurements were conducted twice. Third and fourth measurements were conducted
736 if measurements were discrepant [by 0.1 kg (weight) or 0.5 cm (height)]. The mean of the
737 measures was used.

738 At the middle childhood assessment, weight and height were measured by staff using a
739 Detecto scale (calibrated weekly) and a Seca stadiometer. Similar to the preschool assessments,

children were measured twice and two more measurements were taken if these were discrepant. The mean of the two measures was used. Staff were recertified annually in accurate anthropometry.

1.6. Medications. Medication use was reported by parents at each assessment. Each child was assigned a score from 0-2 at each assessment for regular use of a medication with possible effects on cortisol or sAA, even if they did not take the medication on the day of the assessment (9). Children with no medications or medications with no effect on cortisol/sAA were assigned a value of 0. Children taking medications with a possible effect on cortisol/sAA were assigned a 1. Children taking medications that would likely affect cortisol/sAA, were assigned a 2.

1.7. Puberty. At the middle childhood assessment, parents estimated their child's pubertal development based on a visual Tanner staging scale (Morris & Udry, 1980). For females, parents completed the breast and pubic hair ratings, and for males, parents completed the genital and pubic hair ratings. The ratings ranged from 1 (not started developing) to 5 (fully developed). The genital and pubic hair score was used for males, and the average of the breast and pubic hair scores was used for females.

1.8. Sleep quality. At the preschool assessment, parents reported their child's sleep quality using the overall sleep quality scale of the Children's Sleep-Wake Scale (11). At middle childhood, parents reported their child's sleep quality using the total sleep disturbance scale of the Children's Sleep Habits Questionnaire (12).

1.9. Demographics. The parent reported his or her highest level of education at the first preschool assessment as 1) did not finish high school, 2) high school diploma or US high school equivalency test (General Educational Development test; GED), 3) some college courses, or 4) 2-year college degree. The family's income-to-needs ratio at the preschool (1st assessment) and

middle childhood assessments were calculated by parent report of annual pre-tax income from all sources. Families were sorted into one of 18 categories based on their response, from less than $5,000 to more than $200,000. This midpoint of the category dollar amount was divided by the poverty threshold for a same-sized family, which produced the income-to-needs ratio. The parent reported the child's race and ethnicity, which was coded as non-Hispanic white = 0, Hispanic and/or non-white = 1 for analysis. The parent reported child sex (male vs. female), which was included in all models.

2. Data analytic plan

2.1. Diurnal cortisol and alpha amylase data. In preschool, cortisol and sAA values were excluded if (1) the value was >3 SDs from the mean (13), or (2) the value was >2 SDs from the mean and did not fit the child's diurnal pattern or the child had an unusual experience (i.e. reported to be getting sick) (14). Individual cortisol values were excluded if the child took a medication known to affect cortisol (e.g., steroid) on that day; cortisol values for other days without medication use that affects cortisol were retained. For the stress reactivity assessment in preschool, any value >3 SDs was excluded. At this assessment, medications did not impact cortisol or sAA levels and thus values were not excluded for medication use. In middle childhood, any cortisol or alpha amylase value more than 3 standard deviations from the mean of a specific time point was removed (13). Preliminary analyses were conducted to identify covariates associated with either sAA or cortisol. Informed by these analyses, diurnal sAA values were removed if the child used an inhaler that day, and stress reactivity values were removed if the child was not healthy or had a cold/fever/allergic reaction in the past 24 hours due to preliminary analyses showing that these factors were significantly associated with cortisol values. For both time points, all-values were log-transformed to capture the log-linear pattern of

the cortisol and alpha amylase rhythm and ensure normality of the residuals. Calculations for outliers were made within weight status group for each time point separately as it was hypothesized that patterns might differ by weight status. Children with at least five saliva samples across 2 or more days were included to create diurnal curves that closely represented the child's diurnal pattern on greater than one day.

Hierarchical linear modeling (HLM) was used to capture diurnal cortisol and alpha amylase curves for each participant by producing random parameters with the restricted maximum likelihood method (REML) (15, 16). As these trajectories have a known parametric form, HLM is a powerful technique to estimate individual trajectories (17). HLM can account for differential timing of measurement if sampling times are not uniform, which is done by using the parametric function of the diurnal pattern. Even with missing data, HLM is a robust estimation method. Separate models were used to estimate the cortisol and alpha amylase trajectories. Using parent-reported minutes since awakening as the independent variable and log-transformed cortisol or alpha amylase as the outcomes, the diurnal patterns obtained for cortisol and alpha amylase are linear on time in a log-scale (for time $\geq$ 60 min), and the resulting pattern is captured by the intercept and slope of the derived line. The random intercept generated is an estimate of the 60 min post-awakening cortisol or alpha amylase level for the individual. For cortisol, the random slope generated is the expected rate of cortisol decay from 60 min post-awakening through the end of the day. For alpha amylase, the random slope would represent the expected rate of increase in sAA after 60 minutes post-awakening as sAA typically rises over the course of the day after 30 minutes post-awakening (18).

As each child provided samples for three days, each cortisol or alpha amylase measurement on each day was included in the model, including the corresponding time since

awakening for that day and sample time. Each child's expected cortisol pattern over the three days was estimated with random effect parameters, providing a single predicted intercept and slope for each child using data from all three days. The random cortisol and alpha amylase intercepts and slopes in both preschool and middle childhood were used as individual-level variables for the analyses. At the middle childhood assessment, preliminary analyses indicated that cortisol and sAA values were sensitive to whether the child ate before the sample. Thus, whether the child ate before each of the samples was controlled for in the HLM model for middle childhood cortisol.

2.2. Cortisol and alpha amylase reactivity data. For both the preschool and middle childhood assessments, any saliva sample for which the cortisol or salivary alpha amylase value deviated more than 3 standard deviations from the mean of a specific time point was removed (19). Cortisol and sAA responses to stress were created by calculating the area under the curve (AUCi) using the trapezoidal rule, which reflected the child's cortisol or sAA output increase from baseline. For cortisol, the baseline was the first sample at preschool and the second sample at middle childhood, and for sAA, the baseline was the first sample at both preschool and middle childhood (determined by the highest mean level of increase in cortisol or sAA at that time point). Any samples collected after the baseline sample (through the fifth sample) were used to calculate AUCi for cortisol or sAA at preschool and middle childhood. AUCi is used as an indicator of overall stress response (20). AUCi units were all z-scored for analyses.

3. Results

We conducted additional analyses to be sure that medication use did not change results. We removed any participants who reported medication use that may affect cortisol or sAA even if not taken on the day of the sample. Participants were excluded from these analyses if they had

this type of medication use at either time point. Medication use was also handled statistically when creating the intercept, slope, and reactivity variables at each time point (see Supplement Section 2.1). With these participants excluded, we conducted the same 3 models and found that the paths were all in the same direction with similar magnitude compared to the full sample, leading us to conclude that these findings are not driven by participants taking medications that affect sAA or cortisol.

The overweight/obesity to sAA intercept path had the same direction and similar magnitude (β = -0.16, 95% CI: -0.35, 0.03) as with all participants included (β = -0.18, 95% CI: -0.34, -0.03). Similarly, the overweight/obesity to cortisol intercept path was also in the same direction and had a similar magnitude (β = -0.18, 95% CI: -0.36, 0.007) compared to the full model (β = -0.22, 95% CI: -0.38, -0.06). The overweight/obesity to sAA slope path in the subsample (β = -0.16, 95% CI: -0.34, 0.01) was consistent with the full model (β = -0.18, 95% CI: -0.34, -0.03). The sAA reactivity to overweight/obesity path in the subsample (β = -0.13, 95% CI: -0.30, 0.05) was similar to the full model (β = -0.20, 95% CI: -0.38, -0.01). The overweight/obesity to cortisol reactivity path in the subsample (β = -0.26, 95% CI: -0.46, -0.07) was similar in magnitude and direction to the full model (β = -0.19, 95% CI: -0.35, -0.02).

4. Previous Research

There have been several papers from this cohort that present sAA and cortisol findings (14-16, 21-24), though none examine longitudinal bidirectional associations between overweight/obesity, sAA, and cortisol.

5. Code Availability

Mplus output is available at: https://osf.io/nyk6a/?view_only=3a198e3ddf98456e9c35428ba51d29a8

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