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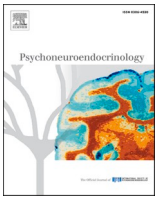

A population-based study of children suggests blunted morning cortisol rhythms are associated with alterations of the systemic inflammatory state

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ABSTRACT

Background: In children, digital media, lifestyle, and the COVID pandemic have impacted sunlight exposure, exercise, and diet patterns - cues that entrain the circadian clock. We hypothesized that low morning cortisol reflects a weak circadian clock, impacting the pro-inflammatory state. The primary objective was to test relationships between diurnal cortisol fluctuations and the inflammatory state in children as a means of providing indirect support for this hypothesis.

Methods: The Cardiovascular Health Intervention Program (CHIP) was a population-based cross-sectional and longitudinal study of circadian health in public elementary school children in Southern Maine, USA (recruitment period 2012–2017). Participants were 689 students in 4th grade (baseline; age=9.2 ± 0.4 years), and 647 students in 5th grade (age=10.5 ± 0.5 years). Nine salivary cortisol measures per child (2 awakening and 1 prior to bed for 3 sequential days) (n = 1336 child phenotype days; n = 7987 cortisol assays), 10 cytokines measured in morning and evening saliva samples (n = 202 child phenotype days), and lipids were measured. Clinical outcomes were blood pressure, weight and height (body mass index [BMI]; BMI = kg/m^2), among others.

Findings: Upon-waking cortisol levels were 0.28 ± 0.13 μg/dL, 30-minute post-waking 0.33 ± 0.15 μg/dL, and evening 0.08 ± 0.10 μg/dL. Salivary cytokine levels (n = 202) showed interleukins (IL) IL-1β and IL-8 were highest in early morning (upon awakening; AM), and IL-6 and tumor necrosis factor (TNF) TNF-α highest before bed (PM) (IL-1β AM > PM [−4.02 fold; $p < 0.001$]; IL-8 AM > PM [−1.36 fold; $p < 0.001$]; IL-6 AM < PM [+1.49 fold; $p < 0.001$]; TNF-α AM < PM [+1.73 fold; $p = 0.03$]. Regression modeling showed high morning cortisol was associated with high morning IL-1β ($p = 3.82 \times 10^{-6}$), but low evening IL-1β ($p = 6.27 \times 10^{-4}$). Regression modeling of BMI z-score as the response variable showed the expected significant relationships to high density lipoprotein (HDL) (negative; $p < 0.001$), mean arterial pressure (positive; $p < 0.001$), and morning cortisol (negative; $p = 0.01$) but only weak relationships to either evening cortisol ($p = 0.1$) or cytokine (positive; $p = 0.02$; from the model with smallest Rsquared) levels.

Interpretation: We provide preliminary data on diurnal fluctuations of inflammatory cytokines in saliva in a population-based cohort of children. Correlation of morning and evening cortisol levels with inflammatory cytokines in the same saliva samples showed that high morning cortisol was associated with high morning IL-1β and low evening IL-1β. Future studies may test the hypothesis that strong diurnal cycling of IL-1β may serve as a homeostatic mechanism keeping the immune system in check, and that low morning cortisol (possible circadian misalignment) may lead to less stringent control of inflammatory networks.

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1. Introduction

Current societal trends have resulted in increased incidence of cardiovascular risk factors and pro-inflammatory disease states at younger ages (Ludwig, 2007). Obesity clearly drives both dyslipidemia, and a more pro-inflammatory state.

Mechanistic relationships between obesity and dyslipidemia are well-studied, and obviously tied to obesity-related changes in metabolism. There are also clear associations between obesity, metabolism and circadian misalignment, where melatonin, glucocorticoids (cortisol), and leptin show circadian-related changes that drive changes in lipid metabolism (Li et al., 2020).

Inflammation in obesity has been best studied in adipose tissue, where both adipocytes and cytokines secrete inflammatory cytokines (Wu and Ballantyne, 2020). In transition from the lean to obese state, macrophages convert from M0 and M2 phenotypes to more pro-inflammatory M1 phenotypes, associated with increased secretion of TNFα, IL-1β, and IL-6, as well as expressing CD11 and MHC-II. While associations between obesity and a pro-inflammatory state are well-established in both humans and animal models, the mechanisms driving the pro-inflammatory state are poorly understood. Most models suggest a complex interplay of increased immune cell recruitment and interactions of immune cells in adipose tissue, including persistent expression of MCP-1, RANTES, fractalkine, KC, and MIP-2α by both adipocytes and immune cells, resulting in persistent systemic inflammation (Rohm et al., 2022).

There is growing pre-clinical evidence that circadian misalignment, typically defined by low (blunted) morning cortisol, can drive a pro-inflammatory state. Exposure of mice to chronic circadian misalignment leads to immune senescence and consequent chronic inflammation, as well as shortened lifespan (Inokawa et al., 2020; Castanon-Cervantes et al., 2010). This is also consistent with pharmacodynamic modeling of cortisol rhythms and effects on inflammatory pathways in mice and rats (Curtis et al., 2014; Almon et al., 2007; Acevedo et al., 2019). However, similar data has not been available in human populations.

As corticosteroids, including cortisol, are potent immunomodulators (Roy et al., 2022), we hypothesized that circadian misalignment measured by low morning cortisol may contribute to the systemic pro-inflammatory state. To test this hypothesis, we focused on studying cortisol cycling at an early age, where less influence of multiple prevalent chronic disorders and co-morbidities, which emerge later in adulthood, and where circadian misalignment might be expected to be increased due to loss of circadian entrainment (sunlight, exercise, healthy diet patterns). If circadian misalignment in children is found to be associated with a pro-inflammatory state, while controlling for obesity, this might suggest that circadian rhythms may directly lead to dysregulation of the immune system. This might help explain the observed increasing rates of pediatric chronic inflammatory disease (asthma, inflammatory bowel disease, cardiovascular risk factors) (Ng, 2014; Beasley et al., 2003; Stemeseder et al., 2017). Evidence for such a model would include blunting of diurnal cortisol fluctuations (low awakening cortisol), as observed in mice. To study circadian alignment, we carried out a population-based study of Maine school children (1336 child phenotype days), including repeated measures of awakening and bedtime salivary cortisol (>10,000 tests).

2. Methods

2.1. Participant recruitment

The Cardiovascular Health Intervention Program (CHIP) was a population-based study of diurnal health and cardiovascular risk factors in school children of Southern Maine (4th and 5th grades; 8–11 years; n = 1354 participant assessments); the 4th grade assessments were done in the Fall, and 5th grade assessments done in the Spring. The study period was 2012–2017. The study design and procedures, inclusive of all assessments, have been previously described in detail, and are not repeated here (White et al., 2017; Dai et al., 2021). Demographics of study population is provided in Supplemental Table 1). The participants were recruited after approval from the superintendent from two local schools that were geographically close to University of New England where the assessments were taken. The study was approved by the Institutional Review Boards of the University of New England.

Anthropomorphic data (weight, height, BMI, fitness), laboratory data (HDL, LDL, glucose), mean arterial pressure data (MAP), and cortisol data (first-awakening [AM], and late evening [PM]) have been previously described (White et al., 2017; Dai et al., 2021; Yeung et al., 2016; Downing et al., 2021).

2.2. Salivary analytes

During their University visits, participants were taught to facilitate under the tongue saliva collection procedures at home three times per day (on school days) at first awakening, 30 min post-awakening and at bedtime over 3 consecutive days using oral collection swabs (9 samples per child). The samples were stored at − 20°C at home, transported to school, then stored at − 80°C until biochemical analysis.

2.2.1. Cortisol assays

All saliva samples were assayed for cortisol in duplicates following manufacturer's protocol (Salimetrics, State College, PA), as we have previously described for this study population (Dai et al., 2021; Yeung et al., 2016). The cortisol assay had a test volume of 25 μl, and standards ranged from 0.01 to 3 μg/dl with lower limit of sensitivity of 0.003 μg/dl.

2.2.2. Cytokine assays

Saliva samples from each participant were pooled into a single morning and single evening sample (maximum of 6 morning samples/subject; 3 evening samples/subject), and the pooled sample then carried forward to cytokine assays. Pooling was done to provide an intra-subject experimental average of morning vs. evening samples (mitigate variables such as diet or activities on 1 specific day), as well as to reduce laboratory time, reagents, and cost. For the participants studied for salivary cytokines, all morning saliva samples were mixed into a single morning sample per participant (awakening, 30 min post-awakening; 3 consecutive days; 6 salivary samples mixed per participant), and a single evening sample per participant (3 consecutive days). Saliva samples were then depleted of amylase, as previously described (Xiao et al., 2016). The morning samples were defined as "AM", and evening samples as "PM" in this current analysis. Samples were assayed for cytokines in duplicate using Meso Scale Discovery (MSD) 10-plex assay. The cytokines assayed were IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α.

2.3. Data analysis and statistical modelling

2.3.1. Total group and sub-group analyses

To study associations of circadian misalignment, obesity and inflammation, we focused on 4th grade children (689 participants; age 9.2 ± 0.4 years) (Supplemental Table 1), and selected a subset of 573 participants with complete data sets (cardiovascular risk factors, cortisol measures, and both morning and evening saliva samples available for cytokine measurements). Missingness of data was due to inadequate saliva samples (no saliva sample provided; 101 participants), or missing BMI data (15 participants). An additional criterion was availability of 4 or more morning salivary cortisol values (of 6 possible - awakening, 30-minutes post-awakening, 3 consecutive days). Of the 573 4th grade participants with a complete dataset, 532 met the additional selection criteria.

Missingness of specific variables has been previously described (Dai

et al., 2021). For BMI, the missing data rates were 2% for 4th grade participants, and 0.9% for 5th grade participants. For cortisol measures, missingness was 9.0%. Cytokine assays were done on a subset (n = 202) of 4th grade participants that were stratified by both BMI and morning cortisol measures to test for associations of cytokines with both BMI and cortisol measures (Supplemental Table 2). The limitation of cytokine assays was due to cost, as it was not possible to assay all 7987 saliva samples for 10 cytokines. The stratification was designed to enrich for more extreme samples that might efficiently test our hypothesis of circadian rhythm cortisol vs. obesity (BMI) as associated with the pro-inflammatory state.

2.3.2. Between and within-group change

For each of the cytokines, descriptive statistics were calculated (mean± SD). Within group change between samples taken upon awakening (upon awakening and 30 min later were mixed into a single "AM" sample over 3 consecutive days; maximum of 6 morning samples mixed per child) vs bedtime samples (samples taken immediately before bed, 3 consecutive days, mixed into a single "PM" sample) was investigated using paired t-tests (within child longitudinal analysis) and Wilcoxon signed rank test with adjustment for multiple testing (Benjamini-Hochberg false discovery method) on log2 transformed data in R (Benjamini and Hochberg, 1995; R Core Team, 2020). Partially quantified datapoints (i.e., at least one of AM or PM samples quantified and others imputed using half of the lower limit of quantitation [LLOQ] value if not quantified) were also tested with paired t-test and Wilcoxon signed rank test (which can deal with such imputed data better) to use more of the available data and to check for consistency of signal.

2.3.3. Regression analyses

To understand the associations of diurnal patterns of cytokines and cortisol and BMI, a multiple linear regression model was done with PM-AM cytokine values as the response, and the following explanatory variables (covariates): AM cortisol level, BMI level, peak cytokine level, change in cortisol from AM to PM, and interactions between AM cortisol level and BMI level, between change in cortisol from AM to PM and BMI level, and lastly, between AM cortisol level and change in cortisol from AM to PM. The rationale for these variables included our hypothesis that the strength of the circadian rhythm could be measured by the difference between morning vs. night assessments of cortisol and cytokines. We also wanted to test baseline inflammation (not morning/night change), so also tested the time point where the cytokine under study showed the highest level across the population (peak cytokine) – for some cytokines this was in the morning, for others at night. We included BMI as a variable as we anticipated the associations with serum lipids and mean arterial pressure as a positive control, while also testing the association of BMI with cortisol signaling and cytokines.

For distributional assumptions, cortisol and cytokine values were first log2 transformed and no imputed values were used when cortisol, TNF-α or IL6 levels were below the limits of detection of the assays. For these regression models, p-values from each cytokine were adjusted for multiple testing using a Benjamini Hochberg false discovery rate (FDR) adjustment in R. BMI z-scores were also modeled using cortisol and high density lipoprotein (HDL) and mean arterial pressure (MAP) as covariates and contrasted to an analysis with only cytokines. Finally, the awakening cortisol values were regressed on cytokine values.

3. Results

3.1. Salivary cytokine assays in morning and evening in CHIP participants

A subset (n = 202) of the complete dataset participants from 4th grade (n = 532) was selected for cytokine assays based upon BMI (high vs normal/low) and morning salivary cortisol levels (high vs low) (n = 47–55 per group; 4 groups) (Group 1. High BMI [>85 percentile], High Cortisol; Group 2. High BMI [>85 percentile], Low Cortisol; Group 3. Normal/low BMI, High Cortisol; Group 4. Normal/low BMI, Low Cortisol) (Supplemental Table 2). The means of BMI in these four groups were: Group 1. 22.7 ± 2.8; Group 2. 24.2 ± 3.9 kg/m2), or normal/low BMI; Group 3. 16.6 ± 1.6; Group 4. 16.9 ± 1.3 kg/m2). Group means of cortisol were: Group 1. 0.49 ± 0.15; Group 2. 0.135 ± 0.05; Group 3. 0.57 ± 0.18 μg/dl); Group 4. 0.148 ± 0.05 μg/dl) (Supplemental Table 2).

Of 10 cytokines studied in first-awakening samples (mixture of upon-awakening and 30 min after awakening over 3 consecutive days per participant) and bedtime samples (mixture of samples taken immediately before bed over 3 consecutive days per participant), 6 cytokines showed > 75% of measures below the LLOQ and these were excluded from further analyses. IL-1β and IL-8 were detected in 100% of morning and evening samples, IL-6 in 47.8% and TNF-α in 31.9%. All four cytokines exhibited circadian patterns, with change in expression from AM to PM significant for each (IL-1β, IL-8, and IL-6 $p < 0.001$; TNF-α $p = 0.03$) (Table 1). IL-1β and IL-8 were higher in morning (4.02-fold and 1.36-fold, respectively), whereas IL-6 and TNF-α were higher in the evening (1.49-fold and 1.73-fold, respectively) (Table 1).

The children were then stratified into four groups based on morning cortisol and BMI (Table 2). All subgroups showed salivary IL-1β levels that were 2–5 fold higher in the morning compared to the evening (all $p < 0.001$), although the high BMI, low morning cortisol group showed the lowest morning IL-1β and highest evening IL-1β levels. Morning IL-1β levels were elevated in high morning cortisol subjects ($p = 0.034$), and showed greater cytokine cycling (drop from AM to PM) ($p = 0.0003$) (Fig. 1).

IL-8 levels were higher in the morning in all groups, but the differences between morning and evening were less pronounced than IL-1β (Table 2). Within group paired comparisons of AM vs. PM values retained significance for all groups except the high BMI low morning cortisol group, again suggesting a blunting of the circadian cycling in

Table 1
Circadian alignment of salivary cytokines. The change in expression of the cytokines from AM (~7 AM prior to school) to PM (~9 PM prior to bed) are also reported along with their significance level from paired t-test analysis on pairwise complete data after adjustments for multiple testing.

Cytokine (LLOQ-ULOQ pg/ml)	Number assessments above LLOQ (% of 202 children)		AM	PM	change in AM vs PM[a]
	AM	PM	Mean ± SD pg/ml	Mean ± SD pg/ml	Mean ± SD pg/ml (fold-change) (p value)
IL-1β (0.65–375)	202 (100%)	202 (100%)	98.8 ± 82.2	24.5 ± 23.6	-74.3 ± 73.6 (−4.0 fold) ($p < 0.001$)
IL-8 (0.59–375)	202 (100%)	202 (100%)	367.4 ± 250.5	268.7 ± 207.0	-98.7 ± 247.7 (−1.4 fold) ($p < 0.001$)
IL-6 (0.63–488), entire dataset	64 (32%)	129 (64%)	2.5 ± 2.9	2.4 ± 2.9	
IL-6 (0.63–488), complete pairwise	54 (27%)	54 (27%)	2.7 ± 3.1	3.8 ± 3.9	1.1 ± 3.2 (+1.5 fold) ($p < 0.001$)
TNF-α (0.69–248), entire dataset	75 (37%)	50 (25%)	1.1 ± 0.7	13.3 ± 19.8	nd
TNF-α (0.69–248), complete pairwise	28 (14%)	28 (14%)	1.3 ± 1.1	4.9 ± 8.9	3.6 ± 8.9 (+1.7 fold) ($p = 0.03$)[b]

[a] Univariate model, adjusted for multiple testing. Fold-changes are back-transformed from log2 transformed calculation.

[b] The relatively low number of values above LLOQ is a limitation in interpretation of TNF-α values. Wilcoxon tests were not significant ($p = 0.09$).

Table 2
Salivary cytokine levels in children stratified for morning cortisol and BMI. For each of the cytokine that had more than 1/4th of the samples in the detection range, p-value of AM (~7 AM prior to school) to PM (~9 PM prior to bed) paired analysis (t-tests; pairwise complete data) for each of the groups (combinations of high and low cortisol and BMI) are reported after adjusting for multiple testing. Multiple testing corrected for each cytokine using Benjamini-Hochberg FDR method.

Cytokine	Low BMI High Cortisol			Low BMI Low Cortisol			High BMI High Cortisol			High BMI Low Cortisol		
	AM mean ± SD pg/ml	PM mean ± SD pg/ml	Paired t-test AM vs PM	AM mean ± SD pg/ml	PM mean ± SD pg/ml	Paired t-test AM vs PM	AM mean ± SD pg/ml	PM mean ± SD pg/ml	Paired t-test AM vs PM	AM mean ± SD pg/ml	PM mean ± SD pg/ml	Paired t-test AM vs PM
IL-1β	104.3 ± 76.3	21.3 ± 17.5	$p < 0.001$	96.5 ± 80.1	24.9 ± 26.5	$p < 0.001$	107.1 ± 79.0	22.4 ± 21.3	$p < 0.001$	86.5 ± 94.3	29.9 ± 28.0	$p < 0.001$
IL-8	354.3 ± 236.9	272.3 ± 212.7	$p < 0.01$	390.4 ± 262.8	272.6 ± 217.1	$p < 0.01$	400.3 ± 238.4	258.4 ± 195.2	$p < 0.001$	323.5 ± 264.1	270.5 ± 206.8	ns
IL-6	3.2 ± 3.7	3.6 ± 3.9	ns	3.3 ± 3.9	4.9 ± 5.5	ns	2.0 ± 2.0	2.7 ± 2.9	ns	2.5 ± 2.5	4.5 ± 3.9	ns
TNF-α	1.1 ± 0.4	4.5 ± 8.5	ns	1.7 ± 2.0	0.8 ± 0.2	ns	1.0 ± 0.5	10.8 ± 14.0	ns	1.7 ± 1.5	3.0 ± 4.2	ns

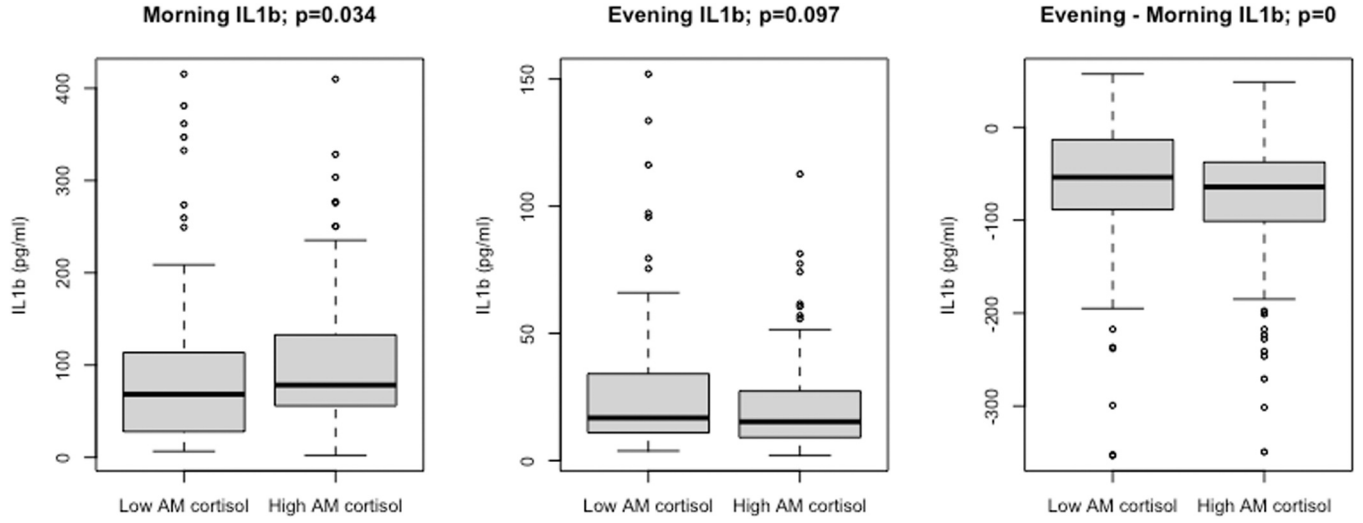

Fig. 1. Comparisons of IL-1β AM (~7 AM prior to school) to PM (~9 PM prior to bed) **and paired change over the day (evening – morning concentration) between high AM cortisol and low AM cortisol subjects.** Independent samples t-tests were used on log2 transformed data.

this group relative to the others as was seen with IL-1β.

IL-6 was higher in the evening for all groups, although none of the subgroups showed significant AM/PM differences as opposed to the high awakening cortisol groups (Table 2). TNF-α levels at nighttime were quite variable from child to child (high variance, coupled with the majority of values under the LLOQ), and showed no retention of statistical significance for morning vs. evening levels in any subgroup (after adjusting for multiple testing).

3.2. Regression modelling

Associations between BMI z-score as the response variable, with HDL, LDL, mean arterial pressure (MAP), AM cortisol, and PM cortisol using the complete 4th grade data set was tested (Model 1; Supplemental Table 3). BMI z-score showed a negative association with HDL ($p < 0.001$), and positive association with MAP ($p < 0.001$) after adjusting for other variables in the model, as expected. To test associations of BMI with awakening and evening cytokine levels, BMI z-scores were regressed on both AM and PM cytokine levels (those with complete data, i.e., IL-1β and IL-8) (Model 2; Supplemental Table 4). This showed BMI to be associated with PM IL-1β levels ($p = 0.02$) and AM IL-1β concentrations ($p = 0.05$), although AM and PM IL-1β explained very little of the variance in BMI z-scores (adjusted $r^2 = 0.036$; ~3% of data).

Using awakening cortisol levels as a surrogate marker for circadian alignment, we used AM cortisol concentrations as a response variable, and modeled morning and evening cytokines (those with complete data) as independent variables (Model 3; Supplemental Table 5). High morning cortisol (circadian alignment) was associated with high morning IL-1β ($p = 3.82 \times 10^{-6}$), and low night IL-1β ($p = 6.27 \times 10^{-4}$), with the model explaining about 14% of AM cortisol or circadian alignment.

We then studied relationships between the diurnal pattern of each cytokine (AM vs. PM intra-subject daily change), and the peak level of that same cytokine (AM values for IL-1β and IL-8; PM values for TNF-α and IL-6) (Table 3). We also adjusted this model for AM cortisol level (high vs low), change in cortisol over the day, BMI (high vs low), and interactions between change in cortisol over the day and BMI, and AM cortisol and change in cortisol over the day. For IL-1β, low and high AM cortisol subjects had significantly different slopes for over the day change in cortisol. After adjusting for other variables, high AM cortisol subjects had a larger during the day reduction in IL-1β. On the other hand, for IL6, as compared to low AM, high AM cortisol subjects had a marginally smaller during the day increase in IL6. For IL-1β, the expected drop in cytokine over the day was lower for high BMI subjects as compared to low BMI subjects. Both IL-1β and IL-8 showed a relationship between peak concentration and diurnal change after accounting for interaction and other terms (respectively, $p = 1.85 \times 10^{-15}$; $p = 1.50 \times 10^{-14}$). IL-6 and TNF-α also showed a positive association, where increased evening (peak) cytokine levels were associated with high diurnal cycling after accounting for interaction and other terms (Table 3).

Importantly, a relatively high degree of the AM/PM differences in IL-1β were explained by this linear regression model ($r^2 = 0.491$; ~50% of variance in response). Given the importance of IL-1β, the superior

Table 3
Regression modeling of diurnal cytokine levels (AM [~7 AM prior to school] to PM [~9 PM prior to bed]); continuous) with BMI levels (high vs low), AM cortisol levels (high vs low), change in cortisol (PM-AM), and salivary peak IL-1β & IL8 (AM), TNF-α & IL-6 (PM). Coefficients of the regression along with their respective p-value of each of the cytokines (log2-transformed) after adjusting for multiple testing are also reported. Both morning cortisol and BMI were analyzed as categorical variables (high vs. low) based on group selection.

Cytokine (PM - AM)	β_1 AM cortisol levels (p)	β_2 BMI levels (p)	β_3 Peak cytokine (p)	β_4 AM vs. PM change in cortisol[a] (p)	β_5 AM cortisol levels *BMI levels (p)	β_6 AM vs. PM change in cortisol* BMI (p)	β_7 AM cortisol * AM vs. PM change in cortisol (p)	Adjusted R^2
IL-1β (n = 202)	-0.019 (ns)	**0.515 (0.02)**	**-0.488 (1.85×10^{-15})**	**0.653(4.59×10^{-7})**	-0.552(ns)	-0.109(ns)	**-0.450(0.005)**	0.491
IL-8 (n = 202)	0.131 (ns)	0.227 (ns)	**-0.518 (1.5×10^{-14})**	0.189 (ns)	-0.430 (ns)	-0.122 (ns)	-0.152 (ns)	0.289
TNF-α (n = 28)	0.925(ns)	0.102 (ns)	**0.831 (5.1×10^{-7})**	1.278 (ns)	0.417 (ns)	0.299 (ns)	-1.442 (ns)	0.809
IL-6 (n = 54)	-0.892(ns)	-0.219 (ns)	**0.284(0.03)**	**-1.272 (0.02)**	-0.704(ns)	0.682(ns)	**-0.915(0.03)**	0.178

[a] This variable was scaled for the main and interaction factors (variance inflation factors from regression were too high otherwise).

performance of IL-1β (TNF-α regression on small subset of data; IL-1β on complete data), and that the interaction between morning cortisol levels (high vs low) and morning vs. evening (PM cortisol - AM cortisol) cortisol levels was significant, we also ran regression analysis stratified on high vs low morning cortisol level groups (Supplemental Table 6). For low morning cortisol subjects, high BMI levels associated with smaller drop in IL1b over the day ($p = 0.014$). Furthermore, the change in cytokine from AM to PM is associated with AM to PM change in cortisol ($p = 9.4 \times 10^{-7}$), with a larger reduction in cortisol during day associated with a larger drop in cytokine during the day, and the higher the morning cytokine level ($p = 2.63 \times 10^{-10}$), the larger the change in cytokine over the day. On the other hand, for high morning cortisol subjects, the only significant predictor of change in cytokine from AM to PM was the peak (morning) cytokine concentration ($p = 1.32 \times 10^{-7}$).

We hypothesized that bedtime (PM) IL-1β levels may best reflect the systemic pro-inflammatory state, as interactions with cortisol levels at the PM nadir would be least pronounced. We then set PM IL-1β as a continuous outcome variable, then tested cortisol, AM IL-1β, BMI, and the interaction between the latter two as explanatory factors (each continuous variables). This showed an inverse relationship between bedtime IL-1β and awakening (AM) cortisol (this remains true if stratifying on morning cortisol level and modeling only low morning cortisol subjects as well; adjusted regression coefficient estimate: −0.44; $p = 0.004$), where lower morning cortisol was predictive of higher evening IL-1β ($p = 0.01$) (Table 4). In contrast, high awakening IL-1β was predictive of high bedtime IL-1β ($p = 1.25 \times 10^{-7}$). Thus, strong diurnal amplitudes of cortisol cycling were associated with strong diurnal amplitudes of IL-1β cycling (high in morning, low at night), but circadian misalignment (defined by blunted or lower levels of awakening cortisol) was associated with a pro-inflammatory state (high IL-1β) at night. Consistent with the categorical modeling of BMI as risk factor for cytokine levels (Table 3), BMI as a continuous variable was likewise associated with evening IL-1β levels (Table 4).

Table 4
Regression modeling of bedtime IL-1β (PM) levels as reflective of the pro-inflammatory state in children. Coefficients of the regression along with their respective p-value of each of the cytokines after adjusting for multiple testing are also reported. All variables were analyzed as continuous variables. Cytokine and cortisol values were log-transformed.

	β_1 AM cortisol (p)	β_2 AM IL-1β (p)	β_3 BMI (p)	β_4 AM cortisol * AM IL-1β (p)	Adjusted R^2
Bedtime IL-1β (PM)	**-0.719 (0.014)**	**0.642 (1.25×10^{-7})**	**0.027 (0.043)**	0.118 (0.076)	0.268

4. Discussion

There is an extensive literature on relationships between cortisol, cardiometabolic factors, inflammation, and stress, with most studying morbid populations (disease states) (Ortiz et al., 2022). It is challenging to differentiate diurnal cortisol and stress-related cortisol, and clearly both impinge on the same glucocorticoid receptor and downstream targets and pathways. Stress-related cortisol has been consistently associated with inflammatory markers and cardiometabolic phenotypes (obesity, blood pressure), but diurnal cortisol less so. For example, a recent systematic review and metanalysis of studies of relationships between cortisol and obesity in children found that there was strong consistent support for association of high cortisol in hair samples with increased obesity, but not morning cortisol (Ma et al., 2022). This suggests that increased cortisol due to chronic stress (measured in hair samples) is associated with objective measures of obesity, but not diurnal (morning peak) cortisol. Cortisol is well-known to have potent immunomodulatory effects, and derivatives of cortisol (glucocorticoid drugs) are extensively utilized as anti-inflammatory therapies. Thus, there is a paradox where high stress-related cortisol is pro-inflammatory, but pharmacologic glucocorticoids are potently anti-inflammatory.

We sought to better define relationships between first-in-morning and evening (prior to bed) cortisol with inflammatory cytokines measured in the same salivary samples, using a population-based cohort of young children. Regression modeling of BMI, blood pressure, blood lipids, and circadian cycling of cortisol showed the expected negative association of BMI and HDL ($p < 0.001$), and BMI and mean arterial pressure (positive association; $p < 0.001$). Addition of diurnal salivary cytokines to the regression models, using BMI Z-score as the response variable, showed only weak associations with early morning or bedtime cytokine levels (Supplemental Table 4). Thus, there were only weak associations between obesity and the pro-inflammatory state as measured by four cytokines in saliva. While BMI can be considered a controversial surrogate for measures of obesity, the strong positive associations with HDL and mean arterial pressure (also markers of obesity) suggest our use of BMI is reasonable in this pediatric population for the current research.

We then sought to interpret our data in the context of an alternative hypothesis, where low morning cortisol (a possible biomarker for circadian misalignment) may serve as a driver of the pro-inflammatory state. Using awakening cortisol levels as the response variable (Supplemental Table 5) we showed negative associations with bedtime IL-1β ($p = 6.27 \times 10^{-4}$), suggesting that high morning cortisol was associated with low evening IL-1β levels. Awakening cortisol was also positively associated with awakening IL-1β ($p = 3.82 \times 10^{-6}$).

As each of the four cytokines studied were significantly changing from morning to night, we carried out linear regression modeling with the difference in AM vs. PM cytokines as the response variable, and

modeled effects of cortisol and BMI on cytokine AM/PM fluctuations (Table 3). This showed that morning and night cortisol fluctuations were relatively more strongly associated with IL-1β morning/night fluctuations as compared to BMI. Interestingly, the peak levels of each cytokine were strongly associated with AM/PM cycling, but in contrasting directions. IL-1β and IL-8 were highest in the morning and high morning levels were negatively associated with AM/PM differences, whereas TNF-α and IL-6 were highest in the evening but positively associated with AM/PM differences.

In terms of biological mechanisms potentially driving morning vs. evening diurnal cycling of these cytokines, it is well-established that cortisol directly binds to the IL-1β gene promoter, and down-regulates IL-1β mRNA expression. This might lead to the assumption that high diurnal cortisol in the morning would lead to low IL-1β protein levels in the morning (the opposite of what we found). However, the pharmacodynamics of IL-1β gene transcription (acute response) vs. IL-1β protein levels (chronic response) are very different. Indeed, high morning cortisol is consistent with a chronic effect on IL-1β expression leading to low IL-1β protein levels in the evening, as we found. The pharmacokinetics (cortisol levels) vs. pharmacodynamics (protein levels of cytokines) are likely complex, and require further studies.

We hypothesized that high awakening cortisol may serve as a surrogate biomarker for circadian alignment, where high morning cortisol reflects circadian alignment (strong circadian rhythm), and low morning cortisol reflects circadian misalignment (weak circadian rhythm). As there are direct and potent regulatory interactions between cortisol and inflammatory cytokines (IL-1β, TNF-α, and many others), including potent auto-regulatory pathways (negative feedback for cortisol; positive feedback for IL-1β), we further hypothesized that circadian misalignment is a risk factor for a pro-inflammatory state. To test this, we carried out regression modeling of awakening vs. bedtime fluctuations in cortisol, and inflammatory cytokines, and further tested if BMI z-score acted as a risk factor for either cytokine or cortisol fluctuations (Tables 3,4; Supplemental Tables 3–5). This showed that morning/night fluctuations in cytokine levels were relatively more strongly associated with awakening cortisol levels (as part of interaction with change in cortisol during the day), and less associated with BMI z-score (Table 3). We then set bedtime IL-1β as response variable reflecting the systemic pro-inflammatory state, with awakening cortisol, BMI and awakening IL-1β as risk factors (Table 4). This model supported our hypothesis, where high bedtime IL-1β levels were explained by low morning cortisol (circadian misalignment; $p = 0.014$) and high awakening IL-1β ($p = 1.25 \times 10^{-7}$), but less so by BMI.

The data presented here supports a model of circadian alignment driving diurnal fluctuations in cytokines, where blunted awakening cortisol (circadian misalignment) may drive a pro-inflammatory state, consistent with what has been seen in rodents. Our data in children (~9 years) show that circadian alignment was robustly associated with diurnal variations IL-1β through regression modeling. Specifically, high morning cortisol was associated with high morning IL-1β ($p = 3.82 \times 10^{-6}$) but low evening IL-1β ($p = 6.27 \times 10^{-4}$). We hypothesize that lower awakening cortisol and IL-1β may promote predominance of the auto-regulatory cortisol loop in the afternoon and evening, leading to higher afternoon/evening IL-1β levels and a pro-inflammatory state. In effect, lower morning cortisol (potential circadian misalignment) may allow inflammatory 'noise' to predominate, as opposed to more strongly regulated morning/night cycling promoted by high morning cortisol. If confirmed by further studies, this model may provide a link between emerging lifestyles of children leading to circadian misalignment and the observed increases in pediatric chronic inflammatory conditions (González-Ruiz et al., 2017; Gibson and Shepherd, 2005; Kalra et al., 2012).

Importantly, our data suggests that cortisol levels (circadian alignment) seems distinct from obesity in children. Rather, there appears to be a more direct relationship between cortisol and pro-inflammatory biomarkers (particularly the central IL-1β that is directly regulated by cortisol/receptor gene promoter binding). We do not see any immediate clinical implications of our findings, other than consideration of morning cortisol as an additional potential systemic health biomarker.

There are limitations of this current study. Part of our original rationale for carrying out this study was that young children are experiencing greater rates of chronic inflammatory disease that might be linked to a reduction in circadian cues (sunlight, exercise, diet). However, we did not directly measure these circadian cues in the target population. Another limitation of our study is that the relatively low level of cytokines detected in the saliva of the target population of young healthy children, where 6 of 10 cytokines showed > 75% of samples below the lower limits of detection (and excluded from analyses). We note that there is a tradeoff between costly exploratory measurement and assaying more cortisol samples that future research should carefully consider. That said, our data is consistent with the literature using the same bioassay used here, where IL-6, IL-8, IL-1β, and TNF-α are most reliably measured in adult volunteer saliva, with TNF-α showing the lowest levels (Riis et al., 2021). Also, a study comparing saliva and serum cytokine levels in older adult volunteers, again using the same bioassay used in our study, found overall higher cytokine levels compared to our study in children, but with many samples below the limits of quantitation for the same cytokines that we found below the limits of quantitation. These authors also found strong correlations between the two sample times (morning, night) (Parkin et al., 2023). There are strong associations between psychological well-being (stress, depression), stress-related cortisol, and the pro-inflammatory state (Sharpley et al., 2019; Miller et al., 2002, 2014). We did not measure levels of stress in this population-based study of school children, and this is a limitation. We collected morning and evening saliva samples on 3 consecutive days (9 saliva samples per child) to mitigate intra-subject variability of diet, and daily activity. However, all morning and all evening samples were pooled for each participant to test morning vs. evening cytokine levels in that participant; there are pros and cons to such a pooling approach. Finally, this study was carried out over multiple years (classes of students), and we did not control for year-on-year effects. Importantly, we do not expect any changes in the biomarkers we are studying due to recruitment effect in this population given the lack of a stressor event, and all data was collected prior to COVID school closures.

Evidence before this study

There are extensive published studies of circadian rhythms in human health, and non-human models of health. A query of PubMed shows 875 publications of "circadian misalignment", including 39 randomized controlled trials, and 73 cohort and case control studies. These studies generally show that disruption of circadian rhythms lead to increased stress and poor health outcomes, and similar findings are shown in non-human models. We sought to test the hypothesis that circadian misalignment in school children (9–10 years) leads to a pro- inflammatory state. We conducted a novel experimental design by engaging parents for obtaining samples for first-awakening and late evening cortisol measures (9 saliva samples per child over 3 days). This population-based study of school children included metabolic syndrome measures of all children at a local university.

Implications of all the available evidence

Current lifestyles of children include low exposure to sunlight and low amounts of exercise – both sunlight and exercise are well-recognized to entrain circadian alignment. Chronic inflammatory states in children have risen over the previous decades, and our findings of relationships between circadian misalignment and inflammatory cytokines may have implications for this increase in disease incidence.

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Declaration of Competing Interest

The authors state that they have no conflicts of interest with the presented research.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2023.106411.

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