Psychoneuroendocrinology 99 (2019) 104–111

Contents lists available at ScienceDirect

Psychoneuroendocrinology

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

Salivary C-reactive protein among at-risk adolescents: A methods investigation of out of range immunoassay data

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ARTICLE INFO

Keywords:
Adolescence
C-reactive protein
Immunoassay
Inflammation
Non-Detects
Saliva
Winsorization

ABSTRACT

Inflammatory markers including C-Reactive Protein (CRP) are increasingly used within research and clinical settings. Yet, varying methodologies for cleaning immunoassay data with out of range (OOR) samples may alter characteristic levels of CRP, thereby obscuring interpretation and reliability. This study investigated the influence of eight immunoassay OOR data treatment techniques on salivary CRP (sCRP) samples from at-risk adolescents. Participants from the 'Sleep and Education: learning New Skills Early' (SENSE) Study were 86 adolescents at-risk for depression (50 female), aged 14.29 years (SD = 1.04). ANOVA results showed no statistically significant differences in average morning ($F(7, 590) = 1.24$, $p = .28$) and evening ($F(7, 599) = 1.29$, $p = .25$) values produced by each OOR data cleaning technique. However, varying techniques produced differences in the magnitude of Pearson's correlations between consecutive saliva samples (r's between 0.27–0.78), and influenced the significance of a sCRP diurnal pattern; two techniques produced statistically higher morning than evening sCRP levels ($t(85) = 2.70$, $p = .01$ and $t(85) = 2.67$, $p = .01$), whereas six techniques failed to find statistical differences between morning and evening sCRP levels (p's $> .05$). Varying techniques also produced statistically divergent associations between sCRP and age and depressive symptoms. Results from this study provide evidence for the temporal stability of sCRP among adolescents, show winsorization as an effective OOR data management technique, and highlight the influence of methodological decisions in cleaning salivary biomarker data and the need for consistency within the field.

1. Introduction

C-reactive protein (CRP) is a rapid systemic inflammatory responder to infection and tissue damage, supporting the immune system by killing and clearing pathogens from the body (Black et al., 2004). Inflammatory markers such as CRP are increasingly used in human clinical research as tools to measure exposure to various forms of psychological stress, biological responses to treatment interventions, and risk for medical issues such as cardiovascular disease (CVD; Ridker, 2003). Although most studies have measured peripheral inflammatory markers in blood, newer research suggests that oral fluids (e.g., saliva, oral mucosal transudate, gingival crevicular fluid) may provide accurate detection of acute phase proteins such as CRP (Byrne et al., 2013), as well as associations between localized inflammatory markers and social stress (Slavich et al., 2010), depression (Delany et al., 2016), systemic inflammation (Megson et al., 2010), cognitive functioning (Cullen et al., 2017), and physical and psychological health (Naidoo et al., 2012; Goodson et al., 2014; Cicchetti et al., 2015). Investigating inflammatory markers via saliva has pragmatic benefits including relative ease of collection and non-invasiveness of sampling, making saliva particularly acceptable for use with clinical populations like anxious or depressed adolescents. Yet despite their utility, levels of salivary inflammatory markers such as salivary CRP (sCRP) may be highly sensitive to methodological decisions arising from the immunoassay process.

Immunoassays are tests used to quantify specific biomarker levels within a sample, using known concentrations of the biomarker (i.e., standards) to generate a standard curve. Sample values are then interpolated onto the standard curve to determine biomarker levels. Out of range (OOR) samples are values that are flagged by the assay procedure as 'non-detects,' samples too high above or too low below the standard

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https://doi.org/10.1016/j.psyneuen.2018.08.035
Received 23 February 2018; Received in revised form 3 August 2018; Accepted 27 August 2018

curve range. These samples represent unique forms of missing data and require informed cleaning before their use in analyses. In the case of sCRP specifically, extreme sample values resultant from endogenous participant factors such as medication use, acute infection, or recent trauma (Posthouwer et al., 2004; Tsai et al., 2005; O'Brien et al., 2006; Prasad, 2006; Haran et al., 2012) are often excluded from analyses (Miller and Cole, 2012; Byrne et al., 2013; Park et al., 2016). However, the question of how to manage OOR data that persist after external factors have been controlled for remains.

Ideally, adjusted dilution and re-assay of OOR samples to fit within the standard curve range would occur, requiring additional resources that may no longer be available such as extra sample and assay kits. Incidentally, researchers investigating biomarkers have utilized numerous methods for OOR data management but often without justification for specific techniques. Some have removed all participants with OOR data (e.g., list-wise deletion, as in Lucas et al., 2016), calculated summary statistics for only detected observations (e.g., pair-wise deletion, as recommended by the United States [US] Environmental Protection Agency [EPA], 2000), imputed OOR data using covariate information (Lubin et al., 2004; Baccarelli et al., 2005), or winsorized OOR data (Patel et al., 2015). Still, others have substituted arbitrary values for low OOR samples (such as one half of the assay sensitivity value, e.g., Meier-Ewert et al., 2001; Tajimi et al., 2005), dichotomized data into 'high' versus 'low' level groups (Visser et al., 1999; Epel et al., 2001; Danner et al., 2003), extrapolated values outside the assay standard curve to incorporate OOR data (Kifude et al., 2008; Breen et al., 2011; Schlaudecker et al., 2013), or, more commonly, not reported OOR treatment procedures at all (e.g., Park et al., 2016).

To date, no consensus exists to address the many methods for treating immunoassay OOR data within psychobiological research despite the growing use of biomarkers such as sCRP. Arriving at such an accord is necessary for diligent research design and cross-study comparisons, as well as for clinical decision making. For example, cutoff points of < 1 mg/L (low), 1–3 mg/L (moderate), and > 3 mg/L (high) of serum CRP levels have been used to assess American Heart Association (AHA) CVD risk (Ridker, 2003), with Out et al. (2012) finding similar results when predicting AHA CVD risk from sCRP levels as well. These findings highlight the utility of salivary measures of inflammation, and the strong needs for consistent processing of immunoassay data to ensure measurement reliability.

Therefore, from a sample of convenience, this paper aimed to evaluate the sensitivity of levels of sCRP to OOR data cleaning techniques in three distinctive ways. First, previous research has shown in sample sizes ranging from 27 to 107 participants that sCRP is highly stable over consecutive days and across time (Out et al., 2012; Izawa et al., 2013). As such, we aimed to first test the influence of eight distinct OOR data treatment methodologies on their capacity to alter the strength of correlation between consecutive, biological duplicate (i.e., "test-retest") samples across two days. Second, because some research has shown that sCRP exhibits a high morning, low evening diurnal pattern (Koc et al., 2010; Out et al., 2012; Izawa et al., 2013; Cullen et al., 2017), we aimed to examine how different OOR data cleaning techniques may influence the relationship between morning and evening levels of sCRP. Third, as CRP has been previously associated with both age (Park et al., 2016) and depression (Howren et al., 2009), we aimed to investigate how varying OOR data treatment techniques could influence the strength of associations between CRP and age, and CRP and depressive symptoms. Hopefully this will lead to more consistent methodological treatment for research with inflammatory biomarkers, greater reproducibility and replication, ultimately resulting in greater vertical movement in this field.

2. Materials and methods

2.1. Participants

A baseline subsample of participants within the 'Sleep and Education: learning New Skills Early' (SENSE) Study who provided both morning and evening saliva samples across two consecutive days and who reported no medication use nor recent physical illness were chosen for inclusion in the present study. Eighty-six adolescents (50 female) aged 14.29 years ($SD = 1.04$) provided 344 saliva samples. Briefly, the SENSE Study ($n = 144$) was a randomized control trial (RCT) investigating the efficacy of a 7-week mindfulness-based cognitive behavior sleep treatment program to prevent depression in at-risk adolescents. 'At-risk' was defined as adolescents with high anxiety and poor sleep, and no current or prior diagnosis of a Major Depressive Disorder. Supplementary Table 1 presents brief participant descriptive data including 'at-risk' characterization as defined by the SENSE Study. A full description of the screening process and protocol for the SENSE Study can be found in Waloszek et al. (2015), and immediate post-intervention treatment effects can be found in Blake et al. (2016) and Blake et al. (2017a, 2017b).

2.2. Salivary C-reactive protein measurement

Participants collected approximately 2 mL per each saliva sample via 'passive drool' and were instructed not to eat, drink, or brush their teeth for at least 30 min prior to sample collection. Consecutive weekday morning 1 (M1) and morning 2 (M2) average collection times were 07:25 ($SD = 79.0$ min), and 07:16 ($SD = 59.1$ min), respectively. Consecutive weekday evening 1 (E1) and evening 2 (E2) average collection times were 20:42 ($SD = 66.0$ min), and 20:09 ($SD = 78.9$ min), respectively. Samples were first stored in a -30 °C freezer for an average of 16.0 months ($SD = 11.3$ months), and in preparation for assay, all samples were removed from -30 °C freezers to centrifuge at 10,000 g for 10 min at room temperature (24 °C). Clarified saliva was aliquoted into 1.5 mL eppendorf tubes and stored in -80 °C freezers until the day of assay, an average of 36.5 months after sample collection ($SD = 10.6$ months). Samples underwent two freeze/thaw cycles. Enzyme-linked immunosorbent assays (ELISA) were conducted with sCRP Salimetrics (State College, PA) research assay kits following protocols. All samples were assayed in duplicate using identical lot number assay kits. Saliva test volume was 15 µL, samples were diluted 1:10 prior to assay, and kits had a standard curve range from 93.75 to 3000 pg/mL with a lower limit of sensitivity of 10 pg/mL. Inter- and intra-assay coefficients of variability were 4.68% and 16.0%, respectively.

2.3. Medication use/physical health

Diaries were used during sample collection to exclude possible influences of recent medication use or physical illness on sample values. Although serum levels of CRP > 10 mg/L indicate acute infection (Ridker, 2003; Pearson et al., 2003), no corresponding acute infection cut-off values for CRP as measured by saliva exist to date. Therefore, participants were chosen for the current methodological study if they reported no current or recent medication use, physical illness or injury, or oral health issue.

2.4. Standard curve constraints

Standard microplate readers used for ELISA include software that supports absorbance detection for bioassays and calculations of corresponding concentrations based on interpolation of the optical density (OD) onto the standard curve. Using an extrapolation method within KC Junior Software® (*Bio-Tek Instruments, Inc.*), a widely used data analysis software for absorbance detection with immunoassays, extrapolated OD values were interpolated on the linear standard curve recommended by Salimetrics for sCRP.

Data outside the immunoassay standard curve range were removed in *strict* standard curve datasets. Data that were software-extrapolated outside the standard curve range were retained within *relaxed* standard curve datasets. Table 1 depicts percent and number of low and high OOR data per *strict* and *relaxed* curve datasets.