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Validation of a high-sensitivity assay for C-reactive protein in human saliva

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ABSTRACT

This study aimed to validate a high-sensitivity assay for C-reactive protein (CRP) in saliva as an alternative medium to study inflammation in large epidemiological cohorts and young people. We measured CRP in saliva and serum in 61 (29.5% males) healthy adult volunteers. We found a moderate-to-strong association between CRP measured in saliva and in serum ($r = .72$, $p < .001$). In agreement with the non-steroidal structure and the high molecular weight of CRP, we observed a low saliva-to-serum CRP ratio (1:1633.64). Furthermore, a dichotomous index of salivary CRP, equivalent to a clinically relevant serum CRP cut-off (3 mg/l), was associated to known correlates of systemic inflammation (IL-6, BMI and smoking). Finally, we showed that CRP in saliva is stable at room temperature up to 8 h after collection. Our study provides initial evidence suggesting that non-invasive assessment of CRP in saliva allows valid prediction of serum CRP. Salivary CRP may thus facilitate and promote research exploring the correlates of low-grade inflammation in epidemiological studies and makes it feasible to expand psychoneuroimmunology research to pediatric populations.

1. Introduction

Research indicates that higher inflammation levels are associated with greater risk of disease, including cardiovascular disease, cancer and depression (Buckley et al., 2009; Erlinger et al., 2004; Howren et al., 2009; Miller et al., 2009; Ridker et al., 1997). It is therefore important to characterize the determinants of individual differences in inflammation levels. Longitudinal-prospective studies found initial evidence suggesting that exposure to childhood maltreatment is associated with enduring elevation in inflammation levels (Danese et al., in press, 2007). Consistent with these findings, inflammation is increasingly hypothesized as a potential physiological mediator of the association between early-life stress and health inequalities (Felitti et al., 1998; Green et al., 2010).

The investigation of the determinants of individual differences in inflammation levels is delayed by the lack of non-invasive methods to assess inflammation, which would enable research in large population-representative samples and in young people. Inflammation levels are commonly measured through venipuncture. However, venipuncture is an invasive procedure and requires skilled professionals, laboratory equipments and considerable financial resources. In contrast, saliva collection is non-invasive, stress- and pain-free and may constitute an alternative strategy to assess immune activity prospectively in large samples. Broadly available and empirically validated methods to assess inflammation in saliva are currently lacking. The present study aimed to validate a non-invasive measure of inflammation in saliva, which could easily be implemented in large epidemiological studies and pediatric populations.

Cumulative evidence suggests no significant association between serum and salivary IL-6 levels, a cytokine regulating the acute phase protein synthesis, in adults under baseline conditions (Sjogren et al., 2006) and before and after strenuous exercise in athletes (Cox et al., 2008; Minetto et al., 2005, 2007). In addition to blood contamination (Suh et al., 2009), the lack of association between serum and salivary IL-6 may be due to local regulatory mechanisms affecting salivary but not systemic IL-6 levels, such as the acinar cells of the salivary glands (Sjogren et al., 2006). Alternatively, C-reactive protein (CRP), a common marker of inflammation secreted by hepatocytes under the transcriptional control of the cytokine IL-6 (Pepys and Hirschfield, 2003), is likely to originate from the liver and to mirror serum CRP levels. Therefore, peripheral (e.g., salivary) CRP measures could offer a more accurate estimate of systemic inflammation than peripheral measures of cytokines, which are locally synthesized. Because of its non-lipophilic structure and high molecular weight, CRP is likely to show limited transfer from blood to saliva (Vining and McGinley, 1987). Consequently, the high-sensitivity commercially available enzyme-linked immunoassay (ELISA) adapted to measure CRP in human saliva may offer a valuable strategy to assess expected low CRP levels in various populations. Only one study has investigated the association between serum and salivary CRP levels

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and reported no significant association between levels measured in the two media (Dillon et al., 2010). However, in contrast to our study, no information was collected about the participants' health (e.g., medications, inflammation diseases, bucco-dental problems), limiting the investigation of conditions that may have affected the relationship between serum and salivary CRP. Moreover, salivary CRP was not tested in association with known correlates of systemic inflammation, such as BMI.

In addition to being non-invasive, the assessment of CRP in the saliva allows data collection to take place in the participants' natural environments (e.g., home, school). Collecting saliva outside the research facilities may however delay the storage of the samples in freezers. Samples returned by research assistants or by regular mail services may thus remain at room temperature for extended length of time before being stored. Previous research has found that leaving saliva at room temperature for a week did not disrupt cortisol measurement (Clements and Parker, 1998). No study has yet explored the stability of CRP concentrations in saliva at room temperature.

The objective of this study was to examine the validity of salivary CRP as a non-invasive marker of systemic inflammation. First, we examined whether salivary CRP was correlated with serum CRP. Second, we tested whether salivary CRP was associated with its main secretagogue IL-6 and known correlates of systemic inflammation, such as BMI and smoking (Danese et al., 2009; Greenfield et al., 2004; Thomas et al., 2008). Finally, we investigated if the stability of CRP was affected by the length of time saliva was left at room temperature.

2. Methods

2.1. Sample

Participants were 61 volunteers (29.5% males) aged between 20 and 54 years (mean (SD) = 31.6 (8.9)). The majority of participants were Caucasian (80.3%) and none reported infectious, immune or salivary gland disorders. Moreover, no participants reported symptoms suggesting that they were sick the day of data collection (e.g., fever, flu, diarrhea), although one participant reported to have a cold and took cold mediation. We did not exclude this participant because serum and salivary CRP levels were in the normal range (serum CRP = 1.70 mg/l; salivary CRP = 1187.8 pg/ml). Participants were recruited through internal emails and public posting at King's College London (UK). Participants were asked to refrain from eating (1 h), drinking coffee, acidic/sweet liquids and smoking (30 min) prior to the visit. Participants gave informed consent. The study protocol was approved by the Psychiatry, Nursing and Midwifery Research Ethics Sub-Committee (King's College London, UK).

2.2. Procedures

Data collection took place between 10 h 00 min and 14 h 00 min at the research laboratory. Participants were first invited to rinse their mouth with water and to fill out a short health questionnaire. We recorded measures of blood pressure and heart rate prior to a 5 ml blood venipuncture using serum separator tubes with inert gel barrier and clot activator coating (BD Vacutainer® SST™, BD Diagnostic). We then collected unstimulated saliva by passive drool through a straw into a polypropylene vial. We recorded the time needed by the participants to obtain 5 ml of saliva to control for saliva flow rate. We measured height and weight.

Blood samples were immediately separated by centrifugation at 3000 rpm (1680 relative centrifugal force) for 10 min. Serum samples were stored at −80 °C until shipped on dry ice to an independent laboratory for CRP and IL-6 assays (Core Biochemical Assay Laboratory, Addenbrooke's Hospital, UK). Saliva was split in four aliquots. One aliquot was immediately stored at −80 °C while the others were left at room temperature (average of 21.4 °C) and placed in freezers after 8, 24, and 96 h. Aliquots were shipped on dry ice to a second independent laboratory for salivary CRP assay (Salimetrics Europe Ltd., UK).

2.3. Measures

2.3.1. High-sensitivity salivary CRP

Salivary CRP was determined in duplicates by enzyme-linked immunoassay with the Salivary C-Reactive Protein ELISA Kit (Salimetrics Europe, UK) using the Tecan Genesis Freedom 150/8 automated liquid handler. Saliva samples were diluted (1:10) in a phosphate buffered solution containing a non-mercury preservative before being transferred (50 μl) to microtitre plate wells precoated with mouse anti-CRP antibodies alongside with the standards and controls. Detection antibodies (goat anti-human CRP antibodies linked to horseradish peroxidise) were diluted (1:250) in a phosphate buffered solution, the resulting solution was added to each well (150 μl) and incubated at room temperature for 2 h mixing constantly using a Dynex AM89B microplate shaker/incubator (500 rpm). The wells were washed four times using a buffered solution containing detergents and a non-mercury preservative. A solution of Tetramethylbenzidine (200 μl) was added to each well for color development prior to sealing and the incubation of the plate in the dark and at room temperature for 30 min mixing constantly. A stop solution (sulfuric acid; 50 μl) was added before the plates were mixed on a plate rotator for 3 min and placed on a standard microplate reader at 450 nm (within 10 min of the addition of the stop solution). The average optical density values of the controls and unknown (minus the background reading) were plotted against the standard curve made according to the following calibrator concentrations: 93.8, 187.5, 375, 750, 1500 and 3000 pg/ml. The lower detection threshold (determined by interpolating the mean optical density plus 2 SDs for 10 sets of duplicates at the 0 pg/ml standard) was 10 pg/ml. All the samples were above the detection range. The coefficients of variation (CV) of all duplicate samples were less than 20%. The limit of quantification, which corresponds to the lowest value with a CV less than 20%, was therefore the lowest value in our sample (117.69 pg/ml; CV = 8.5%). Within-assay CVs at low (178.8 pg/ml) and high (1992.5 pg/ml) concentrations were 5.9% and 1.9%; between-assay CVs at low (238.1 pg/ml) and high (2167.1 pg/ml) concentrations were 11.2% and 3.7%. The linearity of dilution was assessed by diluting two samples by a factor of two four times (1:2, 1:4, 1:8 and 1:16). The observed values ranged from 89.8% to 100.8% of the expected values, with a mean of 96.2%. No cross-reaction for human albumin, alpha 1-antitrypsin, lysozyme and IL-6 was documented by the manufacturer.

2.3.2. High-sensitivity serum CRP

High-sensitivity serum CRP concentration was assayed using a high-sensitivity automated colourimetric immunoassay analyzer (Dade Behring Dimension analyzer; Siemens Healthcare, UK). All samples were run in duplicate in a single batch. The lower detection threshold was .10 mg/l and within- and between-assay CVs were less than 5.1%. In addition to the continuous serum CRP measure, we also considered a clinically relevant cut-off adopted by the Centers for Disease Control and Prevention and the American Heart Association used to define high cardiovascular risk (>3 mg/l; 21.6% of the sample) (Pearson et al., 2003).

2.3.3. Serum IL-6

IL-6 was measured using an electrochemical luminescence immunoassay (MesoScale Discovery, USA). All samples were run in duplicates in a single batch using MesoScale's plates, reagents and

calibrators. The lower detection threshold was .10 pg/ml. Within- and between-assay CVs were less than 5.0% and 7.7%, respectively.

2.3.4. Saliva flow rate

Saliva flow rate was calculated by dividing the volume (ml) of saliva collected per minute required. The saliva flow rate varied from .07 to 2.20 (mean (SD) = .60 (.35)).

2.3.5. Blood contamination in saliva

A competitive immunoassay was conducted to quantify the presence of transferrin, a large protein (molecular weight: 76,000) present in abundance in the blood but normally found in trace in saliva (salivary blood contamination enzyme immunoassay kit, Salimetrics Europe, UK). The lower detection threshold of transferrin in the saliva was .80 mg/l. Within- and between-assay CVs were 5.4% and 5.4% at high concentrations (35 mg/l) and 7.4% and 13.0% at low concentrations (2.5 mg/l) (Schwartz and Granger, 2004).

2.3.6. pH determination in saliva

The pH values of 25 μl undiluted saliva samples and calibrators with a known pH (4, 6, 7, 8 and 9.2) were placed in duplicate in a microplate before 200 μl of pH sensitive assay diluent was added to the wells (Salimetrics, PN: 8005). All liquid handling was conducted on a Tecan Genesis Freedom 150/8. The plate was shaken for 5 min (Tecan MIO) and incubated for 15 min at 22 °C (LMK incubator). The optical density was read using a Tecan Infinite F200 at 560 nm and then analyzed using the Tecan Magellan software.

2.3.7. Body mass index

Body mass index (BMI) was calculated as participants weight in kilograms divided by the square of their height in meters. BMI varied from 18.29 to 38.35 (mean (SD) = 24.27 (4.41)).

2.3.8. Physical health

Participants completed a short health questionnaire assessing participants' smoking, illnesses, medications and allergic diseases. Participants were asked if they had "an active cold sore", "bleeding gums", "tooth ache" or "taken cough syrup", responding "in the last week", "today" or "no". We also asked the participants if they had a chronic disease such as infectious and salivary gland disorders. Finally, we asked the participants if they were smokers or not (19.6% of the sample were smokers).

2.4. Statistical analyses

We investigated the association between salivary and serum CRP using parametric (Pearson *r*) and nonparametric (Spearman *r*) in order to explore and quantify the sensitivity of the reported results to even marginal deviations of CRP levels from the normal distribution. The confounding effects of sex or age were tested using partial correlation analyses. To test whether the accuracy of CRP measures in the saliva depended on the systemic inflammation levels, we explored whether the strength of the association between salivary and serum CRP differed for participants with serum CRP concentrations below or above the mean using Pearson and Spearman correlations. Finally, we tested the capacity of salivary CRP to correctly classify participants' membership to a clinically relevant dichotomous index of serum CRP using the area under the Receiver Operating Characteristic (ROC) curve analysis (Pencina and D'Agostino, 2004). Briefly, the area under the curve (or *C* statistic) is calculated as a function of the sensitivity and specificity of a proposed "predictor" and represents the probability to correctly assign participants to high/low serum CRP groups in comparison to a null model (diagonal of reference: *C* statistic = .5). A *C* statistic of .85 generally indicates a good discrimination (Cook, 2007; Pepe et al., 2004; Polanczyk et al., 2001).

To further validate the measurement of CRP in saliva, we tested if elevated salivary CRP was associated with known correlates of systemic inflammation, such as IL-6, BMI and smoking. Specifically, we used Mann–Whitney U (BMI and IL-6; continuous measures) and chi-square (smoking; dichotomous measure) tests to determine if participants with high levels of salivary CRP had higher IL-6 concentrations, BMI and were in higher proportion smokers. We then tested the sensitivity of these analyses by exploring the associations between the continuous salivary CRP measure and the above correlates of systemic inflammation through Spearman correlations.

To test whether the length of time saliva samples were left at room temperature affected the stability of salivary CRP, we examined the association between CRP measures derived from four experimental conditions (0, 8, 24 and 96 h) using Pearson and Spearman correlations. We also tested whether the average salivary CRP levels varied significantly between these conditions using Wilcoxon signed-rank test. Variations in salivary CRP mean levels between each condition were described using means of percentage of change scores (e.g., $((CRP_{t1} - CRP_{t2})/CRP_{t1}) * 100$).

3. Results

Salivary CRP concentrations ranged from 117.69 to 24156.00 pg/ml (mean (SEM) = 2994.68 (573.35)). From the total sample ($n = 61$), 10 participants had salivary CRP values that exceeded 2 SD from the mean reported in healthy adults by the manufacturer (2 SD = 6163.46 pg/ml) (Salimetrics). For these individuals, salivary CRP values represented up to 10.25 times the SD. Similar high values were not observed for serum CRP (range = .07–11.68 mg/l; mean (SEM) = 2.02 (.31)). We conducted a series of tests to explain these high salivary CRP values. First, we examined whether these values were due to poor accuracy of measurement at high CRP concentration. The 10 extreme values were serially diluted at 10×, 20×, 40× and re-analyzed. The observed values across all dilutions and individuals ranged from 90.82% to 135.66% of the expected values, with a mean of 102.45%. Although most high CRP samples' dilutions followed a linear scale, greater deviations from linearity were found for one individual (mean = 134.52%). Overall, these findings suggest that the high values were not due to poorer precision of the assay for samples with CRP values beyond the dynamic range of the assay. Second, we tested whether saliva flow rate induced higher salivary CRP concentrations. Saliva flow rate did not correlate with salivary CRP levels ($r_s = .07$, $p = .61$). Third, we considered the possibility that small blood leakages could have resulted in large deviations of salivary CRP given the low salivary-to-serum CRP ratio. Blood contamination (measured by transferrin levels) did not correlate with salivary CRP levels ($r_s = .17$, $p = .34$). Fourth, we investigated whether saliva pH compromised the determination of salivary CRP. No significant association between saliva pH and salivary CRP was detected ($r_s = .001$, $p = .99$). Fifth, we examined whether health conditions or medications reported by the participants influenced salivary CRP levels. We did not find such effects. The 10 extreme values could not be explained by biological and clinical variables and were excluded from the rest of the analyses. After the exclusion, salivary concentrations ranged from 113.69 to 6131.40 pg/ml (mean = 1293.28, SEM = 140.61). Excluding those 10 participants did not affect the serum CRP distribution. Mean serum CRP did not differ before and after removing these participants (mean (SEM) = 2.02 (.31) and 2.01 (.30), respectively).

3.1. Associations between salivary and serum CRP levels

As shown in Fig. 1, the correlation between serum and salivary CRP was $r = .72$ ($r_s = .63$). The association between serum and sali-

vary CRP did not vary according to sex (*partial* $r = .73$, $p < .001$) or age (*partial* $r = .73$, $p < .001$). The saliva-to-serum ratio was low (1:1633.64). The following equation can be used to predict serum CRP from saliva: $y = 1553.15\ x - 1413.19$, where y = serum CRP and x = saliva CRP. A lower correlation was found at low serum CRP levels (i.e., ⩽mean; $r = .32$, $p = .06$ and $r_s = .31$, $p = .07$) in comparison to high serum CRP levels (i.e., >mean; $r = .75$, $p < .001$ and $r_s = .66$, $p = .006$), suggesting that the prediction of serum CRP levels from salivary CRP levels is more accurate at higher serum mean levels. Finally, the estimated *C* statistic indicated that salivary CRP discriminated accurately participants below or above a clinically relevant serum CRP cut-off (3 mg/l) ($C = .89$, SE = .05, $p < .001$; 95% CI = .79–.99). This finding indicates that salivary CRP concentrations predicted accurately 89% of participants with high serum CRP levels, which is considered a good measure of discrimination.

We then computed a dichotomous index of salivary CRP corresponding to the serum CRP values below or above 3 mg/l based on the regression equation described above (salivary CRP cut-off = 1629.39 pg/ml; 21.6% of the sample). Serum and salivary CRP dichotomous indices were significantly associated (χ^2 (1, $N = 51$) = 14.67, $p < .001$).

3.2. Associations between salivary CRP and known correlates of systemic inflammation

Fig. 2 shows the associations of salivary CRP with known correlates of systemic inflammation. Participants with high salivary CRP levels had higher serum IL-6 levels than participants with low salivary CRP levels (mean (SEM) = .66 (.13) and .43 (.06) pg/ml, respectively; $U = 129.00$, $Z = 1.92$, $p = .055$). Similarly, individuals with high salivary CRP levels had higher BMI than participants with low salivary CRP levels (mean (SD) = 26.98 (4.09) and 23.38 (3.97), respectively; $U = 104.00$, $Z = 2.66$, $p = .008$). Finally, participants with high salivary CRP levels were smokers in higher proportion than those with low salivary CRP levels (46% vs. 18%; $\chi^2 = 3.75$, $p = .05$).

To further validate the salivary CRP measure, we tested the sensitivity of the previous analyses by exploring the association between the continuous salivary CRP measure and IL-6 levels and BMI. Fig. 3 shows that salivary CRP was positively correlated to IL-6 levels ($r_s = .30$, $p = .04$). Although the continuous measure of salivary CRP was not significantly associated with BMI ($r_s = .19$, $p = .18$), the association followed the same direction than those observed with the dichotomous index of salivary CRP.

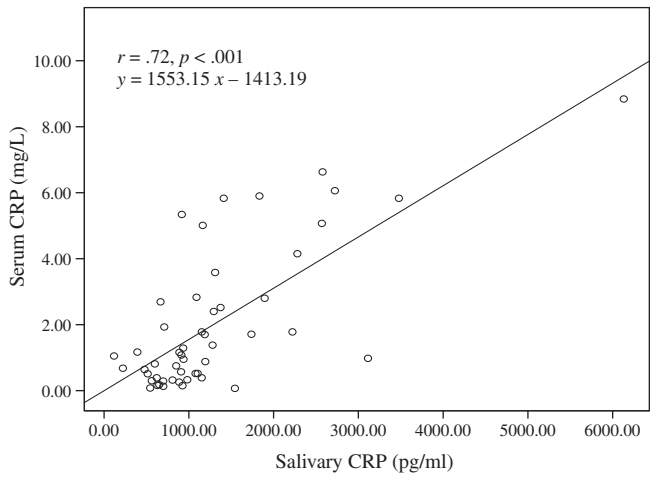

Fig. 1. Association between salivary and serum CRP levels. *Note:* The solid line indicates the regression equation between salivary and serum CRP levels: $y = 1553.15x - 1413.19$, where y = serum CRP level and x = saliva CRP level.

3.3. Salivary CRP levels according to different storage conditions

Table 1 shows Pearson and Spearman correlation coefficients between the four conditions and in association with serum CRP. Specifically, the correlations between serum and salivary CRP remained stable during the first 24 h but declined 96 h after collection, suggesting that freezing the saliva samples on the day of collection is optimal for preserving the participants' rank within the group. Furthermore, the mean salivary CRP levels ascertained from samples frozen immediately upon collection did not differ from those left at room temperature for 8 h (% of decrease = 5.19; $z = 1.79$, $p = .07$). In contrast, lower mean levels were observed subsequently in comparison to the baseline condition (24 h: % of decrease = 11.87, $z = 3.17$, $p = .002$; 96 h: % of decrease = 13.89, $z = 3.14$, $p = .002$). These findings indicate that freezing saliva samples within 8 h from collection may optimize the quantification of salivary CRP.

4. Discussion

The present study aimed to validate the measurement of CRP in saliva, which could constitute a non-invasive alternative marker of systemic inflammation. We observed a moderate-to-strong association between CRP measured in saliva and serum. Furthermore, a dichotomous index of salivary CRP evidenced differences in known correlates of serum CRP (IL-6, BMI and smoking) (Danesh et al., 1999). Our study provides initial evidence suggesting that non-invasive assessment of CRP in saliva allows the valid prediction of serum CRP. Several findings support that salivary CRP is a valid marker of systemic inflammation. We found a moderate-to-strong association between CRP measured in saliva and serum specimens. Consistent with that result, strong saliva and serum CRP correlations were reported in animal studies, such as in healthy ($r = .87$) and diseased dogs ($r = .84$) (Parra et al., 2005) and in pigs ($r = .73$) (Gutierrez et al., 2009). Our finding was however inconsistent with the absence of association found between serum and salivary CRP in medical students (Dillon et al., 2010). According to the range of salivary CRP reported in this study (50–64,300 pg/ml), it is likely that a number of participants had salivary CRP values that exceeded 2 SD from the mean reported by the manufacturer (6163.46 pg/ml) and had values that were well beyond the dynamic range of the assay (3000 pg/ml). Whether an association between serum and salivary CRP levels was present for participants with salivary values falling within the expected range is open to question. Moreover, the stronger association ($r = .71$) reported for participants with high serum CRP levels (10 mg/l) was interpreted as an indication that salivary CRP should be used only with participants with known inflammation-related conditions (Dillon et al., 2010). Alternatively, a stronger correlation may also be observed by including participants with high serum CRP levels in opposition to those with high salivary CRP levels. More research is needed to clarify this issue further.

Salivary CRP was shown to be a good measure of discrimination for the clinically relevant serum CRP cut-off. Participants with high salivary CRP were more likely to have higher IL-6 levels and BMI and to smoke compared to participants with low salivary CRP. Our findings suggest that the dichotomous index of salivary CRP may represent an alternative marker of cardiovascular risk in adults, although more research is needed to establish the relevance and cost-effectiveness of these inflammatory markers in clinical practice (Pearson et al., 2003). More generally, the use of a clinically-relevant index of salivary CRP may facilitate the prospective measurements of CRP in large epidemiological samples and contribute to understand the mechanisms by which inflammation may be associated with later health trajectories. A clinically-rele-

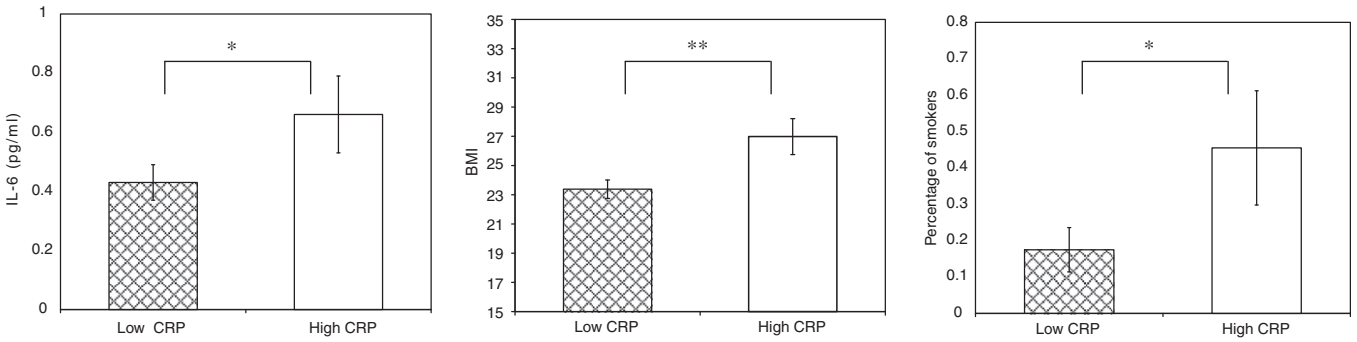

Fig. 2. Statistical descriptives of known correlates of systemic inflammation according to low and high salivary CRP. (A) Serum IL-6 (mean ± SEM), (B) BMI (mean ± SEM) and (C) smoking (yes/no). *Note:* * = $p < .05$; ** = $p < .01$.

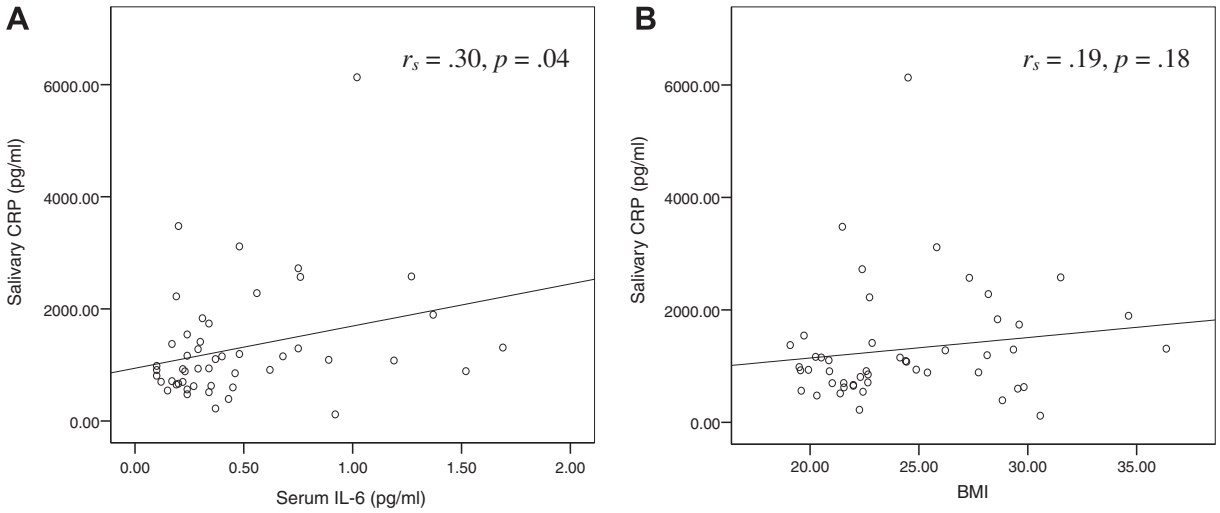

Fig. 3. Association between salivary CRP levels and known correlates of systemic inflammation. (A) Serum IL-6 and (B) BMI.

Table 1
Pearson's (r) and Spearman's (r_s) correlations between CRP measures derived from serum and saliva samples frozen immediately after collection (baseline) or after 8, 24 and 96 h left at room temperature ($n = 51$). *Note:* All Pearson's (r) and Spearman's (r_s) correlations are significant at $p < .001$.

	Saliva CRP (baseline)	Saliva CRP (8 h)	Saliva CRP (24 h)	Saliva CRP (96 h)
Serum CRP (baseline)	$r = .72$ $r_s = .63$	$r = .73$ $r_s = .63$	$r = .70$ $r_s = .64$	$r = .68$ $r_s = .49$
Saliva CRP (baseline)		$r = .91$ $r_s = .75$	$r = .92$ $r_s = .83$	$r = .84$ $r_s = .62$
Saliva CRP (8 h)			$r = .89$ $r_s = .80$	$r = .75$ $r_s = .50$
Saliva CRP (24 h)				$r = .85$ $r_s = .59$

vant index of salivary CRP may also be integrated to a broader multi-systemic characterization of allostatic load in conjunction with other biomarkers (e.g., cortisol, epinephrine, norepinephrine and dyhydroepiandosterone) (Juster et al., 2010). From a more practical point of view, we have shown that CRP in saliva is stable at room temperature up to 8 h after collection, although refrigerating or freezing the samples beforehand may be preferable to optimize the accuracy of salivary CRP quantification. This finding suggests that salivary CRP appears sufficiently stable at room temperature to measure inflammation in the participants' natural settings. Moreover, because previous studies have shown that serum CRP does not have a circadian rhythm (Pearson et al., 2003; Pepys and Hirschfield, 2003), saliva sampling aimed to measure salivary CRP does not require to follow a standardized time collection schedule to avoid diurnal variations.

The validation of salivary CRP measure could be improved in future studies by exploring three unresolved issues. First, we could not explain why 10 participants had salivary CRP values that exceeded 2 SD from the mean reported in healthy adults by the manufacturer. The calibration of the salivary assays, salivary flow rate, blood contamination, saliva pH and health conditions could not explain these extreme salivary CRP values. We speculate that local inflammatory processes could have triggered disproportionately high CRP levels in saliva but not in the blood. Although no cross-reactivity between salivary CRP and IL-6 was detected by the manufacturer (Salimetrics), confounding effect of other salivary biomarkers of periodontal diseases, such as the matrix metalloproteinase-8, cannot be excluded at this point (Herr et al., 2007). Our finding suggests that salivary CRP determination using ELISA requires further validation, especially when values greater than the expected range for healthy adults are expected.

Second, the mechanisms through which CRP is carried from serum into saliva remain unclear. We observed a low saliva-to-serum CRP ratio, which could reduce the precision of salivary CRP measures, especially at low serum CRP concentrations. A low ratio was expected given the CRP chemical proprieties and the mode of entry of CRP in the saliva. CRP is lipid-insoluble and of high molecular weight (Oliveira et al., 1979) which means that entry by diffusion or ultrafiltration (i.e., transfer through the tight junctions between the cells) is unlikely (Kaufman and Lamster, 2002).

For proteins, large molecules or charged steroids, the principal route of entry into the oral cavity is via plasma exudates from gingival crevicular fluid (Vining and McGinley, 1987). As a result, the determination of salivary CRP could be contaminated by small blood leakages or crevicular fluid overflow due to microinjuries or in participants with poor oral health. Future studies should include a complete oral health examination as a part of the participants' health assessment to eliminate the possibility that buccodental features trigger high CRP levels in saliva in the absence of elevations in the serum. Future studies could also provide additional support for the validation of salivary CRP through the demonstration that an experimentally induced inflammation triggers similar changes (direction and magnitude) in serum and salivary CRP.

Third, given the low saliva-to-serum CRP ratio, it is possible that high-sensitivity ELISA may not be sensitive enough to precisely quantify CRP in saliva, particularly at low concentrations. Other analytical methods have been used to determine CRP from saliva, including time-resolved immunofluorometric assays (Gutierrez et al., 2009; Parra et al., 2005), surface plasmon resonance immunosensor (Meyer et al., 2006), magnetic immunosensor (Meyer et al., 2007), microfluidic immunosensor chips (Henares et al., 2008) and lab-on-a-chip devices (Christodoulides et al., 2007, 2005). For example, the lab-on-a-chip system yields five times lower limit of detection than the ELISA technique (Christodoulides et al., 2005). While these analytic strategies may be more sensitive to low-grade inflammation than ELISA, most remain under development and thus are not readily accessible to researchers. It should be explored further whether the collection of larger saliva volume or parotid saliva may optimize the measurement of CRP in the saliva (Kaufman and Lamster, 2002; Quissell, 1993).

Our study provides initial support for the validation of salivary CRP as an alternative marker of inflammation using a broadly available technology adapted for saliva specimens. Saliva sampling is non-invasive, stress-free, can be easily performed in the participants' natural settings and can be repeated over time. Moreover, saliva collection has considerable economical and logistic advantages over venipuncture because it does not require immediate manipulations, access to specialized laboratory equipments and qualified personnel. Replication in larger samples is however needed. Furthermore, future studies should extent the present findings and validate the measurement of salivary CRP in populations with expected high or low CRP levels such as for patients with chronic inflammatory disorders or young children. If replicated, salivary CRP may facilitate the prospective assessment of low-grade inflammation in large epidemiological studies and contribute to better understand the pathways mediating exposure to early adversity and later health inequalities.

Conflict of interest statement

None declared.

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