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Salivary Markers of Inflammation in Response to Acute Stress

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Abstract

There is burgeoning interest in the ability to detect inflammatory markers in response to stress within naturally occurring social contexts and/or across multiple time points per day within individuals. Salivary collection is a less invasive process than current methods of blood collection and enables intensive naturalistic methodologies, such as those involving extensive repeated measures per day over time. Yet the reliability and validity of saliva-based to blood-based inflammatory biomarkers in response to stress remains unclear. We review and synthesize the published studies that have examined salivary markers of inflammation following exposure to an acute laboratory stressor. Results from each study are reviewed by analyte (IL-1β, TNF-α, IL-6, IL-2, IL-4, IL-10, IL-12, CRP) and stress type (social-cognitive and exercise-physical), after which methodological issues and limitations are addressed. Although the literature is limited, several inflammatory markers (including IL-1β, TNF-α, and IL-6) have been reliably determined from saliva and have increased significantly in response to stress across multiple studies, with effect sizes ranging from very small to very large. Although CRP from saliva has been associated with CRP in circulating blood more consistently than other biomarkers have been associated with their counterparts in blood, evidence demonstrating it reliably responds to acute stress is absent. Although the current literature is presently too limited to allow broad assertion that inflammatory biomarkers determined from saliva are valuable for examining acute stress responses, this review suggests that specific targets may be valid and highlights specific areas of need for future research.

Keywords

biomarkers; inflammation; cytokines; saliva; acute stress; psychological stress; exercise; ecological momentary assessment; methods

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1. Introduction

Over the past decade, the relevance of inflammation in multiple health domains has become increasingly apparent. Although inflammatory processes are a critical aspect of immune function (Souëf et al., 2000), their dysregulation (e.g., chronically high levels or slow return to recovery following challenge) is associated with chronic inflammatory and autoimmune disease, as well as a wider range of deleterious outcomes (Miller et al., 2007; Miller et al., 2009; Segerstrom, 2010). Psychological stress and emotional experiences can influence inflammatory responses directly through stimulation of the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympathetic-adrenal-medullary (SAM) system, or indirectly via eliciting dynamic changes in health behaviors (Cohen et al., 2007). In order to better understand the etiology, time course, and consequences of inflammatory processes and to develop better interventions, there is a need for new methods of assessing inflammation that can be conducted in ecological contexts, across time, and at multiple moments throughout the day. Neither the gold standard of obtaining inflammatory measures from venous puncture (drawing blood) nor the relatively new methods involving finger prick (blood spot; McDade et al., 2007) fully meet these needs. The recent advent of determining inflammation from saliva has opened the possibility of studying inflammatory processes in saliva in response to acute stressors. In spite of the promise of this approach, findings on salivary measures of inflammation have been inconsistent, and the evidence available is derived from a very diverse array of methodologies/protocols. A review of the studies conducted in this domain is needed to clarify the degree to which inflammatory processes can be reliably determined from saliva, how and with what timing these inflammatory biomarkers respond to stress, and what additional research is needed.

It is well established that exposure to acute laboratory stressors can influence inflammation; most of these studies, however, have assessed inflammation in serum or plasma (e.g., Altemus et al., 2001; Heesen et al., 2002; Heinz et al., 2003; Steptoe et al., 2001). A small number of empirical studies (n = 13; Campisi et al., 2012; Dugue et al., 1996; Filaire et al., 2010; Groer et al., 2010; Ilardo et al., 2001; Izawa et al., 2013; Lester et al., 2010; Mahmood & Ibrahim, 2013; Mastrolonardo et al., 2007; Minetto et al., 2005; Minetto et al., 2007; Usui et al., 2012; Zefferino et al., 2006) have investigated markers of inflammation in saliva in response to an acute stressor, using a variety of specific analytes: interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-2, IL-4, IL-10, IL-12, and C-reactive protein (CRP). These studies have assessed different biomarkers, employed varying timelines and collection techniques, reported different detectability limits, and utilized different stressors. Thus, a narrative review of stress-induced inflammatory responses in saliva is needed to elucidate patterns in what has been found so far, as well as to highlight inconsistencies and knowledge gaps in the current literature.

An examination of studies assessing salivary inflammatory responses to stress may also identify and help illuminate some validity issues regarding the assessment of salivary inflammatory biomarkers. The degree to which saliva-based inflammatory markers correspond to blood-based markers is unclear in both a resting state and in response to stress. Although it is beyond the scope of the current paper to review all studies that have

examined the association between salivary and blood-derived inflammatory markers, we describe these associations in the small subset of the studies we review that have examined inflammatory responses to acute stress in both saliva and blood (Minetto et al., 2005; Minetto et al., 2007). Even if salivary markers of inflammation do not map clearly onto peripheral blood measures of inflammation, understanding if and how they increase with stress may shed further light on their utility in stress research. We also evaluate some of the broader methodological issues relevant to validating salivary inflammatory markers and provide a checklist of recommendations for future studies.

The inflammatory biomarkers included in this review represent those that have been examined in saliva and in response to stress. Although they serve multiple functions, IL-6, TNF-α, IL-1β, IL-2, and IL-12 have some pro-inflammatory actions (Hawkley et al., 2007; Ito et al., 2014; Watford et al., 2003), whereas IL-4 and IL-10 are typically considered anti-inflammatory in nature (Kindt et al., 2006; Stoner et al., 2013). CRP, an acute phase protein, is well-accepted as an important marker of systemic inflammation (Du Clos, 2000). Only studies that measured one or more of these biomarkers in saliva are included in the primary review. Other biomarkers that can be determined from saliva but which are less relevant to inflammation will not be included in this review. For example, salivary alpha amylase (sAA), which appears to capture components of sympathetic nervous system activity (Nater et al., 2005), is not reviewed; its role in response to stress has been reviewed separately elsewhere (Granger et al., 2007). Studies measuring salivary immunoglobulin A (sIgA), which plays an important role in mucosal immunity (Tsujita & Morimoto, 1999), and neuroendocrine markers like chromogranin A (CgA; Kanamaru et al., 2006; Yamakoshi et al., 2009) are also not reviewed here; biomarkers such as sAA, sIgA, and CgA are relevant to inflammation in the sense that the endocrine and sympathetic nervous systems are connected to the immune system, but are not themselves direct measures of inflammation (Nater & Rohleder, 2009).

To summarize, this review is structured to provide information about which inflammatory biomarkers can be determined in saliva and how these markers respond to stress, with the goal of better understanding the utilization of these biomarkers in stress research. Specifically, the primary aim of this review is to answer the following two questions:

1) Can changes in inflammatory markers be detected in saliva in response to stress and, if so, which appear to respond most reliably?

2) What is the timing of salivary inflammatory biomarker change in response to stress and what are the appropriate assessment time points to best capture stress responses? An additional secondary aim is to enable close examination of factors that influence the detectability of salivary inflammatory biomarkers and their pattern of response to stress – such as salivary flow-rate, saliva collection and handling technique, oral health, hormonal and broad inflammatory confounds, sample size, behavioral and demographic factors, and assay technique – both in terms of interpreting the current literature and with an eye toward future research. These findings will be particularly relevant to research in naturalistic contexts with repeated administration, where traditional methods of sample collection (e.g., blood) are not feasible.

2. Methods

2.1. Search criteria

The present review summarizes all published studies of which we are aware that have assessed salivary inflammatory biomarkers in response to an acute stressor in healthy adult populations. To find these articles, combinations of the search terms “stress,” “acute,” “saliva,” “salivary,” “cytokine,” “inflammation,” “CRP,” and “interleukin” were entered into the US National Library of Medicine National Institutes of Health (PubMed) database. Four of the 13 articles (Groer et al., 2010; Lester et al., 2010; Mastrolonardo et al., 2007; Zefferino et al., 2006) were found from these search terms, and the additional nine references (Campisi et al., 2012; Dugue et al., 1996; Filaire et al., 2010; Ilardo et al., 2001; Izawa et al., 2013; Mahmood & Ibrahim, 2013; Minetto et al., 2005; Minetto et al., 2007; Usui et al., 2012) were found from the references or related references of these first four articles.

2.2 Structure and organization of review

The review is organized by biomarker to best illustrate the manner in which each biomarker may relate to stress. Because the *type* of stressor likely affects the biological response, we further divided our review into two broad categorizations of stressor: social-cognitive stressors and exercise-physical stressors. The social-cognitive stressors included the Trier Social Stress Test (TSST), a commonly used laboratory stressor that involves a combination of mental arithmetic, speech-giving, and social threat (Kirschbaum et al., 1993). Other social-cognitive stressor paradigms included mental arithmetic alone, virtual reality situations, stressful work situations, and cognitively difficult tasks. The exercise-physical stressors included exposure to various types of exercise, competitive rafting, heat (sauna), and the cold pressor task (exposure of a foot or hand to ice water). Studies that examined immune responses in relation to chronic stress (e.g., caregiving) were not included, as chronic stress elicits different inflammatory responses than does acute stress (McEwen, 1998), and such studies would not aid our goal of determining the feasibility of salivary diagnostics for the examination of acute stress responses.

At the start of each section, a brief overview of each biomarker’s immunological function and relation to health is provided. Study results are then reviewed, including sample characteristics, timeline of sampling (time points taken after the stressor are designated as the time after the *completion* of, not the start of, the stressor), and whether, when, and how each biomarker responded to stress. Table 1 is organized by study and provides a comprehensive review that includes information about sample characteristics, what was controlled, the sampling timeline, the stressor type, the sample collection and assay details, the results by biomarker, and study limitations. Following the review of specific studies, we provide a broad overview and synthesis covering the limitations of the summarized studies as well as important factors to take into consideration for future studies that utilize salivary markers of inflammation (Table 2).

2.3 Effect sizes

Effect sizes (Hedge's *g*) were computed for all significant repeated measures results and are reported in Table 1. Hedge's *g* was computed according to published methods, by subtracting the mean of the baseline time point from the mean of the post-stressor time point(s) for each biomarker and dividing that number by the pooled standard deviation (root mean square of the two standard deviations) of the two time points (Hedges, 1981). The Hedge's *g* statistic was used instead of the more conventional Cohen's *d* statistic because it performs better with small sample sizes (Hedges, 1981). Of the 11 manuscripts reporting at least one significant result, six did not have sufficient information within the manuscripts to calculate effect sizes; the corresponding authors from these studies were contacted for more information, yielding enough information for the calculation of effect sizes of two more studies. Therefore, a total of seven studies had significant findings as well as sufficient information to calculate effect sizes. As these are within-subjects effect sizes, where values of greater than three can be obtained when mean differences are large and standard deviations are low, typical conventions for interpreting size of effect are less meaningful; the effect sizes reported in Table 1, however, are standardized and are useful for comparing results across studies (Dunlap et al., 1996; Morris & DeShon, 2002).

3. Results

3.1 Interleukin-1 Beta (IL-1β)

IL-1β is a pro-inflammatory cytokine and is produced by macrophages, microglia, non-immune cells (including muscle cells), and B and T cells as part of the innate immune response(Dinarello, 2009; Kindt et al., 2006). It is an important player in the inflammatory cascade and promotes the release of both TNF-α and IL-6. In serum and plasma, IL-1β has been shown to increase in response to a Stroop and mirror tracing task (gene expression; Brydon et al., 2005), an oral presentation (Heinz et al., 2003), a TSST (Altemus et al., 2001; Bower et al., 2007), and a speech task (Ackerman et al., 1998). IL-1β is implicated in the development of autoimmune diseases (Dinarello, 2009), such as rheumatoid arthritis (RA) (McInnes & Schett, 2007), and appears to be a marker of clinical instability in patients with cardiovascular disease (Stoner et al., 2013). IL-1β is also involved in sleep onset and regulation (Clinton et al., 2011) as well as pain sensitivity (Hutchinson et al., 2008; Mika et al., 2013; Watkins & Maier, 2002) and elevated levels have been observed in individuals with depression (Raison et al., 2006).

3.1.1 Social-Cognitive Stressors and IL-1β—As part of a larger study of patients with and without psoriasis, Mastrolonardo et al. (2007) exposed 50 control participants without psoriasis (matched on age, oral contraceptive use, educational level, smoking use and gender; mean age 39.8 ± 10.6 years; 56% female, $n = 28$ women) to a 5 minute mental arithmetic stressor and a 5 minute Stroop color word naming stressor, a task where participants have to name the color of a color word (e.g., "blue") that doesn't match the color in which it is displayed. Salivary samples were collected immediately before the start of the stressors (after a 5 minute baseline relaxation period) and 10 minutes after the stressors ended. A significant group-by-time interaction ($p < 0.01$) was reported, with increased IL-1β levels following the stressor completion in healthy participants.

Zefferino et al. (2006) measured salivary IL-1β in 30 policemen (mean age of 44.5 ± 5.8 years; all males; average experience of 17.1 ±6.4 years) at the beginning (8:00am) and end (1:30pm) of a shift during a work day and during a vacation period at the same intervals. IL-1β concentrations were lower at the end of the work shift compared to at the beginning of the work shift during the work day period ($p < 0.05$), and there was a non-significant trend for IL-1β reduction during the vacation period compared to work shift period.

Mahmood and Ibrahim (2013) measured salivary IL-1β in 24 healthy dental students (50% female, $n = 12$) during three exam periods-- one month before mid-year exam, during the mid-year exam, and one month after the mid-year exam. All sessions occurred between 8:00am and 12:00pm and were collected via passive drool. IL-1β levels were statistically higher during the mid-year exam period as compared to before and after the exam period ($p < .01$).

3.1.2 Exercise-Physical Stressors and IL-1β—Usui et al. (2012) exposed 10 physically active participants (mean age 23 ± 3 years; all male) to a 60 minute exercise stressor of biking on a stationary bicycle (recumbent ergometer at 75 % V4 O2 max) and to a 60 minute resting control condition (reading or writing silently) on different days at least seven days apart (order was randomized). Saliva samples were taken immediately before, immediately after, as well as one hour (60 minutes), and two hours (120 minutes) after task completion. Levels of IL-1β significantly increased during the exercise stress session compared to the control resting session ($p < .01$), with levels highest immediately after the completion of the 60 minute stressor. Levels remained elevated at 60 minutes post-task completion (though significantly reduced from the above peak) and returned to baseline by 120 minutes post-task completion.

Dugue et al. (1996) exposed 14 “subjectively healthy” participants (mean age 34.5 years; 57% female, $n = 8$) to the heat of a sauna for 10 minutes (at 90°C with 40% humidity) and to a control resting condition on a different day (order was randomized). On the stressor day, saliva samples were taken after a 15 minute resting baseline period, which started at 6:30pm, and then immediately after the completion of the 10 minute sauna heat exposure. On the control day, saliva samples were taken after a 15 minute resting baseline period, which also started at 6:30pm, and then immediately after an additional 30 minute resting period. IL-1β levels showed a non-significant tendency to increase from baseline (pre-sauna) to post-sauna ($p = .11$).

Ilardo et al. (2001) exposed 30 participants (mean age 20.6 years; 33% female, $n = 10$) to control training sessions over the first two days of a three day period and to a one-hour competitive rafting competition (i.e., the stressor; one session on the 3rd day). Saliva samples were collected between 2:00-3:00pm at the first one-hour training session on day 1, between 10:00-11:00am at the second one-hour training session on day 2, and before (at 3:00pm) and immediately after (at 4:00pm) completion of the one-hour competitive rafting session on day 3. To determine circadian rhythms, two saliva samples were also collected at 8:00am & 6:00pm each day. There was a significant increase ($p = .01$) in IL-1β concentrations before and after the stressor (on day 3) but not after the control sessions (days 1-2).

3.2 Tumor Necrosis Factor-Alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine produced by macrophages, natural killer (NK) cells, microglia, and other cell types (Kindt et al., 2006; Mika et al., 2013). It is an important player in the inflammatory cascade and promotes the release of both IL-1β and IL-6. It also plays a role in the acute phase response and systemic inflammation and is involved in the creation of new blood vessels, the regulation of cell differentiation, and programmed cell death (Kindt et al., 2006). In serum and plasma, TNF-− has been shown to increase in response to a TSST (Altemus et al., 2001; Bower et al., 2007), an anger-recall task (Suarez et al., 2006), a speech task (Ackerman et al., 1998), and mental arithmetic, Stroop, and public speech tasks (Heesen et al., 2002). Consistently high levels of TNF-α are often interpreted as evidence of dysregulation and have been shown to be related to a variety of chronic diseases, including Alzheimer's disease (Minagar et al., 2002), major depression (Khairova et al., 2009; Tuglu et al., 2003), certain cancers (Balkwill, 2006), multiple sclerosis (Minagar et al., 2002), cardiovascular diseases (Cesari et al., 2003; Stoner et al., 2013), and general frailty (Hubbard et al., 2009). TNF-α also plays a role in the activation of prostaglandins and other substances related to pain perception (Watkins & Maier, 2002) and is a primary mediator of chronic pain (Mika et al., 2013) as well as an important clinical indicator of inflammation in autoimmune diseases (Sfikakis, 2010). Changes in TNF-α have also been strongly associated with sleep onset and dysregulation (Clinton et al., 2011).

3.2.1 Social-Cognitive Stressors and TNF-α—Filaire et al. (2010) examined TNF-α responses of nine university professors (mean age 40.85 ± 2.45 years; 22% female, $n = 2$) after having them give a two hour university lecture (a presumptive stressor) and on a control day without the lecture stressor. On the lecture day, saliva samples were taken immediately before (10:00am) and after (12:00pm) the completion of the 2 hour lecture (stressor), as well as 120 minutes (2:00pm) and eight hours after completion (8:00pm). On the control day without the lecture, samples were taken at the same time points. TNF-α levels varied across time ($p = 0.04$) on lecture (stressor) days, with significantly higher concentrations 120 minutes after the lecture compared with pre-lecture levels, and a return to near baseline by 8 hours.

3.2.2 Exercise-Physical Stressors and TNF-α—Usui et al. (2012) exposed participants to a 60 minute exercise stressor (cycling on a stationary bicycle; recumbent ergometer at 75 % V4 O2 max) and to a resting control condition (reading or writing silently) on different days at least seven days apart (order was randomized) (for more detailed methods see above in IL-1β section, as well as Table 1). TNF-α levels significantly increased during the exercise stress session compared to the control resting session ($p < .01$), with levels highest immediately after the completion of the 60 minute stressor. Levels returned to near baseline by 120 minutes post-stressor.

Upon exposing 14 participants to the heat of a sauna for 10 minutes (at 90°C with 40% humidity) and to a control resting condition on a different day at the same time of day (order was randomized), Dugue and colleagues (1996; for more detailed methods see above in IL-1β section, as well as Table 1) found that immediately following exposure to sauna heat

there was a statistically significant increase in TNF-α among males ($p < 0.05$) but not among females.

3.3 Interleukin-6 (IL-6)

IL-6 is produced by a diverse range of cells, including T-cells, B-cells, macrophages, and microglia (Kindt et al., 2006; Mika et al., 2013). It serves as the primary signal for CRP release from the liver as part of the acute phase response and is also involved in inducing cell proliferation, differentiation and programmed cell death (Guzmán et al., 2010; Kindt et al., 2006). Although typically conceptualized as inflammatory within the context of psychological stress and depression, IL-6 possesses both pro- and anti-inflammatory properties (see Hawkley et al., 2007 for a review); an important example of the latter is in the context of physical exercise, where IL-6 can have anti-inflammatory effects on other cytokines, including TNF-a (Starkie et al., 2003; Woods et al., 2009). In serum and plasma, IL-6 has been shown to increase in response to a mental arithmetic task (Edwards et al., 2006), the TSST (Bower et al., 2007; Rohleder et al., 2003; von Kanel et al., 2005), a Stroop and a mirror tracing task (Brydon et al., 2005; Brydon et al., 2004; Steptoe et al., 2002; Steptoe et al., 2001), an anger recall task (Suarez et al., 2006), speech tasks (Goebel et al., 2000), and a 100 second cold pressor task (Roupe van der Voort et al., 2000). Like other pro-inflammatory cytokines, IL-6 is involved in the development and maintenance of auto-immune and inflammatory diseases such as RA (Hirano et al., 1988), diabetes mellitus (Pradhan et al., 2001), multiple sclerosis (MS; Minagar et al., 2002), cardiovascular disease (Stoner et al., 2013), and human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS; Breen et al., 1990). There is also a particularly strong body of research linking IL-6 with clinical depression (Raison et al., 2006). Like IL-1B and TNF-a, IL-6 also plays an important role in pain sensitivity (Mika et al., 2013; Watkins & Maier, 2002) as well as sleep and fatigue (Thomas et al., 2011).

3.3.1 Social-Cognitive Stressors and IL-6—Groer et al. (2010) studied 141 (mean age 34.45 years; 19% female; $n = 27$) police officers with three or more years of experience. All officers initially completed a baseline (control) virtual reality scenario, where participants practiced moving (e.g., walking forward/backward, kneeling) based on instructions projected onto a wall. Officers were then randomly assigned to either a two minute virtual reality motorcycle chase or a six minute virtual reality workplace gun confrontation scenario (it was not reported whether these two scenarios were thought to be comparably stressful). Saliva samples were taken immediately after completion of the baseline scenario, and obtained again 10 and 30 minutes after the completion of the stressor scenarios. There were no significant differences in salivary biomarker levels between the two stressor scenarios at either time, and the authors thus combined the data from both scenarios for analysis purposes. Overall, there was high variability in IL-6, but levels did significantly increase at 10 minutes after the completion of the stressor as compared to baseline in the workplace group.

Lester et al. (2010) measured salivary samples of immune function in 36 first year undergraduate students (females: age 20–35 years, males: age 21–22 years, 94% female, $n = 34$) in an anatomy class during a non-exam and three exam periods. Saliva samples were

taken two weeks before their first exam period (baseline) and then within one hour prior to three anatomy practical exams, each scheduled between 12:30 pm and 1:00pm, approximately 3 weeks apart. IL-6 levels at exam 3 were significantly higher than baseline ($p < 0.05$), but there were no differences between comparisons of other given exams. Although not confirmed by the authors, it is possible that the third exam period was more stressful because it may have carried more weight and/or coincided with final exam periods.

Izawa et al. (2013) measured salivary samples of IL-6 in 50 healthy young adults (mean age 21.2 ± 2.3 years; 22% female; $n = 11$) before and after exposure to the Trier Social Stress Test. All sessions occurred between 2:00pm and 7:30pm and samples were collected by means of the passive drool method after 10 minutes of resting baseline, after a 10 minute preparation period, after a 5 minute speech, after a 5 minute mental math task, and 10, 20, 30, 45, and 60 minutes after the math task stressor ended. IL-6 levels were significantly higher than baseline immediately after ($p < .01$) and 10 ($p < .01$) and 20 minutes after ($p < .01$) the completion of the TSST, with levels returning to near baseline 60 minutes after the completion of the TSST.

3.3.2 Exercise-Physical Stressors and IL-6—Upon exposing participants to both an exercise stressor of biking on a stationary bike (recumbent ergometer at 75% V4 O2 max) for 60 minutes and to a resting control condition (reading or writing silently) on a different day at least seven days apart (order was randomized) Usui and colleagues (2012; see above in IL-1β section and in Table 1 for more details) found that levels of IL-6 significantly increased during the exercise stress session compared to the control resting session ($p < .01$), with levels highest immediately after the completion of the 60 minute stressor. Levels remained elevated at 60 minutes post-task completion (though significantly reduced from the above peak) and returned to baseline by 120 minutes post-task completion.

In the sauna heat study by Dugue and colleagues (1996; see above in IL-1β section and in Table 1 for more details) salivary levels of IL-6 were only detectable in 60% of the samples analyzed; thus, no comparisons in IL-6 levels in response to stress in this study were reported.

Minetto et al. (2005) exposed seven athletes (“endurance-trained;” mean age 29.5 ± 8.0 years; 43% female, $n = 3$) to a controlled bicycling activity (“spinning” exercise task) for three hours from 7:00-10:00pm. Ten additional athletes (two marathon runners, one triathlete, two rowers, two alpine skiers, two body builders, one volleyball player; mean age 27.0 ± 6.9 years; all males) were exposed to a 15 minute warm-up cycling activity and then to a 15 minute isokinetic exercise task consisting of 160 maximal contractions of the knee on a Cybex 6000 device; the total exercise (spinning warm-up plus isokinetic exercise) was 30 minutes. For the bicycling spinning activity, saliva samples were collected 15 and 5 minutes before and then immediately after the completion of the 3 hour stationary cycling exercise task. For the isokinetic knee contraction activity, saliva samples were collected beginning at 4:00pm, 15 and 5 minute before the 15 minute warm-up period on a stationary bike, and immediately after, and then 7, 15, 30, 45, 60, 90 and 120 minutes after the completion of the 15 minute isokinetic stressor. Salivary and serum levels of IL-6 were not correlated at any time points during either activity. For the isokinetic task, salivary levels of

IL-6 showed a slight but non-significant increase immediately following the short exercise activity. For the spinning activity, serum IL-6 peaked approximately 60 minutes after the completion of the exercise, whereas salivary IL-6 levels peaked immediately to 7 minutes after its completion.

In a separate study, Minetto et al. (2007) conducted an exercise stressor paradigm with 15 elite athletes (all males, median age of 23 years). The athletes were exposed to a 15 minute warm-up cycling activity and then to a 25 minute isometric exercise stressor of 160 contractions of the knee; the total exercise (spinning warm-up plus isometric exercise) was 40 minutes. Saliva samples were collected immediately before and immediately after, and then 30, 60, 90, and 120 minutes after completion of the exercise. Baseline saliva was collected by Salivette and passive drool. Although IL-6 levels were highly correlated between the two salivary collection methods ($r = .72$, $p < 0.001$), levels of IL-6 were higher in the passive drool method than the Salivette method and were thus deemed more accurate by the authors. No significant correlations were found between serum and salivary IL-6 levels collected at baseline or post-exercise. Levels of salivary IL-6 (obtained via passive drool) peaked immediately after exercise (which was significantly different from baseline, $p < .05$) and were no longer significantly higher by 30 minutes, returning to baseline by 90 minutes post-task. Serum IL-6 levels were significantly elevated immediately after exercise, and remained elevated until 90 minutes post-task.

3.4 Interleukin-2 (IL-2)

IL-2 is a cytokine produced by T-cells during an immune response; it is involved in the development and proliferation of T-cells and natural killer (NK) cells (Ito et al., 2014; Kindt et al., 2006). In serum and plasma, IL-2 has been shown to increase in response to both an exercise alone plus a mental challenge while cycling task (Huang et al., 2010) and the anticipation of an acute surgery stressor (Schulte et al., 1994). Higher levels of IL-2 and evidence of IL-2 up-regulation have been associated with pain in individuals with neuropathic pain (for review, see DeVon et al., 2014; Mika et al., 2013). A relative balance of IL-2 compared to other cytokines appears important for health and to be related to stress. For example, IL-2 deficiency appears to play a role in autoimmune diseases and the ability of other cell types to fight infection and to destroy infected cells (Hoyer et al., 2008). Like chronic psychosocial stress, sleep deprivation or restriction can result in lower levels of IL-2 relative to other cytokines (as part of what is often interpreted as a Th-1 to Th-2 shift) (Axelsson et al., 2013). First investigated for its role in fighting cancer without strong success, IL-2 treatment is currently being investigated for its utility in immunotherapeutic intervention, particularly for autoimmune diseases (Ito et al., 2014).

3.4.1 Social-Cognitive Stressors and IL-2—Following a two hour lecture (presumably a stressor, compared to a control non-lecture day) in a sample of nine university professors, (Filaire et al. 2010; for more detailed methods, see above in TNF-α section and Table 1) higher salivary IL-2 concentrations were found 120 minutes after the completion of the lecture compared with pre-lecture levels ($p < .05$). No such changes were observed on non-lecture days.

In an exam stressor study, Lester et al. (2010) measured salivary samples of immune function in 36 first year undergraduate students during a non-exam (baseline) and within one hour prior to three anatomy exam periods, each approximately 3 weeks apart (see above in IL-6 section and Table 1 for more detailed methods). IL-2 levels at exam 3 were significantly higher than those at both baseline and exam 1 (both $p < 0.05$), with no differences between exams at other time points.

3.4.2 Exercise-Physical Stressors and IL-2—In their sauna heat study Dugue and colleagues (1996; see above in IL-6 section or Table 1 for more detailed methods) were unable to detect IL-2 in saliva at either baseline or following participants' exposure to sauna heat; therefore no comparisons could be made with that cytokine in this study.

3.5 Interleukin-4 (IL-4)

IL-4 is a classic Th-2 type cytokine that is produced by activated T lymphocytes, mast cells, and granulocytes (Kindt et al., 2006; Stoner et al., 2013). IL-4 has strong anti-inflammatory properties; indeed, it has been called a "prototypic immunoregulatory cytokine" in that it inhibits the expression of inflammatory cytokines (such as IL-1β , IL-6, and TNF-α) as well as regulates antibody production and the differentiation of T cells (for reviews, see Brown & Hural, 1997; Mika et al., 2013). In serum and plasma, IL-4 production has been shown to decrease in response to chronic job stress (Nakano et al., 1998). IL-4 is implicated in chronic inflammation and wound repair through the activation of endothelial cells and fibroblasts (Kindt et al., 2006; Salmon-Ehr et al., 2000) and is elevated in patients with cardiovascular disease (Stoner et al., 2013). Overall, IL-4 appears to be associated with lower expression of symptomatology among individuals with chronic disease. Exercise has been shown to up-regulate IL-4 (and the anti-inflammatory cytokine IL-10) among those with type-2 diabetes (for review, see Stoner et al., 2013) and genetic therapy to enhance IL-4 signaling is being studied as a way to reduce pain (for review, see Mika et al., 2013) and breathing difficulties due to obstructive airway diseases (Dasgupta et al., 2013). There also appears to be a linkage between acute stress and IL-4, although the human literature linking stress and other inflammatory cytokines is more robust (Steptoe et al., 2007); for example, anxiety induced by examination stress appears to reduce production of IL-4 and another anti-inflammatory cytokine (IL-10) (Maes et al., 1998).

3.5.1 Social-Cognitive Stressors and IL-4—Filaire and colleagues (2010; for more detailed methods see above in TNF-α section or Table 1) examined responses to giving a two hour lecture in nine university professors. Participants had higher IL-4 concentrations 120 minutes after completion of giving the lecture compared with pre-lecture levels ($p < .05$); no such changes were observed on non-lecture days.

3.5.2 Exercise-Physical Stressors and IL-4—To our knowledge, no studies have been conducted on salivary IL-4 responses to exercise stressors.

3.6 Interleukin-10 (IL-10)

Produced by Th-2 cells, B cells, monocytes, and macrophages, IL-10 is a cytokine that is involved in the proliferation of B-cells as well as the production of IgA, which is the main

immunoglobulin in salivary immunity (Kindt et al., 2006; Stoner et al., 2013). IL-10 has potent anti-inflammatory actions: it acts in an anti-inflammatory manner on macrophages and on vessel walls, and appears to play an important role in atherosclerosis (Stoner et al., 2013). Notably, IL-10 elevations have been related to decreased risk for myocardial infarction and death, although only in those with high CRP (Heeschen et al., 2003). In serum and plasma, IL-10 has been shown to increase in response to an academic examination stressor (Maes et al., 1998). IL-10 also has been observed to rise simultaneously with pro-inflammatory markers in the short-term in response to manipulated stress and pain but then decrease with prolonged pain (e.g., Mika et al., 2013), which may explain why both higher and lower levels of IL-10 have been observed in individuals with greater pain (DeVon et al., 2014). Importantly, IL-10 activation in the central nervous system appears to mitigate pain signaling, and drug therapies to promote IL-10 are being examined to aid in various pain conditions (Shah et al., 2012; Watkins et al., 2007).

3.6.1 Social-Cognitive Stressors and IL-10—Examining responses to giving a two hour lecture (compared to a control non-lecture day) in a sample of nine university professors, Filaire and colleagues (2010; see above in TNF-α section for more detailed methods) found no effect of stress on IL-10 levels.

3.6.2 Exercise-Physical Stressors and IL-10—To our knowledge, no studies have been conducted on salivary IL-10 responses to exercise stressors.

3.7 Interleukin-12 (IL-12)

IL-12 is a cytokine that is produced by monocytes, macrophages, dendrites and other cells, and which strongly regulates adaptive immune responses and induces release of the pro-inflammatory cytokine interferon-γ (IFN-γ) by NK cells and T-cells (Kindt et al., 2006; Watford et al., 2003). Less well-studied than inflammatory cytokines such as TNF-α and IL-1β in human models or even *in vivo* animal models, research is ongoing to determine clinical consequences of IL-12 and its relation to stress and health. Although animal research has suggested that IL-12 and its actions contribute to the development of autoimmune diseases, research suggests that IL-12 and related cytokines have complex anti-inflammatory as well as pro-inflammatory functions (Hunter, 2005; Shaashua et al., 2014; Watford et al., 2003). IL-12 appears to have anti-tumor effects in rodent cancer models (Kanegane et al., 1998) and recent animal research suggests that IL-12 can be suppressed in response to behavioral stress (Shaashua et al., 2014).

3.7.1 Social-Cognitive Stressors and IL-12—In an exam stressor study, Lester and colleagues (2010; see above in IL-6 section and Table 1 for more detailed methods) measured salivary samples of immune function in 36 first year undergraduate students during a non-exam (baseline) period and within one hour prior to three anatomy exam periods, each approximately 3 weeks apart. They found that IL-12 levels progressively increased from baseline to the third exam, with concentrations at exam 2 and exam 3 each being significantly different from baseline and exam 1 ($p < 0.05$).

3.7.2 Exercise-Physical Stressors and IL-12—To our knowledge, no studies have been conducted on salivary IL-12 responses to exercise stressors.

3.8 C-Reactive Protein (CRP)

CRP is an acute phase protein that is secreted by the liver in response to IL-6 (Kindt et al., 2006; Marnell et al., 2005) and which also promotes the production of pro-inflammatory cytokines in response to infection (Du Clos, 2000). CRP activates the complement cascade, which helps mediate protection from bacteria (Marnell et al., 2005). In serum and plasma, CRP has been shown to increase in response to a speech and mirror tracing task (Hamer et al., 2006), an auditory serial addition test while tilted at 64° (Veldhuijzen van Zanten et al., 2005), a mock job interview (Miller et al., 2005), and a Stroop and mirror tracing task (Steptoe et al., 2003). In plasma, CRP is considered a broad biomarker of systemic inflammation of clinical significance outside of the context of infection. Chronically high levels of CRP are a strong independent predictor of cardiovascular disease (Ridker et al., 2000; Stoner et al., 2013), diabetes mellitus (Pradhan et al., 2001) and other chronic diseases, as well as general frailty (e.g., Puts et al., 2005) and depression (Howren et al., 2009). There is a less robust literature showing that CRP rises in response to acute psychological stress than there is for inflammatory cytokines such as IL-1β and IL-6, but a recent meta-analysis showed a marginally significant effect for CRP to increase following exposure to acute stress (Steptoe et al., 2007).

3.8.1 Social-Cognitive Stressors and CRP—Campisi et al. (2012) exposed 15 healthy undergraduate students (18-22 years old; 73% female, $n = 11$) to the TSST. Samples were taken immediately before and immediately following the completion of the stressor and again 30 minutes after the completion of the TSST. Salivary CRP levels were successfully determined, but no significant changes in CRP levels occurred following the TSST.

3.8.2 Exercise-Physical Stressors and CRP—To our knowledge, no studies have been conducted on salivary CRP responses to exercise stressors.

4. Discussion

4.1 Overview

This review focused on the empirical studies conducted to date of stress-induced inflammation as measured in saliva. Its primary goals were to 1) determine if changes in inflammatory markers have been detected in saliva in response to acute stress, and 2) elucidate patterns of change, including which specific biomarkers have been most often shown to respond, the timing of response, and to what types of stressors. This review was also designed to highlight inconsistencies and knowledge gaps in the literature on salivary inflammatory responses to stress and to illuminate what methodological factors and participant characteristics appear most critical in moving this area of research forward. The feasibility of using salivary inflammatory markers to determine physiological stress responses needs to be more fully understood. If determined to be reliable and meaningful, these salivary markers could be used to assess stress responses as they occur in real-time and ecological context, thus better illuminating how psychological stress causes biological

changes as well as new ways to mitigate the often adverse impact that stress can have on physiological health. Provided below is a discussion of the patterns that are apparent from past studies, a review of issues regarding the assessment and interpretation of salivary inflammatory responses, and specific recommended guidelines for future research.

4.2 Does stress induce detectable changes in salivary inflammatory markers; if so, which markers respond most reliably?

The reviewed studies show that certain inflammatory cytokines in saliva appear to increase fairly consistently in response to acute stress. The pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 exhibited the most consistent responses to acute stressors; they showed either a significant or marginally significant increase post-stress in the majority of studies in which they were examined. For example, IL-1β showed a significant increase after social-cognitive stress in one of the two studies in which it was included (Mastrolonardo et al., 2007), during an exam period as compared to two non-exam periods (Mahmood & Ibrahim, 2013), as well as after exercise stress in two of the three exercise studies reviewed (Ilardo et al., 2001; Usui et al., 2012). TNF-α showed a significant increase after giving a lecture (Filaire et al., 2010), after exercise stress (Usui et al., 2012), and after sauna heat exposure among males but not females (Dugue et al., 1996). Significant increases in IL-6 to stress were observed in the majority of studies in which it was successfully measured in saliva; IL-6 showed increases at a final exam time point from an earlier time point (Lester et al., 2010) and 10 minutes after a TSST as compared to baseline (Izawa et al., 2013), as well as in two studies with an exercise stressor (Minetto et al., 2007; Usui et al., 2012). However, IL-6 showed no significant increases in response to a virtual reality stressor (Groer et al., 2010) or to an exercise stressor in a different study (Minetto et al., 2005). IL-2 also increased in response to stress in both studies in which it was detectable: in response to a lecture stress task (Filaire et al., 2010) and from a final exam stressor as compared to an earlier time point (Lester et al., 2010).

In terms of the robustness of the effect of stress on inflammation determined from saliva, there was considerable inconsistency, with Hedges *g* repeated measures effect sizes ranging from .11 (very small) to over 3.0 (very large). It is important to note that some of the stressors utilized in the studies reviewed here were not well-validated. Of the three studies (Campisi et al., 2012; Izawa et al., 2013; Mastrolonardo et al., 2007) that employed a classical laboratory stressor (TSST or similar social evaluative task), two (Izawa et al., 2013; Mastrolonardo et al., 2007) found a post-stress increase of inflammatory cytokines (IL-6 and IL-1β, respectively), with within-subjects effect sizes ranging from .27 to .37 (there was not enough information available from Mastrolonardo et al., 2007 to calculate effect sizes). Of the other ten studies that employed non-validated stressors (Dugue et al., 1996; Filaire et al., 2010; Groer et al., 2010; Ilardo et al., 2001; Lester et al., 2010; Mahmood & Ibrahim, 2013; Minetto et al., 2005; Minetto et al., 2007; Usui et al., 2012; Zefferino et al., 2006), nine (all but Minetto et al., 2005) found a post-stress increase in at least one of the inflammatory cytokines they measured (TNF-α, IL-1β, IL-6, IL-2, IL-4), with within-subjects effect sizes of 1.73 for IL-2 and 1.33 for IL-4 (both from Filaire et al., 2010), and ranging from .12 to 2.53 for TNF-α (Dugue et al., 2006, Filaire et al., 2010, Usui et al., 2012), .16 to 1.65 for IL-6 (Groer et al., 2010 and Usui et al., 2012), and .35 to 9.69 for IL-1β (Mahmood and

Ibrahim, 2013, Usui et al., 2012, Zefferino et al., 2006; there was not enough information from Filaire et al., 2010, Lester et al., 2010 and Minetto et al., 2007 to calculate effect sizes). The type of stressor and the degree to which it successfully evoked an inflammatory response may be critical to understanding such inconsistencies, an issue which is further addressed in section 4.5.1.

In terms of which salivary inflammatory biomarkers can be determined to respond to stress, specific knowledge gaps became apparent from this review. For example, several biomarkers reviewed herein (i.e., CRP, IL-4, IL-10, and IL-12) were assessed in only a single study each, making it impossible to determine whether these biomarkers show consistent patterns in response to stress. IL-12 showed an increase from one sampling point to a final exam point (Lester et al., 2010), as did IL-4 in response to giving a lecture (Filaire et al., 2010); in contrast, IL-10 did not change significantly in response to lecture stress ((Filaire et al., 2010). CRP did not increase following a TSST paradigm (Campisi et al., 2012); however, the time frame of 30 minutes following stress may not have been sufficient to show change in CRP based on what is known of the time-course of CRP detectability in blood (i.e., changes may occur somewhat later, e.g., 120 min post-task) (Hamer et al., 2006; Steptoe et al., 2003). Issues related to the time points of sampling are thus critical, and are discussed further below. It is also possible that the significant overall findings for stress-related increases in inflammation measured through IL-1β, TNF-α, and IL-6 (as compared to less commonly measured markers such as IL-10 and IL-4) may relate to these biomarkers being more commonly measured and/or may represent selective publication of positive findings (publication bias).

Overall, this review suggests that there are detectable changes in salivary inflammatory markers following stress but also that there are a number of specific unknowns that future research could target to help determine which salivary inflammatory markers are most promising and useful. Steptoe and colleagues performed a meta-analysis of blood-derived markers of inflammation in response to acute stress (Steptoe et al., 2007) and concluded that the most robust increases in plasma or serum were in IL-6 and IL-1β (relative to other markers such as TNF-α and CRP). Other studies published since then have shown similar results, particularly among individuals either at risk for or diagnosed with major depression. In a study by Carpenter et al. (2010), after exposure to a TSST, individuals with a history of child maltreatment showed greater plasma IL-6 responses compared to individuals without a history of maltreatment, with both groups' levels of IL-6 peaking 60 minutes after completion of the TSST. A similar study by Pace et al. (2006) showed that exposure to the TSST significantly increased levels of plasma IL-6 along a similar timeline in both individuals with major depression and those without depression (peaking at 60 minutes post stressor completion); depressed individuals had significantly higher levels at this peak. Although the results from the present review examining salivary responses generally match these findings in blood, the limitations of the current data make explicit comparisons somewhat premature. More research is needed using better-validated stressors, longer time-frames, and taking into account additional factors that are discussed below.

4.3 What is the timing of biomarker change in response to stress and what are the appropriate time points for assessment?

More research is needed on the kinetics of inflammatory change in saliva following acute stress to determine the most effective sampling time points, and also to inform specifically how oral inflammation is being elevated/triggered in response to stress. There are two likely routes by which acute stress could lead to an increase in inflammation in saliva. One route is infiltration via blood (for a review see Bosch, 2014), where HPA axis and sympathetic nervous system activation can lead to increases in systemic inflammation (for review see Hawkley et al., 2007).The other route is by acute stress directly affecting oral inflammation in the mouth; numerous cell types in the mouth, particularly in the salivary glands, have functions that are influenced by sympathetic nervous system innervation and are capable of releasing inflammatory cytokines (Schapher et al., 2011). This review highlights the possibility that this latter route may be common. Two of the reviewed studies (Minetto et al., 2005; Minetto et al., 2007) found that acute stress induced an increase in IL-6 levels that occurred earlier in saliva than in blood; levels were higher in saliva as well (Minetto et al., 2007), suggesting that this cytokine may have been secreted locally into saliva, as opposed to it entering saliva by way of blood. Additional research is needed to more clearly identify the mechanisms through which stress increases inflammation in saliva and the timing of these responses.

Many of the reviewed studies obtained only one or two samples, with some collected only up to 30 minutes post-stressor completion. This may not capture the timeframe for peak post-stress responses, and very likely does not capture recovery to baseline. Although the temporal details for salivary assessments of reactivity are as of yet unclear, in blood many inflammatory biomarkers peak anywhere between 60-120 minutes post-stressor completion and take 120-180 minutes to show full recovery to baseline (Steptoe et al., 2007). For example, in serum and plasma, IL-6 has been shown to have stronger effect sizes when samples were taken 30-120 minutes post-stressor completion, as compared to samples taken immediately post-stressor completion (Steptoe et al., 2007). Ideally, a baseline sample should be taken, and additional samples taken immediately after the stressor, and at multiple time points thereafter (optimally for at least two hours post-stressor completion) to demonstrate full recovery to baseline, as was done in several of the reviewed studies (i.e., Filaire et al., 2010; Minetto et al., 2005; Minetto et al., 2007). It is also the case that some cytokines may exhibit different response patterns. For example, anti-inflammatory markers may rise as a compensatory response to inflammation in these models, and thus pro-inflammatory markers may be better for assessing stress effects. Therefore, both the timing of biomarker assessment and the selection of appropriate biomarkers are important for interpretability of findings.

4.4 What might indicators of salivary inflammation signify biologically and clinically?

A secondary goal of the present review was to highlight issues related to the importance and interpretability of findings. Of importance to this effort is to determine what validity or prognostic/clinical value such biomarkers may have for the field. Related to this question are a number of issues that have been brought to light by the current review.

4.4.1 Saliva and blood divergence—For many reasons it would be desirable if salivary inflammation mapped clearly onto blood-derived markers of inflammation and thus yielded a good approximation of what is happening systemically. On the whole, this does not appear to be the case (with some exceptions noted below); several studies have reported poor correlations between saliva and blood for inflammatory markers (Dillon et al., 2010; Riis et al., 2014). Although there is some infiltration of proteins from blood into saliva, as noted above, most proteins do not easily pass the multiple barriers between these two compartments (for a recent review see Bosch, 2014). For example, cytokines are too large to enter saliva via passive diffusion (as cortisol does) and enter instead via leaky patches such as through sites of inflammation or tissue damage (Bosch, 2014). This method of entrance into saliva produces large variability between individuals, which may help to explain the generally poor correlations that have been reported between saliva and blood for inflammatory markers. It is possible that individual differences in oral inflammation may also serve as important indicators of health status.

A number of common (local) issues can substantially increase inflammation in the mouth, and undoubtedly also contribute to a poor overall correlation between inflammation in saliva and blood. These issues include poor oral hygiene, gingivitis, periodontal disease, and mouth injury. Under such conditions, salivary inflammation increases due to higher local production of inflammatory factors and possibly an increased infiltration from blood (Bosch, 2014). Several studies have reported substantially higher levels of inflammatory markers in saliva than in blood (Khan, 2012; Miller et al., 2010; Rahnama et al., 2013), further supporting the notion that salivary cytokines are being produced by local tissues in lieu of (or in addition) to their infiltrating the mouth from the periphery (i.e., from blood). Importantly, a vast majority (>80%) of adults in the United States exhibit gingivitis in one or more teeth, and almost 50% of individuals over age 50 present with some level of periodontal disease (Albandar, 2002). Numerous studies have reported higher salivary inflammation in subjects with periodontal disease compared to healthy controls, as assessed by CRP (e.g., 18.2x higher; Christodoulides et al., 2007) or inflammatory cytokines (e.g., IL-1β, TNF-α 2x higher; Yoon et al., 2012), and treatment of severe gingivitis has been shown to reduce TNF-α and CRP levels in the mouth (Alzahrani et al., 2013). Higher plaque burden (gingivitis) has similarly been associated with higher IL-1β levels in the mouth (Gonzales et al., 2001). Hence, poor oral hygiene (or gum disease) appears to increase salivary inflammation and may thus substantially contribute to reduced associations with these same markers in blood.

A few inflammatory markers have shown correlations between saliva and peripheral blood. For example, IL-1β levels were correlated (r = .11) between saliva and blood assessments in a study of healthy adolescent girls, although a long list of other inflammatory mediators were not (including IL-2, IL-6, IL-8, IL-10, and TNF-α) (Riis et al., 2014). The best example may be salivary CRP, which has been found to correlate with blood levels of CRP in healthy individuals (Ouellet-Morin et al., 2011), women seeking help from domestic violence (Out et al., 2012), as well as in cardiac patients (Floriano et al., 2009; Punyadeera et al., 2011). CRP levels obtained from saliva and blood may be particularly likely to correlate because, unlike cytokines, CRP is synthesized primarily in the liver. Hence, there

is no local production of CRP in the mouth and its most likely route into saliva is via blood; supporting this notion, salivary CRP levels are much more dilute (~1600x) than blood CRP levels (Ouellet-Morin et al., 2011). Another study, however, failed to show a significant association between CRP in saliva and serum (Dillon et al., 2010). One study suggests that salivary CRP is a better indicator of systemic CRP when it is at low levels, whereas at higher levels it is less likely to correlate with levels in blood (Out et al., 2012). A possible explanation for this is that higher levels of CRP in saliva may indicate an abnormally high infiltration from blood due to local inflammation, tissue damage, or poor oral health (Bosch, 2014).

As a final point about saliva and blood divergence, it is important to note that even if saliva-based measures do not map well onto peripheral measures (or have greatly lagged associations) they may yet be predictive of disease and well-being. This is highlighted by research on CRP discussed above. Further research relating salivary inflammation (and salivary inflammatory responses to stress) to health is needed to better determine the clinical relevance of salivary inflammatory biomarkers.

4.4.2 Lagged time associations between systemic and salivary inflammation—

The studies that have focused on correlations between salivary and blood-based inflammation have been conducted with static, baseline levels (i.e., not in response to stress) (Rahnama et al., 2013; Williamson et al., 2012). Salivary inflammatory cytokines may perhaps relate better to blood-based cytokines in the context of stress responses, and/or if examined on a lagged time basis from systemic inflammation. As discussed earlier, some salivary markers of inflammation rise more quickly than blood-derived markers. For instance, two studies (Minetto et al., 2005; Minetto et al., 2007) found that salivary IL-6 levels peaked almost immediately after the completion of an exercise stressor, whereas serum IL-6 levels peaked 60 minutes after the completion of an exercise stressor in one study (Minetto et al., 2005) and had not yet returned to baseline by 90 minutes post-stressor in the other study (Minetto et al., 2007). The finding that these levels rose in saliva *prior* to blood suggests that IL-6 under these conditions is not primarily entering saliva *from* blood. Inflammatory cytokines (e.g., IL-6 or TNF-α) in saliva may map better onto peripheral blood responses that are determined from later time-points (i.e., after saliva collection). In contrast, because it is not produced in the mouth, CRP responses in saliva may best relate to CRP responses in blood, and this may be especially true when saliva collection is somewhat lagged behind blood collection. The present review highlights the need to compare serum and salivary biomarkers not just at the same time points in response to stress but also on a time lagged basis to best assess correlations between these factors.

4.5 Methodological and demographic considerations

There is a need for certain methodological considerations in research dealing with salivary inflammatory responses, several of which may be critical for determining the validity of salivary inflammatory changes in response to acute stress. One factor is to what extent the stressors being utilized are valid and meaningful. The method of saliva collection, salivary flow rates, details relating to the protein assay, pre-existing conditions (such as oral health

and the presence of inflammatory diseases), and a number of demographic variables are also essential factors to consider. Each of these issues is discussed in more detail below.

4.5.1 Stressor type and validation—It is essential to use acute stressors that have been well-documented in terms of their ability to evoke a psychological and physical stress response, and/or collect subjective stress ratings to quantify experiential reactions. Because individuals vary widely in the degree to which they experience any given event as stressful, any new event-related stressor being utilized should be well-validated, ideally with a diverse and large sample. Even public speaking tasks, such as those used by Campisi et al. (2012) and Filaire et al. (2010), which many might assume to be stressful, are likely to evoke a different response in an extrovert versus an introvert, or in a veteran public speaker versus a neophyte. Dickerson and Kemeny (2004) have shown that mental arithmetic alone, for example, may not be sufficient to reliably evoke an HPA-axis response. In terms of social-cognitive stressors, the well-validated and well-known TSST (Kirschbaum et al., 1993) is a good choice for a laboratory-based stressor. The social-evaluative threat component of the TSST, stemming from the perception that one is being evaluated critically on domains of social importance, seems to be critical in activating the HPA-axis, and consequently, evoking cortisol and inflammatory responses (Dickerson et al., 2009). Even with the TSST, however, small variations in protocol can change the degree to which it evokes a physiological response (Dickerson & Kemeny, 2004).

It is also important to acknowledge that differences may exist in the nature of response between physical and social-cognitive stressors. For example, although exercise stressors are associated with an acute rise in IL-6, in the context of exercise, IL-6 appears to have anti-inflammatory effects on other cytokines, including TNF-a, both in peripheral blood and to immune challenge (Starkie et al., 2003; for review, Woods et al., 2009). These and other related findings help explain why those who exercise regularly tend to have significantly lower systemic inflammation, particularly as indexed by broad inflammatory markers such as CRP, even after controlling for demographic factors, mediation use, and pre-existing disease (Woods et al., 2009).

4.5.2 Salivary collection, handling technique, and salivary flow-rate—The method by which saliva is collected can make a difference in terms of values and interpretability of the resulting data. The techniques used to collect saliva varied among the reviewed studies, although the majority (eight) of the 13 studies used Salivettes (Campisi et al., 2012; Dugue et al., 1996; Filaire et al., 2010; Ilardo et al., 2001; Mastrolonardo et al., 2007; Minetto et al., 2005; Usui et al., 2012; Zefferino et al., 2006). Four studies used the passive drool method only (Groer et al., 2010; Izawa et al., 2013; Lester et al., 2010; Mahmood & Ibrahim, 2013), and one study used both Salivette and passive drool methods to collect saliva (Minetto et al., 2007). In comparison to passive drool, levels of salivary testosterone, DHEA, progesterone, and estradiol were found to be higher when collected using Salivettes, whereas salivary IgA levels were lower (Shirtcliff et al., 2001). Another study reported that collection via cotton Salivettes reduced the yield of IL-8 by >80% (Groschl et al., 2008). The materials in these sampling tools (e.g., cotton) may at times partially sequester the analyte of interest. In addition, chewing on a Salivette mechanically

stimulates flow; hence the method of saliva collection appears to alter the obtainable levels for some analytes.

The area of collection in the mouth can affect inflammatory measurements as well. Differences in some cytokines (IL-1α, IL-6, IL-8) have been reported when comparing whole (mixed) saliva to parotid saliva (obtained strictly from the parotid glands) (Ruhl et al., 2004) Collecting unstimulated whole saliva is ideal, as this should minimize the variability in saliva composition that occurs from different glands. Collection of whole unstimulated saliva (e.g., by passive drool as (described by Navazesh, 1993) has another advantage when dealing with inflammatory markers, and particularly when examining stress responses. When saliva is obtained via passive drool, it is possible to determine and control for salivary flow rate (an indicator of how much saliva is produced over a given time). Salivary flow rate is important because it can alter protein concentrations within the sample; higher flow rates typically result in lower protein concentrations. This may have particular relevance in stress-related research because psychological stress (sympathetic activation) slows salivary flow-rate in humans (Matos-Gomes et al., 2010). When possible in stress studies (especially in laboratory-based studies) we suggest controlling for salivary flow rate (by measuring the volume or weight of the sample obtained in a given time) to reduce variability between participants. However, controlling for flow rate is not always possible, particularly in field studies or those employing ecological momentary assessments. It is likely not essential to control for flow rate to have meaningful results, particularly if other factors (see Table 2) are controlled.

Finally, one must consider the potential impact of the specimen collection procedure on sample degradation with regards to the time between sample collection, processing, and storage, and the effects of both long-term storage and repeated freeze-thaw cycles on stored samples. The timing of salivary cytokine degradation is not currently well characterized. However, cytokines are much less stable in saliva than steroids (e.g., cortisol) and have been shown to degrade by up to 70% after a day at room temperature (Groschl et al., 2008). Saliva samples should be refrigerated or placed on ice (in the field) immediately after collection, and frozen as soon as possible. Aliquotting samples into smaller volumes prior to freezing will help to avoid freeze-thaw issues. Because mucins or other salivary components may sequester cytokines (Ng et al., 2007) all saliva samples should be collected, centrifuged, and stored in as consistent a manner as is practical to reduce variability in yields. With the increasing popularity and use of salivary diagnostics in both laboratory and field settings, studies should be conducted to determine the procedures and time lines needed for producing optimum results when quantifying inflammatory markers in saliva.

4.5.3 Assay technique—Most of the assay kits used for saliva analysis in the reviewed studies were not optimized for use with saliva samples but instead were designed for serum or plasma analysis. The majority of studies used an ELISA (Campisi et al., 2012; Dugue et al., 1996; Groer et al., 2010; Ilardo et al., 2001; Izawa et al., 2013; Lester et al., 2010; Mahmood & Ibrahim, 2013; Mastrolonardo et al., 2007; Minetto et al., 2007; Usui et al., 2012; Zefferino et al., 2006), one used an immunoradiometric assay (Minetto et al., 2005), and another used a cytometric bead array kit (Filaire et al., 2010). The saliva matrix is very dissimilar from blood due to factors such as pH, protein load, viscosity, enzymes, and the

presence of various other factors that can increase sample to sample variation, even from the same subject. Optimizing an assay for saliva specifically accounts for the majority of these factors and yields a result that is much more accurate than analyzing saliva using reagents that are optimized for blood. Currently, on the market there are several relatively new kits available that have been optimized for salivary analysis of CRP, IL-6, and IL-1β (Salimetrics). To our awareness, however, Salimetrics assays for IL-6 and IL-1β have not been calibrated against blood; therefore, the correlation between salivary and plasma values for these metabolites is not as clear as it is with CRP. Further salivary-blood validation studies must be conducted to address this limitation in our understanding of what elevations in these biomarkers may mean. Assays for additional analytes, optimized for salivary analyses, are presently under development and should yield more accurate results in salivary research than assays that have been optimized for blood/tissues. Regardless of using a kit optimized for saliva or not, the coefficients of variation (inter- and intra-assay) must be calculated to ensure acceptable reliability of the laboratory measures.

4.5.4 Oral health—As noted in section 4.4.1, oral health is another factor for which it increasingly appears critical to control in studies using salivary inflammatory markers. Only four of the 13 studies controlled for oral health status (Campisi et al., 2012; Filaire et al., 2010; Mahmood & Ibrahim, 2013; Usui et al., 2012), highlighting another need for new standards in this field. Local inflammation (e.g., gingivitis, periodontal disease) may confound measures of salivary inflammation in response to stress. Evidence for this comes from a study by Deinzer et al. (2004) in which 12 medical students in their first clinical semester (mean age 22.3 ± 1.8 years; 50% female, $n = 6$) were randomly assigned to either an “experimental gingivitis” group, where they refrained from brushing different quadrants of the mouth for 28 days, or to a control hygiene condition where they continued dental hygiene as usual. On day 27 and 28 of the protocol, all participants were subjected to either a 30 minute stressor (anticipation period and public speech) or to a magazine reading control condition. Psychological stress and plaque accumulation each independently induced a significant increase in IL-1β concentrations obtained from gingival crevicular fluid (GCF), which is an exudate that occurs in minute amounts between the gingiva (gums) and the teeth and which increases under conditions of local inflammation (Ozkavaf et al., 2000). The effects of stress in this study were amplified under conditions of poor hygiene (Deinzer et al., 2004). Thus, there is some preliminary evidence that poor oral health (plaque accumulation) can inflate stress-related increases in inflammation.

4.5.5 Hormonal and inflammatory confounds—Both menstrual cycle status and hormonal contraceptive use have been shown to influence the HPA-axis and inflammatory responses (Bouman et al., 2005; Engeland et al., 2009; Kirschbaum et al., 1999). Of the eleven studies that had female participants (all but Usui et al., 2012; Zefferino et al., 2006), only one reported controlling for menstrual cycle status (Izawa et al., 2013), and none reported controlling for use of hormonal contraceptives. The presence of inflammatory diseases (e.g., Sjögren’s syndrome, RA, diabetes mellitus) may also alter salivary biomarker levels by increasing levels of inflammation or by reducing salivary flow rate (Kaufman & Lamster, 2002). Seven of the 13 studies (Campisi et al., 2012; Filaire et al., 2010; Ilardo et al., 2001; Izawa et al., 2013; Mahmood & Ibrahim, 2013; Mastrolonardo et al., 2007; Usui et

al., 2012) controlled extensively for the presence of preexisting health conditions, including chronic illness and/or inflammatory diseases. It is important to note that none of these factors – hormonal fluctuations in women, medication use, or chronic disease comorbidities – are easy or straightforward to control. Further, the generalizability of results decreases as more of these factors are controlled (particularly when used as exclusion criteria). Additional research is needed to determine more precisely how important these types of factors are for interpretability of salivary inflammation in response to stress.

4.5.6 Sample size—Studies with greater sample sizes are needed to enable the examination of potential moderators – such as age, gender, personality factors, or socioeconomic status – that may potentially alter stress levels, stress responses, and immune function (Dowd & Aiello, 2009; Engeland et al., 2006; Segerstrom, 2000). Sample size was an issue for the majority of studies reviewed (see Table 1). Ten of the 13 reviewed studies had 36 participants or fewer, and five of those ten studies had 17 participants or fewer. Having so few subjects makes moderation analyses impossible and limits the power of the studies, increasing the chance that certain effects observed were spurious and/or driven by outliers (Type 1 error) or that actual effects went undetected (Type II error).

4.5.7 Behavioral and demographic factors—A review by O'Connor et al. (2009) identified biobehavioral factors that should be considered when assessing blood-based inflammatory markers; such factors include fitness level, dietary patterns, smoking habits, medication use, and menopause status. The authors also suggest controlling for age; gender; socioeconomic status (SES); ethnicity; BMI; use of alcohol, aspirin, statins, anti-hypertensives, and antidepressants; and sleeping patterns. They recommend possibly excluding participants who have sleep disorders or have had recent sleep deprivation, who have partaken in recent acute exercise, who have partaken in acute (e.g., same day) substance use (e.g., nicotine, caffeine, SSRIs), or who are current substance abusers or smokers. Although these guidelines were developed based on studies investigating circulating inflammatory markers in serum or plasma, they very likely also correspond to salivary measurements of inflammatory markers.

4.5.8 Summary of methodological and demographic considerations—Based on the studies reviewed, we provide below a checklist of factors to consider and control for in future studies using salivary markers of inflammation in response to stress (see Table 2). We also note that it is important in studies of inflammatory responses to stress to assess more than a single marker of inflammation when possible, particularly if the marker has anti-inflammatory qualities; with only one marker of inflammation it can be difficult to interpret what a rise in levels means (i.e., more or less inflammation?). Finally, consistent with comments raised in section 4.4.2, in studies where both saliva and blood are obtained and an analytic objective is to determine how the two types of measures are related, we note that it would be ideal to consider lagged time points of measurement to reflect differences in rates and/or processes involved in biological release.

4.6 The value of inflammatory diagnostics obtained from saliva

Inflammatory markers in saliva have great potential value to the field, both clinically and experimentally. Certain questions about how stress is experienced in real time and "gets under the skin" can only be answered with sampling schedules that involve multiple assessments throughout the day (and across days), within the same individual and in "real life" contexts. This combination of needs precludes both the use of venous puncture and even blood spot, which would be either impossible or overly intrusive. For example, although blood spot can be accomplished in the field, participants find it challenging to complete blood spot procedures by themselves (without an experimenter) and it is overly burdensome and potentially painful to perform many times a day.

If the assessment of salivary inflammation in response to stress is demonstrated to be viable, it would enable new approaches in ecological momentary assessment and other ambulatory research to understand stress and health connections. For example, one might signal a subject (e.g., with a mobile device) at any point during the day and have them not only provide self-report data but also provide a biological sample that would reflect their inflammatory state in that moment. Multiple days or weeks of such intensive ecological momentary data, combined with longer term follow-up assessments of physical health status, would enable much greater understanding of how, for example, acute stress experiences are related to acute inflammatory responses that may pose long-term disease risk (see Smyth & Heron, 2012). Alternatively, if information from biological samples could be determined quickly in the field, it could be used to provide relatively real-time feedback to participants as a form of intervention (see Heron & Smyth, 2010). Although we are not there yet, appropriate and careful examination of the utilization for salivary diagnostics makes sense in this context, as will careful examination of other potential ambulatory biomarker assessment collection devices and monitors that can be used in real-time in the natural environment.

4.7 Conclusion

Overall, it appears that some cytokines, notably IL-1β, TNF-α, and IL-6, increase fairly consistently in saliva in response to acute stress. This conclusion, however, is based on a very limited literature; further, there is a dearth of studies with large samples and robust methodologies. Due to such limitations, it is premature to draw firm conclusions about the response patterns of salivary inflammation to acute stress at this time. Moreover, some of the more promising candidate salivary inflammatory markers have not been assessed in the context of acute stress more than once or with an appropriate sampling time frame. Given the need for biomarkers that can be sampled across time in ecological context, and in light of the present limited yet suggestive literature, further research to examine the utility, feasibility, and validity of salivary inflammation in response to acute stress seems warranted. Some guidelines on how to do so have been provided herein.

There are both challenges and opportunities ahead. Collection and assay techniques must be optimized specifically for saliva, and studies must be conducted to determine the most effective time points of sampling. Larger sample sizes are needed to enable investigation of how demographic and behavioral factors affect results. Studies utilizing well-validated acute

stress protocols are critical. Finally, additional research validating salivary inflammation measures to systemic inflammation and to health is needed. Importantly, even if saliva-based measures are not found to map well onto systemic measures (or have greatly lagged associations), they may still be strongly predictive of disease and well-being and have both clinical and research utility. If saliva proves to be as reliable and as meaningful as blood-based measures for assessing stress-induced changes in inflammation, its non-invasiveness would allow researchers to better capture how individuals physiologically respond to stress in more naturalistic settings and as stressors occur.

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Table 1

Summary of Studies

Citation	Sample characteristics	Factors controlled	Stressor type	Sampling timeline	Collection method / assay	Results by biomarker	Limitations
Filaire et al., 2010	*N* = 9 university professors (*M* = 12.1 ± 1.3 years of experience in teaching and lecturing) (males: *n* = 7; age: *M* = 42.5 ± 2.4 years; height: *M* = 181.0 ± 3.8 cm; weight: *M* = 81.2 ± 2.7 kg; experience teaching: *M* = 12.3 ± 1.8 years) (females: *n* = 2; age: *M* = 39.2 ± 2.5 years; height: *M* = 167.1 ± 1.5cm; weight: *M* = 54.2 ± 3.0 kg; experience teaching: *M* = 11.9 ± 0.8 years)	all healthy and free of cardiovascular and inflammatory diseases, allergies, dental issues, and substance abuse; no women pregnant or taking oral contraceptives; no extreme physical activity 48 hours prior; no sports, anti-histamines, or anti-inflammatory medication 24 hours before testing; rescheduled if infection on test day; no teeth-brushing before morning saliva samples; no smoking, eating, or alcohol, caffeine, or fruit juice 60 minutes before sampling	within-subjects design: 2 hour lecture to 200 students (2nd class period of the year) vs. a control day without lecture	right before (10am) , 0 minutes after (12pm), 120 minutes after (2pm), 8 hours after (8pm) the completion of the stressor or the control period	TNF-α, IL-10, IL-2, and IL-4: cotton swab Salivettes (Sarstedt Co., Nümbrecht, Germany; cytometric bead array kit (BD Biosciences Pharmingen, San Diego, CA, USA)	TNF-α: effect for sampling time [*F*(3, 24) = 4.7; *p* = .04, *g* = 2.53], with higher TNF-α concentrations 120 minutes after the completion of the lecture compared with pre-lecture levels IL-10: no effect for day or time of sampling IL-2: effect for sampling time [F(3, 24) = 5.1; *p* = .05, *g* = 1.73], with higher IL-2 concentrations 120 minutes after the completion of the lecture compared with pre-lecture levels IL-4: effect of sampling time [F(3, 24) = 4,2; *p* = .04, *g* = 1.33], with higher IL-4 concentrations 120 minutes after the completion of the lecture compared with pre-lecture levels	very small sample size, primarily men; no control for menstrual status in the two women; stress paradigm not been validated in previous studies; professors likely of uniformly high SES and thus results may not be generalizable; unclear to what degree participants found the manipulation stressful
Usui et al., 2012	*N* = 10 (all males; age: *M* = 23 ± 3 years; height: *M* = 176.4 ± 3.4 cm; body mass *M* = 66.8 ± 7.8 kg; V4 O2max *M* = 49.7 ± 4.8 ml/kg/minute)	non-smokers, active, no respiratory or anti-inflammatory diseases (incl. asthma); no recent psychological issues or traumas; no dental issues; no medication use 4 weeks prior; no caffeine and alcohol 24 hours before task	within subjects design: exercised on recumbent ergometer at 75 % V4 O2 max for 60 minutes (exercise session) or sat reading or writing quietly (resting session)	0 minutes before, 0 minutes after, 60 minutes after, 120 minutes after the completion of the stressor or the control period	TNF-α, IL-6, and IL-1β: cotton swabs; ELISA kits (Bio- Rad, Bio-Plex Pro Assays, CA, USA).	TNF-α: effects of time: at baseline vs. immediately after ($p < .01$, $g = 2.05$) and 60 minutes ($p < .05$, $g = .77$) after the completion of the stressor; effect of condition control vs. stress immediately after the completion of the stressor, ($p < .01$); levels peak immediately after the completion of the stressor IL-6: effect of time: at baseline vs. immediately after ($p < .01$, $g = 1.65$) and 60 minutes ($p < .05$, $g = .39$) after the completion of the stressor; effect of session: control vs. stress	very small sample size; no female participants

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Citation	Sample characteristics	Factors controlled	Stressor type	Sampling timeline	Collection method / assay	Results by biomarker	Limitations
						immediately after ($p < .01$); and 60 minutes ($p < .05$) after the completion of the stressor; levels peak at immediately after the completion of the stressor IL-1β: effect of time: at baseline vs. immediately after ($p < .01$, $d = 9.69$) and 60 minutes after ($p < .05$, $g = 3.03$) the completion of the stressor; effect of session: control vs. stress immediately after ($p < .01$) and 60 minutes after ($p < .05$) the completion of the stressor; levels peak immediately after the completion of the stressor	
Dugue et al., 1996	$N = 14$ (males: $n = 6$, 22-38 years, $M = 33.8$ years) (females: $n = 8$; age 30-39 years, $M = 35.1$ years)	participants were "subjectively healthy"	within subjects design: stress condition: 15 minutes rest period and then 10 minutes sauna exposure (90°C, , 40% humidity) control condition: resting periods for 15 minutes and then an additional 30 minutes	stress condition: 0 minutes after rest period and 0 minutes after completion of the sauna exposure control condition: 0 minutes after the completion of the first rest period and 0 minutes after the completion of the second rest period	TNF-α, IL-6, IL-1β, and IL-2: Salivettes; ELISA kits (Immunotech, Luminy, France)	TNF-α: tendency to increase after completion of the sauna stressor; statistically significant increase in the males ($p < .05$, $g = .12$) IL-6: detectable in only 60 percent of the specimens investigated IL-1β: showed a tendency ($p = .11$) to increase after the completion of the sauna stressor as compared to baseline IL-2: levels not detectable	very small sample size; no objective measurement of health status; no control for periodontal health in all participants; no control for menstrual cycle and hormonal contraceptives in female participants; stress paradigm has not been validated; only one post-stimulus saliva sample taken soon after the stressor
Groer et al., 2010	$N = 141$ ($n = 27$ female), $M = 37$ years old (range, 22–64 years); "motorcycle scenario" ($n = 49$), "workplace scenario" ($n = 92$)	majority of the officers had >3 years or more of police experience; two groups were comparable on demographic variables	between subjects design: two virtual reality scenarios: motorcycle chase (2 minutes) and workplace gun confrontation (6 minutes) scenarios	0 minutes before, 10 and 30 minutes after completion of the stressor	IL-6: passive drool ELISA (eBioscience, San Diego, CA)	IL-6: significant increase at 10 minutes after the completion of the stressor in the workplace group ($t = 2.02$, $p = .05$, $g = .16$)	smaller proportion of women than men; no control for periodontal health; no control for menstrual cycle and hormonal contraception in females; samples only taken up to 30 minutes post-stressor; virtual

Citation	Sample characteristics	Factors controlled	Stressor type	Sampling timeline	Collection method / assay	Results by biomarker	Limitations
							reality simulation not well-validated and may not have been considered stressful
Minetto et al., 2005	*N* = 17 spinning activity: *n* =7 endurance-trained athletes; age *M* = 29.5 ± 8.0 years; 42.9% female, *n* = 3) isokinetic activity: *n* = 10 athletes of different disciplines; age *M* = 27.0 ± 6.9 years; all males	complete medical exam performed; no intense physical activity for 24 h before the study	between subjects design: 7 endurance athletes did controlled spinning activity for 3 hours; 10 athletes from different disciplines did isokinetic exercise test on a Cybex 6000 device (Cybex, Division of Lumex, Ronkonkoma, USA)	spinning activity: 15 and 5 minutes before, and 0 minutes after completion of the exercise isokinetic activity: 15 and 5 minutes before the warm-up, 0 minutes after the completion of the exercise activity, and 7, 15, 30, 45, 60, 90 and 120 minutes after completion	IL-6: Salivettes (Sarstedt, Numbrecht, Germany); ELISA (Quantikine High Sensitivity human IL-6 immunoassay, R&D Systems) and immunoradio metric assay (IRMA; Biosource-Europe, Nivelles, Belgium)	IL-6: not detectable in IMRA methods; for the isokinetic test salivary levels only showed a non-significant increase immediately after the completion of the test; salivary and serum levels were not correlated at any time point	very small sample size; no control for periodontal health; no control for menstrual cycle and hormonal contraception in females
Minetto et al., 2007	*N* =15 elite athletes (all male) median age = 23 years, median weight = 70 kg	none of the subjects was a current smoker or taking any medication; no exercise 24 hours before experiments	within subjects design: (only the athletes completed the exercise task, which was 15 minutes warm-up cycling and three isometric maximal voluntary contractions of the knee extensors; then 160 isometric contractions of the knee extensor muscle completed in ~25 minutes	all samples collected between 10am and 1pm; 0 minutes before exercise (in all 32 participants), and then 0 minutes after, and 30, 60, 90, and 120 minutes after completion of the stressor (in the athletes)	IL-6: passive drool and Salivettes (Sarstedt, Numbrecht, Germany); ELISA (Quantikine High Sensitivity human IL-6 immunoassay, R&D Systems, Abingdon,UK)	IL-6: levels peak immediately after the completion of the stressor (statistically significant from baseline, $p < .05$) and return to baseline by 120 minutes; no significant correlation between serum and salivary levels; cotton-interference effect with cotton Salivettes, passive drool more accurate	small sample size; no female athlete participants; no control for periodontal health; no control for menstrual cycle and hormonal contraception in females
Lester et al., 2010	*N* = 36 first year undergraduate students (males: *n* =2, age = 21–22 years)	excluded if pregnant; no eating or drinking one hour before saliva sampling	within subjects design: 1 baseline period and 3 timed anatomy practical exams	all samples collected between 12:30 and 1:00 pm at a baseline time point (two	IL-6 and IL-12: passive drool; ELISA (R&D Systems, Minneapolis, MN)	IL-6: increase from the first to the third test ($p < .05$) IL-2: increase from the first to the third test ($p < .05$) IL-12: increase from the first to the third test ($p <$	small sample size; no control for periodontal health in all participants; no control for menstrual cycle and hormonal

Citation	Sample characteristics	Factors controlled	Stressor type	Sampling timeline	Collection method / assay	Results by biomarker	Limitations
	(females: *n* = 34, age = 20–35 years)		~3 weeks apart	weeks before the first test) and then within 60 minutes prior to each of the three exams, each ~3 weeks apart		.05)	contraception in females; samples only taken at one time point during the exam stressors
Ilardo et al., 2001	*N* = 30 normal healthy individuals (males: *n* = 20, age = 20-28, M = 20.1 years) (females: *n* = 10, age = 19-23, *M* = 21.2 years)	excluded if: history of psychological disorders, chronic illness, ongoing medical treatment, regular drug intake, alcohol use or smoking	within subjects design: rafting competition; subjects were assigned to six-member rafting teams and paddled for an average time of 1 hour	0 minutes before (at 2pm) and 0 minutes after (at 3pm) the completion of the first one-hour training session on day 1; 0 minutes before (at 10am) and 0 minutes after (at 11am) the completion of the second one-hour training session on day 2; and 0 minutes before (at 3pm) and 0 minutes after (at 4pm) the completion of a one-hour competitive rafting session on day 3; two samples also collected at 8am & 6pm on each day	IL-1β: Salivettes (Sarstedt, Germany); ELISA (Immunotech, Luminy, France)	IL-1β: concentration significantly higher in males (12.6 pmol/l) than in females (5.1 pmol/l) after the completion of the competition period (z = 1.96, $p < .05$); significant increase after the completion of the one hour competitive rafting in both sexes ($p < .05$)	small sample size; no screening for periodontal health in all participants; no control for menstrual cycle and hormonal contraception in females; non-validated stressor paradigm; only one sample was taken immediately post-task; unclear whether participants had any previous experience with rafting
Zefferino et al., 2006	*N* = 30 emergency policemen (all males: age *M* = 44.5 ± 5.8 years; experience *M* = 17.1 ± 6.4	no mention of exclusion criteria; no eating or drinking (except for water) 1 hour before saliva collection	within subjects design: emergency work shift vs. control vacation period	at the beginning and end of the completion of a work shift (8am and 1:30pm, respectively)	IL-1β: Salivettes (Salivette-Sarstedt); ELISA (Roche)	IL-1β: concentrations higher at the beginning of the shift than at the end, ($p < .05$, $g = .35$); non-significant trend for IL-1β reduction during the vacation period compared to work shift period	small sample size; no screening for periodontal health in all participants; no female participants; participants may not have seen the work day situation as

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Citation	Sample characteristics	Factors controlled	Stressor type	Sampling timeline	Collection method / assay	Results by biomarker	Limitations
	years)			or at the same times during the vacation period			particularly stressful
Campisi et al. 2012	*N* = 15 healthy college undergraduates (males: *n* = 4, age = 18-22) (females: *n* = 11, age = 18-22)	no chronic or acute illness (including periodontal disease), no regular medication (with the exception of contraceptives); good health prior to study; no exercise, meals, or beverages at least 1h prior to study	within subjects design: Trier Social Stress Test	0 minutes before stressor, and 0 minutes after and 30 minutes after the completion of the stressor	CRP: Salivettes (Sarstedt, Newton, NC); ELISA (Salimetrics, State College, PA)	CRP: no statistically significant differences in levels between each time point of sampling	very small sample size; no control for periodontal health in all participants; no control for menstrual cycle and hormonal contraception in females; samples only taken 30 minutes post-stressor
Mastro-lonardo et al. 2007	*N* = 50 (as part of a larger study on those with and without psoriasis; only controls used in this review) age- and sex-matched healthy controls: 22 men and 28 women; age *M* = 39.8 ± 10.6)	controls: matched for age, sex, educational level, smoking habits, and use of oral contraceptives; no exercise, smoking, alcohol, or eating for at least 1 h before the session	between subjects design: 5-minute relaxation period, two stressful tasks (mental math and Stroop Color Word Naming Test) of 5-minutes duration each	all sessions occurred between 2:30pm and 3:30pm; collected at 0 minutes before stressor and 10 minutes after the completion of the stressor	IL-1β: Salivettes (Sarstedt, Germany); ELISA (Euroclone Ltd., city, UK)	IL-1β: levels increase after completion of the stressor among healthy controls with a significant group-by-time interaction ($p < .01$)	no control for periodontal health in all participants; no control for menstrual cycle and hormonal contraception in females; only one post-stressor sample was taken
Izawa et al. 2013	*N* = 50 healthy young adults (males: *n* = 39, age *M* = 21.4 ± 2.4 years) (females: *n* = 11, age *M* = 21.6 ± 3.4 years)	no physiological or psychological disorders and no HPA–axis and immune system-affecting drug; all females were in late luteal or early follicular phase of menstrual cycle; no eating , drinking or exercising 1 hour before experiment	within subjects design: Trier Social Stress Test	all sessions occurred between 2:00pm and 7:30pm; collected after 10 minutes of resting baseline, after a 10 minute preparation period, after a 5 minute speech, after a 5 minute mental math task, and 10, 20, 30, 45, and 60 minutes after the completion	IL-6: passive drool; ELISA (R & D Systems, Abingdon, UK)	IL-6: levels significantly higher than baseline immediately after ($p < .01$, $g = .37$), and 10 ($p < .01$, $g = .33$) and 20 minutes after ($p < .01$, $g = .27$) the completion of the TSST; levels return to near baseline 60 minutes after the completion of the TSST	no control for periodontal health in all participants; no control for hormonal contraception in females

Citation	Sample characteristics	Factors controlled	Stressor type	Sampling timeline	Collection method / assay	Results by biomarker	Limitations
				of the stressor			
Mahmood et al., 2013	*N* = 24 dental students (males: *n* = 12, females: *n* = 12)	non-smoking, not taking antibiotics, no chronic diseases or pregnant women; no psychotropic medications	within subjects design: three exam periods--one month before mid-year exam, during the mid-year exam; and one month after the mid-year exam	all sessions occurred between 8:00am and 12:00pm	IL-1β: passive drool ; ELISA (Salimetrics, State College, PA)	IL-1β: levels significantly higher during mid-year exam period as compared to the pre-exam period ($p < .01$, $g = 1.24$) and the post-exam period ($p < .01$, $g = 2.14$)	small sample size; no control for hormonal contraception in females

Table 2

Factors to Measure and Methodological Considerations for Future Studies

Pre-existing factors to measure
• Gender, BMI, age • Other sociodemographic factors, including SES indicators • Pre-existing health status, including oral health • Medication use • Menstrual cycle status and use of hormonal birth control in women
Methodological recommendations:
• Use a well-validated stress paradigm, such as the TSST • Use only those with good oral health, or control for oral health status; ideally as determined by an expert (e.g., dentist), or by excluding those with known periodontal disease • Use immunoassays optimized for saliva whenever possible • Use the passive drool collection method and measure salivary flow rate when possible • Collect saliva at multiple time points to capture full change and recovery (e.g., at baseline; 30, 60, and ideally at least 120 minutes after completion of the stressor)