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Salivary free light chains as a new biomarker to measure psychological stress: the impact of a university exam period on salivary immunoglobulins, cortisol, DHEA and symptoms of infection

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Abstract

Introduction: Measurement of immunoglobulin free light chains (FLCs) in saliva can serve as a non-invasive biomarker in health and behavioural research. FLCs have been explored in relation to physiological stress but FLC responses to psychological stress and their relationship with infections remain unknown. This study aimed to investigate the impact of exam period stress on salivary FLCs alongside other established biomarkers of stress and whether FLCs relate to symptoms of infection.

Methods: 58 healthy adults studying at university completed saliva samples and questionnaires in a period without exams (baseline), and again prior to the start of an exam period. Saliva samples were assessed for FLCs, IgA, cortisol and dehydroepiandrosterone (DHEA). Measures of life events stress, perceived stress, anxiety and depression were completed. Students also reported incidence and severity of symptoms of infection and rated general well-being at baseline, prior to, during and after the exam period. Exercise, sleep and alcohol consumption were also assessed at both timepoints.

Results: FLCs secretion rates were significantly lower at the exam period compared to baseline ($p < .01$), with reductions of 26% and 25% for κ FLC and λ FLC, respectively. In agreement, salivary IgA secretion rate was lower at exams (non-significant trend, $p = .07$). Cortisol concentration significantly increased at exams ($p < .05$) while DHEA did not change, leading to an increase in the cortisol:DHEA ratio ($p = .06$). Depression ($p < .05$) and anxiety increased from baseline to exams and life stress reported in the build up to the exam period was higher compared with baseline ($p < .001$). Well-being significantly decreased from baseline to exams ($p < .01$). The proportion of participants reporting infection symptoms (70%) was unchanged between baseline and prior to exams. No significant relationships were found between FLCs or other saliva parameters and infection symptoms, well-being or stress/psychological measures. Changes in saliva parameters between timepoints were independent of health behaviours.

Conclusions: Salivary FLCs are responsive to life events stress and corroborate with IgA. This preliminary study highlights the potential utility of FLCs as a new salivary biomarker in stress research.

1. Introduction

Salivary biomarkers are well-established in psychoneuroimmunology research as measures of stress. The physiological stress response is orchestrated by the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis and comprises a coordinated release of hormones from the hypothalamus, anterior pituitary gland and adrenal glands, terminating with secretion of glucocorticoids and catecholamines. Several analytes in saliva have emerged as robust markers able to assess an individual's response to stress. HPA axis responses, via salivary cortisol and dehydroepiandrosterone (DHEA), have been widely measured in researching both acute and chronic stress (Glaser and Kiecolt-Glaser, 2005). Salivary alpha amylase has been used as a surrogate marker for autonomic nervous system activity and has been shown to be sensitive to acute and chronic stressors (Engeland et al., 2019). Immunoglobulin A (IgA) in saliva is predominately produced by plasma cells in the salivary glands and its secretion is regulated by autonomic nerves (Brandtzaeg, 2007). In general, acute stress is associated with increases in salivary IgA whereas prolonged stress has been linked to decreases in salivary IgA (Engeland et al., 2019).

IgA exerts anti-microbial properties in a number of ways; it inhibits adherence via agglutination, opsonises bacteria, activates complement and neutralises viruses (Fabian et al., 2012). As such, it plays a key role in the first line of defence against pathogens and assists in controlling carriage of bacteria (Nurkka et al., 2003). Therefore, salivary IgA not only acts as a measure of the body's response to stress but also the impact of stress on the mucosal immune system. Low levels of IgA compromise protection against infections and stress-induced suppression of IgA may mediate vulnerability to infections (Drummond and Hewson-Bower, 1997; Isaacs et al., 1984). Indeed, life events stress has been implicated as a risk factor for upper respiratory tract infections. It's been nearly thirty years since Cohen et al found psychological stress was linked to acute respiratory illness by intentionally exposing study participants to viruses, where the risk of illness increased proportionately with the degree of stress (Cohen et al., 1991). This study forms a key part of the body of literature that has documented associations between stress and infections (Boyce et al., 1977; Cobb and Steptoe, 1996; Graham et al., 1986).

Immunoglobulins comprise two identical heavy chains and two identical light chains and during immunoglobulin synthesis plasma cells produce light chains in excess of intact immunoglobulin; these extra light chains, known as free light chains (FLCs), are released into the circulation.(Suki and Massry, 1998) FLCs are a cancer biomarker for plasma cell malignancies and form a key part of diagnosis and monitoring (Dispenzieri et al., 2009). FLCs dysregulation is also a feature of various inflammatory conditions (Brebner and Stockley, 2013) and have been shown to be predictive of all-cause mortality in individuals without plasma cell disorder (Dispenzieri et al., 2012).Consequently, FLCs are increasingly being viewed as a marker of inflammation, immunosenescence and health and disease in the general population (Dispenzieri et al., 2012; Drayson, 2012 ; Heaney et al., 2016b).

As a result of assays with high sensitivity, FLCs are gaining traction as a new useful salivary biomarker. FLCs in saliva are secreted by local plasma cells and secretion rates correlate with IgA, although FLC levels are over 100-fold lower than IgA (Rapson et al., 2020).Increases in salivary FLCs have been associated with ageing and likely relate to oral inflammation (Heaney et al., 2016a). FLCs have been explored in relation to physiological stress where acute exercise has been shown to induce a transient reduction in concentrations and secretion rates following exercise. In contrast, more prolonged physiological stress in the form of a period of intensified exercise training increased basal salivary FLC parameters (Heaney et al., 2018). However, the impact of psychological stress on salivary FLCs has yet to be examined. Further, it is unclear if FLCs, like IgA, relate to vulnerability to infection, thus implicating FLCs in performing a functional role in immune defence.

Academic stress induced by a period of exams is a common form of life stress mainly experienced throughout adolescence and during young adulthood. University exams take place in January and the summer term each year, often representing the culmination of 3-4 months' work. They may also determine whether a student progresses to the next year of study and determine their final degree classification. As such, university exams can present as a substantial period of stress. The efficacy of university exam periods as a model of psychosocial stress has been demonstrated

using immune and neuroendocrine measurements, including salivary IgA, cortisol and DHEA (Deinzer et al., 2000; Izawa et al., 2012; Jemmott and Magloire, 1988; Weekes et al., 2006).

The aims of the current study were to I) investigate the utility of salivary FLCs as a marker of psychological stress using university exams as a stress model II) compare FLC response to established biomarkers (IgA and stress hormones) and III) examine potential relationships between salivary FLCs, infection symptoms and well-being.

2. Materials and Methods

2.1. Participants and study design

Participants were university students who were due to undertake examinations at the end of semester 1. A total of 63 participants were recruited and 58 completed the study. Key inclusion criteria were no chronic illness or history of chronic illness and aged 18-35 years old. All participants gave written informed consent prior to the study, which had the appropriate Ethics Committee approval from the University of Birmingham and University of Bath. Saliva samples and questionnaires were completed in two waves: in December when students were not taking exams (baseline), and again in January in the days prior to an exam period (exams). Figure 1 depicts the study design and shows the specific measures taken and when.

2.2. Saliva sample collection procedure and assays

Participants were asked to provide 3 saliva samples upon waking on 3 consecutive days at baseline and again at exams. Samples were collected upon waking to avoid any impact of diurnal variation. Each participant was provided with a pack of universal tubes labelled with the sampling days. Participants were briefed concerning the collection procedure and sampling times and performed a practice sample observed by a researcher. Participants were instructed to take the saliva sample immediately after they wake up, prior to eating, drinking or brushing their teeth. Whole saliva samples were collected by passive dribble into pre-weighed tubes for a timed period of 3 min. Participants were asked to immediately store tubes in a freezer in a re-sealable bag that was provided, before collection by a member of the research team. To measure compliance all

participants were given a diary to record any potential delays in sampling times, forgotten samples, or deviations from protocol. Out of 348 samples, 8 (2.3%) were excluded: 5 due to non-compliance (four samples were taken more than a 10 min delay after waking and one where the participant had already eaten) and 3 were forgotten by participants on that day. No other participants reported a time deviations of greater than 10 minutes from waking to taking the sample: this should have avoided the cortisol awakening response that peaks 30-45 min post-waking. Saliva samples were collected from participants within one week and thawed to be processed. Saliva volume was calculated by re-weighing the tube post-collection assuming a density of 1g/mL Saliva flow rates (mL/min) were determined by dividing the volume of saliva by the collection time. Samples were centrifuged to separate cells and insoluble matter and the supernatant was removed and stored at –20°C until assay. Salivary kappa (κ) and lambda (λ) FLCs were quantified using commercially available sensitive sandwich ELISAs (Abingdon Health, York, UK). These assays have an initial measurement range of approximately 0.01–1mg/L with 100µl of sample added to each plate at an initial 1 in 5 dilution. Salivary IgA, cortisol and DHEA were quantitated using sensitive direct (IgA) and competitive (cortisol and DHEA) ELISAs (IBL International, Hamburg, Germany). The salivary IgA assay had a measurement range of 6.9–400 mg/L and required 25µl of sample to be added at an initial 1 in 1000 dilution. The cortisol ELISA had a range of 0.41–110 nmol/L and the DHEA ELISA a range of 0–7.5 nmol/L and both assays required an initial application of 50µl of neat sample. Samples were measured in singlet with control saliva samples (from healthy donors) measured in duplicate across all plates. Any samples measuring above the standard curve underwent repeat measurement at an appropriate dilution factor. All cases with samples available were measured (assay results were generated for all samples, with cases only excluded based on protocol deviations [see above]). Intra-assay coefficients of variation (CV) were: 4% κFLC, 3% λFLC, 9% cortisol and 10% DHEA. Individual participant samples from both timepoints were all measured within the same plate.

2.3. Salivary parameters

The assays generated concentrations for κ and λ FLCs, IgA, cortisol and DHEA. In measures that are affected by flow rates, such as IgA and FLCs, concentrations typically decrease as flow rates increase or vice versa. Accordingly, they should be expressed as secretion. Saliva secretion rates of immunoglobulins reflect the total availability of protein at the oral mucosal surface and serve to control for hydration status (Oliver et al., 2007). Secretion rates of FLCs (μg/min) were calculated as saliva flow rate x κ/λ/IgA/FLC sum concentration and are reported herein. Cortisol and DHEA are not affected by saliva flow rate and thus only concentrations are reported (Kirschbaum and Hellhammer, 1994). The FLC sum (κ + λ) was included as this measure has been employed in the general population as an indicator of total immunoglobulin production (Dispenzieri et al., 2012). The FLC sum is more applicable in healthy populations than the FLC ratio or FLC difference, which are biomarkers used for the diagnosis, prognostication and monitoring of haematological malignancies (Dispenzieri et al., 2008; Siegel et al., 2009). Individual κ and λ FLC secretion rates were investigated alongside the sum to assess if both isotypes followed similar patterns of fluctuation or if differences existed, possibly due to or factors relating to molecular structure or production.

2.4. Questionnaires

2.4.1 Stress measures and psychological variables

At both time-points, the Undergraduate Stress Questionnaire was used to assess less serious life events over the past month (Crandall et al., 1992). The questionnaire lists 83 events ranging from minor daily hassles to major life events, which are rated on a 4–point severity scale. Feelings of general stress were measured using the Perceived Stress Scale where participants were asked to indicate how often they felt or thought a certain way about 14 items on a 5-point scale (Cohen et al., 1983). The Hospital Anxiety and Depression Scale (HADS) was used to measure depression and anxiety (Zigmond and Snaith, 1983). It contains 7 items measuring anhedonic rather than somatic aspects of depression and 7 measuring anxiety with items are scored from 0–3. At the exam period, feelings towards examinations and exam-related anxiety were measured using the Test Anxiety Inventory featuring 20 items measured on a 4-point scale (Taylor and Deane, 2002).

2.4.2 Health behaviours

To investigate if lifestyle factors and health behaviours differed between timepoints (potentially explaining any changes seen in saliva parameters), participants completed a questionnaire pack on the first day of saliva sampling at each time point. Participants were asked about smoking and typical alcohol consumption in terms of frequency of drinking and units consumed per week using a questionnaire adapted from the Whitehall Study (Marmot et al., 1991). Participants were categorised by how often they consumed alcohol (never, special occasions, 1-2/month, 1-2/week, almost daily, ≥2/day) and how much alcohol they drank per week (none, 1-5, 6-10, 11-20, 20-40 and >40 units). Exercise was measured via the International Physical Activity Questionnaire using the last 7-day format (IPAQ) (Craig et al., 2003). This questionnaire measures the time spent physically active over the previous 7 days, including work, housework and leisure time. Activity is categorised into vigorous, moderate and walking. The World Health Organisation (WHO) Oral Health Questionnaire for Adults was used to assess whether participants' oral health and practices were affected by the upcoming examination period (Engeland et al., 2019).

2.4.3. Infection and well-being

Participants were provided with a log book to be completed on the day prior to saliva sampling, on every day during sampling, during exams and after the exam period to record symptoms of infection. Participants recorded if they were experiencing any of the following symptoms: sore throat, runny nose, sneezing, nasal congestion, malaise (general feeling of discomfort, illness, unease) "under the weather", fever, headache, hoarseness, earache, cough, chills, joint aches and pains. If so, symptom severity was rated as 1 (mild), 2 (moderate) or 3 (severe). Participants recorded the number of hours slept the previous night and then rated general well-being using the Hoopers index where participants were asked to rate facets including sleep quality, fatigue, general muscle soreness and stress on a 1-5 likert scale (Hooper and Mackinnon, 1995).

2.5. Data analyses

For saliva biomarkers, at each time point, median values of parameters over the 3-day period were computed for each participant. This was to reduce the effects of day-to-day variability and generate a more accurate reflection of saliva measures at each specific timepoint. Previous studies have shown that FLCs, IgA and cortisol fluctuate day-to-day within-persons (Francis et al., 2005; Rapson et al., 2020; Stalder et al., 2009) and taking multiple measurements over a number of days can help attenuate noise from day-to day variability and determine longitudinal change more accurately. Wilcoxon matched pairs signed rank tests were used to assess if salivary parameters changed between the timepoints. Delta values (change in concentration/secretion between time points) and percentage change of median salivary biomarker parameters between baseline and exams were calculated. Changes between baseline and exams in other continuous variables (health behaviours, stress measures) were also assessed using Wilcoxon matched pairs signed rank tests. To examine changes in categorical variables (alcohol consumption) between the two time points the McNemar test was used. To assess changes over more than two timepoints, in the case of infection symptoms (4 timepoints) the Freidman test was used. To assess differences between groups (for example based on alcohol consumption, presence of infection, gender) and saliva parameters Mann Whitney (2 groups) and Kruskal Wallis (3 or more groups) tests were used. Spearmans' rank correlation was used to assess relationships between continuous variables and saliva parameters at the different time points.

3. Results

3.1. Participants

Participant characteristics are described in Table 1. A total of 58 participants took part in the study (57% male) with a median age of 20 years. Participants had a median BMI of 21.5 kg/m^2 and reported their ethnicity as white British (66%), Asian (17%), Black (3%) or mixed race (10%). Participants were healthy with only two participants classing themselves as smokers and as a cohort the median time spent exercising was 4.3h per week at study entry.

3.2. Salivary immunoglobulins decreased and cortisol increased between baseline and exams. Figure 2 displays salivary biomarkers at baseline and exams for salivary immunoglobulin secretion rates and steroid hormone concentrations. Salivary κ and λ FLC secretion rates significantly decreased from baseline to exams (κ FLC Z = -2.90 and λ FLC Z = -2.89, $p < .01$). Accordingly, the FLC sum was also significantly lower at exams compared to baseline (Z = -3.19 $p < .01$). The percentage reductions in median secretion rates from baseline to exams were 26%, 25% and 13% for κ FLC, λ FLC and FLC sum, respectively. The same findings were also seen for FLC concentrations, with significant decreases seen for κ FLC, λ FLC and FLC sum, $p < .05$. Salivary IgA secretion rate decreased at exams (17% reduction in median IgA secretion rate from baseline to exams); however, this was a non-significant trend ($p = .07$). IgA concentration also decreased but again this was not significant ($p = .09$). While salivary immunoglobulins decreased from baseline to exams, the opposite was found for cortisol, which significantly increased at exams (Z = -2.37, $p < .05$). The increase in median concentrations between baseline and exams was 25%. DHEA on the other hand did not change between time points. This led to an increase in the cortisol:DHEA ratio at exams (36% increase in median ratio between time points), although this was borderline significant (Z = -1.88, $p = .06$). There was no difference in saliva parameters on the basis of gender or ethnicity at either time point and salivary responses over time did not differ between males or females or between reported ethnic groups.

Earlier waking times were associated with higher salivary secretion rates at baseline (FLC sum, $r_s = -.31$, $p < .05$; IgA $r_s = -.37$, $p < .01$) and exams (FLC sum, $r_s = -.26$, $p = .064$; IgA $r_s = -.37$, $p < .01$). Waking time was not associated with cortisol or DHEA levels. The time participants woke up was not significantly different between the two time points and thus change in waking patterns did not account for observed changes in FLCs or IgA between baseline and exams.

3.3. Measures of stress and psychological parameters

Perception of stress did not change between baseline and exams (median score 23.5 baseline vs 24 exams). Depression, anxiety and life events results are shown in Figure 3. Both depression and anxiety increased from baseline to exams, significantly so for depression (Z = -2.27, $p < .05$).

The number of life stresses reported in the last month in the build up to the exam period was significantly higher compared with baseline (Z = -6.38, $p < .001$). However, the perception of the severity of events significantly decreased (Z = -4.50, $p < .001$). Median test anxiety scores were 40 (22-73), 50% of the total possible score of 80.

At baseline, a higher number of life events and higher severity score was associated with significantly lower saliva secretion rates (κ and λ FLC, FLC sum, IgA secretion rate, r_s -.27 to -.34, $p < .05$). Higher anxiety was associated with a lower cortisol (r_s = -.44) and lower cortisol:DHEA ratio (r_s = -.44), p <.01. Although FLC sum alone was not associated with anxiety, when combined with cortisol, a significant association was observed in relation to secretion (r_s = -.35) and concentration (r_s = .30), $p < .05$. There were no significant relationships between depression, perception of stress and salivary measures. These relationships did not maintain significance at the exam period. Nor were delta values or percentage change in saliva parameters between time points related to stress measures at exams.

3.4. Infection symptoms and well-being

Well-being scores (Figure 4) significantly decreased from baseline to exams, both total well-being score for the 4 days recorded (Z = -3.40, p <.01) and average score per day (Z = -2.78, p <.01). There were no significant relationships between well-being and saliva measures at either time point.

Figure 5 displays infection incidence and severity of symptoms across the study.
The proportion of participants reporting an infection symptom/infection symptoms was unchanged between baseline (68%) and prior to exams (68%) but significantly decreased during exams (54%) and post-exams (49%) compared to the previous two timepoints ($p < .05$). Out of those reporting symptoms at baseline, 74% also reported symptoms at exams. Despite a decrease in incidence during the exam period, average severity of symptoms was highest at this timepoint, although average symptom severity did not significantly vary between timepoints. Those with infectious

symptoms reported lower well-being at both timepoints, which was significant at baseline only (p < .05).

No significant differences in saliva parameters were observed between those who did and did not report infectious symptoms, either at baseline or the exam period. Although not significant, there was a difference in percentage changes in salivary parameters based on symptoms at the different timepoints. Participants who reported symptoms of infection at both timepoints had the greatest percentage reductions across all salivary FLC and IgA concentrations and secretion rates, followed by those without symptoms at baseline but symptoms at exams, then those with symptoms at baseline but not at exams, with the lowest percentage reductions seen in those without symptoms at either time point. In participants who reported infection symptoms, there was no difference in salivary parameters between those classed as mild, moderate and severe at either timepoint. There were also no correlations between infection severity and saliva parameters at either timepoint.

3.5. Health behaviours

Health behaviours at both timepoints are reported in Table 1. Overall, time spent exercising did not significantly differ at baseline compared to exams. However, participants reported spending more exercise time carrying out moderate rather than vigorous activities. This increase in moderate activity and decrease in vigorous activity was statistically significant (Z = -2.47 and -3.11, respectively, p < .05). Exercise time, and time spent in moderate or vigorous activity, was not related to salivary concentrations or secretion rates at either timepoint.

Consumption of alcohol reduced in frequency between baseline and exams (p < .01). More participants refrained from alcohol (22% to 34%) at the exam period. At baseline only a few participants fell in the special occasion (9%) and 1-2/month categories (2%), with the majority reported drinking alcohol 1-2/week (57%). At exams, participants were spread evenly across these categories with 21% reported in each. Alcohol units consumed per week decreased, although this was not significant. At baseline, 18% of participants reported consuming < 5 units/week, with 16%

consuming 20+ units/week compared to 35% and 7% at exams, respectively. Alcohol consumption frequency and units of intake had no significant effect on salivary parameters at baseline or exams.

The number of hours slept per night did not significant differ between time points and there was no association between hours slept and salivary parameters at baseline or exams. Overall self reported oral health was at the higher end of the scale, with participants reporting a median score of 121 and 119 (out of a possible 137) at baseline and exams respectively, with no significant change between timepoints. No significant relationships were observed between self-reported oral health and salivary parameters. Only two participants smoked tobacco and excluding these participants made no changes to the main analyses.

3.6. Relationship between infection symptoms, stress measures and health behaviours

At baseline, those reporting infection symptoms reported a greater number of life events and of greater severity compared to those without symptoms (p <. 05). The difference in life events severity on the basis of infection symptoms was also significant at exams (p < .05). There were no differences for any other stress/psychological parameters (perceived stress, anxiety, and depression) on the basis of infection symptoms at baseline or exams. There were no significant differences in any health behaviours between those with/without infection symptoms at either timepoints.

3.7 Correlational analyses

Significant positive associations were observed between FLC sum and IgA secretion rates at both baseline and exams: r_s = 0.80, p <.001 and r_s = .72, p < .001, respectively. This relationship is shown in Figure 6. Significant associations were also found between FLC sum concentration and IgA concentration both at baseline (r_s = .74, p < .001) and exams (r_s .64, p <.001). At baseline, cortisol and DHEA were significantly correlated with each other (r_s = .44, p < .001) but steroid hormones did not correlate with FLC sum or IgA. The same was observed at exams, with a significant relationship between cortisol and IgA (r_s = .50, p < .001) but not between cortisol or DHEA and salivary immunoglobulin. In addition, no significant associations were seen for delta scores or percentage change between salivary parameters.

4. Discussion

This study investigated for the first time salivary FLCs in relation to psychological stress. A significant reduction in FLC parameters were observed at the exam period compared to the period without exams. This finding occurred alongside a significant increase in cortisol, which would be expected if an individual was manifesting stress, and concurs with previous studies looking at periods of exams (Weekes et al., 2006). Lower FLC secretion has previously been associated with a higher cortisol:DHEA ratio (Rapson et al., 2020). The decrease in FLCs at exams in the present study occurred alongside an increase in the cortisol:DHEA ratio, driven by the increase in cortisol. In addition, salivary IgA also decreased at the exam period, which is consistent with previous findings in relation to exam stress (Afrishama et al., 2016; Deinzer et al., 2000; Jemmott and Magloire, 1988). Interestingly, the percentage reduction in IgA was not as large as κ and λ FLCs and the IgA finding also failed to reach statistical significance; highlighting FLCs as a sensitive measure to this type of stress. It demonstrates the importance of not measuring single physiological parameters and using a multi-biomarker approach (Gleeson et al., 2017). FLCs may act as a useful adjunct to IgA to improve reliability of findings.

As a relatively new salivary analyte, physiological stimuli responsible for FLC secretion have yet to be ascertained thus the underlying cause for the observed reduction in FLC secretion in response to exam period stress is currently uncertain. Given FLCs present in saliva are secreted from local plasma cells most of which are also secreting IgA, (Rapson et al., 2020) it is likely that mechanisms responsible may overlap with IgA. Cortisol has been found to inhibit IgA concentrations and cortisol has been proposed as a probable mediator of mucosal immunity (Afrishama et al., 2016; Engeland et al., 2019; Sabbadini and Berczi, 1995). The present study did not reveal any correlation between cortisol and FLCs levels but the potential influence of cortisol activity on salivary FLCs warrants further investigation in laboratory studies.

Depression and anxiety was higher at the exam period compared to baseline. Using the HADS, as employed in the present study, high rates of anxiety and depression have been found previously in students (Webb et al., 1996). HADS anxiety and depression has been shown to significantly

increase from before a course started to mid-course (Andrews and Wilding, 2004). The current findings highlight that exam periods may exacerbate feelings of anxiety and depression. This could be particularly worrying in individuals already suffering from adverse mental health and may have implications for student performance, as previously shown (Andrews and Wilding, 2004).

While the number of life stresses reported in the build up to the exam period increased perceived severity of events decreased. Life events stress was associated with significantly lower FLC and IgA secretion rates at baseline. Stressful life events have previously been inversely associated with salivary IgA in university students (Ng et al., 2004) and in general have been shown to increase the risk of infectious illness (Boyce et al., 1977; Cobb and Steptoe, 1996; Graham et al., 1986). Those reporting infectious symptoms also reported a greater number of life events/more severe life events at baseline, where only severity related to infection at exams. Therefore the burden of life events may indeed have been higher during the period without exams.

It has been found previously that self-reported well-being progressively declines throughout the semester, reaching its lowest during the final exam period (Preoteasa et al., 2016). Here we found a significant decrease in general well-being from baseline to exams. As anticipated, lower well-being was reported by those with infectious symptoms; however, the proportion of patients reporting symptoms did not increase in relation to the exam period. Therefore, the decrease in salivary parameters did not occur parallel to any increase in symptoms of infection. It is currently not known if FLCs play a functional role in oral immunity and health outcomes and here we investigated their link with infectious symptoms for the first time. This preliminary investigation into this topic suggests that FLCs in saliva are not related to infection symptoms or general health/well-being. It should be noted that this study measured self-reported symptoms of infection and were not clinically documented. Indeed, a high proportion of participants reported an infection symptom/symptoms during recording and, although the study was conducted in the winter period and peak cold/flu season, it's likely these symptoms did not reflect a true viral or bacterial infection in a number of cases. To confirm if FLCs have any relationship with infection risk, future research should seek to have infectious symptoms clinically verified. Further research is required to

determine if, like IgA, FLCs play a role in oral defence and if their secretion performs any protection against URTIs.

A range of behaviours were assessed to examine if students changed aspects of health and lifestyle in the build up to exams as potential confounding variables. Overall, health behaviours did not substantially vary. Numbers of hours slept per night did not significantly differ between time points. In addition, there was no difference in self-reported oral health at baseline compared to exams nor did oral health relate to salivary measures (although there wasn't much variation in scores between participants, with most scoring at the higher end of the spectrum). A dental examination would have been more accurate than self-report and could have revealed different findings. Academic stress has previously been shown to increase gingival inflammation (Johannsen et al., 2010). Although if this was the case, an increase, rather than decrease, in FLCs would be expected given oral inflammation increases concentrations of immunoglobulins in saliva (Brandtzaeg, 1998). Time spent exercising did not change but there was a greater contribution of moderate activity vs vigorous activity to overall time at exams. Consumption of alcohol reduced in frequency and volume of units between baseline and exams. There was no relationship between exercise, alcohol consumption or sleep and salivary parameters. This accords with previous findings where reported health behaviours did not significantly impact upon salivary FLC variability (Rapson et al., 2020).

The present study is not without limitations. Participants completed 3 days of saliva sampling to generate a representative picture of levels at each timepoint and avoid comparing single measurements which can be influenced by day to day-variability within individuals. Sampling over a longer consecutive period would have enabled more data to be encompassed when creating a level for each study wave but had to be balanced against burden for participants and resources for laboratory analysis of multiple samples. We explored a range of health behaviours to explore if they changed as a result of the exam period and thus potentially could explain findings rather than stress itself. It is possible that variables not measured as part of this study may have also changed and could be included in future studies. Finally, saliva sampling protocol adherence was measured via self-report. Ideally, when resources allow, diary-based systems should be used alongside

objective measures (i.e atigraphy, electronic monitoring). This is the first investigation into salivary FLCs and psychological stress and included a relatively modest 58 participants. Follow-on studies involving a much larger participant cohort and adopting a multi-level approach to examine a range of variables that could influence salivary responses are required to corroborate the present findings.

Stress arising from examination periods is a common life stressor and the number of exams young people experience seems to be increasing, particularly in countries such as the UK, which can be overwhelming and have negative impact on mental and physical well-being (The National Society for the Prevention of Cruelty to Children, 2019). Salivary FLCs may offer a sensitive tool to detect this type of academic stress and monitor the effectiveness of interventions to tackle stress. Present findings require validation as part of larger studies. Future research should also explore salivary FLCs in relation to other types of psychological stress to determine their potential contribution in this field of research. We, among others, have previously advocated the use of multiple biomarker panels and avoidance of single marker approaches in health and behavioural research to increase robustness of findings (Campbell and Turner, 2018; Rapson et al., 2020). This preliminary study puts forward salivary FLCs as a potential candidate to include alongside other markers including IgA and HPA axis activity to support investigations into the impact of psychological stress on the body.

Author contributions

J. Heaney and J. Campbell contributed to conception and design, data analysis and interpretation and drafted the critically revised the manuscript. L. Irshad contributed to data acquisition, laboratory analysis and critically revised the manuscript. S. Faustini contributed to laboratory analysis and critically revised the manuscript. L. Evans contributed to laboratory analysis. M Drayson contributed to interpretation and critically revised the manuscript. All authors gave their final approval.

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Table 1. Participant characteristics at baseline and health behaviours at baseline and exams

Participant characteristics (N = 58)	Baseline	Exams
Age (years), median (range)	20 (18-26)	
Ethnicity, %		
White	66	
Asian	17	
Black	3	
Mixed race	10	
Did not disclose	3	
BMI (kg/m^2), median (range)	21.5 (17.7-35.7)	
BMI Category, %		
Underweight <18.5	4	
Healthy weight 18.5-24.9	86	
Overweight 25-29.9	6	
Obese 30+	4	
Total exercise per week (hours), median (range)	4.3 (0-11.5)	4 (0-10.5)
Vigorous exercise per week (hours), median (range)	3 (0-10.5)	2 (0-6)
Moderate exercise per week (hours), median (range)	1 (0-7.5)	2 (0-9)
Smokers, %	3.4	3.4
Consumed alcohol, %	78	66
Units per week, %		
1-5	18	35
6-10	14	11
11-20	30	13
20+	16	7
Average hours slept per night	7.1 (4.3-9.5)	7.3 (5.3-9.5)
Oral health score	121 (109-130)	119 (105-132)

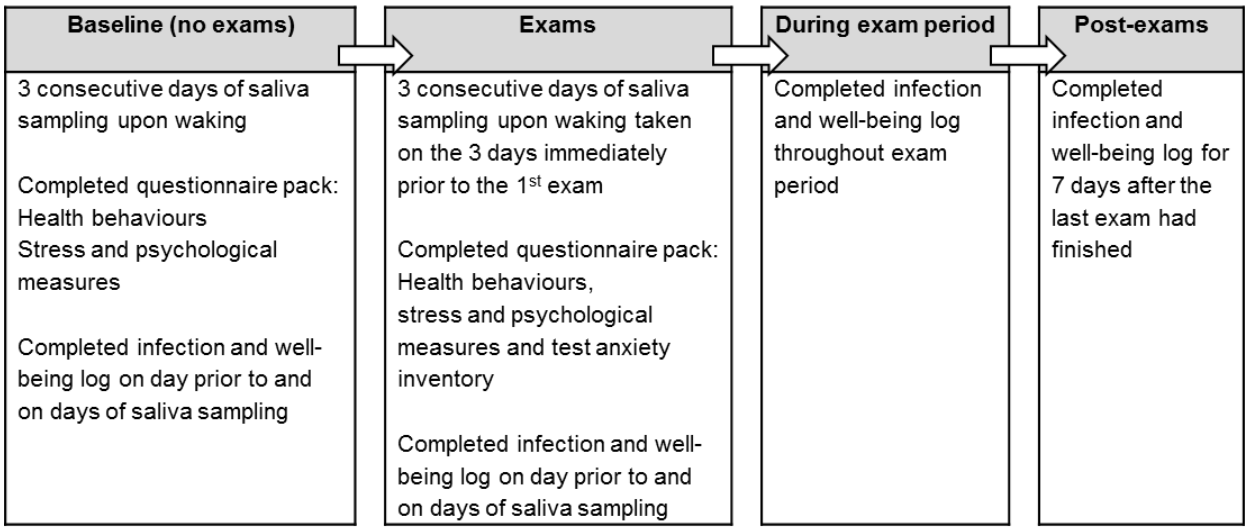

Figure 1. Schematic of the study protocol and timings of all measures

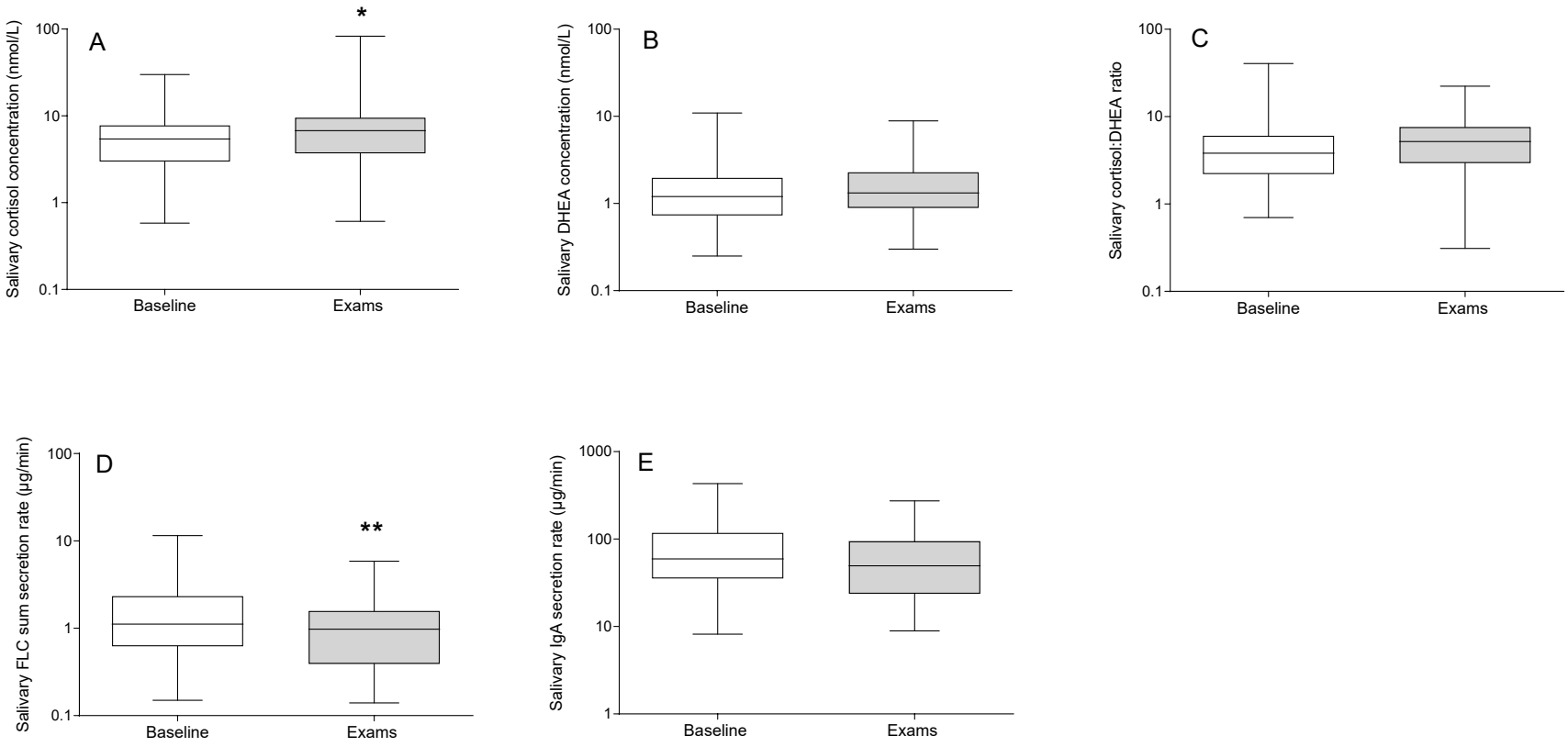

Figure 2. Salivary biomarkers at baseline and exam period

Data is shown for salivary biomarkers during a period of no exams (baseline) and prior to the start of an exam period (exams). Salivary cortisol (A) significantly increased at exams, *$p < .05$. Salivary DHEA (B) did not significantly change. The cortisol:DHEA ratio (C) increased at the exam period although this did not reach statistical significance ($p = .06$). The free light chain sum secretion rate (D) significantly decreased from baseline to exams, **$p < .01$. Salivary IgA secretion rate also decreased although this change was not statistically significant ($p = .07$).

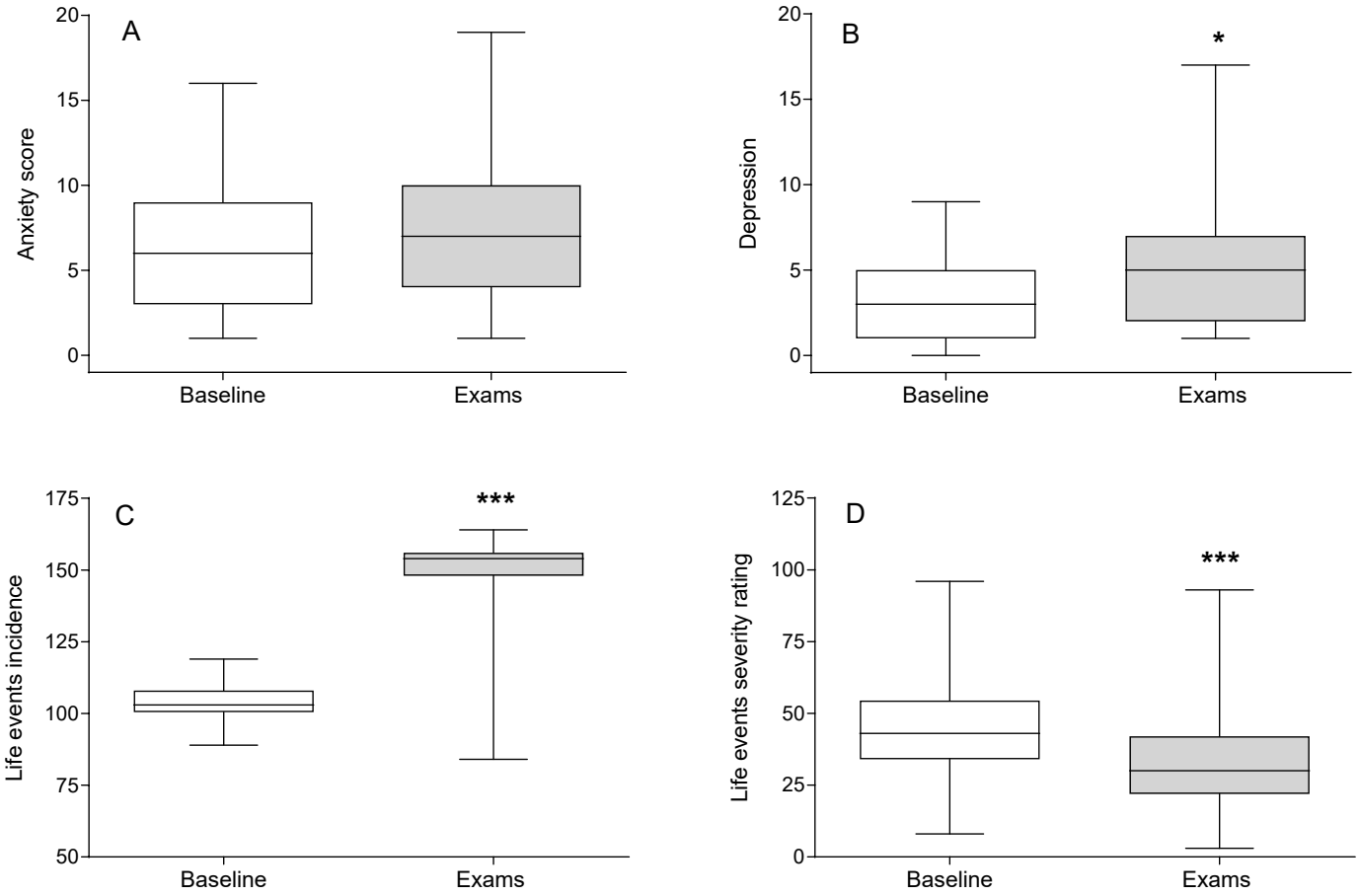

Figure 3. Anxiety, depression and life events at baseline and exam period

Data is shown for anxiety (A), depression (B), number of life events (C) and severity of life events (D). Anxiety, depression and number of life events increased from baseline to exams but perception of severity of life events decreased at exams: *$p < .05$; ***$p < .001$ vs baseline

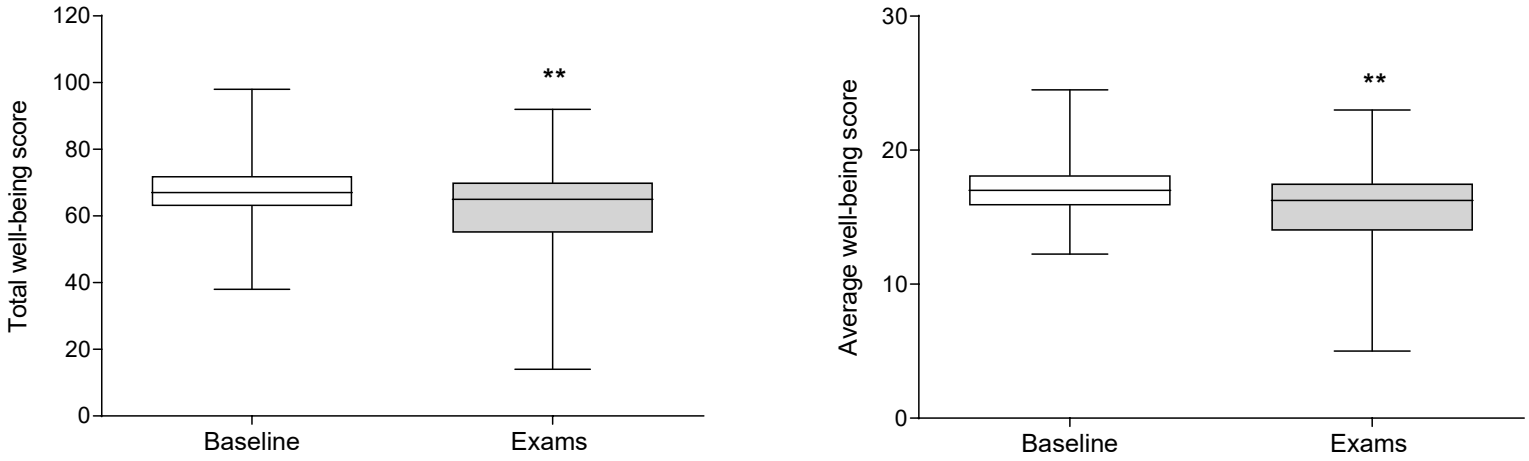

Figure 4. Well-being at baseline and exam period

Total well-being across each period (left) and average well-being each day within each period (right) significantly decreased from baseline to the exam period, $^{**}p < .01$

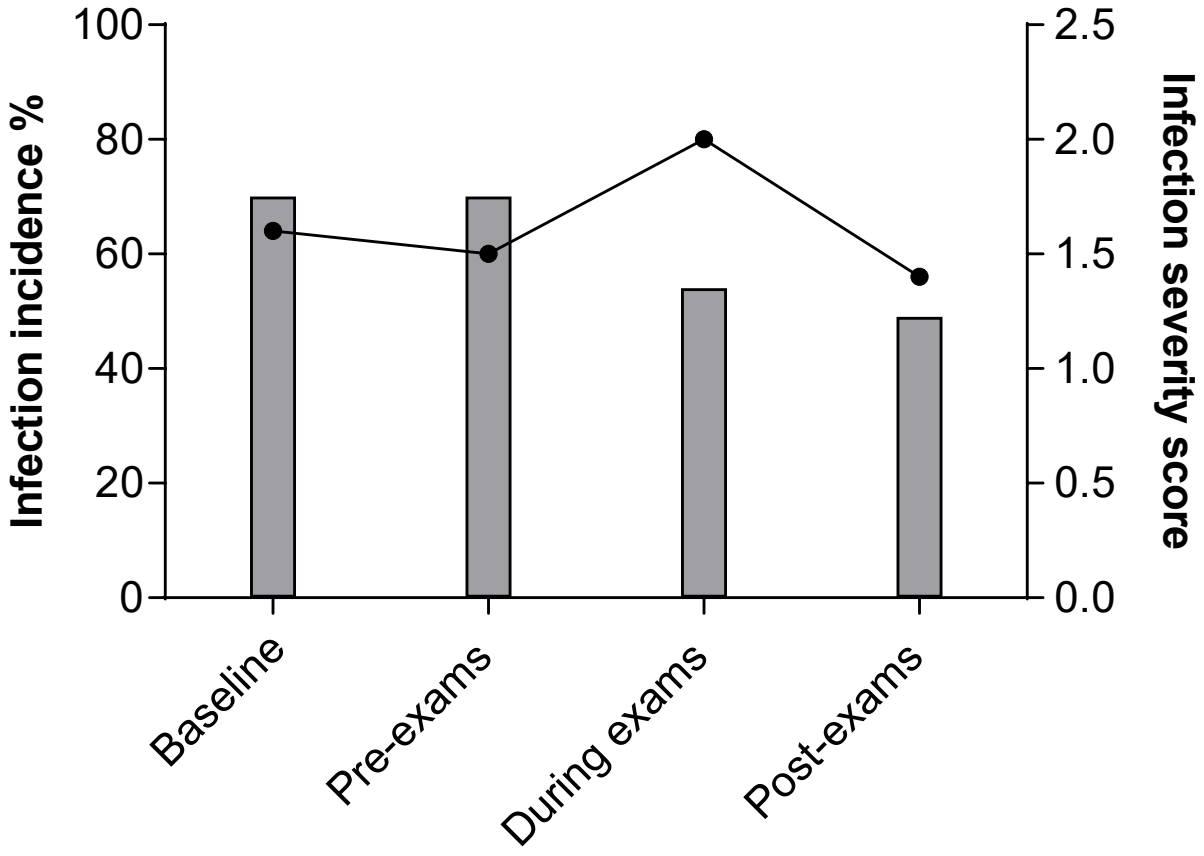

Figure 5. Self-reported infection incidence (bars) and infection severity score (line)

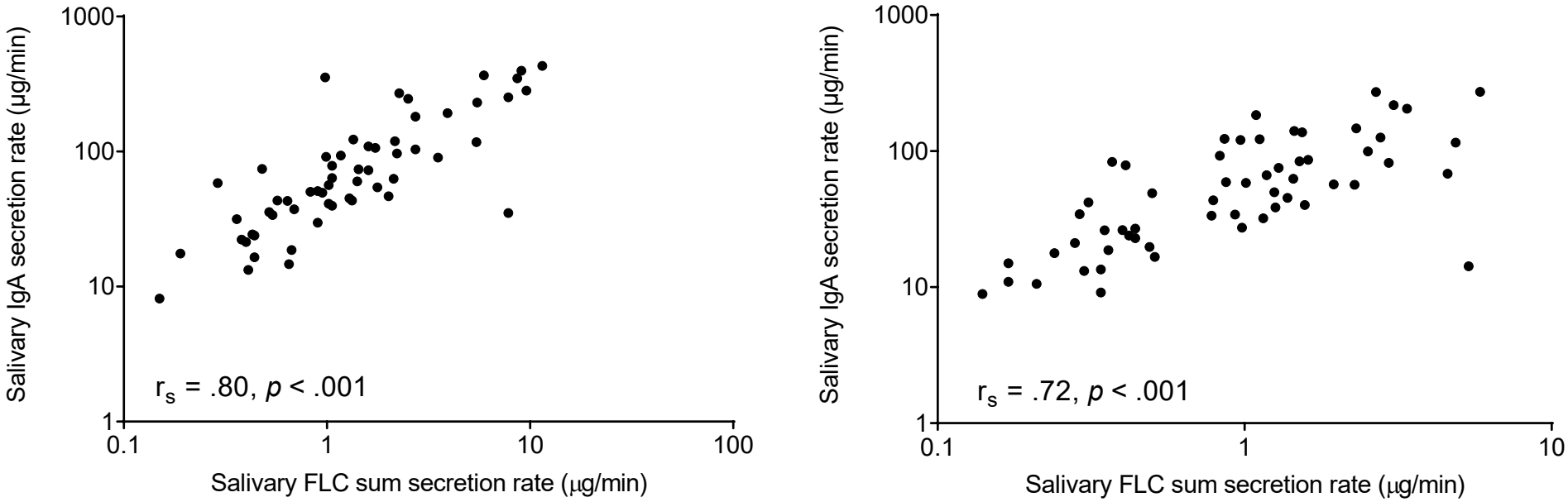

Figure 6. Relationship between FLC sum and IgA secretion rates at baseline (left) and exams (right)