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Free light chains as an emerging biomarker in saliva: biological variability and comparisons with salivary IgA and steroid hormones

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Abstract

Background: Salivary free light chains (FLCs) are an emerging biomarker in health and behavioural research. However, little is known regarding biological variability of salivary FLCs and how they relate to other established salivary biomarkers. This study aimed to investigate the diurnal and day-to-day variation of salivary FLCs and their relationship with salivary IgA and steroid hormones. **Methods:** A total of 46 healthy adults participated in studies exploring the biological variability of FLCs. Diurnal variation was investigated by collecting saliva samples immediately upon waking, 0.5h, 3h, 6h, 9h and 14h post-waking. Saliva samples were assessed for FLCs, IgA, cortisol and dehydroepiandrosterone (DHEA). Between-day variation in FLCs and IgA was assessed by collecting saliva samples immediately upon waking for seven consecutive days. Participants underwent a dental examination to exclude oral health as a potential confounding variable. Within and between-person day-to day variation was explored in relation to a range of different factors: awakening time, sleep, exercise, well-being and alcohol consumption.
Results: Salivary secretion rates of FLCs decreased following waking and up to 3h post-waking and then plateaued. This same pattern was observed for IgA. DHEA was stable upon waking and higher levels were seen in the morning with significantly lower levels thereafter. Cortisol levels significantly increased 0.5h post-waking then continued to decline across the day. FLCs were significantly correlated with IgA but not cortisol or DHEA. Both FLCs and IgA parameters showed day-to-day variability, with coefficients of variation ≥ 40%. Earlier waking time was significantly correlated with higher FLC and IgA secretion rates. Inter-person differences in saliva parameter variability were observed but the degree of variation in FLCs and IgA was related within person. Inter-person day-to-day variation appeared to be uninfluenced by lifestyle or behavioural factors.
Conclusions: Saliva FLCs secretion exhibits diurnal fluctuation that mirrors IgA fluctuation. Findings strongly indicate salivary FLC secretion is orchestrated by local plasma cells. FLCs and IgA both showed notable variability day-to-day, which was similar within person and influenced by awakening time. FLCs offer a promising adjunct to IgA in the measurement of oral immune activation.

1. Introduction

The use of salivary biomarkers is expanding across behavioural immunology research. As a non-invasive measure that can be collected quickly, with ease, and without specialist training, saliva is preferable to blood in a variety of studies, particularly those that include very young or elderly populations, field research or study designs that involve repeated sampling. Salivary immunoglobulin A (IgA) is one of the most widely used salivary biomarkers in psychoneuroimmunology research and studies investigating the immune system in relation to stress, health and exercise. IgA in saliva is an important immune biomarker. As the principle immunoglobulin in the oral cavity, it performs a key role in the first line of defence against pathogens and assists in controlling carriage of bacteria.[1] IgA exerts anti-microbial properties in a number of ways; it inhibits adherence via agglutination, opsonises bacteria, activates compliment and neutralises viruses.[2, 3]

Despite the extensive use and volume of publications concerning salivary IgA as a marker of immune status, there are shortcomings to its utility as a standalone biomarker. Firstly, levels are subject to diurnal variation, which has to be considered for study design in relation to timing of sampling and controlled for in studies with multiple samples. Levels of salivary IgA peak immediately after waking and then decline thereafter.[4, 5] Other commonly used salivary biomarkers show a similar diurnal pattern of higher levels in the morning compared to the evening, such as cortisol and DHEA.[6-8] IgA has been shown to exhibit a high degree of variation both within and between individuals when taking measurements on different days.[9] A study that measured salivary IgA over a 30-day period found that levels varied within individuals, between populations based on exercise activity, and that the degree of within-person variation differed based on group.[10] Fluctuations within individuals day-to-day has also been seen in case studies.[11] For most analytes, reference ranges can be determined, often with respect to age and gender. However, high coefficients of variation (CV) between individuals, often in excess of 60%, prevent the establishment of a healthy reference range for IgA in saliva. Indeed, commercially available assay kits often state ranges across nearly 800µg/mL based on small cohorts of individuals. High CVs within-individuals, in the region of 50%, create difficulties in monitoring individuals as to detect true change it has to be above their normal biological variance. Determination of baseline variance also

requires observation over multiple days or weeks.[10, 11] Therefore, despite its widespread use, the biological variability of IgA make it a challenging marker to interpret. Consequently, it has been suggested that IgA should not be used in isolation[12] and integrated panels of biomarkers may offer a more precise approach to investigating health outcomes in individual people.[13] Salivary free light chains (FLCs) present an interesting new candidate for such models.

Immunoglobulins are produced by plasma cells and comprise two identical heavy chains and two identical light chains, which can be either kappa or lambda isotypes. During the process of immunoglobulin synthesis, plasma cells produce light chains in excess of intact immunoglobulin; these surplus light chains, known as free light chains (FLCs), are released into the circulation.[14] FLCs enable sensitive measurement of disease activity and responses to treatment in plasma cell malignancies, such as multiple myeloma, and form part of International Guidelines for diagnosis and monitoring.[15-19] FLCs are also dysregulated in a range of non-malignant disorders (including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, heart failure, diabetes, renal disease, asthma, chronic obstructive pulmonary disease, inflammatory bowel disease and HIV infection) and may serve as a useful marker of disease activity and prediction of disease course.[20-22] FLCs have been shown to be prognostic for all-cause mortality in the general population, whereby higher levels negatively predicted survival in a large cohort of individuals aged ≥ 50 years without plasma cell disorders. [23] In light of these studies, FLCs have progressed from a cancer marker of monoclonal gammopathies to a biomarker of inflammatory disease, immunosenescence and health and disease in the general population.[23-25]

Recent developments in assay sensitivity have enabled reliable quantitation of FLCs in saliva.[26] Reference ranges of salivary FLCs appear to have much tighter physiological limits across persons compared to IgA.[26] Initial studies have investigated how FLCs in saliva are influenced by age, acute exercise and exercise training.[26, 27] Elderly individuals have been found to have markedly higher levels of salivary FLCs compared with younger adults, likely reflecting poorer oral health and a greater degree of local inflammation and immune activation in this age group.[26] A period of intensified exercise training increased resting salivary FLC parameters, possibly indicative of oral inflammation and highlighting FLCs as a new candidate to measure exercise-induced stress.[27] The

ability to assess FLCs in saliva provides wide scope for their use; however, little is known about fundamental aspects of their physiology in salivary fluid. Specifically, there is a lack of data regarding the natural variation of FLC levels in oral fluid and whether this varies across the day or day-to-day. Further their relationship with IgA, and other commonly measured salivary biomarkers in behavioural immunology research, such as cortisol and dehydroepiandrosterone (DHEA), is currently unknown.

Variation of measurement is an important issue in biomarker research that needs to be taken into account for study design and results interpretation. It is central in order to conclude how salivary measures are affected by particular physical, psychological, environmental stressors or by interventions.[10] In order to utilise salivary FLCs in health research, a greater understanding of fundamental aspects of their biological variability and how levels in saliva relate to established biomarkers is required.

The aims of the present study were to: investigate if saliva FLCs exhibited a diurnal rhythm and compare to other analytes known to exhibit diurnal variation (IgA and steroid hormones – cortisol and DHEA); examine the correlation between FLCs, IgA, cortisol and DHEA; examine day-to-day variation in salivary FLCs in comparison with IgA; and explore and "trait and state" behavioural factors that could influence FLC variability.

2. Materials and Methods

2.1. Participants and study design

Cohort 1 diurnal variation: healthy adults (N = 12, 10 female) with a mean age of 23.7 ± 3.5 with a mean BMI of 23.5 ± 2.2 kg/m^2. Participants were asked to collect a saliva sample over the course of a typical weekday at 6 different time points: immediately upon waking (0h), 30 minutes post-waking (0.5h), and then at 3h, 6h, 9h and 14h post-waking. A 14h rather than 12h sample was included to ensure a sample in the evening period was captured in the case of participants waking early in the morning. Cohort 2 between day variation: healthy adults (N = 34, 22 females) with a mean age of 29.1 ± 8.0 years and mean BMI of 22.0 ± 2.5 kg/m^2. Participants were asked to collect a sample immediately upon waking for seven consecutive days. Key inclusion criteria for the

cohorts were no chronic illness or history of chronic illness. All participants gave written informed consent prior to the study, which had the appropriate Ethics Committee approval from the University of Birmingham.

2.2. Saliva sample collection procedure and assays

Each participant was provided with a pack of universal tubes labelled with the sampling times (cohort 1) or days (cohort 2). Participants were briefed concerning the collection procedure and sampling times and performed a practice sample observed by a researcher. Saliva samples were collected using the same technique across cohorts. 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. For the diurnal variation study, 11% of samples were recorded as late; these samples were from samples later in the day (≥ 6h after waking) and all within 1h of the intended sampling time and thus were not excluded from analysis. For the between day study, 3.3% of samples were missing or excluded due to lateness in sample acquisition (classed as more than a 30 min delay after 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 and lambda FLCs were quantified using commercially available sensitive sandwich ELISAs (Abingdon Health, York, UK). Salivary IgA, cortisol and DHEA were quantitated using sensitive direct (IgA) and competitive (cortisol and DHEA) ELISAs (IBL International, Hamburg, Germany). Intra-assay coefficients of variation (CV) were: 8.5% for kappa FLC, 5.2% for lambda FLC, 8.6% for IgA, 5.7% for cortisol and 8.2% for DHEA. Individual participant samples were measured within the same plate for both cohorts and all fitted on one plate for the diurnal variation study. Regarding the larger between-day study, inter-assay CV's were: 16.6% for kappa FLC, for 13.7% lambda FLC and 12.3% for IgA.

2.3. Salivary parameters

The assays generated concentrations for kappa and lambda 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.[28] Secretion rates of FLCs (µg/min) were calculated as saliva flow rate x kappa/lambda/IgA/FLC sum concentration and are reported herein. Cortisol and DHEA are not affected by saliva flow rate and thus only concentrations are reported.[29] The FLC sum (kappa + lambda) was included as this measure has been employed in the general population as an indicator of total immunoglobulin production and as such is used as a measure of global immune activation and therefore basal health status. [23] 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.[30, 31] Individual kappa and lambda 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

In cohort 2, to investigate if lifestyle factors and health behaviours were related to potential variability in salivary measures, participants completed a questionnaire pack on the first day of saliva sampling. Participants were asked about habitual exercise per week and typical alcohol consumption in terms of frequency of drinking and units consumed per week. [32] At study entry, participants were provided with a log book to be completed on the day prior to sampling and on every day during sampling to record: duration and intensity of exercise (which was multiplied to provide an exercise score), hours slept, time of waking, and general well-being. General well-being was measured 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.[33]

2.5. Oral health exam

To exclude periodontal disease as a confounding factor influencing day to day fluctuations in salivary parameters, at study entry participants from cohort 2 were screened to ensure the study cohort had no periodontitis and no gingivitis. Dental examinations were all performed by the same fully qualified dentist and StR in Restorative Dentistry (BDS, MFDS RCS) and clinical lecturer in the department of Periodontology. The clinician (based at Birmingham Dental Hospital and School of Dentistry) performed the Basic Periodontal Examination (BPE) as a rapid screen for presence of periodontitis or health and supplemented by recording full mouth and bleeding sites (percentage bleeding) for code 1 and 2 scores in order to measure gingival inflammation. Probe depths were determined in relation to probe banding at 3.5-5.5mm. The examination was performed in accordance with British Society of Periodontology guidelines. The BPE divides the mouth into sextants and the worse score within the sextant is recorded in the grid. Scores 0–4 correspond to the following classifications: 0 = health; 1 = gingival inflammation (provided bleeding >10%, in absence of > 10% bleeding they were then classed as healthy); 2 = presence of plaque retaining factors (here % sites bleeding was assessed as for code 1); 3 = mild periodontitis; 4 = moderate/severe periodontitis. A bleeding score less than 10% is deemed to be clinically acceptable.[34] On the BPE 22% scored 0 in all sextants. No participant scored greater than 2 in any sextant, indicating an absence of periodontitis. In participants who did receive codes of 1 or 2, percentage bleeding was < 10% leading to a classification of healthy for all participants.

2.6. Data analyses

Analyses were undertaken using IBM SPSS version 21. One-way repeated measures ANOVA were used to assess changes in salivary parameters over the course of the day and over the course of 7-days. Salivary data was not normally distributed and statistical analysis was performed on the logarithmic transformation of the data using ANOVA. Greenhouse-Geisser corrected *F* values are reported for repeated measures analyses and partial η^2, a measure of effect size, is reported throughout. To assess differences between individuals, median levels in key saliva parameters were calculated for each person over the 7-day period and a between-individual CV% was calculated. To measure day-to-day fluctuations in saliva parameters CV% were calculated for

each saliva parameter for each participant, to provide a measure of within-person variation, with median CV% reported for the study cohort overall. Friedman tests were conducted to assess if waking time, sleep, exercise or well-being differed across the 7-day sampling period. Factors influencing day-to-day-salivary parameters (awakening time, sleep, exercise day prior to morning sample, well-being day prior to morning sample) were correlated with the corresponding sample for all days and all participants using Spearman's rank correlation. Spearman's rank correlation was used to investigate inter-individual variability by correlating CV% and maximum change over the 7-day period in salivary parameters with lifestyle factors at baseline (habitual exercise and alcohol consumption) and behaviour and well-being during the sampling week (sleep, exercise, well-being). Spearman's rank correlation was also used to assess relationships between FLCs, IgA and steroid hormones. Day-to-day variation of FLCs and IgA and variation between sexes was compared using Mann-Whitney tests.

3. Results

3.1. Diurnal variation of salivary FLCs and comparison to IgA, cortisol and DHEA

The mean awakening time for the study cohort was 08:23h (SD ± 1h 11 minutes). Saliva parameters for all time points across the day are shown in Table 1. Saliva flow rates were significantly lower at the start of the day, with a significant difference observed at 0h and 0.5h post-waking vs all other time points. Salivary secretion rates of FLCs decreased following waking and then plateaued. This same pattern was observed for IgA. Typically secretion rates of FLCs and IgA were significantly lower at 3h, 6h, 9h and 14h post-waking compared with 0h and 0.5h post-waking. Kappa secretion rates were consistently higher than lambda but showed the same pattern of variation as each other across the day. The reduction immediately following waking was more pronounced for IgA than FLCs. A 32% decrease in FLC sum secretion rate was seen in the first 30 minutes following waking compared with a 60% reduction in IgA secretion rate. Higher levels of DHEA were seen at 0h and 0.5h post-waking, with significantly lower levels thereafter throughout the day. There was no significant difference between DHEA at the first two time points. Cortisol exhibited an awakening response characterised by a significant increase in levels at 0.5h post-waking. Levels then continued to decline across the day. To visually illustrate these patterns of

diurnal variation of salivary parameters, FLC sum and IgA secretion rates and cortisol and DHEA concentrations across the day are shown in Figure 1.

Table 1. Diurnal variation of salivary free light chains, IgA, cortisol and DHEA across the day

	Time post-waking						
Median (range)	**0h**	**0.5h**	**3h**	**6h**	**9h**	**14h**	
Flow rate (mL/min)	0.32 (0.10–0.93)	0.35 (0.18–1.11)	0.61[ab] (0.17–2.02)	0.61[ab] (0.23–1.83)	0.54[ab] (0.22–1.32)	0.48[ab] (0.28–1.46)	$F(5,55)= 8.78$, $p < .001$, $\eta^2 = .444$
Saliva parameters controlling for flow rate (µg/min)							
Kappa secretion rate	0.25 (0.06–7.05)	0.20 (0.08–2.34)	0.08[ab] (0.02–1.89)	0.04[ab] (0.01–0.13)	0.03[ab] (0.01–0.15)	0.06[ab] (0.01–0.19)	$F(5,55)= 14.2$, $p < .001$, $\eta^2 = .563$
Lambda secretion rate	0.20 (0.03–6.86)	0.09 (0.04–0.73)	0.04 (0.02–0.40)	0.04 (0.01–0.09)	0.03[a] (0.01–0.08)	0.03[a] (0.01–0.10)	$F(5,55)= 12.7$, $p < .001$, $\eta^2 = .536$
FLC sum secretion rate	0.41 (0.11–13.92)	0.28 (0.12–2.97)	0.11[ab] (0.03–2.29)	0.09[ab] (0.02–0.19)	0.06[ab] (0.02–0.22)	0.10[ab] (0.03–0.26)	$F(5,55)= 15.0$, $p < .001$, $\eta^2 = .557$
IgA secretion rate	63.34 (14.03–478.94)	25.59[a] (7.98–102.58)	26.50[a] (10.99–88.24)	23.52[a] (5.83–38.46)	16.00[a] (5.78–45.45)	16.60[a] (6.61–35.79)	$F(5,55)= 8.7$, $p < .001$, $\eta^2 = .443$
Saliva concentrations (nmo/L)							
Cortisol concentration	4.90 (2.07–12.86)	10.24[a] (2.37–32.21)	3.81[b] (0.66–21.17)	2.82[b] (1.21–15.29)	1.78[abd] (0.75–10.85)	0.82[abcde] (0.44–3.01)	$F(5,55)= 14.1$, $p < .001$, $\eta^2 = .563$
DHEA concentration	0.91 (0.31–5.74)	1.22 (0.45–2.07)	0.73[ab] (0.18–2.44)	0.57[ab] (0.20–2.55)	0.39[ab] (0.12–2.89)	0.38[abcd] (0.10–1.80)	$F(5,55)= 9.9$, $p < .001$, $\eta^2 = .473$

[a]indicates a significant difference vs 0h; [b]indicates a significant difference vs 0.5h; [c]indicates a significant difference vs 3h, [d]indicates a significant difference vs 6h and [e]indicates significant difference vs 9h, $p < .05$

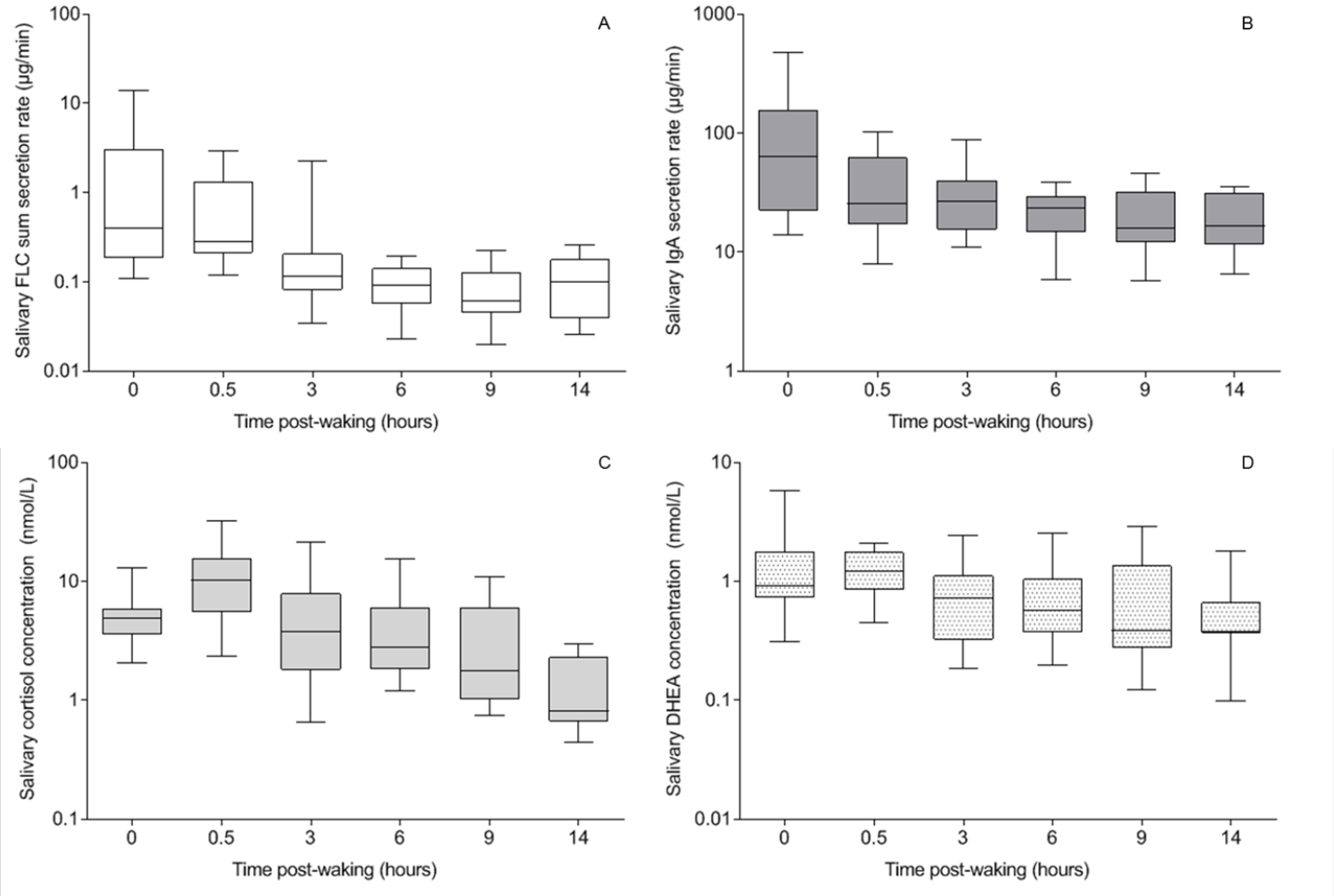

Figure 1. Diurnal variation of salivary FLC sum secretion rate (A), IgA secretion rate (B), cortisol concentration (C) and DHEA concentration (D)

FLC and IgA salivary secretion rates were significantly higher upon waking (0h) compared with 3h post-waking onwards (FLC) or all other timepoints throughout the day (IgA). Salivary IgA secretion rate significantly decreased between 0h and 0.5h post-waking whereas FLCs did not exhibit a significant decline during the same period. Cortisol exhibited a significant increase at 0.5h post-waking, representing the awakening response, followed by an

incremental decrease throughout the day. DHEA concentrations were significantly higher at the first two time points following waking compared with the other time points throughout the day.

3.2. Day-to-day variation of salivary FLCs and IgA

Salivary flow rates and immunoglobulin parameters over the 7-day sampling period are shown in Table 2. As in the diurnal study, kappa secretion rates were higher than lambda but followed consistent patterns of fluctuation across the days. No significant differences were observed between days for any of these salivary parameters. However, when examining individual variability, expressed as CV%, a meaningful degree of variation was seen over the 7-day period. Table 3 reports CV% for the saliva parameters, where CVs% were calculated for each individual participant over the 7-day period and median values are reported. CVs between 52–77.6% were seen for secretion rates. The median variation within-individuals appeared greater for FLC parameters compared with IgA: median CV% for FLC sum secretion rate was significantly higher than IgA secretion rate ($U = 369$, $p < .05$). While the % variation appeared high, when examining the maximum change in units (the highest value minus the lowest value observed over the 7-day period), median absolute differences in µg/min were more modest (Table 3).

Table 2. Between day variation of salivary free light chains and IgA over a 7 day period

Median (range)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Flow rate (mL/min)	0.39 (.03–3.10)	0.30 (0.09–2.70)	0.36 (0.08–2.30)	0.42 (0.05–2.71)	0.42 (0.06–1.67)	0.36 (0.07–1.17)	0.47 (0.06–3.58)
Saliva parameters controlling for flow rate (µg/min)							
Kappa secretion rate	0.48 (0.001–8.88)	0.30 (0.01–4.41)	0.48 (0.01–5.35)	0.44 (0.01–5.94)	0.36 (0.02–8.48)	0.31 (0.01–10.28)	0.46 (0.003–8.25)
Lambda secretion rate	0.25 (0.003–9.79)	0.17 (0.02–4.85)	0.23 (0.02–4.31)	0.26 (0.01–5.43)	0.23 (0.02–7.24)	0.28 (0.01–11.31)	0.25 (0.01–5.94)
FLC sum secretion rate	0.75 (0.004–17.22)	0.42 (0.02–9.26)	0.71 (0.04–7.74)	0.92 (0.02–10.61)	0.63 (0.05–15.72)	0.72 (0.02–21.58)	0.94 (0.01–14.19)
IgA secretion rate	58.02 (3.72–721.75)	58.48 (2.54–581.01)	50.13 (5.30–643.50)	73.35 (5.19–419.15)	59.95 (7.63–561.03)	49.34 (2.82–481.11)	60.11 (2.92–1559.96)

Table 3. Coefficient of variations (CV%) for salivary parameters and maximum difference over the 7-day period

	Median CV%	Median maximum difference*
Flow rate	32.8	0.34
Kappa secretion rate	77.6	1.26
Lambda secretion rate	72.5	0.51
FLC sum secretion rate	69.0	1.81
IgA secretion rate	52	94.70

*mL/min for flow rate; µg/min for secretion rates; maximum difference calculated as the largest observed value minus lowest observed value over the 7-day period

3.3. Factors associated with day-to-day variation in salivary parameters

Average time of waking was between 06:59h and 07:55h across the 7 days, and for the cohort overall there was a significant difference between the awakening time ($X^2 = 40.2$, $p < .001$), due to fluctuations in the average awakening time between days. Awakening time was significantly negatively associated with secretion rates of all salivary parameters ($p < .001$ for all correlations). As shown in Table 4, the earlier the time of waking, the higher the FLC/IgA secretion rate. Only weak correlations ($r_s < .10$) were observed between hours slept and saliva parameters. The number of hours slept before the waking sample did not significantly vary day-to-day for the cohort overall. Well-being scores the day before the sample significantly varied across the 7-day period for the cohort ($X^2 = 16.4$, $p < .05$). A weak significant correlation emerged for kappa secretion rate (-.141, $p < .05$), with lower well-being score the day before associated with higher kappa FLC secretion rates the following morning. Fluctuations in well-being score also showed negative relationships the other saliva parameters but were not significant. Exercise score (duration x intensity) the day prior to the morning sample was found to significantly vary across the days ($X^2 = 14.1$, $p <$

.05). There was no relationship between exercise score and saliva parameters the following morning.

Table 4. Correlations between waking time and saliva parameters for all samples

r_s	Awakening time	Hours slept	Well-being day before	Exercise day before
Kappa secretion rate	-.310***	-.072	-.141*	.069
Lambda secretion rate	-304***	-.078	-.101	-.003
FLC sum secretion rate	-.320***	-.090	-.120	.046
IgA secretion rate	-.376***	-.046	-.105	.028

*$p < .05$; ***$p < .001$

3.4. Inter-individual differences and inter-individual differences in day-to-day variation in salivary variables

Figure 2 illustrates individual FLC sum and IgA secretion rates across the 7-day period for all participants. This figure firstly highlights that between individuals there was a large degree of variability in these saliva measures. When analysing the median secretion rate over the 7-day period for each participant, between-individual CV's were 102% and 116% for FLC sum and IgA, respectively.

While the median percentage variability for saliva parameters was relatively high for the sample overall, there was a high degree of inter-individual variation: some individuals displayed large fluctuations across the 7-day period where others were more stable. For FLC sum secretion rate, the lowest CV was 31% and highest 126%; similarly for IgA secretion rate the lowest CV 26% and the highest 170%. Those with high variability in one parameter were likely to have high variability in the other, with IgA and FLC sum concentrations and secretion rates significantly correlated (r_s = .41 and .39, respectively, $p < .05$). Figure 2 illustrates that some individuals had secretion rates very close together across days whereas others have a much wider distribution of secretion rates.

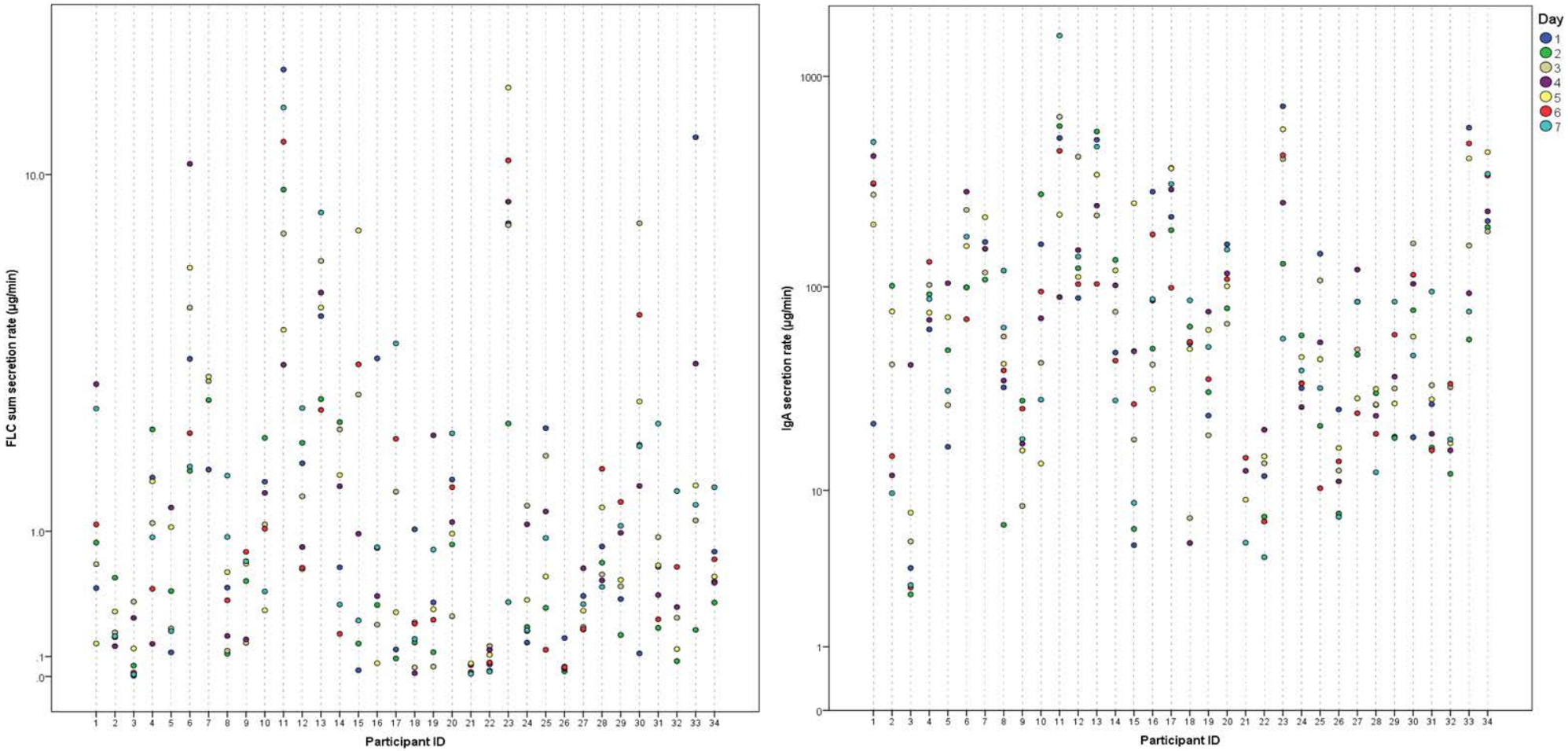

Figure 2. Within-individual variability in FLC sum secretion rates (left) and IgA secretion rates (right) across the 7-day period for all participants

3.5. Baseline factors associated with inter-individual differences in day-to-day-variation

To explore possible reasons why some individuals exhibited high variability between days and others more consistent levels, the association between different factors and measures of variability (CV% and maximum change) were investigated. There was no difference on the basis of sex for CV% or maximum change for any of the salivary parameters. There were no significant associations between habitual exercise (time spent exercising per week) and variability measures. Typical frequency of alcohol consumption and units of alcohol typically consumed per week did not relate to variability of saliva parameters.

3.6. Factors in the sampling week and inter-individual differences in day-to-day variation

The relationship between sleep, exercise and well-being during the sampling week and variability in saliva parameters were assessed. Average sleep was significantly associated with IgA secretion CV% (-.39, $p < .05$) whereby less sleep during the sampling period had higher variability. This pattern was also observed for other saliva parameters but failed to reach statistical significance, but would likely have done so with a larger sample size. No other factors that were measured over the sampling week, average exercise or average well-being, related to variability of saliva parameters.

3.7. Relationship between FLCs and IgA and steroid hormones

In study 1, upon waking median IgA secretion rates were over 200x higher than kappa and lambda FLC secretion rates. These much higher levels of IgA relative to FLCs remained throughout the day. In study 1, significant positive correlations were observed between FLC sum and IgA secretion rates at every time point across the day, $r_s = .63–.86$, $p < .05$.

In contrast, cortisol concentration was negatively associated with FLC sum secretion rates. Higher cortisol related to lower FLC sum values, however, only significantly so upon waking ($r_s = -.59$, $p < .05$) at 14h post-waking ($r_s = -.59$, $p < .05$). The same pattern of negative correlations were observed between cortisol and IgA across the day but were not significant. For both FLCs and

IgA, no consistent relationships were observed with DHEA, with both positive and negative correlations seen at different time points, all of which were non-significant. However, at waking there was a significant association between FLCs and the cortisol:DHEA ratio whereby a higher ratio was correlated with lower FLC sum secretion rate (r_s = -.74), p < .01 . The same pattern was observed at waking for IgA secretion (r_s = -.56) but this failed to reach significance. No other significant correlations with cortisol:DHEA were observed at the remaining timepoints across the rest of the day.

When examining IgA and FLCs during repeated morning sampling in study 2, again, secretion rates of IgA in saliva were much more abundant than FLCs, with levels typically at least 100x higher (Table 3). FLC sum secretion rates were all < 20 µg/min with median values generally < 1 µg/min, whereas median IgA secretion rates were ≥ 50 µg/min with approximately a third of participants showing rates of ≥ 100 µg/min on any given day. FLC sum and IgA secretion rates were significantly correlated on each of the 7 days (r_s = .62–.84, p < .001 for all correlations), indicating that on any given day these measures were strongly associated.

4. **Discussion**

The results of the present study demonstrate for the first time that salivary FLCs exhibit diurnal variation. The diurnal variation flux of saliva FLCs parallels to salivary IgA secretion. The observations of this study strongly indicate that the presence of FLCs in saliva is due to their predominant production from local plasma cells, rather than passive diffusion via their relatively small molecular weight. We also show, using the independent yet complementary biomarkers of salivary FLCs and IgA, that day-to-day variability in local plasma cell immunoglobulin production within-person is associated with awakening time.

The first novel observation from our study herein was that FLCs exhibited diurnal variation. FLCs secretion rates were significantly higher upon waking and 30 minutes post-waking compared to other timepoints throughout the day. The diurnal flux in FLCs was similar to IgA, which showed

higher levels in the morning period and lowest levels in the evening, consistent with previous findings regarding IgA.[4, 5] Fluctuations in IgA and FLCs are not due to saliva volume and are likely under control of a central circadian pacemaker.[35] Similarly, cortisol and DHEA diurnal variation is due to neural regulation of the hypothalamic pituitary adrenal (HPA) axis.[36] As expected, the present study found an increase in cortisol after 30 minutes as a result of the awakening response, thought to be under distinct regulatory influence, and then declining thereafter.[8, 37] DHEA also demonstrated its expected pattern of relative stability upon waking followed by a significant decrease at 3h post-waking.[7] The present investigation reveals the importance of time of day for future studies involving FLCs and re-emphasises time from awaking as a confounding factor for studies that include IgA and steroid hormones. Strict protocols for sampling in morning periods are a particular consideration, as between waking and 30-minutes post waking FLCs decreased by a quarter, IgA decreased by a third, and cortisol doubled in concentration, highlighting a meaningful change in a short time frame.

This study also confirmed for the first time that salivary FLCs and IgA secretion is strongly correlated across the day, and also on consecutive morning samples. The majority of IgA in saliva is from local plasma cell secretion, and IgA concentrations do not correlate well with IgA in peripheral blood.[38] In contrast, cortisol and DHEA enter the saliva via passive diffusion through the cells of the salivary glands,[29] and while much lower than serum, saliva levels are linearly proportional to those in the blood.[39] In a similar manner to IgA, we have previously found that salivary FLCs were not correlated to serum FLCs, despite their relatively small molecular size which may permit passive diffusion.[26] The diurnal variation shown by FLCs in the study herein provides further insight into the biology of salivary FLCs. To our knowledge, any diurnal variation of serum FLCs has not been found, leading to no stipulations regarding time of day of sampling for clinical testing.[40] Taken together, the lack of correlation between serum and saliva and diurnal variation in saliva but not serum, suggests FLCs in saliva are predominantly a product of local secretion from plasma cells within the salivary glands, or entering saliva from gingival crevicular fluid, and reflect plasma cell activity within the periodontal connective tissues. Future studies including paired saliva and serum sampling at multiple timepoints across the day and in conditions

associated with serum FLC dysregulation would provide more understanding on the source of FLCs in saliva but current evidence suggests that FLCs in oral fluid are distinct from FLCs in the systemic circulation.[24, 26]

Greater IgA secretions upon waking have been suggested to be a result of sympathetic activation during sleep, the purpose of which is to protect against bacteria.[41] This could be a response to compensate for a fall in saliva secretion while asleep, which is linked to an increase in bacterial activity.[35, 41, 42] IgA plays an important role in oral immunity and decreases in IgA may increase risk of infections.[43] Thus the diurnal rhythm of IgA may present an important mechanism of oral immunity. It is possible that FLCs and their diurnal rhythm represent another facet contributing to defence of the oral cavity. However, as a new biomarker, the function of salivary FLCs are currently not understood. It has been proposed previously that they may serve as a marker of oral inflammation or immune activation.[26, 27] Or alternatively they may have no functional role per se and may represent pan immunoglobulin secretion, secreted in conjunction with IgA as a result of overlapping stimulation/control mechanisms. Future research exploring the relationship between saliva FLCs, IgA and the oral microbiota is required to explore these possibilities. In this study, FLCs did not correlate with DHEA levels, but lower FLC secretion was associated with higher cortisol upon awakening. Interestingly, upon waking, lower FLC secretion was also associated with a higher cortisol:DHEA ratio, a biomarker that has been linked to ill health and adverse health outcomes in a range of studies;[44-46] this warrants further exploration in future studies.

We observed large inter-person differences in salivary immunoglobulin secretion, as indicated by both IgA and FLCs. These large person-to-person variances in these salivary biomarkers occurred in a population that was fairly homogeneous (young, healthy, with no chronic conditions). The degree of inter-person differences may be found to oscillate to a greater extent in other populations, such as those with chronic disease or in older adults. Indeed, differences in salivary FLCs between differently aged populations have been observed previously and indicate salivary FLCs are strongly and positively associated with age, perhaps due to the cumulative burden of oral inflammation and immune activation.[26]

Another key observation from our study stemmed from the assessment of intra-individual variation in salivary FLCs across a period of 7-days. This is the first study to repeatedly measure salivary FLCs over consecutive days. Both FLCs and IgA showed wide fluctuations across the period (CV% typically > 50%) and it can be concluded that these markers are not stable day-to-day. The variability of these biomarkers was linked within-persons, with high/low variability of FLCs associated with high/low variability of IgA. In contrast, serum FLCs have been shown to have small intra-individual variances (< 10%) when measured at the same time every 2 weeks over the course of 2 months.[47] Again, this yields further evidence that regulation of salivary FLCs is distinct from the systemic circulation and levels do not mirror concentrations seen in blood. In order to monitor a person's salivary FLCs over time, to overcome the issue of within-person variation, we propose that it is essential to assess a baseline level of oscillation over a period of days, and then use absolute change above or below their typical level of oscillation to indicate a meaningful change. Alternatively, oscillation measured over the same number of days in the future (i.e., a secondary timepoint) may indicate a reliable change from the primary timepoint. This strategy is also advised for IgA.[10]

We assessed various factors to try to provide an insight into the day-to-day biological variation in immunoglobulin production observed within persons. Participant samples were all measured within the same assay plate and intra-assay variation was low (< 10%), therefore analytical variability would have made only a small contribution to the variance seen. Awakening time was consistently negatively associated with all salivary parameters, with earlier waking time correlated with greater FLC and IgA secretion rates. Previous studies of salivary analytes have seen relationships with waking time. Indeed, cortisol levels upon waking have been reported to be lower in conjunction with earlier waking[48] and the cortisol waking response has been found to be more pronounced in those who woke earlier.[49] The reason for higher FLCs and IgA with earlier waking times is not clear from the present study and may relate to circadian rhythm or possibly aspects of sleep. Sleep duration showed weak negative associations with immunoglobulin secretions. However, sleep quality and time of going to bed were not captured and the duration of sleep may not have had a sufficiently broad range to fully investigate this aspect, as the number of hours slept did not

significantly vary day-to-day. It could be speculated that lower sleep duration and earlier awakening are representative of a higher state of activation that may lead to increased saliva biomarker activity.

While waking time showed consistent relationships with FLCs and IgA levels, these relationships only explain a proportion of the day-to-day variability observed. The cohort studied were young, active individuals who may have varied activities, behaviours and experiences day-to-day and other factors we did not measure could have influenced daily variation. Variability may be lower in older or sedentary populations: IgA variability has been seen to be highest in elite athletes compared to active or sedentary individuals.[10] IgA secretion has previously been shown to be affected by stress, nutrition, health, physical activity, caffeine, alcohol and light exposure during daytime.[9] A wide variety of additional factors and stimuli need to be explored in future studies to understand their contribution to day-to-day variation of FLCs.

As to why some individuals experienced large variation day-to-day where others had more stable salivary parameters, the factors assessed offered limited explanations. State factors (sex, habitual exercise, typical alcohol consumption) did not influence the degree of variation. Other measures during the sampling week, average exercise or well-being, did not relate to variability measures. As with intra-individual variation, other factors outside of this study may contribute towards inter-individual differences in variation and warrant further investigation. All participants were free from periodontal disease confirmed by a dental examination. In future studies it would be interesting to investigate if those with poor oral health or with chronic inflammation experience different day-to-day variation as a result of ongoing immune activation. To investigate if oral health status and inflammation has an impact on FLC and IgA variability additional data from a cohort with periodontitis, with gingivitis and healthy controls would be required.

Limitations of this study should be recognised. Firstly, adherences to saliva sampling protocols were recorded via a self-reported log. Ideally, diary log systems should be used in conjunction with objective methods, such as actigraphy-based approaches to verify awakening times and electronic monitoring systems to help verify sampling times.[50] Where permitted by resources, future studies

should seek to include objective measures to help verify sampling compliance. Further, while cortisol and DHEA were explored across the day, they were not investigated between days in the present study. Therefore, how the day-to-day variation of FLCs compares to these steroid hormones remains to be verified.

Regular monitoring of salivary IgA is currently recommended by several institutes of sport [51] and is frequently a main outcome variable in behavioural immunology research, specifically in relation to physical and psychological stress. However, the value of IgA as an isolated measure has been questioned[12] and given the inherent variability of measurement, a more integrated approach to salivary biomarker assessment may help make findings more robust. FLCs accord with IgA and fluctuate in a similar magnitude and direction thus they could be used as an adjunct to IgA measurement to provide greater confidence when interpreting results. Single marker approaches to salivary measures in health and behavioural research should be replaced with more integrated biomarker panels in order to demonstrate consistency of findings and provide more robust conclusions.

Measurement of FLCs in saliva could provide a valuable non-invasive tool alongside other salivary biomarkers, such as IgA, cortisol and DHEA. This study adds to the on-going understanding of salivary FLCs and provides novel information regarding diurnal rhythm and variability to inform future studies assessing the utility of this biomarker in stress and behavioural research and as a marker of immune competency.

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