Salivary immunoglobulin free light chains: reference ranges and responses to exercise in young and older adults

Jennifer L J Heaney[1], Michael Gleeson[2], Anna C Phillips[3], Ian M Taylor[2], Mark T Drayson[1], Margaret Goodall[1], Cheng-Shiun He[2], Ida S Svendsen[2], Sophie C Killer[4], and John P Campbell[1]

[1] Clinical Immunology Service, University of Birmingham, Birmingham, UK
[2] School of Sport, Exercise & Health Sciences, Loughborough University, Loughborough, UK
[3] School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Birmingham, UK
[4] English Institute of Sport/ Loughborough Performance Centre, Loughborough, UK

ABSTRACT

Background: *Free light chains (FLCs) have a range of biological functions and may act as a broad marker of immune suppression and activation and inflammation. Measurement of salivary FLCs may provide practical advantages in a range of clinical populations. The aim of the present study was to develop normal reference ranges of FLCs in saliva and assess the effects of acute exercise on FLC levels in younger and older adults.*

Methods: *Saliva FLC concentrations and secretion rates were measured in young (n = 88, aged 18–36) and older (n = 53, aged 60–80) adults. To assess FLC changes in response to acute exercise, young adults completed a constant work-rate cycling exercise trial at 60% VO_{2max} (n = 18) or a 1 h cycling time trial (TT) (n = 10) and older adults completed an incremental submaximal treadmill walking exercise test to 75% HR_{max} (n = 53). Serum FLCs were measured at baseline and in response to exercise.*

Results: *Older adults demonstrated significantly higher levels of salivary FLC parameters compared with young adults. Median (5–95th percentile) concentrations were 0.45 (0.004–3.45) mg/L for kappa and 0.30 (0.08–1.54) mg/L for lambda in young adults; 3.91 (0.75–19.65) mg/L for kappa and 1.00 (0.02–4.50) mg/L for lambda in older adults. Overall median concentrations of salivary kappa and lambda FLCs were 10-fold and 20-fold lower than serum, respectively. Reductions in salivary FLC concentrations and secretion rates were observed immediately post- and at 1 h post exercise, but were only significant for the older cohort; FLCs began to recover between post and 1 h post-exercise. No changes in serum FLCs were observed in response to exercise.*

Conclusions: *The ability to assess FLCs in saliva and the reference ranges provided will likely broaden the use of this biomarker in healthy and clinical populations. The elevated salivary FLCs in older adults may relate to a deterioration of oral health and be important in the context of inflammatory processes and diseases associated with ageing. Exercise did not affect serum FLCs, but reduced salivary FLCs, most notably in older adults, which may reflect reduced transport of FLCs from serum into saliva.*

Key words: Free light chains, saliva, serum, age, exercise

INTRODUCTION

Introduction to free light chains and their production in healthy individuals

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, surplus light chains are produced at a rate of 40% above heavy chains (1). These excess light chains that do not form whole immunoglobulins, known as free light chains (FLCs), are released into the circulation. In healthy individuals approximately 500 mg of FLCs are produced each day, with kappa production outweighing lambda by a ratio of 2:1. In a healthy state, serum FLC reference ranges are 3.3–19.4 mg/L for kappa and 3.7–26.3 mg/L for lambda (2).These reference ranges are based on the first FLC assay developed ten years ago, and new methods for FLC quantitation are now available, leading to a slightly broader range (1.2–55.2 mg/L) in values reported for healthy individuals (3). FLCs are metabolised by the kidneys where up to 10–30 g of FLC can be processed per day (4). Lambda FLCs are cleared from the circulation at a slower rate than kappa FLCs; consequently, the ratio of kappa to lambda FLC (FLC ratio) in serum in healthy individuals is between 0.26–1.65 (2). The short half-life of FLCs in serum (2–4 hours for kappa and 3–6 hours for lambda) compared with whole immunoglobulins (5–8 days for IgA and IgM and 20 days for IgG) enables real time monitoring of immune suppression and stimulation, or disease progression and responses to treatment in conditions involving FLC dysregulation (5, 6).

Corresponding author:
Jennifer Heaney, Clinical Immunology Service
College of Medical and Dental Sciences, University of Birmingham, Birmingham, West Midlands, UK, B15 2TT
Email: j.l.j.heaney@bham.ac.uk

Free light chains as a biomarker in clinical populations

FLCs have become a key haematological biomarker in the diagnosis and monitoring of plasma cell disorders. In these conditions, monoclonal light chains are secreted due to clonal plasma cell proliferation, usually resulting in overproduction of one type of light chain and subsequently a perturbed FLC ratio. The use of serum FLC analysis is recommended internationally for the screening, prognosis and monitoring of multiple myeloma (7, 8). Serum FLCs are also used in the identification and prognostication in a range of other related disorders including pre-myeloma states, such as the non-malignant precursor to myeloma (monoclonal gammopathy of undetermined significance), smouldering myeloma and solitary bone plasmacytoma; and other haematological conditions, including chronic lymphocytic leukaemia, non-Hodgkin lymphoma, Waldenstrom's macroglobulinaemia and AL amyloidosis (6, 8, 9). In plasma cell disorders such as myeloma, the FLC ratio is a key marker for diagnosis, prognosticating and monitoring and the monoclonal light chain level or the difference between the monoclonal light chain and uninvolved light chain is also employed for monitoring over time (10, 11).

Serum FLC measurement has been proven to be important in non-malignant disorders where polyclonal FLCs can be used as a broad marker of immune activation, inflammation and infection. Increases in FLCs have been noted in a range of diseases including: rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, heart failure, diabetes, renal disease, asthma, chronic obstructive pulmonary disease, inflammatory bowel disease and HIV infection (3, 12, 13). Data from these studies suggest that FLCs are useful as a marker of severity and/or risk for certain diseases, monitoring disease activity and potentially predicting disease progression. In conditions relating to polyclonal light chains, both kappa and lambda FLCs typically increase, resulting in minimal or no change in the FLC ratio. In chronic kidney disease, the FLC ratio increases with reference ranges of 0.37–3.1 reported in these individuals (14). This is due to an alternative pathway of clearance being used with renal impairment, resulting in similar serum half-lives of kappa and lambda. As kidney function declines, the ratio becomes further biased towards kappa, as a result of greater production relative to lambda, but similar clearance time (14).

Free light chains as a biomarker in the general population

The use of FLCs as a biomarker in non-clinical populations has attracted attention in recent years due to a pivotal study by Dispenzieri (15). In a large longitudinal data set of individuals aged ≥ 50 years without plasma cell disorders, the sum of kappa and lambda FLCs (FLC sum) negatively predicted survival. In this sample of the general population, those with the highest FLC sum levels had an increased risk of all-cause mortality. This increased risk of death remained at 2:1 after controlling for age, sex and renal insufficiency. Higher activation of the immune system and inflammatory markers relate to cardiovascular and cancer deaths; however, the exact mechanism linking polyclonal light chains with mortality risk is unknown, as is the extent to which the relationship is causal or correlational (10).

Biological role of free light chains

The diverse biological functions of FLCs have been highlighted in several publications, reviewed in detail elsewhere (3, 12). FLCs have been shown to interact with neutrophils and mast cells, which are both involved in inflammatory processes. Neutrophil apoptosis has been shown to be inhibited by FLCs (16) and FLCs can also bind to neutrophils and stimulate release of IL-8 (17), a pro-inflammatory chemokine that promotes neutrophil migration. FLCs have been implicated in the activation of mast cells to induce allergic responses (3). Due to their ability to bind to monocytes, which are antigen presenting cells, FLCs have been suggested to offer an alternative pathway to support antigen uptake and assist the related immune response (12, 18). Due to these activities, FLCs have been proposed to stimulate chronic inflammation via activation of specific immune cells (19). This mechanism would account for the numerous relationships observed between FLCs and inflammatory and autoimmune diseases. Other evidence suggests that FLCs could have beneficial effects; administration of FLCs have also been shown to be anti-inflammatory and have anti-viral properties in the context of viral myocarditis (20).

Free light chains and exercise

It has been hypothesised that acute exercise could affect FLCs via immune activation. Kappa FLCs have been shown to be significantly elevated in runners following a marathon compared with pre-exercise levels. No accompanying change in lambda FLCs was observed, as such some participants experienced an abnormal increase in the FLC ratio after exercise (21). The authors suggested these changes may be due to acute reductions in renal function with prolonged exercise, or alternatively due to immune stimulation or immunoglobulin redistribution. In elderly individuals, endurance walking over a period of 4 days did not significantly affect serum FLC levels (22). It may be that the nature of this exercise, being of relatively low intensity, was not a significant stimulus to elicit immune changes or alternatively impact upon renal function and subsequently FLC clearance. To our knowledge, no other investigations into the effects of exercise on FLCs have been conducted. Given the role of FLCs in immune and inflammatory processes, FLCs could be a useful marker of inflammation in relation to not only acute exercise but also exercise or physical activity interventions; they could also serve as a potential future marker of infection risk or overtraining. Thus, further studies are warranted in order to understand the relationship between exercise and FLCs and their utility in exercise immunology research.

Potential of free light chains as a salivary biomarker

The advantages of saliva testing have been well established in biomarker research. For example, non-invasive measurement may be more appropriate for repeated measures, long term sampling, or for certain populations or study designs where blood sampling may not be feasible.The broad value of FLCs in a range of diseases and the general population make them a suitable candidate for translation into salivary measurement. As the constituents of serum are capable of contributing to the oral environment, salivary FLCs may reflect systemic levels

thus could also provide an insight into local immune activation and inflammation. However, the relationship between FLCs in serum and saliva has yet to be explored.

Salivary FLCs may offer a convenient and accessible method of identifying elevated levels of polyclonal light chain production and monitoring FLCs over time in various diseases and the wider population. However, to date, an assay sensitive enough to detect FLCs in saliva has not been available. We have recently developed assays that sensitively and reliably detect very low levels of kappa and lambda FLCs (< 0.01 mg/L), appropriate for use in saliva. In order to investigate their potential as a future biomarker, normal reference ranges of salivary FLCs need to be established across a range of ages. The aim of the present study, therefore, was to develop normal ranges of FLCs in saliva in healthy individuals of different ages and assess the relationship between FLCs in saliva and serum. Secondly, this study sought to define how salivary and serum FLCs respond to exercise and if this varies in relation to age.

METHODS

Participants

Four separate cohorts (N =141) of participants were used as part of this investigation: 3 groups of young adults (aged ≤ 36 years) and 1 group of older adults (aged ≥ 60 years).

Cohort 1: Healthy young adults (n = 60, 35 males) with a mean ± SD age of 26.2 ± 3.7 years donated a saliva sample to inform saliva FLC reference ranges among this age group.

Cohort 2: Young men (n = 18, aged 22.9 years ± 3.4 years) who engaged in regular sports training (maximal oxygen uptake, VO_{2max}= 55.8 ± 13.6 mL/kg/min) participated in an acute bout of submaximal exercise. Participants were included if they participated in at least 3 sports training sessions per week and ≥ 3 h of total moderate/high-intensity training per week. Their self-reported training loads (determined by a pre-screening questionnaire) averaged 12 ± 5 h/week.

Cohort 3: Trained male cyclists (n = 10) with a competitive cycling background of at least 3 years (VO_{2max}= 73.1 ± 4.7 mL/kg/min), participated in an acute bout of intense exercise. Athletes were cycling ≥ 3 times per week for a minimum of 2 h/day and reported a mean training load of 9.4 ± 2.2 h/week. Key inclusion criteria for cohorts 1–3 were no chronic illness or history of chronic illness.

Cohort 4: Older adults (n = 53, 32 males) aged 67.2 ± 4.9 years participated in an acute bout of submaximal exercise. Their VO_{2max} was 37.8 ± 9.84 mL/kg/min, predicted based on heart rate (HR) and VO_2 during submaximal exercise. Twenty percent of participants reported suffering from a chronic illness, which were hypertension and asthma, and 38% reported taking medication, such as antihypertensives, non-corticosteroid inhalers, statins and gastrointestinal medications.Participants were excluded if suffering from any immune or endocrine disorder or any condition that precluded them from exercise. For all cohorts, inclusion criteria stipulated that participants were not suffering from any acute illness in the two weeks prior to, or during the study.

Baseline reference ranges of free light chains in saliva in healthy adults

To establish reference ranges for FLC in saliva in relation to age, baseline saliva levels from cohorts 1–3 were pooled to form a 'younger' cohort and cohort 4 was used for reference ranges in 'older' adults. Samples in the younger cohort were taken at various times of day: cohort 1, either between 07:00–9:00 or 18:00–20:00; cohort 2, approximately 12:00; cohort 3: 06:30–8:30. All older adult samples were collected in the morning between 08:00–09:00. A subset of 38 young adults (from cohorts 1 and 3) and 40 older adults (from cohort 4) had serum samples available to compare systemic concentrations of FLCs in serum with salivary levels. Again, all serum samples were taken under resting conditions prior to any exercise-and all were taken during the same morning period.

FLC response to exercise in young and older adults: exercise protocols

Cohort 2: Approximately 1 week before the acute exercise trial, participants completed a continuous incremental test on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) to volitional exhaustion to determine their VO_{2max}. Expired gas samples were collected in Douglas bags (Harvard Apparatus, Edenbridge, UK) during the final minute of each work-rate increment, and HR was measured continuously using short-range radio telemetry (Polar, Kempele, Finland). An oxygen/carbon dioxide analyzer (Servomex 1400, Crowbridge, UK) was used along with a dry gas meter (Harvard Apparatus, Edenbridge, UK) to determine V_E, VO_2, and VCO_2. From the VO_2–work-rate relationship, the work-rate equivalent to 60% VO_{2max} was determined. After a 15 min recovery, participants cycled for 20 min at a steady state work rate equivalent to 60% VO_{2max} with expired gas samples collected after 10 and 20 min in order to ensure that the calculated work rate elicited the desired VO_2. For the acute submaximal exercise trial, participants arrived at 12:00 following a 3 h fast. A saliva sample was collected then participants cycled for 2 h at 60% VO_{2max} on a stationary cycle ergometer. Immediately after completing the exercise and also 1 h post-exercise saliva samples were again collected.

Cohort 3: Participants visited the laboratory for pre-trial exercise testing to confirm suitability to participate. During this visit, participants underwent an incremental cycle test to exhaustion on an electronically braked ergometer (Lode Excalibur Sport, Groningen, Netherlands). Expiratory gases were collected continually throughout the test and breath-by-breath analysis was performed automatically with a Moxus metabolic systems analyser (AEI Technologies Inc., Naperville, IL, USA). HR was recorded continually using short range telemetry (Polar, Kempele, Finland). Participants had to achieve a VO_{2max} of ≥65 mL/kg/min to be included in the study. On the day of the acute exercise bout, participants arrived at the laboratory between 06:30–8:30 following an overnight fast of ≥ 8 h. Blood samples were collected followed by a saliva sample. After a 10-min warm up, participants performed an all-out 1 h time trial (TT) on their own bicycles on a turbo trainer (CycleOps Flow). HR was recorded continually during the TT with short range telemetry (Suunto, Vantaa, Finland). Participants were blinded from their power, cadence and HR during the TT and were only

provided with a stop clock to monitor time. Post-TT blood and saliva samples were collected within 5 min of exercise cessation.

Cohort 4: Participants arrived to the laboratory between 08:00 and 09:00 where they were fitted with a HR monitor (Polar, Kempele, Finland).They were then familiarised with the procedure for collection of expired gas using Douglas bags. They then rested for 15 min before a blood sample was taken followed by saliva sample collection. Participants then completed an acute bout ofexercise in the form of an incremental submaximal treadmill test.Participants began walking on the treadmill and speed was gradually increased until the participant reached a pace they considered 'brisk walking'. The gradient was increased every 4 min and during the final min of each stage expired gas samples were collected into Douglas bags (Cranlea, Birmingham, UK). HR was monitored continuously throughout the exercise and the test was terminated once the participant had reached 75% of their predicted maximum HR, as determined by the formula: 208-(age×0.7) (23). A blood and saliva sample was taken immediately after exercise and 1 h post-exercise. VCO_2 and VO_2 were determined using O_2/CO_2 analyser (Servomex 1440, Crowborough, UK) and expired gas volumes were measured using a dry gas meter (Harvard Apparatus, Edenbridge, UK). Using a regression equation created from plotting the relationship between HR and VO_2 during the final three stages of exercise, VO_{2max} was predicted.

Saliva sample collection and analyses

Saliva samples were collected using the same technique across cohorts. Participants were instructed to refrain from eating, drinking (accept water) or brushing their teeth for a minimum of 1 h before arriving to sample collection; no drinking was permitted 10 min before samples were collected. Unstimulated whole saliva samples were collected by passive dribble into pre-weighed tubes for a timed period of 2–4 min. 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 highly sensitive sandwich ELISAs developed by the Clinical Immunology Service at the University of Birmingham. These assays use monoclonal antibodies (mAbs) that have been characterised and validated previously (24).The microtitre wells were coated with mAbs that specifically target either human kappa or lambda FLC and thus do not bind light chain in whole immunoglobulin (Abingdon Health, York, UK). After a blocking period of 1 h to prevent non-specific binding, standards, controls and saliva samples were added to the plate in duplicate. After 1 h incubation, plates were washed to remove any unbound sample. Kappa or lambda detection antibody labelled with horseradish peroxidase was then added to the plate and left to incubate for 1 h. Detection antibodies were mAbs specific for kappa or lambda light chains either free or bound, that recognise an epitope distinct from the one used to capture the FLCs from saliva (Abingdon Health, York, UK). Plates were washed again and substrate solution was added; after 10 min incubation, the reaction was stopped and the optical density was measured at 450 nm. The intra-assay CVs were 9.9% for kappa and 6.7% for lambda and inter-assay CVs were 15.3% for kappa and 16.4% for lambda. The limits of detection were calculated by serially diluting normal serum 1 in 2 in assay buffer and selecting the lowest concentration determined by the assays above the blank (well containing only assay buffer). Limits were 0.004 mg/L and 0.001 mg/L for kappa and lambda FLCs, respectively. The calibration ranges used in the assay were 0.00028–2.82 mg/L for kappa and 0.0003–2.96 mg/L for lambda; saliva samples were diluted 1 in 15 with 0.01M phosphate buffered saline.

Serum sample collection and analyses

Venous blood was collected from an antecubital vein into plain tubes (BD Vacutainer, Plymouth, UK). Blood was allowed to clot at room temperature before being centrifuged and the separated serum was stored at −20°C until analysis. Serum kappa and lambda FLCs were quantified using a multiplex bead-based assay using a Luminex platform (Bio-plex systems, BioRad Laboratories, California, USA). This assay was developed by the Clinical Immunology Service at the University of Birmingham and uses mAbs specific for either kappa or lambda FLC in a competitive inhibition format. Both intra- and inter-assay CVs were < 10%. Full details for this assay have been described previously (24).

Free light chain parameters

A range of FLC parameter outputs were analysed as part of this study. In addition to concentrations of kappa and lambda FLC, the ratio of kappa to lambda FLC (K:λ ratio) and the difference between kappa and lambda FLC (FLC difference) were examined. The K:λ ratio and FLC difference are classically used in conditions involving perturbed levels of FLC, such as plasma cell disorders (11, 25). The sum of kappa and lambda FLCs (FLC sum) was also investigated as this measure has been employed in the general population in relation to non-clonal light chains (15). As this study is the first comprehensive investigation into FLC in saliva, all the above parameters were included to compare saliva to conventional serum markers and enable any future comparisons between healthy and clinical populations. In addition to concentrations, saliva secretion rates of immunoglobulins are typically reported to reflect the total availability of protein at the oral surface and control for hydration status (26). Secretion rates of FLCs (µg/min) were calculated as saliva flow rate x kappa/lambda concentration. The other parameters, sum, difference and ratio, were also additionally expressed in this way to control for any impact of flow rate upon these variables.

Statistical analyses

Analyses were undertaken using IBM SPSS version 21. Univariate ANOVA was used to examine differences between young and older age groups for all saliva and serum FLC parameters. As certain medications can impact upon salivary flow rate regulation (27, 28), subsequent univariate ANCOVA was then performed for saliva variables to control for any confounding effects of chronic medication usage in relation to

age and FLC parameters. As saliva samples within the younger cohort were taken at varied times, to assess time of day as a potential confounder the effects of sample time (morning, midday and evening time groups) on FLCs were analysed using univariate ANOVA. Sex as a confounding variable was also explored by testing the effects of sex and any sex x age interactions on serum and saliva FLC parameters via two-way ANOVA. Spearman's rank correlation was used to assess the relationship between FLC parameters in serum and saliva. Correlational analyses were carried out for the study cohort as a whole and separately within young and older age groups. For cohorts 2–4, repeated measures ANOVA was used to analyse FLC responses to exercise. When significant main effects of time were observed, Bonferroni post-hoc tests were applied. For these exercise studies, percentage change in FLC concentration/secretion rates between pre- and post- exercise time points were calculated and compared across studies using univariate ANOVA. If data were not normally distributed, statistical analysis was performed on the logarithmic transformation of the data. As data was generally skewed, and to provide information regarding the full spread of data, tables report medians along with 5–95th percentiles; for analyses of smaller groups (cohorts 2 and 3) full ranges are reported. Greenhouse-Geisser corrected *F* values are reported for repeated measures analyses and partial η^2, a measure of effect size, is reported throughout.

RESULTS

Ranges of free light chains in healthy young and older adults

Participant characteristics and FLC parameters in serum and saliva in relation to age are reported in Table 1. Saliva flow rates were significantly higher in young adults compared to older adults. Older adults demonstrated significantly higher levels of both kappa and lambda FLCs in saliva; consequently, older adults exhibited higher FLC sum values. Older adults also registered a significantly higher κ:λ ratio and FLC difference as a result of higher kappa FLC levels relative to lambda in older individuals. When examining saliva parameters expressed as secretion rates, significant age differences were again observed for all saliva parameters: kappa and lambda secretion rates, FLC sum, FLC difference and the κ:λ ratio. Age differences between kappa and lambda secretion rates and the κ:λratio controlling for flow rateare illustrated in Figure 1. In serum, there was no significant difference between age groups for kappa FLCs. However, lambda levels were significantly higher in young adults, resulting in a significantly lower κ:λ ratio and greater FLC difference compared with the older adults. Statistics for age group differences for serum and saliva are reported in Table 1.

In the study population as a whole, in comparison to saliva, the median concentrations of FLCs in serum were over 10-fold greater for kappa and 20-fold greater for lambda. A significant positive correlation was present between serum and saliva for the κ:λ ratio, $r_s(76) = 0.33$, $p = 0.004$, and FLC difference, $r_s(76) = 0.40$, $p < 0.001$. No significant correlations emerged between serum and saliva for kappa or lambda FLC concentrations, the FLC sum, nor serum concentrations and saliva secretion rates. Further, there were no significant relationships for serum and saliva FLCs within age groups.

Free light chain parameters controlling for potential confounding variables

None of the younger adults reported taking any on-going medication whereas twenty older adults (38%) reported taking chronic medication. All age x saliva parameter findings withstood adjustment for medication use, with *p* values all remaining ≤ 0.001 with the exception of flow rate ($p = 0.002$) and lambda secretion rate ($p = 0.009$). Similarly, all age x serum findings remained significant at the 0.01 level when controlling for medication use. No sex or sex by age group interactions were observed for any saliva or serum parameters. Within the young cohort, there was no significant difference in saliva FLC concentrations or secretion rates between samples collected in the morning, at midday or evening.

FLC responses to acute exercise in young and older adult populations

I. The effects of 2 h cycling at 60% VO_2max on salivary free light chains in healthy exercise-trained young men

Table 2 describes the salivary FLC responses to acute exercise in cohort 2. In general, saliva parameters were reduced post-exercise, and then recovered at 1 h post-exercise. However, no significant effect of exercise was observed for flow rate, kappa or lambda concentration and FLC sum. For the FLC difference and K:λ ratio, significant effects of time were observed, $F(2,34) = 3.98$, $p = 0.031$, $\eta^2 = 0.190$ and $F(2,34) = 4.84$, $p = 0.015$, $\eta^2 = 0.221$, respectively. These indices increased at 1 h post-exercise in comparison to immediately post-exercise.

These results were replicated when examining FLCs controlling for flow rate, with FLC difference ($F(2, 34) = 4.01$, $p = 0.035$, $\eta^2 = 0.191$) and K:λ ratio ($F(2, 34) = 7.96$, $p = 0.002$, $\eta^2 = 0.319$) producing significantly higher results at 1 h compared with immediately post-exercise. Kappa and lambda secretion rates and FLC sum did not exhibit any significant changes in response to exercise. Percentage changes in FLC concentrations and secretion rates in response to exercise are shown in Table 5.

II. The effects of a 1 h all-out cycling time trial on free light chains in trained young male cyclists

Saliva

Overall, saliva variables registered lower post-exercise values compared with pre exercise, although these did not translate into significant changes in salivary kappa or lambda concentrations or flow rates, FLC sum, FLC difference and K:λ ratio (Table 3) in response to exercise. These results were mirrored for secretion rates and other saliva variables controlling for flow rate (Table 3). The median % changes in salivary FLC concentrations and secretion rates are summarised in Table 5. Although not statistically significant, % reductions in FLC post-exercise were typically higher in response to the 1 h TT than 2 h of submaximal cycling.

Table 1. Participant characteristics and free light chain (FLC) parameters in saliva and serum in healthy young and older adults. All values are median (5–95 percentile) unless stated

	Young adults (N = 88)	Older adults (N = 53)	*F*	η^2
Age (years), median (range)	24 (18–36)***	67 (60–80)	2988.15	.957
BMI (kg/m^2), median (range)	23.15 (18.30–28.70)*	23.90 (19.10–33.26)	6.14	.064
Males, n (%)	63 (72%)	30 (60%)		
Saliva flow rate (mL/min)	0.44 (0.12–0.92)**	0.31 (0.10–0.80)	8.67	.059
Saliva concentrations (mg/L)				
Kappa	0.45 (0.004–3.45)***	3.91 (0.75–19.65)	99.10	.416
Lambda	0.30 (0.08–1.54)***	1.00 (0.02–4.50)	55.17	.284
FLC sum	0.80 (0.11–4.84)***	4.80 (1.06–23.49)	113.48	.449
FLC difference	0.03 (-0.43–1.89)***	2.92 (0.09–15.80)	148.94	.517
K:λ ratio	1.13 (0.05–4.68)***	4.20 (1.22–9.37)	65.48	.320
Saliva parameters controlling for flow rate (μg/min)				
Kappa secretion rate	0.18 (0.003–1.35)***	1.38 (0.16–5.67)	71.45	.340
Lambda secretion rate	0.14 (0.03–0.68)***	0.35 (0.07–1.24)	25.61	.156
FLC sum	0.38 (0.04–1.91)***	1.86 (0.03–4.79)	74.04	.348
FLC difference	0.01 (-0.17–0.86)***	1.03 (0.03–4.79)	117.06	.457
K:λ ratio	0.47 (0.02–3.01)***	1.40 (0.25–5.69)	26.81	.162
Serum FLC concentration (mg/L)	n = 38	n = 40		
Kappa	10.41 (7.41–14.35)	11.36 (5.11–16.70)	0.50	.006
Lambda	13.16 (8.61–20.51)**	9.50 (5.90–18.81)	8.79	.104
FLC sum	23.62 (16.91–33.79)	20.73 (10.68–35.38)	2.29	.029
FLC difference	-2.58 (-7.21–1.87)***	0.77 (-4.02–4.20)	23.65	.235
K:λ ratio	0.81 (0.58–1.29)***	1.07 (0.77–1.52)	27.83	.268

Significant differences between age groups are indicated by *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$
Degrees of freedom for saliva parameters $F(1,139)$ and $F(1,76)$ for serum

Serum

No changes were observed in serum FLCs in response to exercise (Table 3). Haematological measures were not assessed in this study; however, participants consumed water *ad libitum* during exercise in a temperature controlled environment (18–20°C), therefore any changes in plasma volume would be expected to be minor (< 5%) based on previous observations under similar conditions.

III. The effects of an incremental submaximal treadmill test to 75% HRmax on free light chains in healthy older adults

Saliva

There was no significant effect of exercise on saliva flow rates in the older adult cohort (Table 4). There was a significant main effect of time for both kappa, $F(2,104) = 23.12$, $p< 0.001$, $\eta^2 = 0.308$ and lambda, $F(2,104) = 24.54$, $p< 0.001$, $\eta^2 = 0.321$, FLC concentrations. As shown in Figure 2, salivary FLC concentrations were significantly reduced post-exercise and 1 h post-exercise compared with pre-exercise in saliva. Levels then significantly increased at 1 h post-exercise compared with immediately post-exercise. The same response pattern was observed for FLC sum and difference: decreasing post-exercise, followed by an increase at 1 h, but remaining below pre-exercise values: $F(2,104) = 24.22$, $p< 0.001$, $\eta^2 = 0.318$ and $F(2,104) = 19.63$, $p< 0.001$, $\eta^2 = 0.274$, respectively. There was no significant effect of exercise on the K:λ ratio.

Saliva secretion rates (Figure 3) followed the same response profile as concentration, with kappa ($F(2,98) = 29.58$ $p< 0.001$, $\eta^2 = 0.376$) and lambda ($F(2,98) = 33.56$ $p < 0.001$, $\eta^2 = 0.406$) secretion reducing post-exercise, followed by an increase at 1 h post-exercise. Differences across all time points were also observed for FLC sum ($F(2, 98) = 31.793$ $p< 0.001$, $\eta^2 = 0.394$) and FLC difference ($F(2,98) = 19.51$ $p< 0.001$, $\eta^2 = 0.285$) when controlling for flow rate. There was a significant differenced in the K:λ ratio controlling for flow rate ($F(2,98) = 4.84$ $p = 0.012$, $\eta^2 = 0.090$, Table 4), where the ratio significantly decreased post-exercise, followed by a significant increase 1 h post-exercise compared with immediately post; this recovery in the ratio was above pre-exercise levels. Percentage changes in FLC in response to exercise for older adults are reported in Table 5.

Serum

As shown in Figure 2, there were no significant effects of exercise on kappa or lambda FLC concentrations in serum, nor was there any impact of exercise on the serum FLC sum, difference or K:λ ratio (Table 4). The median plasma volume

Table 2. Salivary free light chain (FLC) responses to 2 h cycling at 60% VO_2max in healthy exercise-trained young men

Median (range)	Pre-exercise	Post-exercise	1 h post-exercise
Saliva flow rate (mL/min)	0.40 (0.11–0.84)	0.30 (0.07–0.78)	0.31 (0.11–0.77)
Saliva concentrations (mg/L)			
Kappa	0.41 (0.004–1.96)	0.20 (0.004–2.19)	0.38 (0.004–3.49)
Lambda	0.25 (0.03–1.46)	0.32 (0.07–0.97)	0.40 (0.003–2.11)
FLC sum	0.81 (0.03–2.75)	0.55 (0.10–3.05)	0.75 (0.01–4.99)
FLC difference	-0.02 (-0.82–1.38)	-0.07 (-0.71–1.33)	0.02 (-0.37–1.99)*
K:λ ratio	0.83 (0.03–4.29)	0.66 (0.02–2.54)	1.15 (0.02–3.39)*
Saliva parameters controlling for flow rate (μg/min)			
Kappa secretion	0.12 (0.001–0.69)	0.06 (0.001–0.54)	0.12 (0.001–1.175)
Lambda secretion	0.14 (0.02–0.40)	0.08 (0.02–0.29)	0.12 (0.001–0.66)
FLC sum	0.32 (0.02–1.02)	0.13 (0.02–0.74)	0.23 (0.002–1.62)
FLC difference	-0.01 (-0.26–0.53)	-0.01 (-0.21–0.32)	0.01 (-0.12–0.73)*
K:λ ratio	0.28 (0.001–3.03)	0.13 (0.00–0.90)	0.36 (0.01–1.83)**

Significantly different to post-exercise indicated by * $p < 0.05$, ** $p < 0.01$

Table 3. Free light chain (FLC) saliva and serum parameters in response to an all-out 1 h cycling time trial in young, well-trained, male cyclists

Median (range)	Pre-exercise	Post-exercise
Saliva flow rate (mL/min)	0.51 (0.28–1.25)	0.48 (0.24–0.83)
Saliva concentrations (mg/L)		
Kappa	1.19 (0.13–4.07)	0.74 (0.14–1.51)
Lambda	0.60 (0.14–3.65)	0.33 (0.22–2.03)
FLC sum	1.99 (0.27–7.07)	1.23 (0.48–3.54)
FLC difference	0.37(-0.23–2.90)	0.03 (-0.81–1.27)
K:λ ratio	2.70 (0.97–3.91)	1.09 (0.15–6.83)
Saliva parameters controlling for flow rate (μg/min)		
Kappa secretion	0.80 (0.17–1.60)	0.41 (0.03–0.71)
Lambda secretion	0.37 (0.08–1.04)	0.21 (0.07–0.88)
FLC sum	1.26 (0.27–2.07)	0.55 (0.26–1.52)
FLC difference	0.25 (-0.08–1.14)	0.02 (-0.23–0.42)
K:λ ratio	1.30 (0.27–3.25)	0.75 (0.04–2.26)
Serum concentration (mg/L)		
Kappa	10.24 (7.91–12.29)	10.68 (7.28–13.96)
Lambda	13.04 (10.44–17.25)	12.89 (8.45–16.74)
FLC sum	23.38 (18.64–28.44)	24.26 (15.74–29.86)
FLC difference	-2.37 (-6.06–0.96)	-2.53 (-5.87–0.02)
K:λ ratio	0.75 (0.58–1.09)	0.76 (0.65–1.00)

reduction post-exercise was -3.9% and by 1 h post-exercise had increased above pre-exercise levels by a median of 3.7%. Plasma volume increased by a median of 7.5% between immediately post- and 1 h post-exercise. Following adjustment for plasma volume changes, there were still no significant effects of exercise on serum FLC.

IV. Comparison of the effects of acute exercise on free light chains in young and older adults

Table 5 summarises the % FLC changes in serum and saliva in response to exercise across studies. When comparing the degree of percentage change between studies, a significant difference was observed for the % change in lambda salivary FLC from pre- to post-exercise between the older adults and young adults who exercised at 60% VO_2max for 2 h, $F(2,78) = 4.31$ $p = 0.017$, $\eta^2 = 0.099$. There were no other significant differences % changes in response to exercise between studies.

DISCUSSION

For the first time we have established reference ranges for FLCs in saliva for healthy individuals through application of newly developed highly sensitive ELISAs. Previously it was not possible to reliably detect FLCs in saliva due to the sensitivity and technical limitations of existing commercially available assays. The total sum of FLCs in saliva was typically < 1 mg/L for young and < 5 mg/L for older adults, which is below the lower range of other assays.The lowest calibration point of Freelite™, the first and most widely used FLC assay, is typically 3 mg/L for kappa and

Table 4. Free light chain (FLC) responses to an incremental submaximal treadmill test to 75% HRmax in healthy older adults

Median (5–95th percentile)	**Pre-exercise**	**Post-exercise**	**1 h post-exercise**
Saliva flow rate (mL/min)	0.31 (0.09–0.80)	0.29 (0.10–0.86)*	0.32 (0.11–0.90)
Saliva concentrations (mg/L)			
FLC sum	4.80 (1.06–23.49)	2.84 (0.44–17.17)	3.83 (0.61–16.97)*** †††
FLC difference	2.92 (0.9–15.80)	1.72 (-0.37 –13.49)***	2.14 (0.11–12.77)*** †††
FLC ratio	4.20 (1.23–9.37)	4.16 (0.37–9.05)	4.29(1.14–8.57)
Saliva parameters controlling for flow rate (μg/min)			
FLC sum	1.86 (0.28–6.67)	0.65 (0.08–4.44)***	1.11 (0.22–6.34)*** †††
FLC difference	1.03 (0.02–4.80)	0.40 (-0.03–3.12)***	0.65 (-0.002–4.36)*** †††
FLC ratio	1.39 (0.25–5.69)	1.01 (0.05–4.62)*	1.45 (0.19–4.97)†
Serum concentrations (mg/L)			
FLC sum	20.73 (10.68–35.38)	20.98 (11.00–34.88)	20.42–35.60)
FLC difference	0.77 (-4.02–4.20)	0.63 (-3.87–4.30)	1.15 (-3.87–4.41)
K:λ ratio	1.07 (0.71–1.52)	1.07 (0.70–1.43)	1.13 (0.71–1.45)

∗ Significantly different to pre-exercise; † significantly different to post-exercise
*** and ††† $p < 0.001$; * and † $p < 0.05$

5 mg/L for lambda. The precision around these concentrations has been accepted as being poor and the 'gap' in quantitation at the lower end of the assay has been highlighted previously (24, 29). The development of these new highly sensitive FLC assays opens the door to improved detection and accuracy for a range of other biological specimens that characteristically have low concentrations of FLCs, such as cerebral spinal fluid and urine.

Overall median concentrations of salivary kappa FLCs were 10-fold lower, and lambda FLCs 20-fold lower compared with serum. Interestingly, no significant correlations emerged between FLCs in saliva and serum for any parameters, with the exception of the FLC ratio and FLC difference. This suggests that the proportion and difference between FLC isotypes in saliva broadly reflects serum, but saliva may not be representative of individual kappa and lambda concentrations or total polyclonal FLC levels in serum. Consequently, these data do not provide evidence that salivary FLC levels are able to identify those with higher/lower FLC levels in serum, but could be useful in detecting those with altered FLC ratios or large differences between isotypes. This would be particularly applicable in plasma cell dyscrasias. Future research in disease populations is required to develop salivary FLC reference ranges in patients and explore the relationship between FLCs in serum and saliva to assess disease-specific utility.

Identifying the source of FLCs in saliva was not examined as part of the present investigation; however, possible pathways have been highlighted by other studies involving salivary bio-

Table 5. Summary of median percentage changes in free light chain parameters in serum and saliva in response to acute exercise in young and older adults

	Young adults			**Older adults**	
	Maximal exercise: 1 h all-out cycling time trial	*Submaximal exercise*: 2 h cycling at 60% VO_2max		Submaximal treadmill test to 75% HRmax	
	% pre–post	**% pre–post**	**%pre–1 h post**	**%pre–post**	**%pre–1 h post**
Saliva concentration					
Kappa	-33.9	-9.6	-6.0	-50.6	-27.9
Lambda	-6.2	5.6	-3.9	-46.3*	-37.6
Saliva Secretion					
Kappa	-34.0	-29.8	-10.4	-59.0	-26.8
Lambda	-8.2	-12.0	-28.8	-53.5	-31.0
Serum concentration					
Kappa	2.4			1.3	0.7
Lambda	0.6			0.3	-2.3

* Significantly different to young adults submaximal exercise pre-post change value, $p < 0.05$

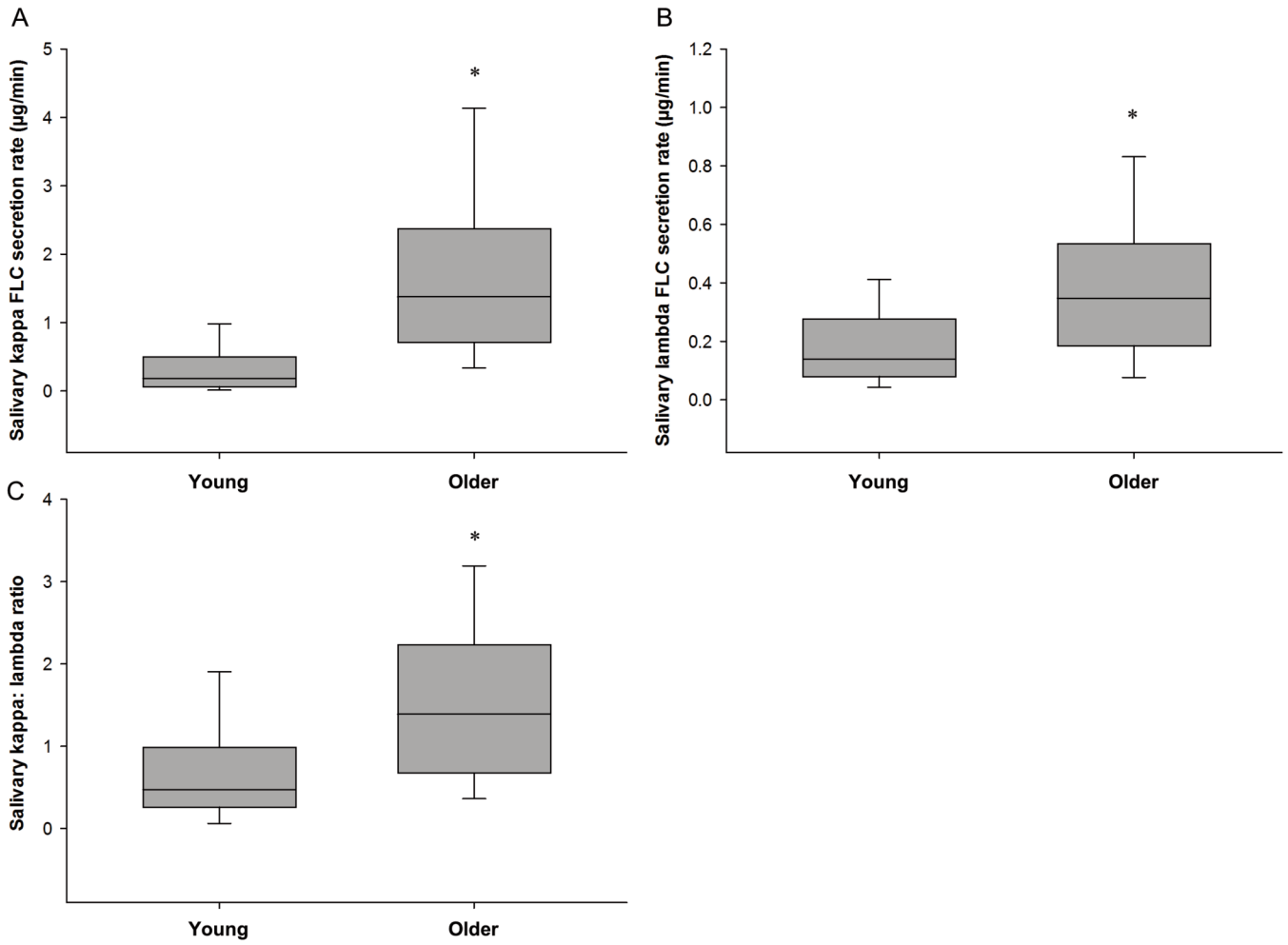

Figure 1. Salivary kappa (A) and lambda (B) free light chain (FLC) secretion rates and the kappa:lambda ratio controlling for flow rate (C) in young and older adults. Boxes represent the 25–75th percentile, with the line indicating the median, and whiskers show the 10–90th percentile. * Significantly higher FLC secretion rate and kappa: lambda ratio in the older adults compared with the young adults, $p < 0.001$

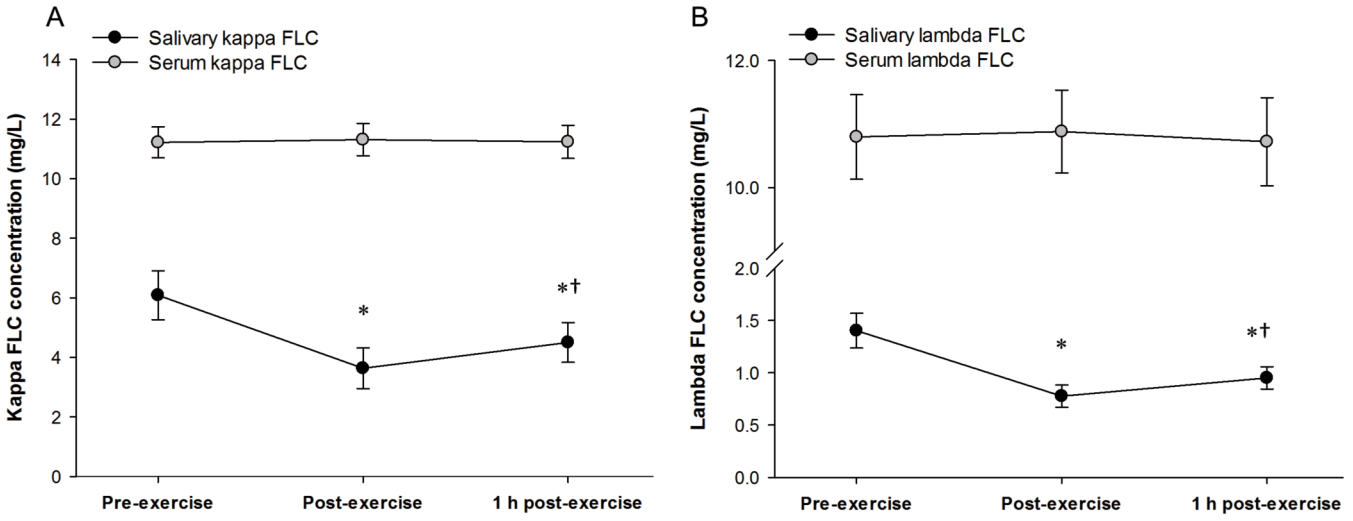

Figure 2. Kappa (A) and lambda (B) free light chain (FLC) concentrations in serum and saliva in response to submaximal exercise in older adults * Kappa (A) and lambda (B) FLC concentrations were significantly reduced post-exercise and 1 h post-exercise compared with pre-exercise in saliva, $p < 0.001$; † Kappa and lambda FLC concentrations increased 1 h post-exercise compared with immediately post-exercise in saliva, $p < 0.05$. Serum FLC levels did not change in response to exercise. Values are means ± SEM

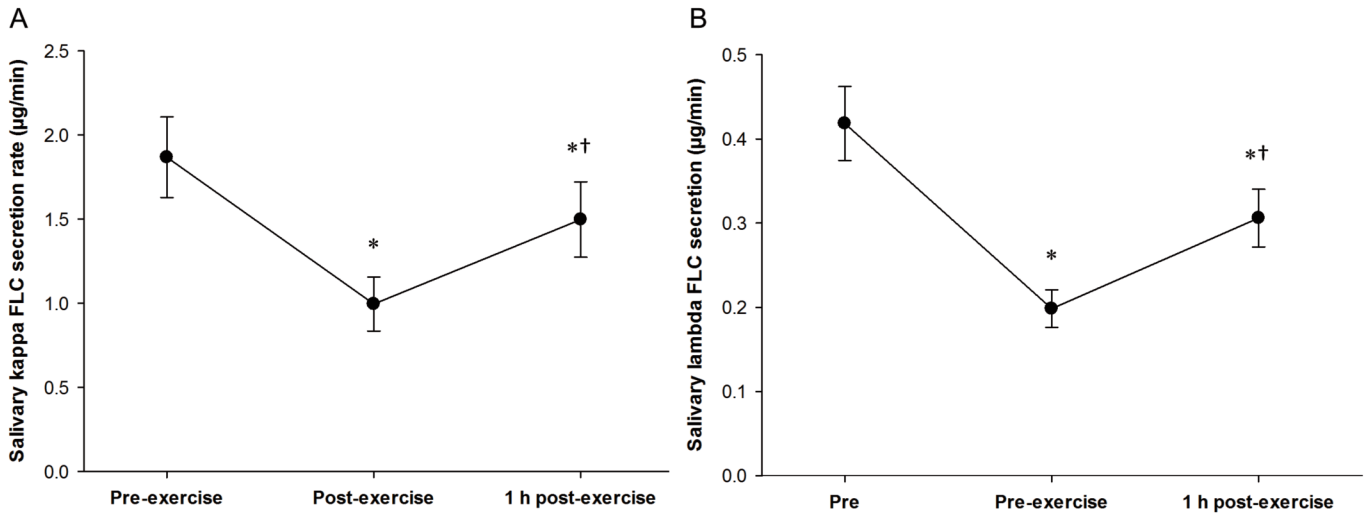

Figure 3. Salivary kappa (A) and lambda (B) free light chain (FLC) secretion rates in response to submaximal exercise in older adults. * Kappa (A) and lambda (B) FLC secretion rates were significantly reduced post-exercise ($p < 0.001$) and 1 h post-exercise ($p < 0.01$) compared with pre-exercise in saliva; † Kappa and lambda FLC secretion rates increased 1 h post-exercise compared with immediately post-exercise, $p < 0.001$. Values are means ± SEM

markers. The predominant immunoglobulin in saliva is secretory IgA, which is secreted by local plasma cells and synthesised in dimeric form. Alternatively, IgG, and the majority of monomeric IgA, present in saliva is derived from serum rather than local production; these likely enter the saliva through gingival cervices, via gingival crevicular fluid (GCF) (30, 31). GCF is an oral fluid that consists of serum transudate and tissue exudates, and has been shown to include immunoglobulins (32). Other serum factors, such as hormones, can enter saliva via passive diffusion through cells or filtration mechanism between cells, depending on the size of the molecule (33). As FLCs concentrations and secretion rates in saliva did not correlate well with serum levels, a proportion of FLCs in saliva may be a result of local production and/or dependent upon variable levels of periodontitis. FLCs in saliva could be from serum or may reflect local production, or a combination of both these possibilities. Further studies are required to determine the exact source and mode of entry of FLCs in saliva.

Older adults demonstrated significantly higher levels of all FLC parameters in saliva compared with young adults. Saliva flow rates were significantly lower in older adults compared to young adults. This is consistent with previous studies and has been proposed to be driven by medication use (28, 34), in fact, flow rate may not be affected by age in healthy individuals who are not taking medication (35). In the present study, all findings withstood correction for chronic medication usage and when adjusting for flow rate all saliva parameters remained higher in the older adults, suggesting the observed age differences are not simply reflecting flow rate differences. The pattern of elevated FLCs in the older cohort was not observed for serum, where higher lambda FLC levels were seen in the younger adults, resulting in a lower FLC ratio and higher FLC difference. Although these findings were statistically significant, it should be noted that all participants had FLC levels and ratios within the normal range, and these age differences in serum are therefore unlikely to have any clinical implications. There were no significant sex effects on FLC levels, although it should be noted the majority of participants were male, therefore possible gender effects should still be considered in future studies. Further, age differences in FLCs were independent of time of day: there were no differences in saliva samples based upon time of day and all serum samples were taken in the same time period. However, repeated sampling of individuals across the day would be required to conclusively determine if FLCs exhibit any form of diurnal variation.

As age-related increases were observed across saliva indices rather than serum, these findings may be indicative of age specific changes at an oral level. Physiological changes due to ageing have been hypothesised to result in salivary gland atrophy and in turn affect secretory capacity, although available evidence suggests that this postulation may not necessarily be true (36). The present study suggests that the concentration and secretion of FLCs is elevated rather than diminished with age; this may be due to oral factors related to ageing. Ageing is associated with higher rates of gum disease, decay and consequently tooth loss. In the UK, less than 10% of adults over 65 years of age have no signs of pocketing, calculus or bleeding (indicators of periodontal health) and only 1% of adults aged over 55 can be classified as having excellent oral health (37). Nearly 50% of adults aged 65–74 display signs of gingivitis and 65% of 75–84 year olds have indications of more advanced periodontal disease (38). Similarly, a large study in the US found that 64% of adults aged ≥ 65 had moderate or severe periodontitis (39). Periodontitis is associated with low molecular weight serum proteins in the crevivular fluid, proportional to the degree of inflammation (40). Low molecular weight proteins would encompass FLCs rather than whole immunoglobulins. The higher levels of salivary FLCs observed in the elderly cohort may reflect poorer overall oral health and accompanying greater degree of immune activation and inflammation compared with the young cohort. Although no participants in the present study reported any specific dental problems or gum disease, underlying processes may be

taking place that have not yet manifested with any noticeable signs to the individual, or may occur prior to the presentation of periodontal disease. Broader ranges of FLCs were observed in older adults for saliva variables compared with young adults, suggesting a higher degree on inter-individual variability in this age group; this may reflect larger individual differences in oral health.

Elevated salivary FLC levels in older individuals may have implications for wider aspects of health. The immune decline and dysregulation that occurs with ageing favours a pro-inflammatory profile, which is associated with various chronic diseases (41, 42). Oral inflammation has been suggested to impact upon systemic inflammation, and consequently effect vital organs and contribute to or exacerbate inflammatory conditions (43); this concept is particularly important in the context of immunosenescence and age-related diseases. Although FLCs have been implicated in immune and inflammatory processes, as this is the first study to explore salivary FLCs their specific roles within the oral environment is currently unknown. Further, in the present sample all participants had serum FLC levels within the normal range and were in good health. It would be interesting to examine the relationship between salivary FLCs and serum FLCs in patients with periodontitis or chronic diseases where polyclonal FLCs may be elevated. Alternatively, as evidence suggests that FLCs may have anti-viral properties (20), raised FLCs may also confer some degree of protection against pathogens, such that having elevated levels may in fact be advantageous. Whole immunoglobulins were not measured as part of the present study; however, salivary IgA secretion rates have been shown to be significantly lower in elderly individuals and decrease with increasing age (34, 44). The balance between secretory immunity and FLCs in saliva should be explored as part of future investigations.

In younger adults, 2 h of submaximal exercise resulted in modest reductions in salivary FLC concentrations and secretions rates immediately post-exercise. However, significant findings only emerged in relation to the FLC difference and K:λ ratio between post- and 1 h post-exercise; these were driven by slightly higher recovery of kappa FLC concentrations and secretions at 1 h relative to lambda FLCs. For the 1 h TT, percentage reductions in salivary parameters post-exercise were greater than observed after 2 h of cycling, but no significant findings were observed for this exercise trial. In contrast, older adults demonstrated significant reductions in kappa and lambda FLC concentrations and secretion rates, in addition to sum, difference and ratio parameters, post-exercise. These variables then significantly increased 1 h post-exercise compared to immediately post-exercise, although generally remained significantly below pre-exercise values. These findings suggest that salivary FLCs are significantly reduced after exercise in older adults, where only minor alterations occur in young individuals.

Stimulation of the sympathetic nervous system can result in vasoconstriction of salivary glands. This, in addition to hyperventilation causing evaporation and dehydration during exercise, may lead to a decrease in saliva volume (45, 46). Parasympathetic withdrawal is also thought to contribute significantly to the reduction in saliva flow rate with exercise (44). It may be that blood flow to the saliva glands during exercise is reduced to a greater extent in older adults. However, given there was no significant effect of exercise on saliva flow rates, in either age group, and changes were also observed when variables were expressed as secretion rates, the mechanism responsible for the decrease in salivary FLCs does not appear to be linked to saliva volume. These changes are also unlikely to reflect differences in exercise duration or intensity. Older adults exercised for one third or sixth of the time as the young adult cohorts, and although the maximum intensity was higher than the 2 h cycling bout, the TT exceeded 75% HR_{max}. Prolonged and intense exercise has been shown to decrease secretion of salivary IgA, which has been attributed to changes in transport into the saliva, rather than changes in local plasma cell activation and immunoglobulin synthesis (47). It is likely that this is also the case for FLCs due to the transient nature of exercise-induced changes, and the mechanisms of FLC transport into the saliva are reduced in response to exercise, without any significant accompanying change in volume. As the roles of salivary FLCs are yet to be determined, the implications of a reduction in FLCs post-exercise are unclear at present, nor if acute changes translate into altered resting levels as a result of exercise training.

Serum FLCs did not change in response to exercise in either age group. This is consistent with findings in elderly individuals in relation to endurance walking (22). In contrast, increases in FLCs, resulting in minor elevations in FLC sum and K:λ ratio, have been observed after marathon running (24). It may be that only high intensity exercise of a sufficiently prolonged nature is capable of eliciting perturbations in serum FLC parameters. Including the present study, only three investigations into FLCs and exercise have taken place to date. Further studies are required to fully characterise the FLC response to acute exercise, including resistance exercise.

CONCLUSIONS

We have generated reference ranges for FLCs in saliva in healthy individuals. Polyclonal FLCs in serum have been shown to be able to identify and monitor disease in a range of conditions and be prognostic of mortality in the general population. The ability to reliably assess FLCs in saliva and the reference ranges provided will potentially broaden the use of this biomarker in healthy and clinical populations. We have demonstrated that older adults have higher salivary FLC parameters compared with young adults. This may be important in the context of inflammatory process and diseases associated with ageing. Additional research is required to explore connections between serum and saliva FLCs in disease and understand the biological roles of salivary FLCs. Exercise did not affect serum FLCs, but significantly reduced salivary FLCs in older adults. These changes appear to be transient and FLC concentrations and secretion rates began to recover at 1 h post-exercise, although remained below pre-exercise values. Future studies are needed to appreciate the relationship between FLCs and acute exercise and chronic exercise training. Given what we already know about serum FLCs and their diverse applications as a biomarker, salivary FLCs have

a range of exciting prospects within aspects of ageing, disease and exercise immunology research.

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