



LJMU Research Online

Hanstock, HG, Walsh, NP, Edwards, JP, Fortes, MB, Cosby, SL, Nugent, A, Curran, T, Coyle, PV, Ward, MD and Yong, XH

Tear Fluid SIgA as a Noninvasive Biomarker of Mucosal Immunity and Common Cold Risk

<http://researchonline.ljmu.ac.uk/id/eprint/12472/>

Article

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

Hanstock, HG, Walsh, NP, Edwards, JP, Fortes, MB, Cosby, SL, Nugent, A, Curran, T, Coyle, PV, Ward, MD and Yong, XH (2016) Tear Fluid SIgA as a Noninvasive Biomarker of Mucosal Immunity and Common Cold Risk. *Medicine and Science in Sports and Exercise*. 48 (3). pp. 569-577. ISSN

LJMU has developed [LJMU Research Online](http://researchonline.ljmu.ac.uk) for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

<http://researchonline.ljmu.ac.uk/>

Medicine & Science IN Sports & Exercise

The Official Journal of the American College of Sports Medicine

www.acsm-msse.org

. . . Published ahead of Print

Tear Fluid SIgA as a Noninvasive Biomarker of Mucosal Immunity and Common Cold Risk

Helen G. Hanstock¹, Neil P. Walsh¹, Jason P. Edwards¹, Matthew B. Fortes¹, Sara L. Cosby²,
Aaron Nugent², Tanya Curran³, Peter V. Coyle³, Mark D. Ward¹, and Xin Hui Aw Yong¹

¹College of Health and Behavioural Sciences, Bangor University, Bangor, Gwynedd, United Kingdom; ²Centre for Infection and Immunity, Queen's University Belfast, Northern Ireland, United Kingdom; ³Regional Virus Laboratory, Royal Victoria Hospital, Belfast, United Kingdom

Accepted for Publication: 15 October 2015

Medicine & Science in Sports & Exercise® Published ahead of Print contains articles in unedited manuscript form that have been peer reviewed and accepted for publication. This manuscript will undergo copyediting, page composition, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered that could affect the content.

Copyright © 2015 American College of Sports Medicine

Tear Fluid SIgA as a Noninvasive Biomarker of Mucosal Immunity and Common Cold Risk

Helen G. Hanstock¹, Neil P. Walsh¹, Jason P. Edwards¹, Matthew B. Fortes¹, Sara L. Cosby²,
Aaron Nugent², Tanya Curran³, Peter V. Coyle³, Mark D. Ward¹, and Xin Hui Aw Yong¹

¹College of Health and Behavioural Sciences, Bangor University, Bangor, Gwynedd, United Kingdom; ²Centre for Infection and Immunity, Queen's University Belfast, Northern Ireland, United Kingdom; ³Regional Virus Laboratory, Royal Victoria Hospital, Belfast, United Kingdom

Corresponding Author:

Prof. Neil P. Walsh FACSM

College of Health and Behavioural Sciences,

Bangor University,

Bangor,

LL57 2PZ,

UK.

Email: n.walsh@bangor.ac.uk

Telephone: + 44 1248 383480

Helen Hanstock's PhD was supported by a 125th anniversary research scholarship from Bangor University. Study one and two received no external funding. Study three was supported by a graduate student research grant from the European Hydration Institute. None of the authors had a conflict of interest. The results of the present study do not constitute endorsement by the American College of Sports Medicine.

Running Title: Tear fluid SIgA and common cold risk

ABSTRACT

Purpose: Research has not convincingly demonstrated the utility of saliva secretory Immunoglobulin-A (SIgA) as a biomarker of upper-respiratory-tract-infection (URTI) risk and disagreement exists about the influence of heavy exercise ('open-window-theory') and dehydration on saliva SIgA. Prompted by the search for viable alternatives, we compared the utility of tear and saliva SIgA to predict URTI prospectively (study-one) and assessed the influence of exercise (study-two) and dehydration (study-three) using a repeated-measures-crossover design. **Methods:** In study-one, forty subjects were recruited during the common-cold season. Subjects provided tear and saliva samples weekly and recorded upper-respiratory-symptoms (URS) daily for 3-weeks. RT-PCR confirmed common-cold pathogens in 9 of 11 subjects reporting URS (82%). Predictive utility of tear and saliva SIgA was explored by comparing healthy samples with those collected the week pre-URS. In study-two, thirteen subjects performed a 2-hour run at 65% VO_{2peak} . In study-three, thirteen subjects performed exercise-heat-stress to 3% body-mass-loss followed by overnight fluid restriction. **Results:** Tear SIgA concentration and secretion rate were 48% and 51% lower respectively during URTI and 34% and 46% lower the week pre-URS ($P<0.05$) but saliva SIgA remained unchanged. URS risk the following week increased 9-fold (95% CI: 1.7 to 48) when tear SIgA secretion rate $<5.5 \mu\text{g}\cdot\text{min}^{-1}$ and 6-fold (95% CI: 1.2 to 29) when tear SIgA secretion rate decreased $>30\%$. Tear SIgA secretion rate $>5.5 \mu\text{g}\cdot\text{min}^{-1}$ or no decrease $>30\%$ predicted subjects free of URS in $>80\%$ of cases. Tear SIgA concentration decreased post-exercise (-57% ; $P<0.05$) in line with the 'open-window-theory' but was unaffected by dehydration. Saliva flow rate decreased and saliva SIgA concentration increased post-exercise and during dehydration ($P<0.05$). **Conclusion:** Tear SIgA has utility as a non-invasive biomarker of mucosal immunity and common-cold risk. **Key words:** Infection; Immune; Exercise; Stress; Dehydration; Contact-lens

INTRODUCTION

Saliva secretory IgA (SIgA) is known to exhibit broad-spectrum antimicrobial activity against a range of viral pathogens through inhibition of pathogen adherence and penetration of the mucosal epithelia, providing a ‘first line of defence’ against common infections (8). On this premise, and sparked by Tomasi and co-authors’ landmark paper on saliva SIgA in cross-country skiers (40), there has been widespread attention (~200 papers) and optimism that saliva SIgA may serve as a non-invasive biomarker of mucosal immunity and upper respiratory tract infection (URTI) risk in athletes and military personnel (9, 15, 22, 30, 40). It is somewhat disappointing then that research has not convincingly demonstrated the utility of saliva SIgA as a predictive biomarker for URTI by identifying atypically low values for both an on-the-spot application (against the population reference range) and a monitoring application (against the individual reference range).

Numerous studies have assessed the saliva SIgA response to prolonged strenuous exercise (>1.5h), to explore the ‘open window’ hypothesis that immunity may be temporarily compromised after strenuous exercise (44). These studies often show a post-exercise decrease in saliva SIgA, lasting for an hour or more (40, 42) but discrepancies exist as studies have also shown no change (5) or even an increase in saliva SIgA (7). Numerous factors are thought to contribute to these conflicting findings, including: methodological differences in saliva collection and analysis (33); the chosen method to report saliva SIgA (concentration, secretion rate or relative to protein/osmolality) (7, 43); diurnal variation (42); psychological stress (10); hydration status (18, 32); nutritional status (32), smoking (3) and sex (20). Amid the noise of

potential confounders, it is perhaps not surprising that studies have fallen somewhat short of convincingly demonstrating the utility of saliva SIgA to predict URTI.

Tear fluid collection and analysis has been widely employed by ophthalmologists to examine specific aspects of ocular immunity (45). However, assessment of mucosal biomarkers through non-invasive tear sampling is a novel concept in exercise and stress immunology, and the relationship between tear SIgA and URTI has not been explored. Transmission of common viral pathogens from the ocular surface to the upper respiratory tract has been demonstrated in both animals (2, 6) and humans (4), and thus it is reasonable to postulate that a reduction in locally produced SIgA may compromise host defence beyond the eye and increase susceptibility to URTI. The aqueous component of the tear film is secreted by the lacrimal gland; a single gland as opposed to the multiple glands that contribute to saliva composition, and thus we suggest that the composition of tear fluid might be more uniform than saliva, providing a clearer signal amid the noise of potential confounders. Recent advances in nanotechnology and microfluidic fabrication have enabled a new generation of ocular measurement devices for both on-the-spot measurements (e.g. the TearLabTM osmolarity device) and continuous monitoring by contact lens sensors. Indeed, we recently showed the utility of tear osmolarity to track changes in whole body hydration status (17). It remains unknown whether the increase in tear osmolarity with dehydration was the result of a dehydration-driven decrease in tear flow rate (concentrating effect) that might also alter the availability of SIgA in tear fluid in the same way as dehydration influences SIgA in saliva (18, 32). A wearable device that continuously monitors one or more biomarkers offers advantages for the identification of atypical values over on-the-spot measurement. For example, contact lenses have been developed for continuous assessment of

glucose and intraocular pressure and have the potential to measure a range of other biomarkers in tear fluid including antibodies (16). With this information in mind, an exploratory investigation of the potential for tear SIgA to assess mucosal immunity and common cold risk is timely and warranted.

To this end, here we present results from three studies. Firstly, in a prospective study we investigated the utility of tear SIgA and saliva SIgA to predict upper respiratory symptoms (URS) and pathogen-identified URTI in a mixed-sex cohort of contact lens and non-contact lens wearers (study one). Then, using a repeated measures cross-over design, we investigated the effects of prolonged, moderate-intensity exercise stress (study two) and progressive, modest dehydration (study three) on tear SIgA and saliva SIgA. We hypothesized that tear SIgA and saliva SIgA would be lower during and in the days before the common cold and that tear SIgA would have greater predictive utility than saliva SIgA.

METHODS

All studies received local ethics committee approval and all protocols were conducted in accordance with the Declaration of Helsinki.

Study One

Subjects. Forty recreationally active volunteers gave written informed consent to participate in the study (Fig. 1). Subjects were 26 males and 14 females (males: age 22 ± 4 years; height 179 ± 5 cm; body mass 73 ± 10 kg; females: age 22 ± 6 years; height 169 ± 4 cm; body mass 67 ± 20 kg). Subjects were recruited in Bangor, UK in September at the beginning of the common cold

season. Eight of these subjects were contact lens (CL) wearers. Subjects did not self-report URS lasting ≥ 48 h in the previous month, did not have any underlying health conditions, had no recent diagnosis or test for mononucleosis (within 1 year) and were not regularly taking medication known to influence immune indices. Subjects were asked to go about their normal daily routines, but avoid structured exercise 2 h prior to sample collection and food and drink 1 h prior to sample collection.

Experimental procedures. Subjects provided weekly tear and saliva samples and completed the Jackson common cold questionnaire each day (25). The questionnaire included one dichotomous global question and eight symptom items (headache, sneezing, chills, sore throat, nasal discharge, nasal obstruction, cough) scored on a 4-point Likert scale (0 = not at all; 1 = mild, 2 = moderate, 3 = severe). If subjects answered “Yes” to the dichotomous question “Do you think you are suffering from a common cold today?” or reported a symptom score of ≥ 6 for two consecutive days (equivalent to 3 moderate or 2 severe symptoms) they were asked to report to the laboratory as soon as possible at a similar time of day to their initial sample (± 1 h) for tear and saliva samples and nasopharyngeal and throat swabs. These subjects continued to complete the Jackson common cold questionnaire each day and returned to the laboratory for healthy samples after 4 consecutive weeks symptom-free (RECOVERY). For subjects who remained healthy, nasopharyngeal and throat swabs and final tear and saliva samples were collected at the end of the 3-week monitoring period (CON).

Nasopharyngeal and throat swabs. Swabs were collected from subjects using standard procedures (38) both during URS and at the final visit for all subjects. Swabs were immediately

placed in viral transport medium and stored at $-80\text{ }^{\circ}\text{C}$ prior to real-time PCR (RT-PCR) analysis, as described (26). RT-PCR was used to screen for a battery of common upper respiratory pathogens: Influenza types A and B, Respiratory Syncytial Virus types A and B, Metapneumovirus, Adenovirus, Coronavirus, Parainfluenza virus types 1, 2, 3 and 4, Human Rhinovirus (HRV), Bocavirus, *Pneumocystis jirovecii*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Bordetella pertussis*.

Study Two

Subjects. Thirteen healthy, recreationally active adult males gave written informed consent to participate in the study (age 23 ± 5 years; height 179 ± 8 cm; body mass 79 ± 9 kg; $\dot{V}O_{2\text{peak}}$ 52.8 ± 5.6 mL \cdot kg $^{-1}\cdot$ min $^{-1}$). Subjects were non-smokers, who did not take prescription medication or use dietary supplements for the duration of the study or within the preceding month. Subjects were asked to refrain from alcohol, caffeine, over-the-counter medication and heavy exercise for 24 h preceding all experimental trials and did not self-report URS in the 7 days preceding experimental trials.

Preliminary measures. To determine $\dot{V}O_{2\text{peak}}$, subjects performed a ramped treadmill exercise test to volitional exhaustion and speed verification, in line with a protocol detailed in an earlier study (14).

Experimental procedures. Subjects completed two experimental trials in a randomised, crossover design separated by at least one week. The exercise trial (EX) involved 2 h continuous treadmill running at 65% $\dot{V}O_{2\text{peak}}$ in temperate conditions ($19\text{ }^{\circ}\text{C}$, 43% RH) whereas the control

trial (REST) constituted 2 h seated rest in the same environment. Subjects reported to the lab at 0730 h on day 1 of both trials, where they were provided with a standardised breakfast and a fluid allowance of $35 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ *pro rata* for the pre-exercise period. Subjects remained in the laboratory and engaged in sedentary activities such as reading, watching television or browsing the internet after breakfast until pre-exercise samples. The EX or REST took place from 1100 - 1300 h. Tear and saliva samples were collected Pre-EX (1040 h), Post-EX (1300 h), 30 min post-EX (1330 h), 1 h post-EX (1400 h) and 24 h later (1040 h, day 2), and at equivalent time points on REST. Nude body mass was recorded pre- and post- EX to estimate fluid losses. During EX, subjects were provided with $3 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ plain water to partially offset fluid losses through sweating; during REST fluid intake remained at $35 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ *pro rata*. Heart rate (HR) was monitored continuously throughout the 2 h EX or REST period. During EX only, Borg's rating of perceived exertion (RPE) was recorded and 60 s expired gas samples collected at 10 min intervals, except at 30 min, 60 min and 90 min where fluids were provided instead. Following exercise, subjects rested for 1 h and were provided with a standardised meal at 1415 h. Subjects recorded and replicated caloric intake from 1430 h until sleep, and were provided with the same breakfast for the following morning (day 2) before returning to the laboratory at 1040 h day 2 for 24 h follow-up samples.

Study Three

Subjects. Thirteen healthy, recreationally active adult males gave written informed consent to participate in the study (age 24 ± 4 years; height 181 ± 5 cm; body mass 80 ± 10 kg; $\dot{V}\text{O}_{2\text{peak}}$ $56.4 \pm 7.8 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). Subjects took part according to the same criteria outlined in study two.

Preliminary measures. Prior to the main trials, an uphill walking protocol in line with the subsequent exercise to be undertaken during the main trials was employed to estimate $\dot{V}O_{2\text{peak}}$ (4 km·h⁻¹ at 4% gradient, increasing by 2 km·h⁻¹ to 14 km·h⁻¹, then by 2.5% gradient; 3 min intervals). $\dot{V}O_{2\text{peak}}$ data were interpolated to determine a treadmill speed to elicit exercise at 50% $\dot{V}O_{2\text{peak}}$. Whole body sweat rate was determined by calculating nude body mass loss (BML) during 30 min treadmill exercise at 50% $\dot{V}O_{2\text{peak}}$ in an environmental chamber set to 40°C dry-bulb temperature and 40% RH. Sweat rate was used to prescribe an exercise duration expected to elicit 1%, 2% and 3% BML in the main experimental trials.

Experimental trials. The effect of dehydration on tear and saliva SIgA was investigated using a randomised crossover design. Euhydration (EUH) and dehydration (DEH) trials were undertaken by subjects with each trial lasting ~28 h and separated by at least 7 days. Subjects reported to the laboratory at 0800 h on day 1 of the trial, where 40 mL·kg⁻¹·day⁻¹ fluids *pro rata* were provided until 1400 h to ensure euhydration at the start of each trial. Standardised meals were provided at 0830 h, 1200 h, 1800 h and 2100 h on day 1 and 0900 h on day 2 of each trial. Saliva, tear and blood samples were collected at four time points: day 1 at 0% BML (1400 h) and 2% BML (1630 h) and on day 2 after overnight fluid restriction (0800 h) and after rehydration (1100 h). Starting at 1400 h, an exercise-heat protocol (40°C, 40% RH) was employed to evoke dehydration through sweating; subjects walked uphill (4% gradient, 50% $\dot{V}O_{2\text{peak}}$) for three bouts of equal duration, each bout estimated to elicit 1% BML (36 ± 7 min). During EUH, subjects were provided with fluids to offset BML. Exercise to seated rest ratio was 1:1; subjects rested at 20°C in between each exercise bout. Following the final exercise bout, subjects remained in the laboratory overnight and engaged in sedentary activities before going to bed at 2300 h. During

DEH subjects were provided with fluids at a rate of $4 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ from 1800 h to 2300 h; during EUH they received $40 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ fluids *pro rata*. The following morning subjects woke at 0730 h and, following sample collection, received fluids throughout the course of the morning. During DEH, subjects were provided with their net fluid losses and during EUH subjects received $40 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ fluids *pro rata*.

Assessment of plasma osmolality. To assess hydration status, whole blood samples were collected from an antecubital vein into a 6 ml heparinised vacutainer (Becton Dickinson, Oxford, UK). Samples were centrifuged (1500 g , 4°C , 10 min) to obtain plasma for the immediate determination of osmolality (P_{osm}) in triplicate using a freezing point depression osmometer (model 3300 MO, Advanced Instruments, Norwood, Massachusetts, USA).

Tear sample collection, handling and analysis. In all studies, timed, unstimulated tear samples ($\sim 1 \mu\text{L}$; collection time $36 \pm 30 \text{ s}$, minimum 15 s) were collected using $10 \mu\text{L}$ fire-polished glass microcapillary pipets (Sigma-Aldrich, St. Louis, MO, USA) from the inferior marginal tear strip of the left eye (19). Following collection, tear samples were expelled into a pre-weighed microcentrifuge tube and sample volume assessed by calculating mass change. Assuming the density of tears to be $1.00\text{g}\cdot\text{mL}^{-1}$, tear flow rate was calculated by dividing the volume collected by the collection time. Tear samples were diluted 100x in phosphate-buffered saline and stored at -80°C until analysis. After thawing, tear samples were centrifuged and the concentration of SIgA determined using ELISA (Cat. No. 1-1602, Salimetrics, Pennsylvania, USA). Mean intra-assay CV was 1.6% from duplicate standards and samples across all plates and studies. For each study,

samples from a single subject were analysed on the same plate. Tear SIgA secretion rate was determined as the product of tear flow rate and SIgA concentration.

Saliva collection, handling and analysis. Unstimulated whole saliva samples were obtained using the passive drool method for 5 min (32). Following collection, tubes were weighed to the nearest 1.0 mg, and saliva flow rate estimated by dividing the sample volume by collection time, assuming $1.00 \text{ g}\cdot\text{mL}^{-1}$ saliva density. Immediately after collection saliva samples were centrifuged, aliquoted into Eppendorf tubes and frozen at $-80 \text{ }^{\circ}\text{C}$ for later analysis. Concentration of SIgA was determined using the same ELISA kits as used for the tear analysis. Saliva SIgA secretion rate was determined as the product of saliva flow rate and SIgA concentration.

Statistical analyses. Data are presented as mean \pm SD unless otherwise stated. For sample size estimation we used repeated baseline data from a previous study (18) where a deviation in salivary SIgA concentration outside the normal variability ($\pm 1 \text{ CV}$) occurred with an effect size of 0.98. With α set at 0.05 and power 0.8, a minimum sample of 11 was calculated to detect a change of this magnitude (G*Power 3.17). Statistical analyses were performed using common statistical software packages (SPSS 22, IBM, Chicago, IL and GraphPad Prism 5.0, San Diego, CA). Data were checked for normal distribution and in cases where the assumption of normality was violated, data were natural log (Ln) transformed prior to analysis. One-tailed paired and independent t-tests were performed to compare within- and between- group effects in study 1, with Welch's correction applied for unequal variance where applicable. Repeated measures ANOVA was used to assess main effects and trial x time interactions in studies 2 and 3. *F* values refer to a time x trial interaction unless otherwise stated. Post-hoc Tukey's HSD was used to

explore interaction effects. Effect size (ES) was calculated (Cohen's *d*) for the difference between means for main outcome variables. Cohen's *d* effect sizes greater than 0.2, 0.5 and 0.8 represent small, medium, and large effects respectively. For one-tailed tests, 90% confidence intervals (CI) of the difference were calculated, and 95% CIs for two-tailed analyses. Area under curve receiver operating characteristic (ROC) analysis was used to assess global diagnostic and predictive utility of secretory immune parameters, with sensitivity, specificity, accuracy, and the diagnostic odds ratio (OR) also calculated. For t-test, ANOVA and ROC analyses, significance was accepted as $P < 0.05$.

RESULTS

Study One

Laboratory identification of respiratory pathogens. Of the 33 subjects who completed the study, 11 reported with URS (33%, Fig. 1) during the 3-week period (6 females: 5 non-CL and 1 CL; 5 males: 2 non-CL and 3 CL). Of these 11 subjects, nine returned positive swab results following RT-PCR screening (82%); all 9 were positive for HRV, and one of these subjects was also positive for coronavirus (URTI). Of the 22 subjects who did not report URS during the monitoring period, five returned positive swab results at their final visit (3 HRV, 1 influenza, 1 *Pneumocystis jirovecii*), leaving 17 subjects who were non-viral shedders and did not report symptoms; this group served as healthy controls (CON: females: 4 non-CL, 2 CL; males: 10 non-CL, 1 CL).

Effect of URTI on tear and saliva SIgA. Tear SIgA concentration was 48% lower in subjects with current URTI than CON ($P < 0.05$, CI of the difference: -0.10 to -3.98 $\mu\text{g}\cdot\text{mL}^{-1}$; ES: 0.58;

Fig. 2A). Tear SIgA secretion rate was 51% lower in subjects with URTI but this trend did not reach statistical significance ($P < 0.10$; ES: 0.62; Fig. 2B). There was no difference in saliva SIgA concentration (Fig. 2C) or saliva SIgA secretion rate (Fig. 2D) between URTI and CON.

Change in tear and saliva SIgA prior to URS and URTI. In the 11 subjects who reported URS during the 3-week monitoring period, 1-week pre-URS samples and recovery samples were compared to explore whether tear or saliva SIgA were altered in an individual prior to URS. Of these 11 subjects, recovery tear SIgA secretion rate was comparable in the 4 subjects who wore CL ($9.3 \pm 8.6 \mu\text{g}\cdot\text{min}^{-1}$) and 7 who did not ($10.8 \pm 6.6 \mu\text{g}\cdot\text{min}^{-1}$). Tear SIgA concentration was 34% lower during the week preceding URS ($P < 0.05$, CI of the difference: -0.10 to $-2.16 \mu\text{g}\cdot\text{mL}^{-1}$; ES: 0.82; Fig. 3A). Tear SIgA secretion rate was 46% lower in the week prior to URS ($P < 0.05$, CI of the difference: -1.32 to $-8.37 \mu\text{g}\cdot\text{min}^{-1}$; ES 0.79; Fig. 3B). No change in saliva SIgA concentration or secretion rate was observed prior to URS (Fig. 3C-D). A sub-analysis of the nine subjects with pathogen-identified URTI revealed 32% lower tear SIgA concentration and 40% lower tear SIgA secretion rate pre-URT I compared with recovery; although not quite statistically significant, the ES calculations represent medium towards large effects (concentration: $P = 0.08$, ES: 0.72; secretion rate: $P = 0.07$, ES: 0.59). There was no change in salivary SIgA concentration or secretion rate prior to URTI.

Utility of tear SIgA to predict subsequent URS. For tear SIgA concentration and secretion rate, ROC analysis was performed to explore the utility of population cut-off values (on-the-spot application) and % change values from healthy samples (monitoring application) to predict subsequent URS. Tear SIgA secretion rate performed well as area under curve (AUC) was 0.81

(CI: 0.64 – 0.96; $P < 0.01$) for absolute tear SIgA secretion rate and 0.74 (CI: 0.57 – 0.92; $P < 0.05$) for change in tear SIgA secretion rate. For an on-the-spot application, subjects with a tear SIgA secretion rate $< 5.5 \mu\text{g}\cdot\text{min}^{-1}$ were 9 times more likely to develop URS in the following week compared with subjects with a tear SIgA secretion rate above this cut-off value (sensitivity: 0.73; specificity: 0.77; accuracy: 76%; OR: 9.1, CI: 1.7 to 47.7). A tear SIgA secretion rate $> 5.5 \mu\text{g}\cdot\text{min}^{-1}$ (negative test) successfully predicted absence of URS in 85% of cases. For a monitoring application, individuals who experienced a $> 30\%$ reduction in tear SIgA secretion rate were 6 times more likely to develop URS in the following week compared to those whose tear SIgA secretion rate did not decrease $> 30\%$ (sensitivity: 0.64; specificity: 0.77; accuracy: 73%; OR: 6.0, CI: 1.2 to 29.0). A negative test successfully predicted absence of URS in 81% of cases. AUC was only 0.66 ($P = 0.12$; CI: 0.48 – 0.85) for absolute tear SIgA concentration and 0.65 ($P = 0.17$; CI: 0.46 to 0.84) for change in tear SIgA concentration and thus not significantly greater than chance.

Study Two

Effect of prolonged moderate-intensity exercise on tear and saliva SIgA. All thirteen subjects completed both experimental trials. Mean HR during EX rose from 136 ± 15 bpm at 10 min to 160 ± 4 bpm at 2 h (mean of all time points: 147 ± 7 bpm) while mean HR throughout REST was 59 ± 6 bpm. During EX, RPE rose from 8 ± 2 ('very light') at 10 min to 15 ± 2 ('hard') at 2 h. Mean BML was $1.8 \pm 0.4 \%$ during EX and $0.3 \pm 0.5 \%$ during REST. EX caused an immediate 57% reduction in tear SIgA concentration compared to pre-EX ($F = 5.2$, $P < 0.01$; ES: 1.31; Fig. 4B). Saliva SIgA concentration was elevated on EX (main effect of trial: $F = 15.1$, $P < 0.01$; ES: 0.55; Fig. 4E). Both tear and saliva flow rate were influenced by exercise; tear flow rate showed

a delayed reduction at 1 h post-EX vs. pre-EX ($F = 3.5$, $P < 0.05$; ES: 1.12; Fig. 4A). Saliva flow rate was lower than REST immediately and 30 minutes post-EX ($F = 5.2$, $P < 0.05$; ES: 1.37 post-EX, 0.97 at 30 min post-EX; Fig. 4D). There was a reduction in tear SIgA secretion rate post-exercise (44% decrease, ES: 0.47) and at 30 min post-EX (55% decrease; ES: 0.67) vs. pre-exercise values (Fig. 4C) although ANOVA did not indicate an interaction. Despite a 23% decrease from pre- to post-exercise (ES: 0.45), there was no significant effect of EX on saliva SIgA secretion rate (Fig. 4F).

Study Three

Effect of dehydration on tear and saliva SIgA. All thirteen subjects completed the prescribed exercise duration on both trials. An elevation of P_{osm} occurred at 2% BML and at 0800 h on day 2 (3.0 ± 0.5 % BML), compared with 0% BML and EUH ($F = 22.7$, $P < 0.001$; Table 1). Neither tear SIgA concentration nor secretion rate were significantly influenced by DEH; however, tear SIgA concentration was higher at 1630 h than at 0800 h indicating diurnal variation (main effect of time: $F = 6.34$; $P < 0.01$; Table 1). DEH did not significantly influence tear flow rate, but tear flow rate was lower at 1630 h than 0800 h (main effect of time: $F = 4.7$; $P < 0.001$; Table 1). Saliva SIgA concentration increased during DEH at 2% BML and at 0800 h on day 2 (3% BML) and returned to baseline upon rehydration ($F = 11.0$, $P < 0.01$; Table 1). Saliva flow rate was reduced during dehydration at 2% BML and at 0800 h on day 2 ($F = 8.6$, $P < 0.001$; Table 1). There was no effect of DEH on saliva SIgA secretion rate.

DISCUSSION

The three studies presented here are the first to explore and demonstrate the utility of tear SIgA as a non-invasive biomarker of mucosal immunity and common cold risk. In study one we observed that, unlike saliva SIgA, tear SIgA concentration was lower during pathogen-identified URTI (-48%) and in the week before URS (-34%). Availability of SIgA at the ocular surface, reported as tear SIgA secretion rate, tended to be lower during pathogen-identified URTI (-51%) and was significantly reduced the week before URS (-46%). Our data suggest that tear SIgA may have potential for both on-the-spot assessment of an individual's risk of URS, and for a monitoring application whereby a change in tear SIgA secretion rate could be indicative of an individual's URS risk. Regarding on-the-spot application, absolute tear SIgA secretion rate $< 5.5 \mu\text{g}\cdot\text{min}^{-1}$ increased the risk of URS the following week by 9-fold. Given the multi-factorial underpinning of upper respiratory illness, assessing the likelihood of protection against URS is of great practical importance. Accordingly, tear SIgA secretion rate above $5.5 \mu\text{g}\cdot\text{min}^{-1}$ predicted subjects free of URS in 85% of cases. For a monitoring application, a decrease in tear SIgA secretion rate $> 30\%$ resulted in a 6-fold increased risk of URS the following week. Absence of a 30% or more decrease in tear SIgA secretion rate predicted subjects free of URS in 81% of cases in our otherwise healthy male and female cohort during the common cold season. In study two, prolonged exercise caused a transient decrease in tear SIgA (concentration -57% and secretion rate -44%) in line with the 'open window theory' (31). In study three, dehydration did not significantly influence tear SIgA. Both prolonged exercise (study two) and dehydration (study three) brought about a decrease in saliva flow rate and increase in saliva SIgA concentration as shown previously (18, 27).

In study one we demonstrate that tear SIgA, but not saliva SIgA, was lower during URTI compared with CON. A strength of study one was the use of RT-PCR analysis to confirm URTI by identification of common viral pathogens in 9 of 11 subjects (82%) presenting with self-reported URS. HRV was detected in all cases of confirmed URTI, in line with previous evidence that HRV caused 80% of common colds in adults during the seasonal autumn peak (1). We recognize the limitation that we did not screen for the presence of viral pathogens at enrolment so we cannot discount viral reactivation and conclude that the URTIs were all due to a primary infection (23). However, given that we chose the common cold season, that none of our subjects self-reported URS in the month prior to enrolment, and none had been diagnosed with mononucleosis in the previous year, it is plausible that primary infection was prominent in the reported cases of URTI. Our findings exceed rates of confirmed pathogen detection in 70% of URS episodes in 200 Finnish students (28); moreover, we detected a substantially higher proportion of pathogen-confirmed URTI from self-reported URS than the ~30% reported in elite athletes (12, 38). This discrepancy likely occurred since those studies were conducted year-round (12) or during the southern hemisphere summer (38) when average ambient temperature was ~28°C and the common cold incidence is low (likely higher allergy in summer). Athletes engaging in heavy training may be more susceptible to developing URS of non-infectious origin than our cohort, given the high prevalence of airway inflammation and reactivity to airborne allergens in athletes (36). Self-reported URS, irrespective of origin, are a significant economic and social burden, causing absence from work, education and athletic training, poorer performance in work-related tasks (37), and performance decrements in athletic competition (35). Thus, we were principally interested in the utility of tear and saliva SIgA to predict self-reported URS. In the week before URS, both tear SIgA concentration and secretion rate were

lower by 34% and 46% (*vs.* healthy), respectively: lower tear SIgA was also evident in the week before URTI in the nine participants with HRV. As the incubation period for HRV is less than 12 hours (24), it is unlikely that tear SIgA decreased as a consequence of virus presence, but instead represented compromised host defence and an increased susceptibility to the common cold.

Considering the potential for developing wearable technology to monitor ocular biomarkers, the influence of CL wear on tear SIgA secretion and indeed URTI risk are important considerations. Numerous studies have investigated the influence of CL wear on tear AMPs, but the findings are conflicting, with studies reporting a decrease (34, 41) or an increase in tear SIgA with CL wear (29). Moreover, different CL types may have different effects on the tear protein profile; Temel and colleagues reported an increase in tear SIgA in rigid lens wearers but no effect of soft lenses on tear SIgA compared with non-wearers (39). Typically, only tear SIgA concentration has been reported, whereas our findings suggest that SIgA secretion rate, taking into account tear flow rate, may be an important factor in preserving the integrity of mucosal defences at the ocular surface. Moreover, the influence of CL on URTI risk remains unexplored; although CL wearers typically experience increased risk of ocular infections, a very recent report suggests that poor hygiene is a likely contributing factor (11). Our preliminary findings suggest that tear SIgA secretion is comparable in CL and non-CL wearers but we acknowledge the small N for this comparison and the need for future work to verify this finding.

In contrast to the encouraging results for tear SIgA, we observed no change in saliva SIgA prior to URS; saliva SIgA concentration and secretion rate before URS were comparable to saliva SIgA in healthy samples. This finding agrees with a study showing no meaningful relationship

between saliva SIgA concentration and URS incidence during the winter months in athletes (21) but disagrees with another study showing a progressive decline in saliva SIgA concentration in elite sailors in the 3-weeks prior to URS (30). Though the predictive utility of saliva SIgA concentration in the sailing study (30) should be tempered because the increased frequency of URTI in those experiencing a 30% or greater decline in saliva SIgA concentration was only 2.5-fold compared with 6-fold for tear SIgA secretion rate in the present study.

Prolonged, moderate intensity exercise in study two caused a transient decrease in tear SIgA concentration (-57%) that exceeded in magnitude the reduction in tear SIgA concentration prior to URS. This 'open window', though short lasting, is thus likely to compromise mucosal defence at the ocular surface. In study three, we demonstrated that unlike saliva, tear SIgA was not affected by dehydration immediately post-exercise or following overnight fluid restriction. Whilst a diurnal change in tear SIgA concentration was highlighted, tear SIgA secretion rate was unaffected by hydration status or time of day, providing a potentially stable signal from which to identify atypical deviations in tear SIgA secretion rate to indicate compromised immunity. Saliva responses to prolonged exercise in study two and dehydration in study three consisted of the well-characterised decrease in flow rate, concurrent increase in saliva SIgA concentration (concentrating effect) and no overall change in SIgA secretion rate (5, 18, 27). The discrepancy between the response of tear and saliva SIgA in study two may be explained in part by the differences in the neural regulation of tear and saliva secretion. The lacrimal gland receives primarily parasympathetic innervation, with only a minor contribution from the sympathetic nervous system (13). Parasympathetic withdrawal attenuates the rate of tear protein secretion in tears, with likely little effect on electrolyte or water output (13). Thus, the transient post-exercise

decrease in tear SIgA concentration may be accounted for by parasympathetic withdrawal. The rate-limiting step for SIgA secretion in mucosal glands is the availability of the polymeric IgA receptor (pIgAR), which transports SIgA across the acinar cell membrane into mucosal secretions. Since alterations in transcriptional regulation of either SIgA or the pIgAR would be expected to occur over a longer timescale (minutes to hours) the transient post-exercise decrease in tear SIgA concentration was unlikely immunologically driven. Nevertheless, the decrease in tear SIgA after prolonged exercise likely represents a meaningful, albeit temporary, reduction in host defence at the ocular surface.

We conclude that tear SIgA has potential as a non-invasive biomarker of mucosal immunity and common cold risk for those working in the fields of exercise, stress and nutritional immunology and others. Further research is required to confirm these findings, to determine normative population values, to extend tear fluid analysis beyond SIgA to include other antimicrobial proteins, and to explore the utility of other candidate biomarkers in tears to monitor psychological and physiological status (e.g. stress hormones). Studies are also required to fully understand the influence of contact lens wear, age, sex, diet, psychological status and training status, amongst others on tear fluid biomarkers. In tandem, further advances in nanotechnology and microfluidics will likely afford the possibility for on-the-spot tear fluid measurement devices and continuous bio-monitoring by contact lenses.

Acknowledgements

Helen Hanstock's PhD was supported by a 125th anniversary research scholarship from Bangor University. Study one and two received no external funding. Study three was supported by a graduate student research grant from the European Hydration Institute.

None of the authors had a conflict of interest. The results of the present study do not constitute endorsement by the American College of Sports Medicine.

ACCEPTED

References

1. Arruda E, Pitkäranta A, Witek TJ, Doyle C, Hayden FG. Frequency and natural history of rhinovirus infections in adults during autumn. *J Clin Microbiol.* 1997; 35:2864-2868.
2. Belser JA, Wadford DA, Xu J, Katz JM, Tumpey TM. Ocular infection of mice with Influenza A (H7) viruses: a site of primary replication and spread to the respiratory tract. *J Virol.* 2009; 83:7075-7084.
3. Bennet KR, Reade PC. Salivary immunoglobulin A levels in normal subjects, tobacco smokers, and patients with minor aphthous ulceration. *Oral Surg Oral Med Oral Pathol.* 1982; 53:461-465.
4. Bischoff WE, Reid T, Russell GB, Peters TR. Transocular entry of seasonal Influenza-attenuated virus aerosols and the efficacy of N95 respirators, surgical masks, and eye protection in humans. *J Infect Dis.* 2011; 204:193-199.
5. Bishop NC, Blannin AK, Armstrong E, Rickman M, Gleeson M. Carbohydrate and fluid intake affect the saliva flow rate and IgA response to cycling. *Med Sci Sports Exerc.* 2000; 32:2046-2051.
6. Bitko V, Musiyenko A, Barik S. Viral infection of the lungs through the eye. *J Virol.* 2007; 81:783-790.
7. Blannin AK, Robson PJ, Walsh NP, Clark AM, Glennon L, Gleeson M. The effect of exercising to exhaustion at different intensities on saliva immunoglobulin A, protein and electrolyte secretion. *Int J Sports Med.* 1998; 19:547-552.
8. Brandtzaeg P. Secretory immunity with special reference to the oral cavity. *J Oral Microbiol.* 2013; 5:1-24.

9. Carins J, Booth C. Salivary immunoglobulin-A as a marker of stress during strenuous physical training. *Aviat Space Environ Med.* 2002; 73:1203-1207.
10. Clow A, Hucklebridge F. The impact of psychological stress on immune function in the athletic population. *Exerc Immunol Rev.* 2000; 7:1-13.
11. Cope JR, Collier SA, Rao MM et al. Contact lens wearer demographics and risk behaviors for contact lens-related eye infections - United States, 2014. *MMWR Morb Mortal Wkly Rep.* 2015; 64:865-870.
12. Cox AJ, Gleeson M, Pyne DB, Callister R, Hopkins WG, Fricker P. Clinical and laboratory evaluation of upper respiratory symptoms in elite athletes. *Clin J Sport Med.* 2008; 18:438-445.
13. Dartt D. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res.* 2009; 28:155-177.
14. Diment BC, Fortes MB, Edwards JP et al. Exercise intensity and duration effects on in vivo immunity. *Med Sci Sports Exerc.* 2015; 47:1390-1398.
15. Fahlman MM, Engels HJ. Mucosal IgA and URTI in American college football players: A year longitudinal study. *Med Sci Sports Exerc.* 2005; 37:374-380.
16. Farandos NM, Yetisen AK, Monteiro MJ, Lowe CR, Yun SH. Contact lens sensors in ocular diagnostics. *Adv Healthc Mater.* 2015; 4:792-810.
17. Fortes MB, Diment BC, Di Felice U et al. Tear fluid osmolarity as a potential marker of hydration status. *Med Sci Sports Exerc.* 2011; 43:1590-1597.
18. Fortes MB, Diment BC, Di Felice U, Walsh NP. Dehydration decreases saliva antimicrobial proteins important for mucosal immunity. *Appl Physiol Nutr Metab.* 2012; 37:850-859.

19. Fullard RJ, Snyder C. Protein levels in nonstimulated and stimulated tears of normal human subjects. *Invest Ophthalmol Vis Sci.* 1990; 31:1119-1126.
20. Gleeson M, Bishop N, Oliveira M, McCauley T, Tauler P. Sex differences in immune variables and respiratory infection incidence in an athletic population. *Exerc Immunol Rev.* 2011; 17:122-135.
21. Gleeson M, Bishop N, Oliveira M, McCauley T, Tauler P, Muhamad AS. Respiratory infection risk in athletes: association with antigen-stimulated IL-10 production and salivary IgA secretion. *Scand J Med Sci Sports.* 2012; 22:410-417.
22. Gleeson M, McDonald WA, Pyne DB et al. Salivary IgA levels and infection risk in elite swimmers. *Med Sci Sports Exerc.* 1999; 31:67-73.
23. Gleeson M, Pyne DB, Austin JP et al. Epstein-Barr virus reactivation and upper-respiratory illness in elite swimmers. *Med Sci Sports Exerc.* 2002; 34:411-417.
24. Harris JM, Gwaltney JM. Incubation periods of experimental rhinovirus infection and illness. *Clin Infect Dis.* 1996; 6:1287-1290.
25. Jackson GG, Dowling HF, Spiesman IG, Boand AV. Transmission of the common cold to volunteers under controlled conditions. *AMA Arch Intern Med.* 1958; 101:267-278.
26. Johnston NW, Lambert K, Hussack P et al. Detection of COPD Exacerbations and compliance with patient-reported daily symptom diaries using a smart phone-based information system. *Chest.* 2013; 144:507-514.
27. Killer SC, Svendsen IS, Gleeson M. The influence of hydration status during prolonged endurance exercise on salivary antimicrobial proteins. *Eur J Appl Physiol.* 2015; 115:1887-1895.

28. Mäkelä MJ, Puhakka T, Ruuskanen O et al. Viruses and bacteria in the etiology of the common cold. *J Clin Microbiol.* 1998; 36:539-542.
29. Maurya RP, Bhushan P, Singh VP et al. Immunoglobulin concentration in tears of contact lens wearers. *J Ophthalmic Vis Res.* 2014; 9:320-323.
30. Neville V, Gleeson M, Folland JP. Salivary IgA as a risk factor for upper respiratory infections in elite professional athletes. *Med Sci Sports Exerc.* 2008; 40:1228-1236.
31. Nieman DC. Exercise, infection, and immunity. *Int J Sports Med.* 1994; 15:S131-S141.
32. Oliver SJ, Laing SJ, Wilson S, Bilzon JLJ, Walters R, Walsh NP. Salivary immunoglobulin A response at rest and after exercise following a 48 h period of fluid and/or energy restriction. *Br J Nutr.* 2007; 97:1109-1116.
33. Papacosta E, Nassis GP. Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science. *J Sci Med Sport.* 2011; 14:424-434.
34. Pearce DJ, Demirci G, Willcox MD. Secretory IgA epitopes in basal tears of extended-wear soft contact lens wearers and in non-lens wearers. *Aust N Z J Ophthalmol.* 1999; 27:221-223.
35. Pyne DB, Hopkins WG, Batterham AM, Gleeson M, Fricker P. Characterising the individual performance responses to mild illness in international swimmers. *Br J Sports Med.* 2005; 39:752-756.
36. Robson-Ansley P, Howatson G, Tallent J et al. Prevalence of allergy and upper respiratory tract symptoms in runners of the London marathon. *Med Sci Sports Exerc.* 2012; 44:999-1004.

37. Smith AP. Effects of the common cold on mood, psychomotor performance, the encoding of new information, speed of working memory and semantic processing. *Brain Behav Immun.* 2012; 26:1072-1076.
38. Spence L, Brown WJ, Pyne DB et al. Incidence, etiology, and symptomatology of upper respiratory illness in elite athletes. *Med Sci Sports Exerc.* 2007; 39:577-586.
39. Temel A, Kazokoglu H, Taga Y, Orkan AL. The effect of contact lens wear on tear immunoglobulins. *CLAO J.* 1991; 17:69-71.
40. Tomasi TB, Trudeau FB, Czerwinski D, Erredge S. Immune parameters in athletes before and after strenuous exercise. *J Clin Immunol.* 1982; 2:173-178.
41. Vinding T, Eriksen JS, Nielsen NV. The concentration of lysozyme and secretory IgA in tears from healthy persons with and without contact lens use. *Acta Ophthalmol.* 1987; 65:23-26.
42. Walsh NP, Bishop NC, Blackwell J, Wierzbicki SG, Montague JC. Salivary IgA response to prolonged exercise in a cold environment in trained cyclists. *Med Sci Sports Exerc.* 2002; 34:1632-1637.
43. Walsh NP, Blannin AK, Clark AM, Cook L, Robson PJ, Gleeson M. The effects of high-intensity intermittent exercise on saliva IgA, total protein and alpha-amylase. *J Sports Sci.* 1999; 17:129-134.
44. Walsh NP, Gleeson M, Shephard RJ et al. Position statement part one: Immune function and exercise. *Exerc Immunol Rev.* 2011; 17:6-63.
45. Zhou L, Beuerman RW. Tear analysis in ocular surface diseases. *Prog Retin Eye Res.* 2012; 31:527-550.

Figure Legends

FIGURE 1. Flow chart depicting study protocol and final subject numbers in study one.

FIGURE 2. SIgA in tear and saliva in pathogen-verified common cold (URTI; n = 9) and healthy subjects (CON; n = 17). Mean \pm SEM. * $P < 0.05$ tear SIgA concentration significantly lower in subjects experiencing URTI vs. CON.

FIGURE 3. SIgA in tear and saliva in eleven subjects in the week prior to onset of upper respiratory symptoms (PRE URS) and > 3 weeks following URS (RECOVERY). Mean \pm SEM. * $P < 0.05$ tear SIgA lower PRE URS vs. RECOVERY.

FIGURE 4. Tear and saliva flow rate, SIgA concentration and secretion rate response to 2 h treadmill running at 65% $\dot{V}O_{2\text{peak}}$ (closed circles) vs. seated rest (open circles). Mean \pm SEM. * $P < 0.05$ and ** $P < 0.01$ vs. pre EX; † $P < 0.05$ and †† $P < 0.01$ vs. REST.

Figure 1

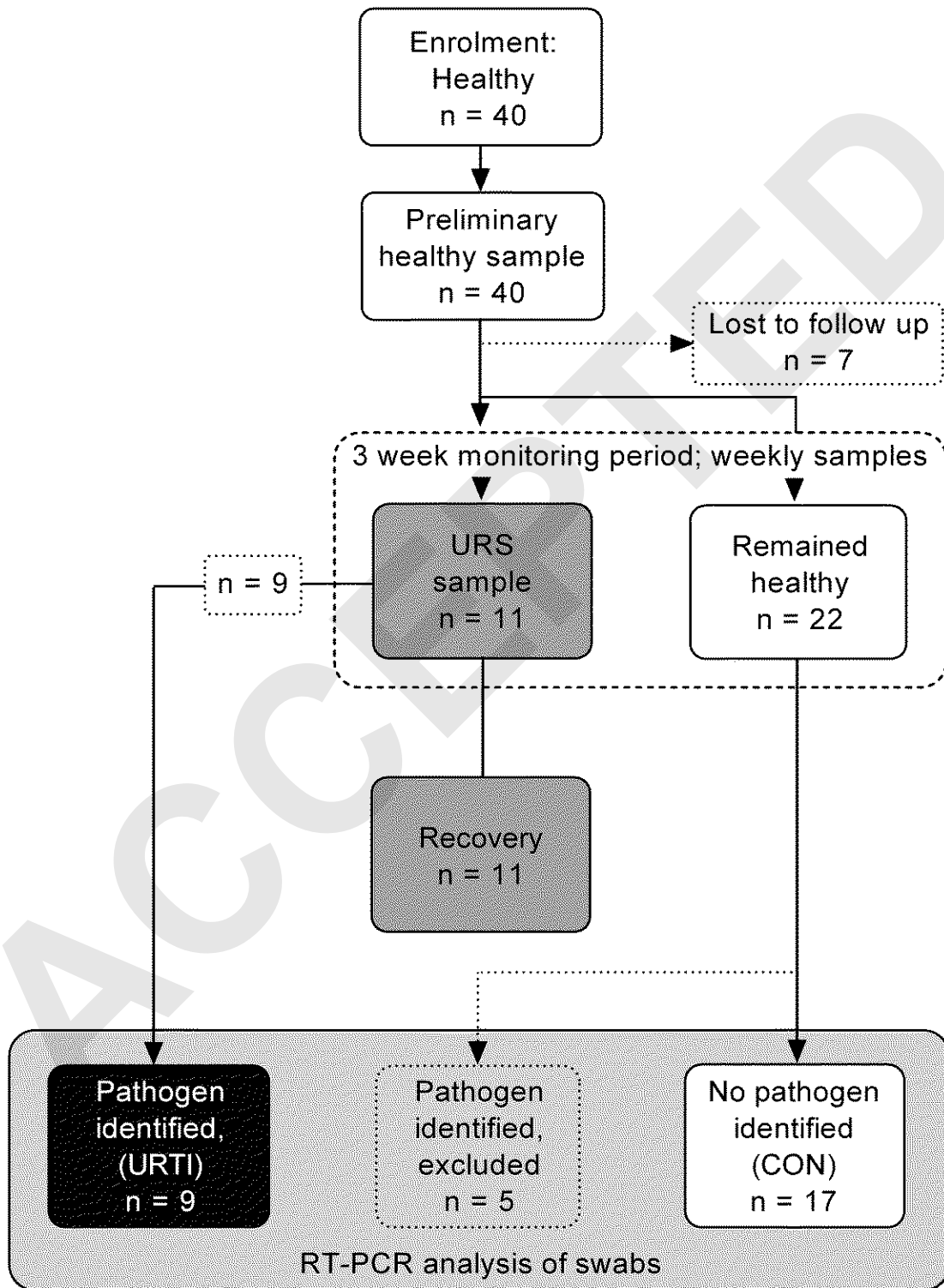


Figure 2

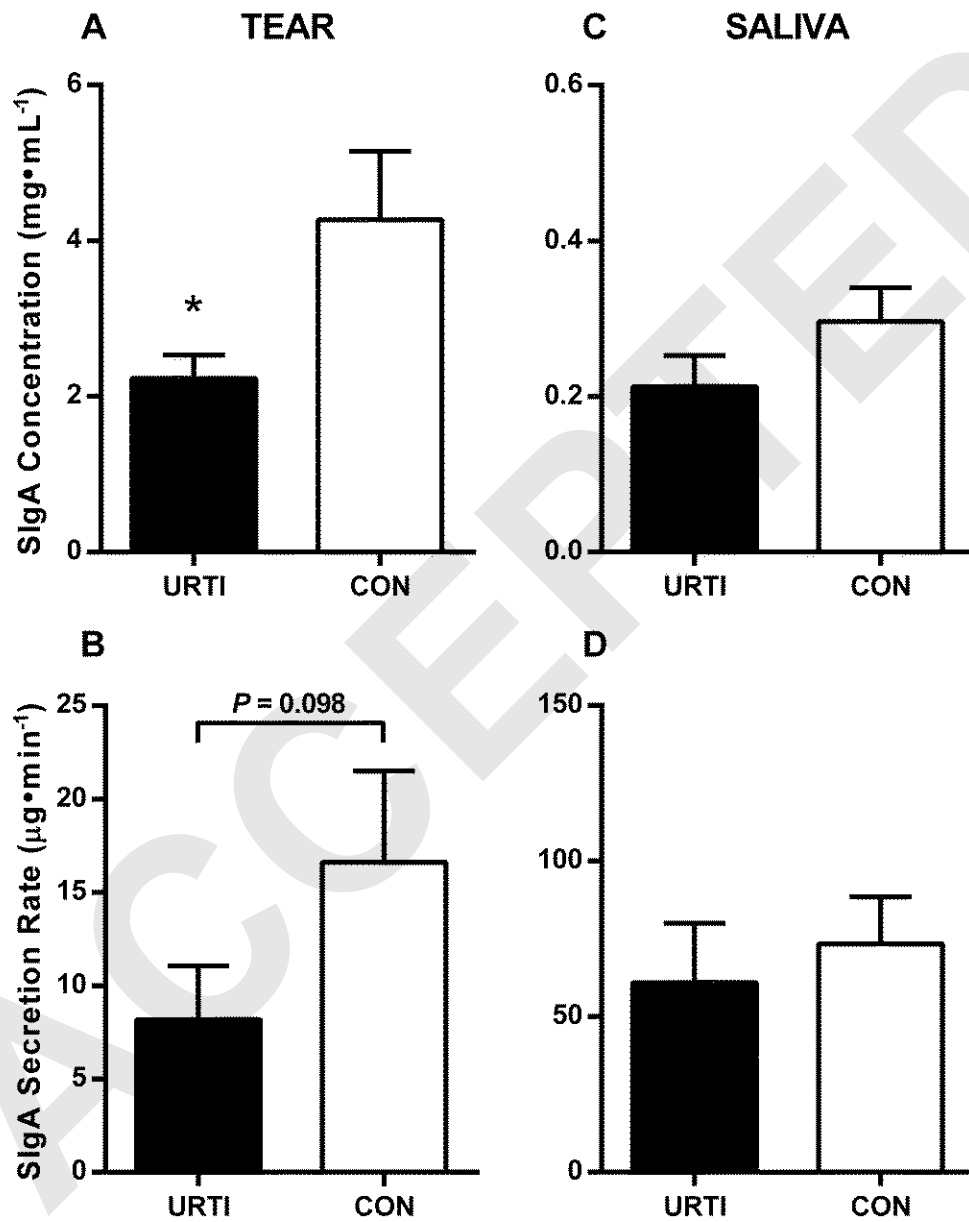


Figure 3

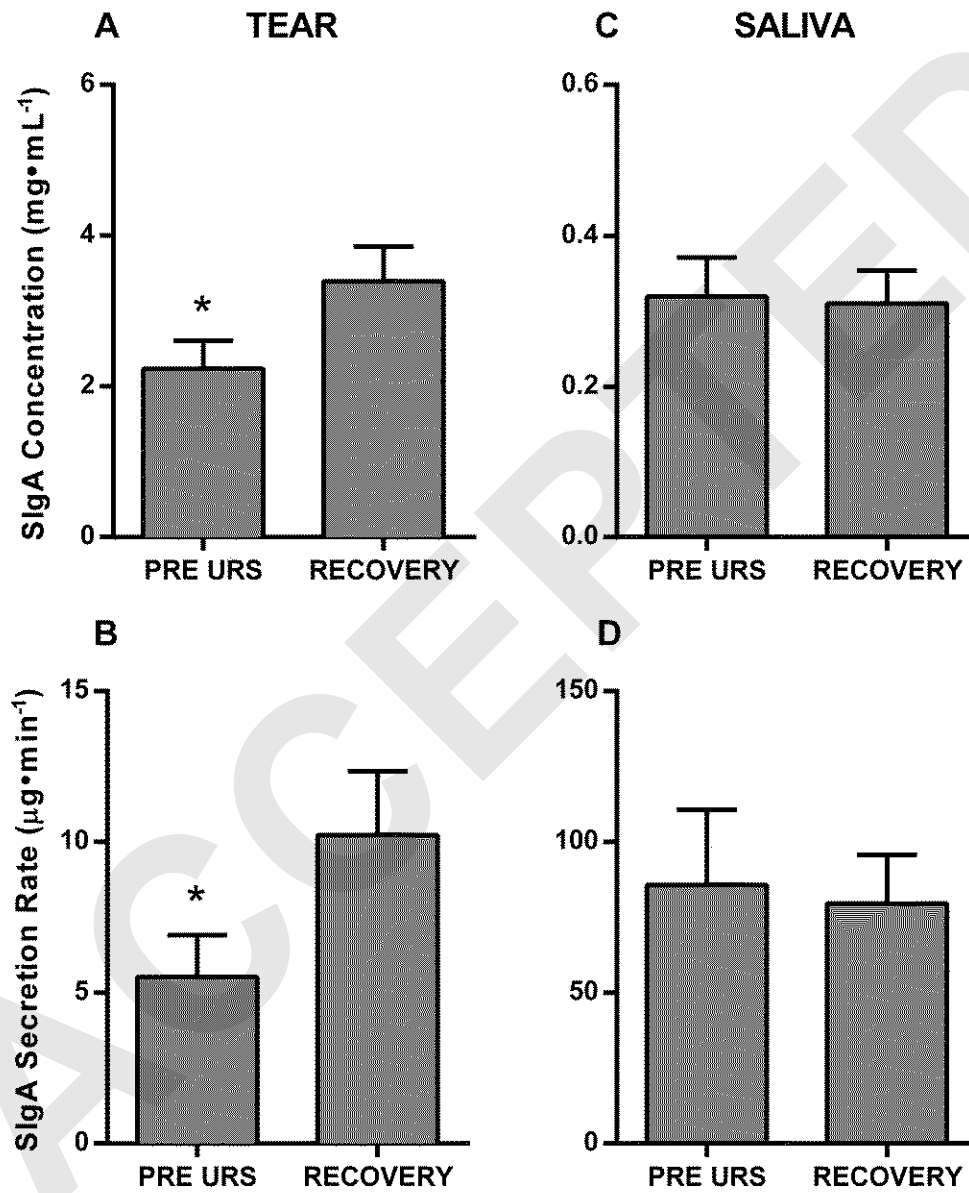


Figure 4

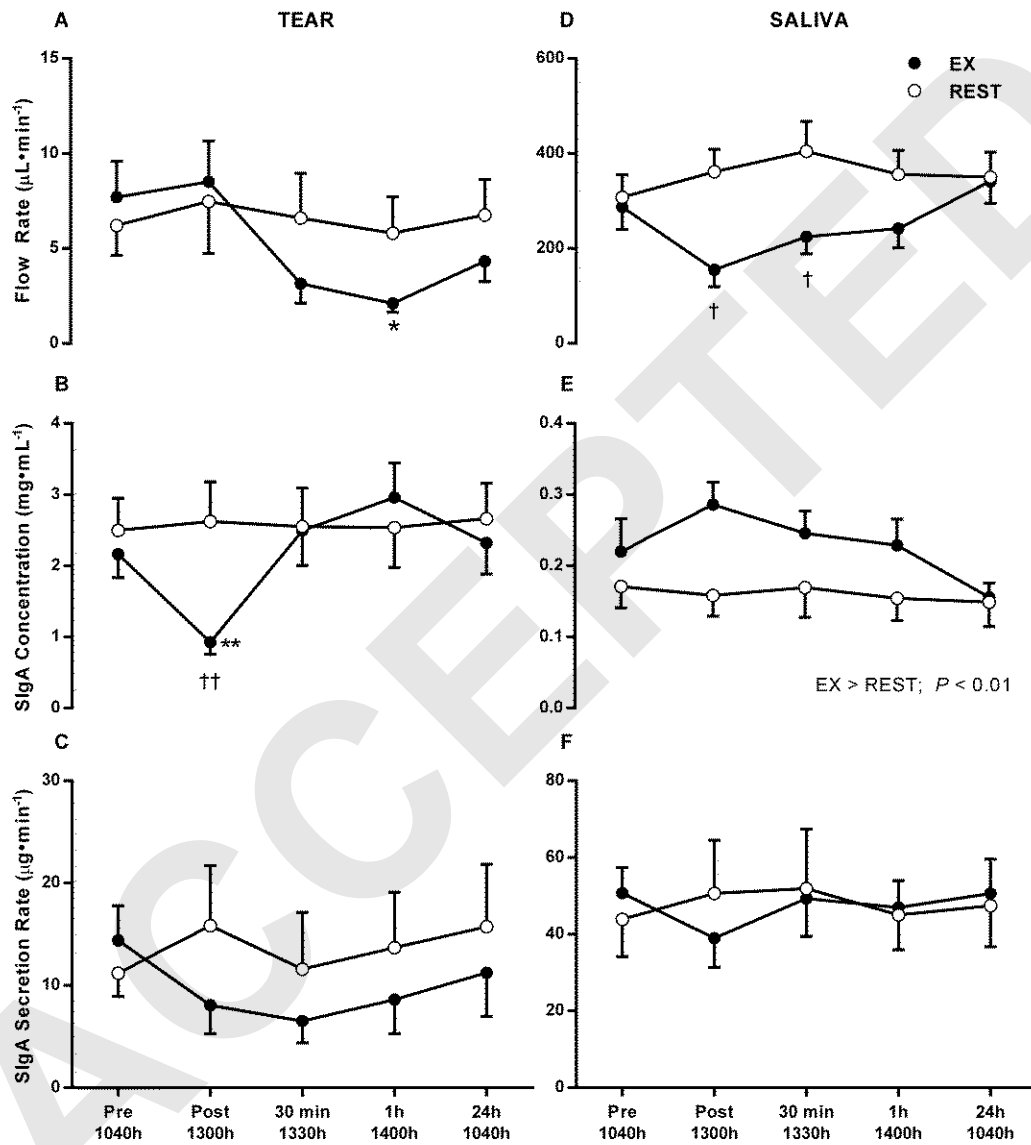


Table 1. Effect of progressive dehydration (DEH) and subsequent rehydration on plasma osmolality (Posm) and SIgA concentration (conc.) and secretion rate (sec.) in tears and saliva.

		Day 1		Day 2	
		1400 h	1630 h	0800 h	1100 h
		0 % BML	2 % BML	3 % BML ¹	Rehydration
Posm (mOsm · kg ⁻¹)	DEH	289 ± 5	294 ± 4 ** ††	298 ± 4 ** ††	288 ± 5
	EUH	289 ± 4	284 ± 4 **	289 ± 4	288 ± 4
Tear Flow Rate (µL · min ⁻¹)	DEH	2.9 ± 3.6	1.7 ± 1.8 §	4.7 ± 6.8	3.4 ± 4.8
	EUH	3.0 ± 2.8	3.0 ± 3.7	4.9 ± 6.0	4.6 ± 5.0
Tear SIgA Conc. (mg · mL ⁻¹)	DEH	6.1 ± 4.5	7.7 ± 4.3 §	4.7 ± 3.5	5.1 ± 1.9
	EUH	6.1 ± 4.2	7.5 ± 3.9	3.6 ± 1.5	5.0 ± 2.5
Tear SIgA Sec. (µg · min ⁻¹)	DEH	14 ± 17	13 ± 15	13 ± 12	18 ± 25
	EUH	16 ± 22	20 ± 21	15 ± 19	22 ± 28
Saliva Flow Rate (µL · min ⁻¹)	DEH	372 ± 226	121 ± 76 ** ††	144 ± 150 ** ††	417 ± 171
	EUH	349 ± 215	330 ± 219	291 ± 251	321 ± 135
Saliva SIgA Conc. (mg · mL ⁻¹)	DEH	0.15 ± 0.10	0.34 ± 0.22 ** ††	0.39 ± 0.30 ** ††	0.13 ± 0.04
	EUH	0.14 ± 0.04	0.18 ± 0.08	0.24 ± 0.20 *	0.17 ± 0.06
Saliva SIgA Sec. (µg · min ⁻¹)	DEH	45 ± 31	32 ± 17	41 ± 27	54 ± 30
	EUH	46 ± 29	50 ± 25	47 ± 21	51 ± 24

Data are mean ± SD.

Significant difference vs. 1400 h: *, $P < 0.05$; **, $P < 0.01$.

Significant difference vs. EUH: ††, $P < 0.01$.

Significant main effect of time vs. 0800 h: §, $P < 0.05$.

¹BML at 0800 h on day 2 in DEH was 3.0 ± 0.5 %.