EXERCISE TRAINING, SALIVARY EPSTEIN-BARR VIRUS DNA AND UPPER RESPIRATORY ILLNESS IN ATHLETES

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Declaration and statements

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other university or institute of learning.

All research within this thesis was conducted according to the guidelines laid down by the Declaration of Helsinki (2008, including 2013 amendments), and all procedures were approved, in advance, by the University of Kent's ethics committee.

General Abstract

A single bout of prolonged and/or strenuous exercise can cause a transient change in the number and function of circulating cells of the innate and acquired immune system, with recovery to pre-exercise levels occurring within 24 hours post-exercise. The clinical relevance and utility of *in vitro* methods has been questioned, and the use of *in vivo* measures has been highlighted as the *in vivo* response to an antigenic challenge involves a multi-cellular response that is believed to be more clinically relevant than findings from *in vitro* work.

Epstein-Barr Virus (EBV) is a human herpes virus that establishes latent infection after primary infection (approximately 90% of the adult population are seropositive for latent EBV infection) via colonisation of the lymphoid system and subsequent expansion of virally infected B-cells in peripheral blood. The virus can exist in a latent state and avoid detection from T-cells, or it can reactivate into the lytic viral lifecycle resulting in infection of epithelial cells of the oropharynx and shedding of viral DNA into saliva (Knipe & Howley, 2013). Lytic reactivation of EBV is controlled by a subset of viral specific cytotoxic T-cells, which may become depressed during periods of intensified training or after an acute bout of prolonged strenuous exercise. Monitoring the viral status of EBV via measurement of viral DNA in saliva may be a useful tool for assessing *in vivo* immune status in athletes throughout a training cycle and also after acute exercise.

The aim of this thesis was to investigate the timing and magnitude of EBV reactivation alongside occurrence of upper respiratory symptoms (URS) and changes in mucosal immune markers (secretory immunoglobulin A, s-IgA) over a period of exercise training, and also after an acute bout of prolonged exercise.

Study 1 (Chapter 3) monitored salivary EBV, incidence of URS, and s-IgA in a group of male professional football players (n=15) over the first four months of an English competitive season. s-IgA was found to be sensitive to changes in physical load (weekly competitive match play), however, the occurrence of just two URS episodes over the 4-month monitoring period prevented full statistical analysis of any relationship between URS and EBV reactivation and/or s-IgA levels. The presence of EBV latent genes in 100% of saliva samples suggests that EBV serostatus can be identified from a saliva sample, which may be useful in an applied sport science setting when venous blood sampling is not possible.

Study 2 (Chapter 4) also monitored changes in salivary EBV, s-IgA, and incidence of URI, but in a group of male and female non-elite endurance athletes (n=30). There was no clear evidence of a relationship between EBV reactivation and URS incidence/risk. Baseline s-IgA levels were significantly lower for individuals who experienced at least one URS episode, however there was no evidence of a significant decline in resting s-IgA in the weeks before or during URS. There was also no relationship between training load and EBV reactivation or s-IgA. Study 2 also suggests that there is a high variability in individual EBV shedding frequency that may limit the ability to use EBV reactivation as a reliable marker of *in vivo* immune monitoring for groups of non-elite endurance athletes.

Study 3 (Chapter 6) investigated the change in salivary EBV load after an acute bout of prolonged cycling (2.5 h) in a group of well-trained male cyclists (n=10). There was no clear evidence of an increase in salivary EBV DNA levels from pre to post exercise (measured at 1, 24 and 48 h post exercise), which could suggest that this

specific exercise bout did not result in significant loss of function in the cytotoxic T-cells that control actively replicating viral cells in the oropharynx.

Keywords: Epstein-Barr virus, secretory immunoglobulin A, URTI, endurance exercise, football.

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Publications

Conference contributions:

Study 1 (Chapter 3)

Hynes, E. & Davison, G. (2017). Mucosal immune markers in professional English football players. British Association of Sport and Exercise Science Student Conference, Plymouth, UK, April 2016.

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Study 3 (Chapter 5)

Hynes, E. & Davison, G. (2017). Reactivation of Epstein-Barr virus following prolonged cycling. International Society of Exercise Immunology Symposium, Coimbra, Portugal, July 2017.

Hynes, E. & Davison, G. (2017). Reactivation of Epstein-Barr virus following prolonged cycling. UK Society of Exercise Immunology Symposium, Loughborough, UK, April 2018.

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List of abbreviations

ANOVA Analysis of variance

CD cluster of differentiation

CD4+ T helper cell

CD8+ T cytotoxic cell

CHO carbohydrate

CMIS common mucosal immune system

CV coefficient of variation

DNA deoxyribonucleic acid

DPCP diphenolcyclopropenone

DTH delayed type sensitivity

EBV Epstein-Barr virus

h hour(s)

Human-rhinovirus (HRV)

HR heart rate

IFN interferon

Ig immunoglobulin

IL interleukin

IQR Interquartile range

K3EDTA tripotassium ethylene-tetraacetic acid

kg kilogramme

L litre

m meter

min minutes

mL millilitre

mg milligram

MHC major histocompatibility complex

NK natural killer

PCR polymerase chain reaction

qPCR quantitative polymerase chain reaction

RPE rating of perceived exertion

s-IgA secretory immunoglobulin A

SD standard deviation

SEM standard error of the mean

TLR toll-like receptor

URI upper respiratory illness

URS upper respiratory symptoms

URTI upper respiratory tract infection

$\dot{V}O_2$ oxygen uptake

$\dot{V}O_{2max}$ maximal oxygen uptake

β beta

Δ delta

γ gamma

μg microgram

μl microlitre

Chapter 1. Literature Review

1.1 Exercise and Risk of Upper Respiratory Illness

Upper respiratory tract infections (URTIs) can occur through bacterial or viral infection, however bacterial infection is rare, with viral infection most commonly caused by human-rhinovirus (HRV) (Turner, 2007). Viral infection at the mucosa of the structures of the upper respiratory tract can include the middle ear, nose, paranasal sinuses, pharynx, larynx, and the trachea. Viral infection can spread from person-to-person through inhalation of respiratory droplets or by direct contact with infected secretions (Makela et al., 1998). The majority of visits to general practitioners worldwide are made by patients presenting with symptoms of URTI (sore throat, blocked nose, runny nose, headache, joint aches and pains) (Eccles, 2005). While the number of reported URTI episodes per year is similar in athletes and the general population (Fricker et al., 2000), there is evidence of a difference in the seasonal occurrence of URTI. For example, athletes can experience a greater rate of URTI during periods of heavy training or competition (Cunniffe et al., 2011; Pyne et al., 2001; Svendsen et al., 2015) or during the post-competition period (Walsh et al., 2011).

Upper respiratory illness (URI) can also be the result of non-infectious causes, with individuals experiencing similar symptoms to an episode of URTI. For example, allergies can result in similar symptoms to those of infectious URI (Robson-Ansley et al., 2012; Schwellnus et al., 2010). Airway irritation can damage the epithelial cells of the upper respiratory tract and also result in non-infectious URI symptoms. For elite swimmers training predominantly in indoor swimming pools, inhaling large volumes of air above the water surface that have

become polluted with chlorine is a common cause of airway irritation (Bougault et al., 2009; Piacentini et al., 2007). Similarly, endurance runners/cyclists/triathletes frequently experience prolonged periods of time with high ventilation rates that can also lead to non-infectious URI symptoms, with airway irritation further exacerbated when exercising in a cold/dry environment (Bermon, 2007; Cox et al., 2008). In the illness monitoring study from Spence et al. (2007) 70% of reported illness episodes were not the result of infectious origin. This study tested throat and nasopharyngeal swabs against a limited panel of known pathogens so it is possible that some of those 70% of negative infectious illness episodes were in fact caused by an infectious agent. Nevertheless, this highlights the fact that not all URI episodes are the result of infection, and therefore the term URTI should only be used when infection has indeed been confirmed by laboratory analysis. The use of self-report illness questionnaires without clinical diagnosis of URI from a medical professional limits researchers to the use of the term upper respiratory symptoms (URS) as the cause of symptoms cannot be confirmed as infection or illness (i.e. may be the result of airway inflammation or allergy).

The proposed J-shaped model for risk of upper respiratory tract infection (URTI) and level of exercise training (Nieman, 1994) suggests that recreationally active individuals (engaging in moderate intensity exercise) are at a lower risk of experiencing URTIs in comparison to sedentary individuals. As the level of exercise training increases from recreationally active towards that of elite athletes engaging in strenuous exercise training and/or prolonged-high intensity exercise, so too does the risk of experiencing URTI. This model is based on evidence from studies reporting the incidence of URTI in the weeks before and after endurance running events, and has been supported more recently by illness surveillance studies encompassing varying levels of physical activity (Gleeson et al., 2011;

Spence et al., 2007). One of the earliest of these studies, from Peters and Bateman (1983), reported that 33% of participants completing the Two Oceans 56 km race in South Africa experienced URTI symptoms during the 14-day post-race period. Whereas the control group (aged matched and shared a home with the race competitors) reported half the number of URTIs than the ultramarathon runners over the same time period. Similarly, in a seven-day post-race period, symptoms of URTI were reported by 12.9% of runners completing the 1987 Los Angeles Marathon in comparison to just 2.2% of runners who withdrew from the race and did not run for reasons not related to illness (Nieman et al., 1990). After analysing the training programmes of the runners who completed the race, Nieman et al. (1990) identified training volume (specifically, running less than 32 km in the week before the race in comparison to running more than 96 km) as a stronger risk factor for URTI than other factors that included: illness at home; stress levels; and age.

Adding support to the J-shaped model was the study from Spence et al. (2007) that reported a higher incidence of URTI in elite athletes (30%) compared to recreational athletes and sedentary controls (10%) over a 5-month period. Unlike earlier work carried out with participants at mass participation endurance events, Spence et al. (2007) were able to confirm the presence of URTI through laboratory analysis of throat and nasopharyngeal swabs collected from participants within 24 hours of reporting symptoms of URTI. A total of 37 URTI episodes were reported across all participants during the study period, with just 30% confirmed as being viral, bacterial, chlamydial, or mycoplasmal in nature. It is, however, possible that participants had been infected by a pathogen that had not been included in the screening panel used by Spence et al. (2007) so there is no guarantee that all of the negative samples were indeed negative. By comparison, a smaller scale study

from Hanstock et al. (2016) found a relatively higher rate of positive laboratory confirmation of respiratory pathogens with HRV being identified in 9/11 (82%) episodes of URS. A lack of laboratory diagnosis and subsequent confirmation of URTI alongside the self-report and/or physician diagnosis of upper respiratory illness (URI) symptoms is a commonly recurring limitation in exercise immunology literature.

In more recent years the publication of research involving elite international athletes has provided evidence to suggest that the traditional J-shaped model should be extended in to an S-shaped model (Malm, 2006). The modified S-shaped curve reflects the fewer number of URTIs that are reported by the very top-level elite athletes. For example, in a retrospective study of 39 Norwegian elite cross-country skiers covering a period of eight years, Svendsen et al. (2016) found that skiers who had won an Olympic and/or World Championship medal reported significantly fewer URI symptom days per year than the national level skiers (mean (range) 14 (6-29) vs 22 (8-43)). Similarly, Hellard et al. (2015) monitored 28 professional French swimmers over a four year period and found that international level swimmers experienced less URI episodes than the national level swimmers (odds ratio 1.40) in the same training group. Finally, in a study of a smaller group of 11 endurance athletes (cross-country skiing, biathlon, and long-distance running) Martensson et al. (2014) reported that the number of training hours per year was significantly and negatively correlated to the number of training days lost due to presence of URI symptoms. Based on the J-shaped model of training load and infection risk, the high volume of training required to compete and be successful in endurance sports at an international level should result in top level elite athletes experiencing more illness episodes per year than sedentary, recreationally active, and national level athletes, which does not appear to be the

case. As such, there is some suggestion in the literature that top level elite athletes are in some way better adapted to coping with the demands of a high training load throughout the year either. This may be due having a more robust immune system (Malm, 2006) or, perhaps more likely, due to having a better understanding of how to reduce infection risk through behaviour and lifestyle (Walsh, 2018).

At times of the year when elite athletes are competing at major championships, there does appear to be an increased risk of URS (Svendsen et al., 2015) with 30-50% of all illnesses reported to medical staff at both winter and summer international events being related to symptoms of URTI (Alonso et al., 2012; Engebretsen et al., 2013; Mountjoy et al., 2010; Soligard et al., 2014). However, given the increased risk of infection with long-haul travel, disruption to sleep routines, and increased psychological stress of competing at a major championship (Svendsen et al., 2016; Walsh, 2018) it is possible that the apparent increased risk of experiencing URS during a major international championships is due in some part to factors other than exercise training and competition (Campbell & Turner, 2017).

1.2 Exercise and The Cellular Immune System

The immune system functions to protect the body from pathogens (viruses, bacteria and parasites) and maintain body homeostasis. The integrated systems of non-specific innate and specific acquired immunity work to recognise, attack, destroy and ultimately protect the body against infection. The innate immune system is the first line of defence against invading pathogens and does not form pathogen-specific actions. By comparison, the acquired immune system is capable of targeting specific pathogens and will form immune memory to strengthen for future attack against previously encountered pathogens (Walsh et al., 2011). Leukocytes of the innate immune system found in circulation include granulocytes (neutrophils, basophils and eosinophils), monocytes and dendritic cells. Lymphocytes include natural killer cells (NK) (innate), and T-cells and B-cells (acquired). T-cells can be further identified as T helper, T cytotoxic, and T memory cells. At rest, the number of total leukocytes in circulation is similar between athletes and healthy controls. Some endurance athletes can experience lower leukocyte counts, but only very few athletes fall below the threshold for clinically low cell counts (Horn et al., 2010). Similarly, when in a true rested state (i.e. after at least 24 h recovery from an acute bout of exercise) immune cell function in athletes is believed to be similar to sedentary individuals (Gleeson, 2007). A single bout of prolonged and intense exercise can disturb leukocyte cell number and function resulting in a period of immunodepression that is thought to increase the risk of infection for several hours post exercise (Pedersen, Rohde & Ostrowski, 1998).

1.2.1 Acute Exercise and Leukocyte Counts

A single bout of exercise causes a profound increase in circulating leukocyte number (leucocytosis), which is mostly due to an increase in the number of neutrophils and lymphocytes (Walsh et al., 2011). During exercise, a combination of haemodynamic factors (increased cardiac output and blood pressure) and release of catecholamines (that bind to β_2-adrenergic receptors expressed on the surface of leukocytes) lead to the mobilisation of leukocytes from the vascular wall to the blood (demargination).

Neutrophils make up the greatest proportion of total leukocytes in the circulation (50-70%) and are highly responsive to acute exercise. During prolonged exercise the initial neutrophilia is primarily the result of immediate demargination, with elevated cortisol levels also contributing to a later rise in circulating neutrophil number via stimulated release of immature neutrophils form bone marrow (Allsop et al., 1992). Exercise induced neutrophilia can continue for several hours post-exercise and is highly dependent upon exercise intensity and duration (Gleeson, 2007), with neutrophil number returning to pre-exercise levels within 24 hours of exercise cessation. In comparison to short intensive exercise (~ 30 minutes), prolonged moderate intensity exercise (> 2 hours) causes a greater peak in neutrophil number that occurs almost instantly after the cessation of exercise. Whereas, short high intensity exercise is followed by a neutrophilia that can take up to three hours to reach its peak due to the delayed effects of cortisol (Robson et al., 1999).

The lymphocyte response to acute exercise is characterised by a well-established transient biphasic response (Gleeson, Bishop & Walsh, 2013). During and immediately after exercise there is an increase in lymphocyte number in blood

(lymphocytosis) that is followed by a decrease to below pre-exercise levels (lymphocytopenia) as lymphocytes move from the blood to surrounding tissues in the early hours of exercise recovery (Simpson et al., 2006), with full recovery to baseline levels occurring within 24 hours of exercise cessation. There appears to be preferential mobilisation within the lymphocyte pool; NK cells exhibit the greatest biphasic response, followed by cytotoxic T-cells (CD8+), B-cells, and finally helper T-cells (CD4+) (Shek et al., 1995). Mature CD4+ and CD8+ T-cells with history of antigen exposure are also more responsive to exercise induced mobilisation (Gleeson, Bishop & Walsh, 2013).

Monocytes account for a relatively small proportion of total leukocyte number at 5-15%. The pro-inflammatory subset of monocytes expressing the CD16 cell marker will preferentially increase immediately after exercise, and typically return to baseline within 1-2 hours (Simpson et al., 2009).

1.2.2 Acute Exercise and Leukocyte Function

The most abundant leukocyte, neutrophils, perform several functions that are affected by exercise. A single bout of acute exercise can stimulate spontaneous neutrophil degranulation (measured as the release of elastase per cell) causing neutrophils to enter into a refractory period and reducing their ability to respond to bacterial stimulation (Bishop et al., 2002). Neutrophil phagocytosis (ingestion of microbes) is also negatively affected by acute exercise. The number of neutrophils engaging in phagocytosis increases in line with leukocytosis, but the overall phagocytic capacity of all neutrophils in circulation is reduced (Chinda et al., 2003). Finally, the ability of neutrophils to release reactive oxygen species

(oxidative/respiratory burst) is affected differently according to exercise intensity and duration. Short duration/high intensity exercise has a suppressive effect, while longer duration/moderate intensity exercise can in fact enhance oxidative burst capacity (Pyne et al., 1994).

The phagocytic activity of monocytes in circulation has been shown to increase following acute prolonged exercise (Hong & Mills, 2008). However, the expression of toll like receptors (TLRs) on the surface of monocytes are reduced immediately after and for up to 2 hours post-exercise (Lancaster et al., 2005a; Oliveira & Gleeson, 2010). TLRs allow monocytes to function as antigen presentation cells via recognition of pathogens and subsequent presentation to T lymphocytes, and therefore play an important role in the activation of acquired immunity.

NK cells destroy microbes through exocytosis of perforin and granzymes that induce apoptosis (cell death) in the target microbe (Smyth et al., 2005). NK cell cytotoxicity exhibits a biphasic response to acute endurance exercise that is a reflection of the changes in number of circulating NK cells (Gannon et al., 1995). Total NK cell cytotoxicity is initially increased immediately post-exercise, but decreases to below baseline levels in the hours during recovery. This should however be interpreted with caution as the level of cytotoxicity per cell is relatively unchanged from pre to post-exercise, and up to 3.5 hours post and therefore changes in total NK cytotoxicity are due to a redistribution of lymphocytes (Nieman et al., 1993).

The acquired immune system comprises of T-cells (T helper, T cytotoxic, T memory and T regulatory cells) and B-cells that perform several functions in order to destroy invading micro-organisms and prevent colonisation of pathogens. Activation of T-cells by antigen presentation cells (dendritic cells, monocytes and

macrophages) stimulates the cell-mediated immune response to invading pathogens. This process can also be referred to as delayed type hypersensitivity (DTH). A primary immune response is initiated when dendritic cells present antigens to the cell surface receptors of T-cells by (Mellman & Steinman, 2001). A secondary immune response is activated when previously encountered antigens are presented by monocytes and macrophages to T memory cells (Gallucci & Matzinger, 2001). Toll like receptors (TLRs) on the surface of these antigen presentation cells will bind to antigens, which initiates expression of Major Histocompatibility Complex (MHC) class I and II molecules which in turn allows the dendritic cells, monocytes, and macrophages to function as antigen presentation cells to T-cells (Banchereau and Steinman, 1998). All T helper cells can be identified by the glycoprotein CD4 (often referred to as CD4+ cells) are can be divided into sub-classes of type 1 and type 2 helper cells (Th1 and Th2). Th1 cells produce cytokines IL-2 and IFNγ, which have specific anti-viral actions and can stimulate production of more helper and cytotoxic T-cells. Th2 cells are responsible for stimulating B-cell antibody production via release of IL-4.

Cytotoxic T-cells, which express the glycoprotein CD8 and are therefore often referred to as CD8+ cells, will release perforin, granzymes and granulysin upon recognition of the MHC surface proteins and antigen. Perforin initiates cell lysis of the target viral cell, which allows the granzymes entry into the infected cell to begin the process of apoptosis (Bennett et al., 1998). This response to antigen presentation cells is a key antiviral action of the CD8+ cells as a viral survival in a new host relies on the ability to infect host cells. Interaction of the CD4+ and CD8+ cells with antigen presentation cells begins the process of CD8+ cell maturation and formation of memory T cells (Hicroz et al., 2012). The signalling protein CD40, which is released from the infected cell whilst bound to the CD8+ cell, mediates

this process to initiate CD8+ T-cell differentiation from a naïve to a mature CD8+ T-cell (Bennett et al., 1998). Cytotoxic T-cells will experience clonal expansion once the cell becomes fully activated following antigen presentation. IL-2 production by Th1 cells mediates this process and acts as a growth differentiation factor to increase the total number of cytotoxic T-cells for destruction of the antigen (Milstein et al., 2011).

Prolonged and strenuous exercise appears to have no effect on the proportion of Th2 cells in circulation but does significantly reduce the proportion of Th1 cells for up to two hours post-exercise (Steensberg et al., 2001) and also significantly reduces the production of IL-2 (Tvede et al., 1993). This negative effect of acute exercise could indicate a depression of cell-mediated immunity in the hours after prolonged strenuous exercise which may increase susceptibility to viral infection. T-cell proliferation (cell division) has been assessed *in vitro* using mitogen and antigen stimulation. When the redistribution of T-cells post-exercise (initial lymphocytosis) has been accounted for (i.e. mathematical adjustments, or use of a fixed number of T-cells in culture) there is a significant reduction in proliferation that reflects a genuine decrease in T-cell function (Bishop et al., 2005). Furthermore, the observed reduction in proliferation per cell is sensitive to increases in exercise intensity as highlighted by Niemen et al. (1994) as 45 minutes of treadmill running at 80% $\dot{V}O_{2max}$ resulted in a significant decrease in mitogen stimulated T-cell proliferation whereas running at 50% $\dot{V}O_{2max}$ resulted in no change from baseline. T-cells migrate towards areas of infection when stimulated by the proinflammatory cytokines and chemokines that are produced by virally infected cells. Bishop et al. (2009) reported a significant reduction in the ability of T helper cells to migrate towards human rhinovirus-infected epithelial

cells after two hours of treadmill running. At 1-hour post-exercise, T-cell migration remained lowered to roughly 40% of pre-exercise levels.

B-cells constitute the smallest proportion of all circulating lymphocytes (5-15%). A surface immunoglobulin (Ig) on B-cells acts as the receptor for activation by T helper cells or direct stimulation from microbes (Janeway et al., 1999). Activated B-cells will proliferate and differentiate into memory cells and plasma cells, with the differentiated plasma cells present capable of secreting Igs into circulation (LeBien & Tedder, 2008). The three most abundant serum Igs secreted by plasma cells include IgG, IgA and IgM (Gleeson, Bishop & Walsh, 2013). The effect of acute exercise on serum Igs has received relatively little attention in comparison to all other aspects of innate and acquired immunity, and has produced some conflicting results. Serum IgG, IgM and IgA concentrations have been found to increase after 45 minutes of walking in comparison to a rest condition (Nehlsen-Cannarella et al., 1991), which conflicted with a previous suggestion that a diurnal rhythm for Ig secretion caused the small increase in Ig concentration that was observed after an incremental exercise test (Nieman et al., 1989). *In vitro* IgM mitogen-stimulated secretion has been found to decrease after prolonged moderate intensity exercise, whereas IgA and IgG remain unchanged (Shek et al., 1995).

1.2.3 Exercise Training and Cellular Immunity

An increase in exercise training load can result in depression of both innate and acquired immune cell functions. In a study of elite swimmers, neutrophil oxidative burst activity (but not resting neutrophil cell count) significantly declined during a

12-week period of intensified training, with neutrophil function being at its lowest during a peak endurance training phase (Pyne et al., 1995). Studies of elite swimmers have also shown a significant decline in resting NK cell number over the course of a training season (Gleeson et al., 2005), with NK cell cytotoxicity found to significantly decrease after one month of intensified volleyball training (Suzui et al., 2004). In the true resting state (i.e. after at least 24-hours of rest) blood lymphocyte counts appear to be similar between athletes and non-athletes (Nieman, 2000). However, stimulated T-cell proliferation and B-Cell Ig production are sensitive to an increase in cycling training load over a period of three weeks (Verde et al., 1992). The clinical relevance of this observed decline in cellular immune function with intensified training is however unclear, as URTI incidence does not necessarily increase around times of lowered cell functions (Gleeson et al., 2005; Pyne et al., 1995).

1.3 Exercise and The Mucosal Immune System

The mucosal immune system provides a first line of defence against external pathogens (Corthesy & Kraehenbuhl, 1999). The Common Mucosal Immune System (CMIS) is an extensive network of structures covering 400 m^2 surface area that protect the mucosal surfaces in the body (Brandtzaeg et al., 1999). This includes the respiratory tracts (including the bronchus associated lymphoid tissue, salivary glands and nasal-associated lymphoid tissue), gut-associated lymphoid tissue, urogenital tracts, lacrimal glands, and lactating mammary glands (Gleeson & Pyne, 2000; Brandtzaeg et al., 1999). The production of Ig, and specifically immunoglobulin A (IgA), by mucosal B-cells adjacent to the salivary glands is the main effector function of the CMIS. Other Igs found in mucosal secretions include IgM and IgG, but these are significantly less dominant in the protection of mucosal surfaces (Brandtzaeg et al., 1999).

Secretory IgA (s-IgA) exists as a dimeric molecule that is comprised of two individual monomers of IgA joined together by a small protein structure known as a J-chain and surrounded by a covalently bonded secretory component (Bishop and Gleeson, 2009). The presence of the J-chain is essential for successful binding of IgA to the polymeric Ig receptor (pIgR), which is responsible for endocytosis and transcytosis of IgA across mucosal epithelial cells from the basolateral to the epical cell membrane (Lamm, 1998). Proteolytic separation of the pIgR-IgA complex after transepithelial transport results in the secretory component of pIgR remaining covalently bound to IgA (Johansen, Braathen and Brandtzaeg, 2001). (Figure 1.1).

s-IgA forms the first line of defence against microbial pathogens via three mechanisms. Firstly, during transepithelial transport the pIgR-IgA complex can

prevent replication and assembly of viruses present within epithelial cells (Yan et al., 2002). Secondly, through a process known as immune exclusion, IgA can prevent adherence of pathogens to the mucosal epithelium (Corthesy, 2009), and finally, IgA also binds to antigens that have crossed the mucosal barrier and excretes them at the luminal surface (Lamm, 1998).

Mucosal secretions of the upper respiratory tract also contain small antimicrobial peptides and proteins (AMPs) including lysozyme, lactoferrin, and alpha-amylase (Bishop & Gleeson, 2009; West et al., 2006). Each of these AMPs exhibit antibacterial properties, with the two most abundant being lysozyme and lactoferrin, (Singh et al., 2000). Lysozyme is able to hydrolyse polysaccharides in bacteria call walls and prevent adherence (Bosch et al., 2002), while lactoferrin prevents bacterial cell growth by competing for and binding with free iron in saliva (Legrand et al., 2004). Lactoferrin also exhibits antiviral properties, specifically targeting adenovirus and respiratory syncitial virus. Alpha-amylase also directly inhibits bacterial cell growth and adherence (Humphrey & Williamson, 2001).

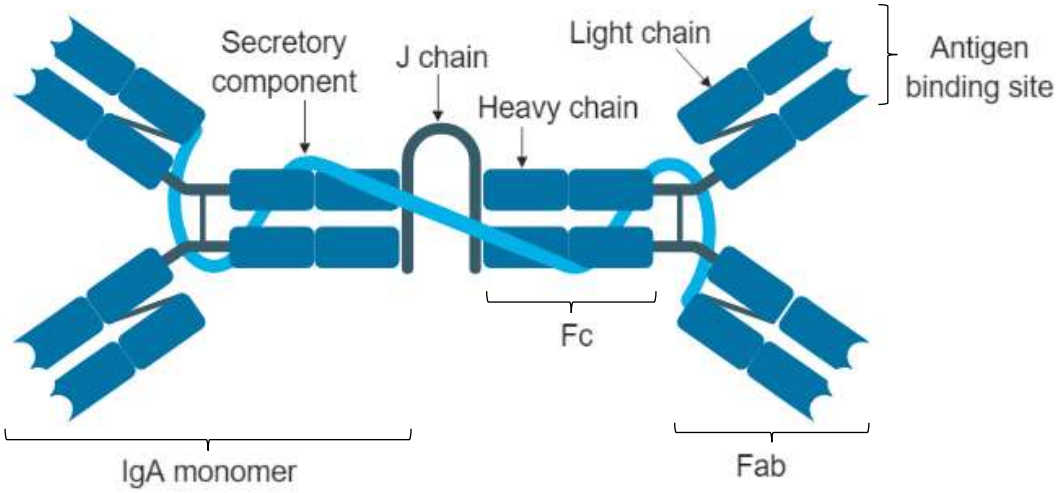

Figure 1.1 Secretory IgA dimer comprised of two individual IgA monomers connected at the J-chain and covalently bound to the secretory component (cleaved from pIgR after transepithelial transport). Fab: fragment antibody binding portion. Fc: fragment crystallisable portion. (Bishop & Gleeson, 2009). Created with BioRender.com.

1.3.1 Acute exercise and s-IgA

Individuals who are deficient in s-IgA are believed to experience URTI episodes more frequently (Gleeson & Pyne, 2000) and as such s-IgA is regularly used as the marker of choice by researchers wanting to examine the effect of acute exercise on mucosal immunity. The intensity and duration of exercise can directly influence s-IgA levels in saliva via changes in sympathetic and parasympathetic nervous stimulation, with other exercise-related factors such as dehydration also capable of influencing total saliva volume and water content of saliva secretions (Bishop & Gleeson, 2009; Walsh et al., 2011). The salivary glands are innervated by both the sympathetic nervous system (SNS) and parasympathetic nervous system (PNS), which can influence the volume and protein content of saliva secretion during exercise. An increase in sympathetic nervous stimulation results in vasoconstriction of salivary glands, which reduces the watery content and volume of saliva, and active transport of proteins into saliva secretions (Proctor & Carpenter, 2007). Whereas, an increase in parasympathetic nervous stimulation results in a greater volume of saliva (due to vasodilation of salivary glands) and a lower protein content (Bishop & Gleeson, 2009). During exercise there is a decrease in saliva flow rate that is the result of a withdrawal of parasympathetic nerve stimulation and not an increase in sympathetic nerve stimulation (Bosch et al., 2002; Bishop and Gleeson, 2009). This response to prolonged exercise may cause an artificial increase in s-IgA concentration (due to total saliva volume decreasing) and as such the secretion rate of s-IgA should also be considered as this measure accounts for changes in saliva flow rate.

The effect of acute exercise on s-IgA has received considerable attention over the last 30 to 40 years. The first research to investigate the changes in s-IgA levels

from pre to post exercise was published by Tomasi et al. (1982). In this study of national level Nordic cross-country skiers, s-IgA concentration decreased by 20% after 2-3 hours of cross-country skiing. Following on from this, several other research groups also observed a decrease in s-IgA concentration and/or secretion rate after a single bout of prolonged exercise (> 2 h). Mackinnon et al. (1989) reported a 63% decrease in s-IgA concentration immediately after two hours of cycling at 70-75% $\dot{V}O_{2max}$. This was found to be a transient response as s-IgA concentration returned to pre-exercise levels by 24 hours post exercise. After completion of a standard Olympic distance triathlon race, s-IgA secretion rate was found to have decreased significantly in a mixed group of both competitive and recreational level triathletes (Steerenberg et al., 1997). Following an ultramarathon race of 160 km, s-IgA secretion rate decreased by 50% in the 31 runners that completed the race (Nieman et al., 2003). This study provided the first indication towards the clinical relevance of s-IgA levels and prolonged exercise as low levels of s-IgA secretion rate at the 90 km checkpoint were found to be the best predictor (from several other markers of immune status and oxidative stress) of URTI occurrence in the two weeks following the race.

Intermittent high intensity exercise, and shorter bouts of continuous exercise, do not seem to have the same negative effect on mucosal immunity as bouts of prolonged exercise. For example, s-IgA levels were unchanged following an acute bout of high intensity interval training (Walsh et al., 1999) and 30 minutes of moderate intensity training (Reid, Drummond & Mackinnon, 2001), and were found to increase following a bout of sprint interval training (Davison, 2011).

The substantial body of work that has investigated the acute effect of exercise on s-IgA levels generally concludes that s-IgA levels decrease following prolonged

exercise (> 2-3 h) and recover to baseline by 24-hours post exercise, or may remain unchanged after shorter bouts of interval training (Walsh et al., 2011). The mechanisms responsible for the changes in s-IgA levels after acute exercise seem to involve mobilisation of the pIgR receptor for transcytosis of IgA into saliva. Animal studies have provided evidence of an adrenaline induced increase to the rate of IgA transcytosis via an increased mobilisation of the pIgR receptor (Carpenter et al., 2004). In humans, ingestion of caffeine pre-exercise elevated plasma adrenaline levels above that of placebo, and resulted in increased levels of s-IgA post-exercise (Bishop et al., 2006). The increased SNS activity associated with caffeine ingestion and prolonged exercise was believed to exceed a first threshold of SNS activation that is required to increase the rate of IgA transport by pIgR. Further SNS stimulation above a second threshold during prolonged exhaustive exercise with rats was believed to be the cause of a decrease in levels of pIgR mRNA that was associated with decreased levels of s-IgA (Kimura et al., 2008). This highlights the role of exercise intensity and duration on the mucosal immune system and may explain why s-IgA levels were unchanged by short duration moderate intensity exercise (Reid, Drummond & Mackinnon, 2001), increased following sprint interval training (Davison, 2011), and decreased following prolonged strenuous endurance exercise bouts (Nieman et al., 2003; Steerenberg et al., 1997).

The use of s-IgA as an isolated marker of immune status in the hours after prolonged exercise has been questioned (Campbell & Turner, 2017). Inconsistency in the method used to report changes in s-IgA (e.g. concentration, secretion rate, ratio with albumin or protein concentration etc) has made it difficult to make direct comparisons between studies of acute exercise (Bishop & Gleeson, 2009) with a view to establishing reference markers for mucosal immune status.

Furthermore, without evidence of the incidence of URS in the 7-14 days after a bout of prolonged exercise alongside acute changes in s-IgA levels (i.e. decrease in s-IgA) in the hours after exercise, it is very difficult to determine the clinical relevance of a decrease in s-IgA levels with prolonged exercise. Novel research in to the levels of IgA present in tear fluid secretions has recently shown a relationship between low levels of tear IgA and increased incidence of URS (Hanstock et al., 2016). However, this area of mucosal immunity has not yet been fully explored.

1.3.2 Exercise Training and s-IgA

The mucosal immune system appears to be susceptible to the physical stress of long-term training in elite athletes. In a group of elite Australian swimmers, s-IgA concentration decreased continuously over a period of 7-months from the pre-season phase through to the taper phase (Gleeson et al., 1999b). Furthermore, in this study low levels of s-IgA were significantly related to the number of illness episodes. In team sport athletes, periods of intense conditioning work (when match time was reduced due to having no competitive fixtures) were found to have a negative effect on s-IgA concentration, with periods of decreased s-IgA concentration followed by an increase in URTI incidence in the following 2-3 weeks (Cunniffe et al., 2011). A significant and negative relationship between training load and s-IgA secretion rate has also been identified in a group of GB wheelchair rugby player (Leicht et al., 2012). However, for this group of para-athletes there was no relationship between s-IgA levels and URS.

Lowered levels of s-IgA have consistently been shown to be related to the number of illness episodes in groups of moderately active adults, recreational runners, and elite athletes (Gleeson et al., 2011; Ihalainen et al., 2016; Neville et al., 2008 Walsh et al., 2011). However, s-IgA is also known to be highly variable within and between individuals (Neville et al., 2008), and as such there are currently no established clinical reference values for absolute s-IgA concentration or secretion rate, and level of risk of imminent URI. There were early suggestions that a s-IgA concentration of less 40 mg/L (Gleeson et al., 1999b) or a secretion rate of less than 40 µg/min (Fahlman & Engels, 2005) may indicate a critical threshold for increased illness risk. A more recent examination of this value as a critical threshold could not confirm an increased risk of URI below 40 mg/L (Gleeson et al., 2017), however this may have been due to low participant numbers.

Despite the high variability of s-IgA, longitudinal research examining changes in resting levels of s-IgA alongside URI has provided evidence of a direct link between URI and mucosal immune depression in athletes. A promising study from Neville et al. (2008) proposed a model for monitoring s-IgA on an individual basis in order to assess the risk of imminent infection. By calculating healthy baseline s-IgA levels for each individual professional yachtsman in the study (mean s-IgA concentration for all saliva samples when no URI symptoms were present), Neville et al. (2008) were able to provide the first indication towards reference values for URI risk. A reduction to less than 40% of individual healthy levels resulted in a 48% increase in risk of experiencing URI within the next three weeks. Other retrospective investigations of s-IgA and URI in athletes have not reported relative changes in s-IgA levels, possibly due to low sampling frequency and/or shorter monitoring periods. For example, Morgans et al. (2014) monitored professional players for just 30-days, and Ihalainen et al. (2016) collected saliva samples at just

two time points pre and post a 12-week training period. In a study of professional rugby players, Cunniffe et al. (2011) did identify a 15% decrease in s-IgA from a URI-free state to when URI was present, alongside the observation of lower resting s-IgA levels in players who experienced URI in comparison to those that remained healthy throughout the 11-month monitoring period. However, neither of these findings reached statistical significance. The collection of saliva samples on a monthly basis could have resulted in a missed opportunity to collect samples during the peaks and troughs of s-IgA around times of URI, and ultimately played a major role in limiting the statistical analysis of s-IgA levels and URI. Nevertheless, this study provides some indication towards the utility of monitoring s-IgA on a relative basis in team sport athletes. More recently, Gleeson et al. (2017) reported a trend towards lower levels of absolute s-IgA concentration of <40 mg/L and higher risk of upper respiratory symptoms (URS) in elite swimmers over a 9-month period. However, these results did not reach significance and as such the authors highlighted the need to monitor changes in s-IgA on an individual level.

The mechanisms responsible for the observed decrease in s-IgA levels in elite athletes could be due to downregulated production of IgA by the plasma B-cells or reduced rate of transcytosis of IgA. The SNS is unlikely to have an influence on resting s-IgA levels during intensified training periods as tetraplegic wheelchair athletes with a spinal cord injury above the level of SNS output are known to experience lowered s-IgA levels (Leicht et al., 2011). The negative effects of cortisol on translocation by pIgR have been suggested to be responsible for the decreased levels of s-IgA with intensified training. The long-term monitoring study from Cunniffe et al. (2011) identified an increase in salivary cortisol levels during a heavy month of training that proceeded the decline in s-IgA levels. Animal studies

have provided evidence of decreased expression of pIgR mRNA that is associated with increased levels of cortisol (Rosato et al., 1995) and as previously discussed lowered s-IgA levels after prolonged exhaustive exercise have also been associated with reduced expression of pIgR mRNA (Kimura et al., 2008). It is therefore possible that repeated bouts of strenuous exercise with insufficient recovery result in repeated exposure to elevated cortisol levels that over time can cause a depletion to the available pIgR receptor for transcytosis of s-IgA.

1.4 *In Vivo* Immunity

The majority of exercise immunology research to date has examined changes in antigen/mitogen stimulated *in vitro* cell function, leucocyte counts, and immunoglobulin concentrations. The clinical relevance of these findings and their utility in determining immune status and imminent URI risk has been questioned (Walsh et al., 2011). The majority of leukocytes within the body are not in the circulatory system and therefore the analysis of leucocyte function in artificial cultures after isolation from peripheral blood does not truly represent the status of leukocytes that remain in the tissue-specific environment (e.g. lymph nodes) of the human body post-exercise (Akbar et al., 2013). The importance of using *in vivo* measures has therefore been highlighted because the *in vivo* response to an antigenic challenge involves a multi-cellular response that is believed to be more clinically relevant than findings from *in vitro* work (Albers et al., 2005). Furthermore, the use of multiple immune markers that provide information on immune function as well as offering clinical relevance has been highlighted (Albers et al., 2013).

1.4.1 *In Vivo* Cutaneous Assessment of Immune Function

In recent years, *in vivo* exercise immunology research has involved the measurement of T-cell-mediated immunity after exercise via the application of antigens to the surface of the skin. This *in vivo* cutaneous method represents a more clinically relevant method for examining T-cell-mediated responses to antigenic challenge following exercise.

Early work from Bruunsgaard et al. (1997) used a method of delayed type hypersensitivity (DTH) to examine the effect of ultra-endurance exercise on *in vivo* cell-mediated immunity. The Merieux CMI Multitest™ used by this research group involves the intra-dermal injection of seven antigens (tetanus, diphtheria, streptococcus, tuberculin, proteus, candida and trichophyton) and a negative control (glycerin/saline diluent) with measurement of the immune response at the skin 48-hours after application. A stronger antigen stimulated immune response is characterised by a greater diameter of induration at the site of the injection on the skin surface. In this study, the exercise group received the antigen challenge 30 min after completing 3 km of swimming, 130 km of cycling, and 21 km of running, which took on average 6.5 hours to complete. Two non-exercising control groups (11 trained triathletes and 22 moderately trained males) also received the antigen challenge. Both of these control groups displayed a greater cumulative response (i.e. greater number of positive test spots at the skin, and greater magnitude of induration) to the antigen at the skin surface than the exercise group. Despite not conducting baseline measurements of the Merieux CMI Multitest™, these results did indicate an exercise induced reduction in *in vivo* cell-mediated immunity after prolonged exercise.

The same test was later used by Gleeson et al. (2004) to investigate the differences in cell-mediated immunity between a group of elite level swimmers and moderately active controls over a period of 5 months. In contrast to the previously discussed findings, there was no difference in the response to the Merieux CMI Multitest™ between the swimmers and the control group at any timepoint. Furthermore, the swimmers showed no evidence of a reduced immune response to the test at the peak of a high intensity training block in comparison to the end of a 5-6-week rest period. Therefore, suggesting that despite evidence of an acute

reduction in *in vivo* cell-mediated immunity immediately after prolonged exercise (Bruunsgaard et al., 1997), long periods of heavy training do not negatively affect *in vivo* immunity at rest.

The Merieux CMI Multitest™ test, which is no longer commercially available, only permitted the investigation of the elicitation phase of the immune response (recall of existing immune memory) as it stimulated a response to previously encountered antigens. More recently, experimental contact sensitisation with the antigen diphenylcyclopropenone (DPCP) has been shown to be a robust and relatively non-invasive protocol that can be used to examine *in vivo* immunity after exercise (Harper Smith et al., 2011). Furthermore, application of DPCP has also been shown to stimulate an antigen specific *in vivo* T-cell-mediated response that is not a reflection of local cutaneous inflammatory processes (Diment et al., 2013). Unlike the Merieux CMI Multitest®, this method allows for investigation of both the induction (establishment of new immune memory) and elicitation phases of *in vivo* T-cell-mediated immunity via two different protocols.

The effect of exercise on the induction of antigen-specific immune memory can be investigated by applying a known sensitising dose of a never previously encountered antigen (DPCP) to the surface of the skin via a patch that remains on the skin for 48-hours. After a period of four weeks (the time period typically allowed for the establishment of immune memory), DPCP is applied again in a dose series and the strength of the immune response to this specific antigen challenge is then quantified through measurement of skin fold thickness (oedema - inflammatory swelling) and erythema (redness). In order to investigate the effect of exercise on the elicitation phase it is necessary to first expose participants to repeated challenges with the same antigen (DPCP) so that a reproducible

response plateau may be achieved. The nature of this type of investigation allows for a repeated measures research design. Alternatively, the induction phase can be investigated using a between-groups research design, which negates the need to establish a plateau in immune response and the response to the first antigen exposure is examined.

Harper Smith et al. (2011) first reported the use of experimental contact sensitisation to examine the effect of prolonged moderate-intensity exercise on both the induction and elicitation phases of *in vivo* T-cell-mediated immunity. In comparison to a rested control condition, both the induction and elicitation phases were significantly impaired by two hours of treadmill running at 60% $\dot{V}O_{2peak}$. The skinfold thickness measured at the skin surface was 53% and 19% lower for the exercise condition (compared to the rest condition) in the induction and elicitation trials respectively. The induction of new immune memory was therefore suggested to be more sensitive to the physical stress of prolonged exercise than the recall of existing immune memory.

Previous *in vitro* work has shown that both short duration high intensity exercise and prolonged duration moderate intensity exercise have a negative effect on immunity (Robson et al., 1999). However, *in vivo* work from Diment et al. (2015) reported somewhat conflicting results. As hypothesised, prolonged moderate intensity exercise (two hours treadmill running at 60% $\dot{V}O_{2peak}$) significantly impaired the immune response with skinfold thickness being 67% lower at recall, in comparison to the control group. However, 30 minutes running at 80% $\dot{V}O_{2peak}$ had no negative effect on *in vivo* immune response. Interestingly, circulating adrenaline, noradrenaline, and cortisol were all significantly elevated after two hours of moderate intensity running and also crucially after 30 minutes of high

intensity running. The authors therefore questioned the role of stress hormones in this specific *in vivo* immune challenge as catecholamines and cortisol were elevated after 30 minutes of high-intensity running, but there was no evidence of a reduction in the *in vivo* immune response.

The protective effect of CHO ingestion against the exercise induced perturbations to immune cells (Bermon et al., 2017; Gleeson et al., 2006; Walsh et al., 2011) was not replicated in a study utilising *in vivo* techniques (Davison et al., 2016). Using a matched groups design, all participants consumed a standardised breakfast before completing two hours of moderate intensity treadmill running with either CHO or placebo drinks provided before, during and after exercise. The CHO group received 40 g CHO before and after exercise, and 60 g per hour whilst running. There was no difference in skin fold thickness between placebo and CHO groups with both groups exhibiting a significantly smaller immune response (~46%) compared to a control condition taken from the previous study by Diment et al. (2013). As previously shown in *in vitro* studies (Gleeson, Bishop & Walsh, 2013), supplementation with CHO did blunt the typical exercise induced rise in cortisol and leukocyte trafficking, but there was no effect of CHO on the *in vivo* cutaneous immune response. These results continue to raise questions over the role of cortisol in the mechanisms of *in vivo* immunity, which adds further strength to the notion that *in vitro* methods of assessing immune function do not capture the full integrated immune response to exercise stress.

Cutaneous assessment of *in vivo* immunity provides a robust and feasible protocol for use in field studies where collection and transport of human tissue samples may not be feasible. For example, Oliver et al. (2013) sensitised a group of 22 mountaineers to DPCP whilst at altitude (3777 m) to examine the effect of hypoxia

on immune induction. The effect of altitude exposure on elicitation was examined 4-weeks later after returning to sea level. At recall, the mountaineers exhibited a significant reduction in skinfold thickness response (52%) and erythema (36%) in comparison to a control group who received their first sensitisation in a laboratory at sea level. The most likely cause of the reduced ability to develop new immune memory at altitude was systemic hypoxia as indicated by a moderate correlation between arterial oxygen saturation (measured before initial DPCP sensitisation) and the size of the immune response measured at recall. These authors' findings support previous *in vitro* work that has shown a decrease in T-cell-mediated immune function at altitude using *in vitro* methods (Facco et al., 2005; Pyne et al., 2000) and demonstrate that *in vivo* methods can be used in field investigations to assess cell-mediated immune function.

The recent *in vivo* work carried out using experimental contact sensitisation has consistently shown that a single bout of prolonged moderate-intensity exercise can reduce both the induction and elicitation phases of the T-cell-mediated immune response (Harper Smith et al., 2011; Diment et al., 2013; Davison et al., 2016). However, the link between measures of *in vivo* immunity and incidence/risk of respiratory infection is yet to be examined.

1.5 Epstein-Barr Virus Infection and Reactivation

Epstein-Barr Virus (EBV) is a human herpes virus that is carried by approximately 90% of the general adult population (Pottgiesser et al., 2006). Primary infection is asymptomatic and occurs via salivary contact most commonly during childhood (Rickinson & Moss, 1997). EBV has the potential to develop into infectious mononucleosis (when infection occurs after childhood) and also to induce tumours and cause diseases such as Hodgkin's disease and B-cell lymphoproliferative disease, but for the majority of the seropositive population EBV rarely results in disease after primary infection (Macsween & Crawford, 2003). After initial infection, the virus establishes life-long persistence through colonisation of the lymphoid system and subsequent expansion of virally infected B-cells in peripheral blood (Yao, Rickinson & Epstein, 1985).

EBV exhibits a dual tropism that allows the virus to infect both B-cells and epithelial cells via different glycoproteins (Shannon-Lowe and Rowe, 2014). EBV can bind to the surface of B-cells when the viral glycoprotein gp350 binds with the B-cell receptor CD21, after which the three-part viral glycoprotein gHgL gp42 will interact with the B-cell major histocompatibility complex II (MHC II) allowing EBV to enter and infect the B-cell (Speck, Haan and Longnecker, 2000). Infection of epithelial cells of the oropharynx occurs after interaction of β1 integrins on the surface of epithelial cells and the EBV BMRF-2 cell surface protein, and then fusion of the viral envelope via interaction between the two-part viral glycoprotein gHgL and the epithelial αvβ6 and αvβ8 integrins (Chesnokova, Nishimura, & Hutt-Fletcher, 2009). In seropositive individuals, memory B-cells form the reservoir of infected cells in the latent lifecycle (non-productive lifecycle) that are required in order for EBV to maintain viral persistence. In healthy seropositive individuals the

levels of memory B-cells in the blood will remain constant over long periods of time (Khan et al., 1996). EBV infected B-cells can switch to the lytic lifecycle (productive lifecycle) (i.e. viral reactivation) and infect epithelial cells of the oropharynx, which triggers an immune response from a subset of viral specific CD8+ T-cells that constitute up to 2% of the total cytotoxic T-cell numbers (Hislop et al., 2007).

When in the latent life cycle, EBV remains dormant in infected B-calls with restricted lytic gene expression. The virus is able to evade immune surveillance and remain undetected by CD8+ T-cells via expression of latent genes that are essential to survival of the virus. For example, Epstein-Barr viral nuclear antigen (EBNA) 1 is a viral protein that interacts with the proteasome within B-cells to prevent degradation of viral proteins into peptides that would otherwise elicit a CD8+ T-cell response (Janeway et al., 1999) and is also required to maintain the EBV genome within a host cell (Knipe & Howley, 2013). Other latent genes expressed from the *Bam*HI-A region of the EBV genome do not have a clear role in viral persistence during the latent cycle, but are consistently detected in infected B-cells of healthy seropositive individuals (Chen et al., 1999).

After establishing latency, the viral lifecycle of EBV infected B-cells involves highly complex molecular pathways that enable the virus to switch from latent to lytic gene expression (Hatton et al., 2014; Murata & Tsurumi, 2014). Epithelial cells can then become infected with EBV upon reactivation and lytic replication of the latent B-cells. The stimulus for the switch from latent to lytic lifecycles *in vivo* is not precisely understood (Odumade, Hogguist, and Balfour, 2011) but is integral to viral survival as transmission of new viral cells to a seronegative host via saliva can only happen after production of new viral cells in the epithelial cells of the

oropharynx (Babcock et al., 1998). As previously discussed, infection of epithelial cells of the oropharynx occurs via interaction of different cell surface receptors to that of B-cell infection. New viral cells formed inside the memory B-cells contain the two-part glycoprotein gHgL that is required to infect epithelial cells, but not the three-part glycoprotein gHgL gp42 that is required to infect B-cells. As such, new viral cells that have been made inside a memory B-cell are capable of infecting epithelial cells of the oropharynx but not new B-cells within the host (Wang and Hutt-Fletcher, 1998). The opposite is true of viral cells that are then produced inside the infected epithelial cells as these cells express the three-part glycoprotein gHgL gp42, but not the two-part glycoprotein, and as such these new viral cells are capable of infecting new B-cells (in a new seronegative host or in the current host) but not new epithelial cells (Wang and Hutt-Fletcher, 1998).

Viral reactivation and subsequent infection of epithelial cells will result in shedding of new viral cells from the epithelial cells directly into saliva (Hadinoto et al., 2009), with EBV reactivation typically being determined via detection of EBV DNA in a saliva sample (Knipe & Howley, 2013). One of the first viral genes to be expressed after the transition from latent to lytic life cycle is BALF5 (Halder et al., 2009), which encodes the viral DNA polymerase (Lin et al., 1991). Analysis of saliva for the presence of fragments of the BALF5 gene has commonly been used as a means to determine whether or not seropositive individuals are currently experiencing non-primary EBV replication (Gleeson et al., 2002; Gleeson et al., 2017; Reid et al., 2004; Yamouchi et al., 2011).

EBV reactivation is thought to be linked to both physical and psychological stress. Increased levels of adrenaline and noradrenaline have been linked to EBV reactivation in astronauts pre and post-spaceflight (Stowe et al., 2000; Stowe,

Pierson & Barrett, 2001). The suppressive effect of catecholamines on EBV specific CD8+ T-cell function (Dobbs et al., 1993) was the proposed cause of increased viral activity in the astronauts. EBV reactivation has also been linked to diminished cell mediated immunity in Antarctic expeditioners (Mehta et al., 2000). During a period of winter isolation, the frequency of viral shedding in to saliva was significantly greater when DTH responses where diminished in comparison to when DTH results were classified as normal. Based on this evidence, there may be a link between monitoring *in vivo* immune status via DTH assessments and EBV shedding. If so, monitoring EBV reactivation via collection of saliva samples may be a useful and practical method for monitoring *in vivo* immunity in situations that impose high levels of physical and/or psychological stress. In the work from Mehta et al. (2000), all 16 of the expeditioners that were monitored during this study had provided at least one saliva sample that was positive for EBV DNA, but did not report any URI symptoms that could be attributed to the decrease in cell mediated immunity. Therefore, the clinical significance of viral shedding in populations experiencing high levels of psychological and/or physical stress, and by extension the ability to predict likelihood of imminent URI, could not be elucidated from this work.

1.5.1 Epstein-Barr Virus Reactivation in Sport and Exercise Science

Over the last two decades research of EBV reactivation within exercising populations has grown, possibly due to the ease of investigation (i.e. collection of saliva samples) and the ability of the virus to replicate intermittently from within the oropharynx (Faulkner et al., 2000). Reactivation of EBV, and subsequent shedding

of viral DNA in to saliva, has been linked to the presence of URS in elite athletes (Walsh et al., 2011).

Longitudinal studies of elite athletes have suggested a link between seropositivity and an increased incidence of URI in athletes. Gleeson et al. (2002) monitored a group of 14 elite Australian swimmers for URS over a 30-day period of intensive training, with saliva samples provided every two-to-three days. The authors identified a consistent pattern of lowered s-IgA levels and detection of EBV DNA that preceded the appearance of URS, making this research group the first to provide evidence of a link between viral shedding, s-IgA, and URS in elite athletes. However, this should perhaps be interpreted with caution as a relatively low number of illness episodes were reported by the seropositive swimmers with EBV DNA being detected in 6/9 episodes. More recently, Gleeson et al. (2017) identified a trend for a higher detection rate of EBV DNA in saliva alongside lower concentrations of s-IgA in illness prone endurance athletes (≥ 3 illness episodes over a nine-month monitoring period). However, these results did not reach statistical significance, and the study may have been underpowered with just four athletes from 16 being identified as illness prone.

In a study of team sport athletes during a 1-month intensive training camp, Yamauchi et al. (2011) collected saliva samples from 32 collegiate rugby players on a daily basis and recorded all URS. EBV DNA was detected more frequently in players who reported symptoms of sore throat and runny nose than in players who reported no symptoms at all (32 vs 20% of all saliva samples provided). Similar to Gleeson et al. (2002), this group also found evidence of a link between lowered s-IgA concentration and detection of EBV DNA in saliva. However, this study was also statistically underpowered with just six illness episodes reported during the

training camp. Furthermore, in this instance the authors failed to report whether or not the players were tested for prior infection with EBV. Despite previous suggestions that EBV reactivation may be more common during periods of physical and/or psychological stress, these authors found no relationship between EBV reactivation and subjective measures of fatigue measured as a rating of subjective fatigue on a scale of 1 to 5.

In contrast to the early work from Gleeson et al. (2002), when a relatively large cohort of 239 athletes were monitored over a period of 16-weeks EBV serostatus alone was found to have no influence on URI incidence (He et al., 2013). Furthermore, Cox et al. (2004) provided evidence to suggest that detection of EBV DNA in saliva, and therefore lytic EBV reactivation, is not directly responsible for URS in elite athletes. When an antiviral agent (Valtrex™) with specific actions against herpes viruses was administered to a group of elite Australian distance runners the EBV viral load in saliva was significantly reduced by 82% during the antiviral treatment month (in comparison to the baseline, placebo, and wash out months). However, there was no reduction in URS during the antiviral treatment month. These results suggest that during the treatment month non-primary EBV replication was not the infectious agent responsible for URS in this group of seropositive elite athletes. Unlike the study from Spence et al. (2007), previous longitudinal studies of EBV reactivation and incidence of URS discussed in this thesis have not included laboratory analysis of infectious agents. Therefore, any suggestion that non-primary EBV infection is directly responsible for URS is highly speculative. Alternatively, it is possible that detection of EBV DNA in saliva may instead be an *in vivo* marker of immune suppression with URS being caused by an alternative infectious agent that was able to overcome the compromised immune system.

There is currently limited evidence on the acute time course of EBV reactivation, and appearance of EBV DNA in saliva, after a bout of exercise. Recently, Gleeson et al. (2017) analysed saliva samples for the presence of EBV DNA that had been collected pre, post, and 24-hours post two exercise bouts each lasting 60 minutes. The authors did not report any quantitative measures for the amount of EBV DNA that was in the saliva samples (i.e. results were "positive" or "negative"), making it impossible to consider the changes in viral load from pre-to-post exercise. This may have been particularly insightful in this instance as all but one of the participants that produced a positive post-exercise saliva sample also produced a positive pre-exercise saliva sample. Furthermore, collection of additional saliva samples between the immediately-post and 24-h post timepoints may provide further insight into any acute changes in viral activity after exercise cessation.

1.6 Summary and Aims

Over the last decade researchers working with professional athletes have attempted to examine the relationship between s-IgA levels at rest and the risk of imminent URS. Evidence of a link between lowered s-IgA levels and increased risk of URS in elite athletes has been reported. However, low sampling frequency, short monitoring periods, and/or low subject numbers have limited the interpretation of results for s-IgA and URS incidence. The model for monitoring individual relative changes in s-IgA levels, as proposed by Neville et al. (2008), seems to present a promising method for assessing individual illness risk. However, there has since been no further investigation in other sports (e.g. team sport athletes or endurance athletes) or with a different level of athlete (e.g. recreational active or national level athletes). Therefore, this thesis will aim to investigate the use of this model with a group of professional team sport athletes, and sub-elite endurance athletes.

The recent cutaneous *in vivo* work carried out using experimental contact sensitisation has consistently shown that a single bout of prolonged moderate-intensity exercise can reduce both the induction and elicitation phases of the T-cell-mediated immune response (Harper Smith et al., 2011; Diment et al., 2013; Davison et al., 2016). However, the link between measures of *in vivo* immunity and risk of respiratory infection is yet to be examined. While this method of *in vivo* immune assessment may represent a more clinically relevant model, compared to commonly used *in vitro* methods, it is not without limitations. After the first sensitisation to the antigen DPCP, the induction phase of immunity can only be assessed once. After which, researchers and sport scientists are limited to examining the elicitation phase of their study participants and athletes. A further

limitation to the utility of these *in vivo* techniques lies in the ability to integrate cutaneous *in vivo* assessment in to an illness risk monitoring model with elite athletes and professional sports teams. The antigen (DPCP) is applied to the skin via a patch that remains on the skin surface for 48-hours, which has obvious limitations for use with water sport athletes (e.g. swimmers, triathletes etc) and may be perceived as unnecessarily cumbersome by other professional athletes.

Monitoring EBV reactivation via the collection of saliva samples would be a less invasive and less time-consuming method (on the part of the athlete/study participant) for monitoring *in vivo* immune status than the previously discussed cutaneous options. The growing evidence base of EBV reactivation in elite athletes seems to suggest a link between increased viral shedding, lowered levels of s-IgA, and URS. However, low numbers of individual URS episodes and/or study participants have limited the ability to make definitive conclusions regarding EBV shedding and URS incidence (Gleeson et al., 2017; Yamouchi et al., 2011). Furthermore, the ability to combine EBV DNA and s-IgA monitoring as a model to assess immune status requires further investigation. Therefore, this thesis will further investigate the temporal relationship between s-IgA, EBV reactivation, and URS.

The mucosal immune response to acute exercise has been extensively investigated across different exercise modalities and sports settings. There appears to be a general consensus that s-IgA levels decrease following a single bout of strenuous and prolonged exercise. However, the clinical relevance of lowered s-IgA in the hours post exercise is unclear, and the consistency of reporting method for s-IgA is lacking. The only previous investigation of acute exercise and EBV reactivation was limited to qualitative analysis of EBV DNA

detection. Therefore, the final aim of this thesis is to examine EBV reactivation following acute exercise using quantitative methods, alongside the mucosal immune response and occurrence of URS.

Chapter 2. General Methods

2.1 Ethics approval

Study 1 was approved by the University of Kent Faculty of Science Research Ethics committee, and studies 2 and 3 were approved by the School of Sport and Exercise Science Research Ethics Committee. All participants were non-smokers and not taking long term medication. Prior to commencing with a study, all participants completed the Physical Activity Readiness Questionnaire (PAR-Q). Both verbal and written informed consent were provided by all participants.

2.2 Saliva analytical methods

2.2.1 Saliva collection

Saliva was collected using the unstimulated, passive drool method. Participants first rinsed their mouth with plain water then sat quietly for at least 10 minutes. Participants sat with their head tilted forward and passively dribbled in to pre-weighed sterile pots for two to three minutes. The exact time was recorded for calculation of saliva flow rate. All three studies required transport of saliva samples to the laboratory under ice (frozen samples for studies 2 and 3, fresh samples for study 1 that were transported to the laboratory under ice and processed for storage within 60 min). All saliva samples were weighed and then centrifuged at 13,400 *xg* for 5 min and the supernatant was stored as aliquots at -80°c for later analysis. Assuming a saliva density of 1 g/ml (Cole and Eastoe, 1988) saliva flow rate was calculated by dividing sample mass by the collection time.

2.2.2 Epstein-Barr virus DNA

DNA extractions were carried out using a commercially available genomic DNA extraction kit (Quick-DNA mini prep, Zymo Research, Irvine, California, U.S.A). The presence of EBV DNA in saliva could then be determined via quantitative (real-time) polymerase chain reaction (qPCR). In all three studies the extracted saliva samples were analysed for the presence of BALF5 DNA fragments, which will be referred to as EBV DNA for the duration of this thesis. For study 1, the extracted saliva samples were also analysed for the presence of *BamHI* DNA fragments. In this case the results specifically name *Bam*HI DNA.

Forward and reverse primers for the BALF5 gene were designed using the Roche Universal Probe Library (UPL) (https://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html) and ordered from Eurogentec (Liège, Belgium). The primers produce an amplicon that is 60 nt in length (GGAG CTGG ACAT GCTC TACG CCTT CTTC CAGC TCAT CAGA GACC TCAG CGTG GAGT TTGT). Probe 11 (cat. no. 04685105001) from the UPL (Roche, Basel, Switzerland) was used for BALF5 qPCR.

7.5 µL of qPCR mix containing 0.2 µl of both forward and reverse primers with a concentration of 400 nM, 0.2 µl fluorescent probe with a concentration of 200 nM, 5.0 µl PCR master mix (FastStart Essential DNA Probes Master (cat. no. 06402682001; Roche, Basel, Switzerland), and 1.9 µl PCR grade (DNase and RNase free) water was added to each well of a 96-well PCR plate. 2.5 µL of extracted saliva samples were then added to wells in the plate. All analyses were undertaken in duplicate. If the difference in duplicate Ct values was greater than one cycle for any sample, that sample was re-analysed in duplicate.

A standard curve was constructed using a positive control stored as a working stock solution of 1 ng/µl that had been prepared in house (Chidley, 2018 https://kar.kent.ac.uk/69956/). Ten-fold serial dilutions were performed from the top standard with PCR grade water. For the negative control, PCR grade water was used in place of samples/standards.

A LightCycler 96 (Roche, Basel, Switzerland) instrument was used for amplification and detection under thermal cycling conditions of: one pre incubation cycle of 10 min at 95 ◦C; 45 amplification cycles of 10 s at 95◦c (denaturation), 30 s at 60◦c (annealing), and 1 s at 72◦c (extension); and finally, one cooling cycle of 30 s at 40◦c. Fluorescence from the probe is measured at the end of each amplification cycle (during extension). The end point fluorescence measured after 45 amplification cycles allows for calculation by the LightCycler 96 software of the quantification cycle (Cq) and concentration of EBV DNA for each individual well. Samples could be classified as positive if the auto-calculated Cq value for both duplicates did not exceed the threshold Cq value that had been calculated across all wells on each plate (determined as ten multiplied by the SD of the baseline fluorescence calculated from cycles 3 - 10).

The qPCR process for detection and quantification of EBV DNA in saliva used throughout this thesis previously been shown to be a reliable and valid method (Chidley, 2018 https://kar.kent.ac.uk/69956/). Intra-assay CV for all BALF5 plates was 5.4 ± 2.7 %.

2.2.3 Human Genome

The presence of human DNA in saliva for studies 2 and 3 was determined using a commercially available qPCR kit (cat. no. g-DNA-q; Primerdesign Ltd, Camberley, UK.) According to the manufacturer's instructions, 7.5 µL of qPCR mix containing gDNA primer/probe mix, master mix, and PCR grade (DNase and RNase free) water was added to each well of a 96-well PCR plate. 2.5 µL of extracted saliva samples were then added to the plate in duplicate. The LightCycler 96 was used for amplification and detection under thermal cycling conditions of: one pre incubation cycle of 2 min at 95 ◦c; 40 amplification cycles of 10 s at 95◦c and 60 s at 60◦c (fluorescence from the probe measured at the end of each extension cycle); and finally, one cooling cycle of 30 s at 40◦c. A standard curve was constructed using four-fold serial dilutions of a provided top standard with a concentration of 5 ng/µl. PCR grade water was used in place of samples/standards as a negative control.

2.2.4 Secretory Immunoglobulin A

All saliva samples from studies 1, 2 and 3 were analysed for s-IgA concentration using an in-house enzyme-linked immunosorbent assay (ELISA) method that was based on the protocol from Leight et al. (2011). Flat bottomed 96-well microtiter plates (Nunc-Immunoplate, Thermo Fisher Scientific, Denmark) were first coated with capture antibody (mouse anti-human IgA; Sigma, code I6635) that had been diluted in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6) with 1:1740 dilution factor to obtain a concentration of 5 µg/ml (100 µl per well). After an overnight incubation at 4◦C plates were washed four times (200 µl per well:

phosphate-buffered saline (PBS), 0.3 M NaCl, 0.1% Tween 20) then blocked with a blocking protein (100 µl per well: 2% BSA, bovine serum albumin, Fraction V, Sigma-Aldrich, St. Louis, Missouri, U.S.A.) in PBS for 60 minutes at room temperature. All defrosted samples were centrifuged for two minutes at 1,700 × g then diluted (1:750) with PBS. A standard curve was constructed via two-fold serial dilution with PBS of a top standard concentration 1 µg/ml of IgA from human colostrum (Sigma-Aldrich, St. Louis, MO, U.S.A.). Seven standards were produced with the eighth as PBS (i.e. 0 µg/ml). Plates were washed four times before adding 50 µl of each sample and standard in duplicate to the plate. Plates were then sealed and incubated overnight at 4°C. Plates were washed again four times before adding 50 µl per well of detection antibody (Polyclonal Rabbit Anti-Human IgA/HRP, Dako, Glostrup, Denmark) diluted 1:2000 in PBS. Following a 90-minute room temperature incubation plates were washed for a final four times before adding 50 µl per well of OPD substrate (Dako, Glostrup, Denmark) to each well. Plates were covered to protect from light and incubated for 7.5 minutes. The colour change reaction was stopped by adding 75 µl per well of 1 M sulphuric acid followed immediately by reading the absorbance of each well using an automated plate reader with a 490 and 630 nm filter (ELx808 Absorbance Reader, BioTek, Winooski, VT, U.S.A.). The background absorbance readings at 630 nm were subtracted from the absorbance readings at 490 nm, and mean of duplicate wells was calculated. A graph of target standard concentrations plotted against measured standard absorbances was then plotted in Microsoft Excel, and a polynomial standard curve was fitted to allow for calculation of the s-IgA concentration of the samples. These calculated concentrations were then multiplied by 750 to account for the saliva sample dilution factor. s-IgA secretion rate could then be calculated by multiplying saliva flow rate by s-IgA concentration.

The intra-assay CV of this method based on analyses of all plates was 2.9 ± 1.6 %.

2.3 Blood collection and analysis of EBV serostatus

EBV serostatus of all participants in studies 2 and 3 was determined from blood samples that were provided at the start of the study period. Finger-tip capillary blood was collected in to K_2EDTA microcuvettes (Microvette®CB 300 K2E, Germany) and then centrifuged at 1500 *xg* for 2 min at 5°c. The supernatant was stored at -80°c for later detection of viral capsid antigen IgG antibodies with commercially available ELISA kits (Epstein Barr Virus (VCA) IgG ELISA; cat. no. EIA-3475; DRG Instruments GmbH, Marburg, Germany). All samples were analysed in duplicate using a plate reader (ELx808 Absorbance Reader, BioTek, Winooski, VT, U.S.A.). According to the manufacturer's instructions, samples were considered seropositive for previous EBV infection if the mean absorbance value of duplicate wells was more than 10% above the cut-off control for each individual plate.

2.4 Monitoring upper respiratory symptoms

2.4.1 Recording symptoms

For studies 2 and 3, a modified version of the Jackson upper respiratory illness questionnaire (appendix A) as described by (Gleeson et al., 2011) was used by participants to record URS on a daily basis. All participants were fully educated

with respect to the symptoms listed on the questionnaire. For study 1, the same questionnaire was provided to participants on the same day of each week (Monday morning), and participants were asked to recall URS from the previous seven days.

The self-report questionnaire asked participants to record the presence and severity for several symptoms of URTI. The symptoms included: fever, persistent muscle soreness, sore throat, catarrh in the throat, runny nose, cough, repetitive sneezing, joint aches and pains, weakness/fatigue, and headache, as well as a loss of sleep or inability to train. The severity of each URTI symptom that was present was recorded as either light, moderate, or severe with a score of 1, 2, or 3 applied to each rating to allow for quantification of illness symptoms. The sum of all severity scores was calculated on a daily basis.

2.4.2 Criteria for illness episodes

The criteria for an individual URS episode was defined as a total symptom score of ≥12 over at least a three-day period with at least one week between that and another three-day period with a total symptom score of ≥12 (Gleeson et al., 2011). If symptoms (3-day ≥ 12) were recorded by an individual less than 1 week apart, they were classified as the same episode.

2.5 General statistical analysis

All statistical analyses were carried out using SPSS (IBM SPSS Statistics for Windows, version 24.0, Armonk, NY:IBM Corp). Data shown in tables, figures, and

the text are mean ± standard deviation (SD) unless otherwise stated, with the level of significance set at $P < 0.05$. All data were checked for normal distribution with the Shapiro-Wilk test. Any data found to be not normally distributed were normalised with log transformation before carrying out further statistical analysis.

For studies 1, 2 and 3, individual EBV DNA shedding frequency was calculated as the percentage of positive samples for each individual participant.

For studies 1 and 2, individual healthy baseline scores for s-IgA concentration and secretion rate were calculated according to the methods of Neville et al. (2008). Any saliva samples provided 14 days pre or post day one of an illness episode were not included in the healthy baseline calculation. Individual relative s-IgA values were then calculated for each saliva sample as the percentage of individual healthy baseline scores. Reliability of s-IgA concentration and secretion rate was calculated within and between participants with the coefficient of variation (CV).

Chapter 3. Study 1 – Epstein-Barr virus and mucosal immune markers in professional English football players

Abstract

Introduction: Team sport athletes appear to be more susceptible than normal to upper respiratory symptoms (URS) during periods of intensified training and match play. Reactivation of Epstein-Barr Virus (EBV) and detection of lytic DNA fragments (within a region of the BALF5 gene) in saliva has been linked to episodes of URS in elite athletes. Furthermore, a decrease in individual relative concentration of salivary immunoglobulin A (s-IgA) has been shown to be associated with an increased risk of upper respiratory illness (URI) in professional athletes. **Aim:** To investigate 1) the utility of monitoring changes in relative s-IgA levels as a tool for evaluating risk of URS in professional team sport athletes 2) reactivation of EBV alongside changes in s-IgA levels, and incidence of URS. **Methods:** Over a period of 16-weeks, 15 male football players from a professional English Football League 1 club provided unstimulated saliva samples and recorded URI symptoms on a self-report questionnaire. Saliva samples were analysed for s-IgA (ELISA) and EBV DNA (qPCR). Individual healthy baseline s-IgA was calculated as the average across all weeks when no illness symptoms were present. Data are expressed as mean ± (SD). **Results:** Whole squad median (IQR) baseline s-IgA concentration was 107 (76 - 150) mg/L and secretion rate was 51 (30-78) mg/min. Whole squad s-IgA concentration and secretion rate significantly decreased during a period of intensified competitive match play from week 8 to week 12 compared to week 1 ($P < 0.05$). Two individual URS episodes occurred during week 10, both when s-IgA was lower than 40% individual healthy baseline, with symptoms lasting 4–7 days. For the two players experiencing URS and six additional healthy players, latent EBV DNA was detected in 100% of saliva samples that underwent qPCR analysis (n = 70). Overall mean shedding frequency of lytic EBV DNA was 40%, with individual shedding frequency ranging from 11-78%. **Conclusion:** The low number of URS episodes has limited the ability to fully investigate any temporal relationship between reactivation of EBV, changes to s-IgA levels, and appearance of URS. Analysis of saliva for presence of latent EBV DNA, specifically *Bam*HI DNA fragments, can be used to determine EBV serostatus in the absence of serum samples.

3.1 Introduction

For team sport athletes, upper respiratory illness (URI) can negatively affect performance either directly via players being unavailable for selection on match days, or indirectly via a loss of training days (Cunniffe et al., 2010; Raysmith & Drew, 2016). Increased incidence of URI in professional athletes has been shown to be associated with decreased resting saliva secretory immunoglobulin A (s-IgA) concentration (Gleeson et al., 1999b; Neville et al., 2008) and secretion rate (Fahlman and Engels, 2005).

Due to the high inter-individual variability of s-IgA there are currently no established clinical reference values for absolute s-IgA concentration or secretion rate, and the level of risk of imminent URI. A model for monitoring changes in s-IgA levels on an individual basis has been provided by Neville et al. (2008) with the risk of URI increasing by 50% when s-IgA levels fall below 40% of the calculated individual healthy baseline. However, other longitudinal investigations involving team sport athletes have not reported relative changes in s-IgA levels. This is possibly due to low sampling frequency, such as the study of professional rugby union players from Cunniffe et al. (2011) where saliva samples were collected once a month over nine months of a competitive rugby season, or shorter monitoring periods such as Morgans et al. (2014) where saliva samples were collected from English Premier League football players over a 32-day period.

Reactivation of Epstein-Barr virus (EBV) has also been linked to upper respiratory symptoms (URS) in elite athletes (Gleeson et al., 2002). After initial primary infection EBV exhibits a latent lifecycle phase (non-productive) by remaining dormant in infected B-cells and a lytic lifecycle phase (productive) when the

dormant B-cells reactivate and infect epithelial cells of the oropharynx (Chesnokova, Nishimura, & Hutt-Fletcher, 2009).

Analysis of saliva for the presence of EBV DNA from the BALF5 gene (early lytic gene) has typically been used as the marker to determine current EBV status (Gleeson et al., 2017; Reid et al., 2004; Yamouchi et al., 2011). When in the latent life cycle, EBV is able to evade immune surveillance and remain undetected by CD8+ T-cells via expression of latent genes that are essential to survival of the virus. For example, Epstein-Barr viral nuclear antigen (EBNA) 1 is a viral protein that interacts with the proteasome within B-lymphocytes to prevent degradation of viral proteins into peptides that would otherwise elicit a CD8+ T-cell response (Janeway et al., 1999) and is also required to maintain the EBV genome within a host cell (Knipe & Howley, 2013). Other latent genes expressed from the *Bam*HI-A region of the EBV genome do not have a clear role in viral persistence during the latent cycle, but are consistently detected in infected B-cells of healthy seropositive individuals (Chen et al., 1999). EBV Serostatus is typically determined via measurement of viral capsid antigen IgG antibodies in serum (Gartner et al., 2003). In an applied sport science setting, collection of blood samples for analysis of EBV serostatus may not always be possible. Given the consistent shedding of *Bam*HI fragments into saliva, analysis of saliva samples for the presence of this specific latent gene may have potential as a surrogate marker for serostatus.

The aims of this study were to investigate 1) the utility of monitoring changes in relative s-IgA levels as a tool for evaluating risk of URS in professional team sport athletes 2) the temporal relationship between changes to s-IgA levels, detection of lytic EBV DNA (BALF5), and incidence of URS, and 3) the shedding frequency of

latent *Bam*HI DNA fragments in saliva, and ability to use detection of *Bam*HI fragments as a salivary marker for EBV serostatus.

3.2 Methods

3.2.1 Participants

22 male football players from a professional English Football League 1 club volunteered to participate in the study. Players were excluded from the final analyses if they were unable to provide a saliva sample on more than 25% of the sampling time points (two players) or if they did not provide a sample on more than two consecutive sampling time points (five players) during the study period. 15 players were included in the final analyses (age 27 ± 4 years; 2 goal keepers, 3 defenders, 8 midfielders, and 2 strikers). Players were retrospectively classified as URS if they experienced at least one URS episode over the 16-week period, or as HEALTHY if they remained free from URS.

3.2.2 Study Design

Over a 16-week period (August to November 2016) players provided saliva samples and completed self-report illness questionnaires on a weekly basis.

3.2.3 Saliva collection

Timed, unstimulated saliva samples were collected the morning after a full rest day (at least 36 h post-match) and before training between 8:00 and 9:00 am on the same morning of each week at the football club's training facility.

3.2.4 Saliva analysis

All saliva samples were analysed for s-IgA concentration and secretion rate according to the methods outlined in chapter 2.

For the URS players (n=2), DNA extractions were carried out on all saliva samples that were collected during the illness episode, as well as the four saliva samples collected before the appearance of symptoms and also the three samples collected after the cessation of symptoms. An additional six players' saliva samples that were time-matched to the URS players' illness episodes, as well as pre-season samples for all eight participants, also underwent the DNA extraction process. All extracted saliva samples were analysed for presence of BALF5 DNA according to the methods outlined in chapter 2.

The presence of *Bam*HI fragments in saliva was determined via qPCR. Forward and reverse primers for the *Bam*HI gene were designed using the Roche Universal Probe Library (UPL) (https://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html) and ordered from Eurogentec (Liège, Belgium). The primers produce an amplicon that is 74 nt in length (GCTAGGCCACCTTCTCAGTCCAGCGCGTTTACGTAAGCCAGACAGCAGCCA ATTGTCAGTTCTAGGGAGGGGGA). Probe 66 (cat. no. 04688651001) from the UPL (Roche, Basel, Switzerland) was used for *Bam*HI qPCR.

7.5 µL of qPCR mix containing 0.2 µl of both forward and reverse primers with a concentration of 400 nM, 0.2 µl fluorescent probe with a concentration of 200 nM, 5.0 µl PCR master mix (FastStart Essential DNA Probes Master (cat. no. 06402682001; Roche, Basel, Switzerland), and 1.9 µl PCR grade (DNase and RNase free) water was added to each well of a 96-well PCR plate. 2.5 µL of

extracted saliva samples were then added to the plate in duplicate. No standard curve was included in this assay; therefore, the results are limited to identification of positive or negative for detection of *Bam*HI fragments. The LightCycler 96 was used for amplification and detection under thermal cycling conditions of: one pre incubation cycle of 10 min at 95 ˚c; 45 amplification cycles of 10 s at 95˚c, 30 s at 60˚c, and 1 s at 72˚c; and finally, one cooling cycle of 30 s at 40˚c. Fluorescence from the probe was measured at the end of each cycle with the end point fluorescence for each well used by the LightCycler 96 software to calculate Ct values and DNA concentration.

3.2.5 URS reports and criteria for URS

Players retrospectively recorded illness symptoms on the same morning of each week using the questionnaire detailed in chapter 2. For study weeks when players were unable to provide a saliva sample their illness symptoms for that week were still recorded.

3.2.6 Match load

The number of times that a player was named as a starter or as a substitute, and the number of minutes played in first team matches were recorded for each player for all matches played over the 16-week period. Players were retrospectively allocated in to one of two groups of "regular starters" (players who started >50% of games during the monitoring period and played >45 mins in each of the games that they started) or "non-starters" (players named as substitutes for >50% of games during the monitoring period and played <45 mins of those matches, or not named at all in the first team squad for >50% of matches).

3.2.6 Statistical Analysis

Normally distributed data are expressed as mean (± SD) with non-normally distributed data expressed as the median and interquartile range (IQR). The level of significance is set at $P < 0.05$.

Healthy baseline s-IgA values for concentration and secretion rate were calculated for each player according to the methods outlined in chapter 2.

A one-way repeated measures ANOVA was used to analyse whole squad weekly absolute and relative s-IgA concentration and secretion rate. Additionally, a two-way repeated measures ANOVA was used to examine differences in s-IgA levels between the starters and non-starters. Any data found to be non-normally distributed were log transformed (whole squad s-IgA absolute concentration, and whole squad absolute and relative secretion rate) and checked again for normal distribution before running an ANOVA.

An independent samples t-test was used to analyse the difference in healthy baseline s-IgA concentration and secretion rate values between regular starters and non-starters.

3.3 Results

3.3.1 URS

Two players experienced one episode of URS over the 16-week monitoring period, with symptoms lasting for 4 days (Player ID 5) and 7 days (Player ID 8). For both of these players, the illness episode occurred during week 10 when both relative s-

IgA concentration (Fig. 3.1A) and secretion rate (Fig. 3.1B) were <40% of the calculated individual healthy baseline. A further seven players experienced one study week when their relative s-IgA concentration and secretion rate scores were less than 40% of the calculated healthy baseline but did not report any illness symptoms. Therefore, 9 players presented with a decrease in relative s-IgA to <40% of baseline and 22% (2/9) experienced a URS. For comparison 0% (0/6) of the players who maintained relative s-IgA >40% of baseline experienced a URS.

3.3.2 Secretory Immunoglobulin A

For the 15 players that were included in this study, a total of 222 saliva samples were collected and analysed for s-IgA concentration and secretion rate over the 16-week study period. A total of 18 out of a possible 240 (7.5 %) saliva samples were not collected due to players being unavailable at the time of collection. Illness questionnaires were completed by all players on all study weeks.

For s-IgA concentration, the whole squad median and IQR across all 16 study weeks was 107 (76 - 150) mg/ml, with a mean within CV of 52%. The between participants CV was 61%. Both absolute and individual relative s-IgA concentration were significantly lower in all weeks from and including weeks 8 to 12 in comparison to week 1 (Fig. 3.2A, $P < 0.05$).

For s-IgA secretion rate, the whole squad median and IQR across all 16 study weeks was 51 (30 - 78) mg/min, with a mean within CV of 58%. The between participants CV was 72%. Both absolute and individual relative s-IgA secretion rate were significantly lower in all weeks from and including weeks 9 to 12 in comparison to week 1 ($P < 0.05$).

3.3.3 EBV DNA

A total of 70 saliva samples were analysed for the presence of *Bam*HI and BALF5 DNA fragments. 100% of saliva samples were positive for presence of *Bam*HI DNA, with 40% being positive for BALF5 DNA (28/70). The whole group mean (± SD) for individual shedding frequency of BALF5 DNA for all eight players was 40 (± 24%). Individual shedding frequency values ranged from the lowest at 11% (1/9 positive samples) to the highest at 78% (7/9 positive samples).

For the two URS players, BALF5 DNA was detected in the weeks before and after URI but not when symptoms were present (table 3.1).

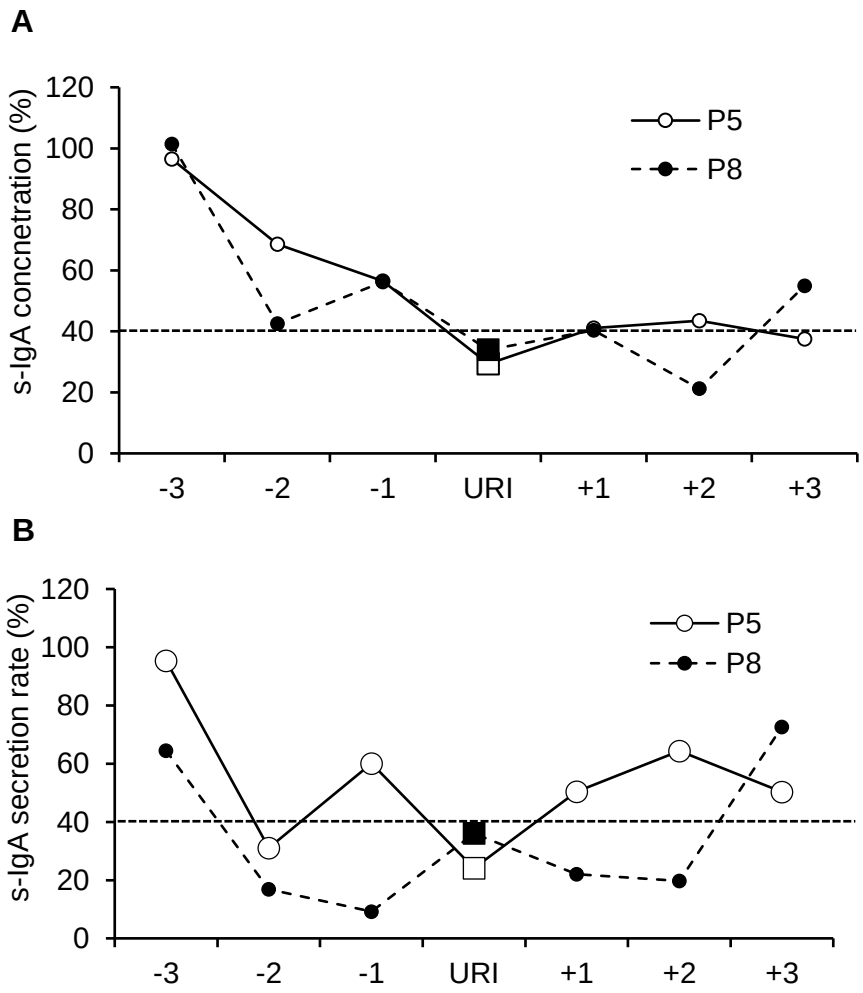

Figure 3.1 Relative s-IgA concentration (A) and secretion rate (B) for the three weeks pre-URS, during URS, and three weeks post URS for players 5 (solid line) and 8 (dashed line).

3.3.4 Match Load

26 competitive matches were played during the 16-week monitoring period. The number of matches played in the seven days preceding each sampling time point are shown in figure 3.2A. Nine players were classified as regular starters (1 goal keeper, 3 defenders, 4 midfielders, and 1 striker) and six players were classified as non-starters (1 goal keeper, 4 midfielders, and 1 striker). There was no difference between regular starters (n=9) and non-starters (n=6) for s-IgA concentration or secretion rate across the 16-week study period ($P > 0.05$) (absolute s-IgA concentration shown in Fig. 3.2 A and B). There was also no difference between starters and non-starters for healthy baseline s-IgA concentration (122 ± 8 vs 128 ± 20 mg/L, $P = 0.548$) (Fig. 3.4A) or secretion rate (62 ± 9 vs 52 ± 9 mg/min, $P = 0.325$) (Fig. 3.4B).

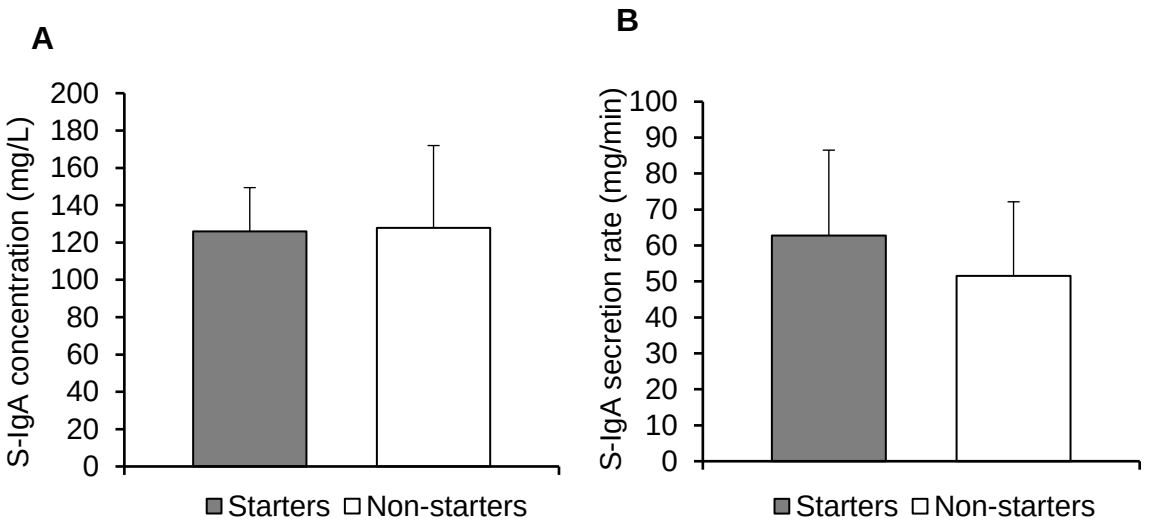

Figure 3.4 Healthy baseline salivary IgA concentration (A) and secretion rate (B) for starters (n=9) and non-starters (n=6) throughout the 16-week monitoring period. Values are mean (± SD).

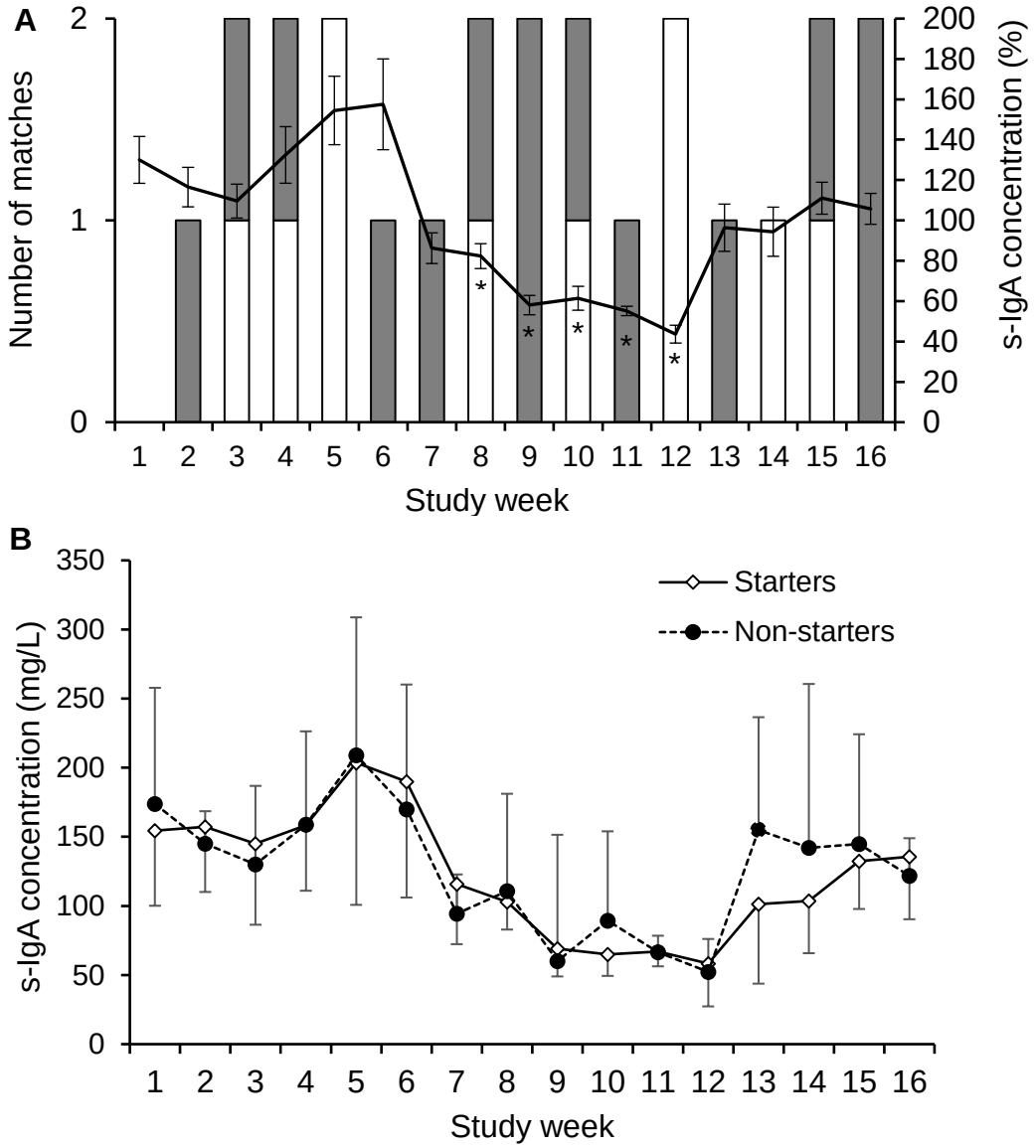

Figure 3.2 (A) Whole squad s-IgA concentration expressed as a percentage of individual healthy baseline values. **P* < 0.05 vs. week 1. White bars indicate home matches. Grey bars indicate away matches. (n=15). (B) S-IgA concentration (mg/L) for starters (n=9) vs non-starters (n=6). Values are mean (± SD).

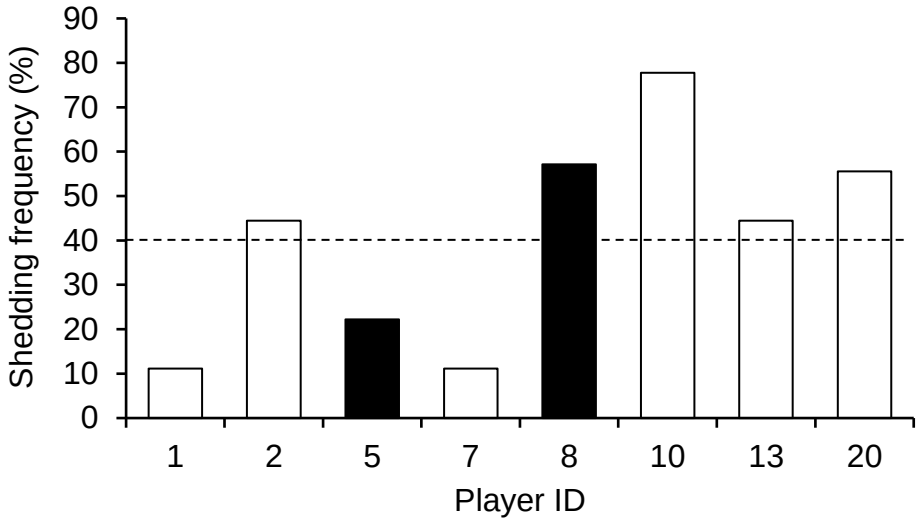

Figure 3.3 Individual shedding frequency for BALF5 DNA fragments. Black bars indicate players who experienced URS. Players 1 and 2 were “non-starters”. Dashed line indicates mean shedding frequency (40%).

Table 3.1 Detection of BALF5 DNA in saliva provided during study weeks 6-13 for the two players experiencing URS.

ID	Study week								
	Pre-season	6	7	8	9	10	11	12	13
5	-	-	-	Positive	-	- *	-	-	Positive
8	-	No data	Positive	-	Positive	- *	Positive	Positive	No data

*indicates week when players experienced URS.

3.4 Discussion

The primary aim of this study was to investigate the utility of monitoring changes in relative s-IgA levels as a tool for evaluating risk of URS in professional team sport athletes. The main findings show that s-IgA concentration and secretion rate significantly decrease during periods of increased match frequency when the number of recovery days between matches decreases, and will recover towards

healthy baseline values when the number of recovery days between matches increases. However, the low number of URS episodes experienced by the football players in this study (n = 2) made it impossible to carry out any statistical analysis to fully examine the relationship between relative s-IgA levels and risk of imminent URS. By extension, any relationship between EBV reactivation, s-IgA levels and URS (the secondary aim of this study) could not be further investigated due to the low number of URS episodes.

The third aim of this study was to investigate the shedding frequency of latent EBV genes into saliva. Fragments of the *Bam*HI gene were found in 100% of saliva samples that were analysed for the EBV section of this study. Therefore, suggesting that detection of this specific gene can be used as a surrogate salivary marker for identifying serostatus when a blood serum sample cannot be collected.

There is good agreement in the literature that periods of intensified training can result in decreased levels of s-IgA at rest (Walsh et al., 2011). For team sport athletes specifically, a congested fixture schedule or heavy periods of training can lead to a reduction in whole squad resting s-IgA levels, which is thought to be linked to increased levels of cortisol during intensified training (Cunniffe et al., 2011; Fahlman & Engels, 2005; Morgans et al 2012; Yamauchi et al., 2011). In the present study, s-IgA concentration and secretion rate were significantly reduced during weeks 8-12 when the team played nine matches over a period of 35 days. In a previous investigation from Morgans et al. (2012), a group of English Premier League football players were monitored over a period of 32 days, which included seven competitive matches. During the most congested period, when the number of recovery days between matches was reduced, the Premier league players

experienced a similar decrease in mucosal immunity to the League One players in the present study.

The low number of URS episodes experienced by the players in this study (n=2) meant that it was not possible to fully examine the relationship between relative changes in s-IgA levels and risk of experiencing URS using statistical tests. The two illness episodes did occur when relative s-IgA concentration and secretion rate fell below 40% of each player's individual healthy baseline, which is in agreement with the model of illness risk and relative s-IgA levels that was reported by Neville et al. (2008). However, seven other players in this study also experienced one week when their s-IgA concentration and secretion rate were less than 40% of their individual healthy baseline, with several additional weeks when their s-IgA levels were below 70% of baseline. This demonstrates the multifactorial nature of URS risk as mucosal immune status is just one factor that can influence risk of experiencing URS (Campbell & Turner, 2018). It is possible that the other seven players with reduced mucosal immunity did not come in to close contact with external infectious agents during those weeks, and as a result did not become ill with URS when their s-IgA levels were at the lowest. It is important to note that the two players who did experience URS during the monitoring period lived with young children. Exposure to infectious pathogens via young children in the home may be a key factor to consider when assessing an individual athlete’s level of risk for URS alongside markers of mucosal immunity. For future studies, a longer monitoring period covering all of the winter months, and the full competitive football season, may capture more illness episodes and subsequently allow for full statistical analysis of relative s-IgA, incidence of URS, and other possible risk factors for URS such as living with young children.

The low number of URS episodes experienced by players has also limited the ability to further examine a relationship between detection of EBV DNA in saliva, s-IgA levels, and URS as previously reported by Yamouchi et al. (2011). The role of non-primary EBV reactivation as the direct cause of URS has been questioned (Cox et al., 2004), with some suggestion that monitoring EBV reactivation could instead function as an *in vivo* marker of immunodepression in professional athletes. The results presented here for the two individual URS episodes do indicate viral shedding and lowered s-IgA that precede the onset of URS, with the absence of EBV DNA detection during illness. However, given the high variability of individual shedding frequency between the six healthy players and the two players who experienced URS (ranging from 11 to 78%), it is difficult to evaluate the role of EBV reactivation as an *in vivo* marker of T-cell mediated immune function. Therefore, positive EBV saliva samples should be interpreted with caution as viral DNA detection does not necessarily infer a state of immunodepression and an increased risk of imminent infection.

The physical load of training and match play was not measured in this study, which is an obvious limitation. Players could therefore only be identified as either regular first team starting players or regular substitutes based on the number of minutes played in first team matches. There was no difference in s-IgA concentration or secretion between the two groups of players at any timepoint throughout the monitoring period, and there was also no difference in mean healthy baseline values between starters and non-starters. These findings, despite a lack of GPS, HR and/or RPE data, could suggest that changes in s-IgA levels are responsive to more than just fluctuations in the physical load of competitive match play. All players in the first team squad experienced a reduction in relative s-IgA concentration and secretion rate during weeks 8-12 when the fixture

schedule was most congested. Furthermore, the significant decline in s-IgA levels seen in week 8 was preceded by two consecutive away fixtures. Interestingly, after a period of two home games in week 12 the non-starters experience a greater rise in s-IgA concentration from week 12 to 13 in comparison to the regular starters, although this did not reach significance. Travelling for away fixtures could have a negative effect on the mucosal immune system via a disruption to nutrition routines and a reduction in sleep quality (as well as the psychological stress of pressure to win that is present with all matches) that is likely to be consistent across all players within a squad irrespective of their physical stress from training and matches. Sleep, in particular, has been shown to be disturbed following evening matches with a significant decrease in objectively measured sleep quality in professional rugby players (Eagles et al., 2014) and subjectively reported sleep duration in professional football players (Fullagar et al., 2016). Psychological stress is known to be a significant factor in the regulation of s-IgA levels due to the secretion of s-IgA being under control of the sympathetic and parasympathetic nervous system (Engeland et al., 2016). Measurement of these additional sources of stress and immune suppression could be included in future investigations in order to further understand the impact of each individual risk factor on overall immune health and URI risk. The inclusion of a non-playing control group (e.g. support staff and coaches at the football club) that would be exposed to the same psychologically stressful environment, disruptions to sleep and nutrition routines, as well as the same changes in season and climate, and exposure to infections during work hours would be useful additions to future studies.

s-IgA is known to be highly variable within and between individuals. The within-player variability for absolute s-IgA concentration reported here is similar to other investigations including professional athletes (Gleeson et al., 1999; Neville et al.,

2008). Other authors have indeed discussed the importance of monitoring changes in s-IgA levels on an individual basis as a result of finding such high variability within individual study participants (Neville et al., 2008; Cunniffe et al., 2011; Gleeson et al., 2017). High sampling frequency is key to individual monitoring when variation is high. A strength of this study is the protocol of weekly sampling that was used throughout the 16-week monitoring period, and also the exclusion of any players missing two consecutive weeks of samples or more than 25% of sampling time points. Furthermore, s-IgA production follows a circadian rhythm of peak production in the morning that declines throughout the day (Li & Gleeson, 2004). Our ability to collect samples at the same time of day each week, and after at least 36 hours of match recovery, therefore also helped to reduce a potential source of additional variation in the saliva samples. This is not always possible to control in studies involving professional athletes.

The present study reported s-IgA levels as both concentration and secretion rate, whereas previous longitudinal work has commonly been limited to reports of just s-IgA concentration (Cunniffe et al., 2011; Neville et al., 2008; Morgans et al., 2012). In an acute exercise setting, a reduction in saliva flow rate due to a removal of parasympathetic sympathetic activation during exercise can indirectly effect s-IgA concentration (Proctor & Carpenter, 2007). For this reason, calculation of s-IgA secretion rate has been used to determine the actual availability of s-IgA in saliva secretions during and immediately after exercise. While dehydration should be less of a confounding factor in longitudinal research (saliva samples are collected after at least 24 hours of rest), it is still possible that players were dehydrated and without analysis of urine or plasma osmolality it is impossible to say for certain that all players were fully re-hydrated at the point that samples were collected. As there was a similar response to both concentration and secretion rate of s-IgA at the

whole group level, the results of this study possibly suggest that either measure of s-IgA levels can be used in longitudinal research to monitor changes on an individual level (provided regular samples are collected, and individuals have had at least 24 hours recovery).

In summary, the results of this investigation show that s-IgA is responsive to intensified periods of match play. As such, monitoring s-IgA on an individual basis can be used as a non-invasive and objective tool to monitor the mucosal immune status of professional team sport athletes, provided that samples are collected on a regular basis and with necessary controls in place to reduce the high level of variation that is known to occur when measuring levels of s-IgA. Inclusion of additional measures to monitor other potential immune modulating factors such as physical load from training and matches, sleep quality, nutrition, and psychological stress may add to our understanding of the physical and psychological stressors that influence risk of upper respiratory illness. Analysis of saliva samples for the presence of EBV *Bam*HI can be used to determine EBV serostatus from a saliva sample. However, identification of current viral status with qualitative measures should be interpreted with caution and requires further investigation with higher numbers of participants and URS episodes.

Chapter 4. Study 2 - Epstein-Barr Virus Reactivation, Salivary Immunoglobulin A, and Upper Respiratory Symptoms in Sub-Elite Endurance Training Adults

Abstract

Introduction: Reactivation of Epstein-Barr virus (EBV) has been linked to immune depression, and incidence of upper respiratory symptoms (URS) in elite endurance athletes. Conflicting evidence as to the exact role that EBV reactivation plays in the occurrence of URS does exist. However, evidence of lowered s-IgA levels and detection of EBV DNA in saliva that precede URS has raised the possibility of combining the two salivary immune markers in a risk monitoring model for URS in athletes. **Aim:** To examine the relationship between detection of EBV DNA in saliva, changes in s-IgA levels, and incidence of URS in a group of sub-elite endurance training adults. **Methods:** Over a 6-month monitoring period 30 participants (19 male, 11 female) provided weekly saliva samples, recorded URS using a self-report questionnaire, and recorded all training sessions. Participants were retrospectively assigned to either URS (experienced at least one URS episode during study period) or HEALTHY (no URS episodes) groups. Saliva was analysed for s-IgA (ELISA) and EBV DNA (qPCR). The mean training hours per week was calculated. **Results:** All 30 participants were seropositive for previous EBV infection. 17 participants experienced at least one URS episode, with a total of 27 individual URI episodes reported. Individual EBV DNA shedding frequency was not significantly different between URS and HEALTHY groups (32 ± 22 vs 53 ± 35 %, $P > 0.05$), and was not correlated to s-IgA baseline levels or URS incidence ($P > 0.05$). Mean healthy baseline scores for s-IgA concentration and secretion rate were significantly higher in the HEALTHY group ($P < 0.05$). Mean weekly training load had no effect on baseline s-IgA scores, EBV DNA shedding frequency, or URI incidence ($P > 0.05$). **Conclusion:** Individual shedding frequency of EBV DNA is highly variable between individuals and does not appear to be linked to incidence of URS in sub-elite endurance training adults.

4.1 Introduction

The general adult population experience one to three individual episodes of upper respiratory tract infection (URTI) per year (Bayer et al., 2014; Fendrick et al., 2003), with illness incidence peaking during winter months in the Northern Hemisphere (Heikkinen & Jarvinen, 2003). Research into the occurrence of upper respiratory symptoms (URS) in elite athletes has shown a similar frequency, but the timing of these URS episodes seems to be related to periods of intensive training and/or competition in cross-country skiers (Svendsen et al., 2016) and swimmers (Hellard et al., 2015). URS can result in a loss of training days and a performance decrement (Cunniffe et al., 2011; Reid et al., 2004; Svendsen et al., 2016), therefore the ability to monitor athletes' immune status in order to assess the risk of imminent URS may be of interest to sport scientists and coaches.

Increased risk of URS in professional athletes has been shown to be associated with decreased levels of secretory immunoglobulin A (s-IgA) in saliva (Gleeson et al 1999; Neville et al 2008). However, due to the high inter-individual variability of s-IgA there are currently no established clinical reference values or critical thresholds for s-IgA concentration or secretion rate, and level of risk of imminent URS. Previous longitudinal investigations into s-IgA levels and upper respiratory illness (URI) in athletes have been limited by low sampling frequency (Cunniffe et al., 2011; Leicht et al., 2012), shorter monitoring periods (Morgans et al., 2014), and/or low incidence of URI (Gleeson et al., 2017).

Neville et al. (2008) were able to overcome these common limitations via the collection of saliva samples on a weekly basis over a period of 12 months. These authors proposed a model for monitoring URI risk that requires the calculation of a healthy baseline level for s-IgA concentration for each individual athlete, and

subsequent monitoring of percentage changes in s-IgA concentration on a weekly basis. A fall in individual s-IgA levels to less than 40% of the calculated bassline was associated with a 50% chance of experiencing URI over the following two weeks. This method of monitoring s-IgA levels has not been further investigated in endurance sports.

Reactivation of Epstein-Barr virus (EBV) has also been linked to immune depression (Mehta et al., 2000) and proposed as a possible cause of URS in elite athletes (Gleeson et al., 2002). Conflicting evidence as to the exact role that EBV reactivation plays in the occurrence of URS does exist, as Cox et al. (2004) found no evidence of EBV reactivation being associated with occurrence of URI episodes in a group of elite runners. However, evidence of lowered s-IgA levels and detection of EBV DNA in saliva that precede URS (Gleeson et al., 2002; Yamauchi et al., 2011) has raised the possibility of combining the two salivary immune markers in a risk monitoring model for URS in athletes.

Therefore, the primary aim of this study was to examine changes in s-IgA levels, detection of EBV DNA in saliva, and incidence of URS over a 6-month training period. The secondary aim was to further investigate relative changes in s-IgA levels alongside URS in a group of endurance training adults. It was hypothesised that URS incidence would be greater in this study than in study 1, and that the frequency of EBV reactivation would increase immediately before URS.

4.2 Methods

4.2.1 Participants

Of the forty-seven participants who volunteered to take part in this study, 17 were lost to follow-up (six participants provided less than 10 saliva samples, and 11 participants did not return any saliva samples). The remaining thirty adults (19 male, 11 female adults: age 41.3 $\pm$ 14.0 years) that had been engaging in endurance training for at least three years (23 runners, 3 cyclists, 4 triathletes) returned their completed illness logs and frozen saliva samples. Participants were retrospectively identified as URS if they reported at least one illness episode, or as HEALTHY if they remained free from URS throughout the monitoring period. All participants provided written informed consent and were free to withdraw at any time. Ethical approval was provided by the University of Kent School of Sport and Exercise Sciences Research Ethics Committee.

4.2.2 Study Design

Over a 6-month monitoring period that included autumn and winter months, participants provided weekly saliva samples, reported URS using a self-report questionnaire, and recorded the duration of all training sessions in a hand written diary. Participants were fully familiarised with the protocol for providing a rested saliva sample, and recording URS using the self-report illness questionnaire.

4.2.4 EBV serology

EBV serostatus of all participants was determined according to the method detailed in chapter 2.

4.2.5 Saliva collection

Timed, unstimulated saliva samples were collected upon waking after at least 24 h of no exercise training. Samples were stored in participants' home freezers and transported to the laboratory under ice at the end of the monitoring period.

4.2.6 Saliva analysis

For participants in the URS group, DNA extractions were carried out on all saliva samples that were provided when URS were present, as well as three saliva samples that were provided before the appearance of symptoms and also two samples provided after the cessation of symptoms. Participants from the HEALTHY group were matched as closely as possible to those in the URS group (according to gender, age, and training mode). DNA extractions were then also carried out on saliva samples from the HEALTHY group that were time matched to periods of illness from the URS group. The presence of EBV DNA and human DNA in saliva was determined via quantitative polymerase chain reaction (qPCR) according to the method outlined in chapter 2.

All saliva samples provided by participants in URS and HEALTHY groups were analysed for s-IgA concentration and secretion rate according to the method outlined in chapter 2.

4.2.7 Training Load

Participants recorded the duration for each training session throughout the monitoring period. The mean number of hours per week for endurance training sessions was calculated and participants were retrospectively assigned to groups of moderate (6-10 h/wk) or high (>11 h/wk) weekly training load for further statistical analysis.

4.2.8 Statistical Analysis

Normally distributed data are expressed as mean (± SD) with non-normally distributed data expressed as the median and interquartile range (IQR). The level of significance is set at $P < 0.05$.

Un-paired samples t-test was used to assess differences in healthy baseline values for s-IgA concentration and secretion rate between the URS and HEALTHY groups. Repeated measures ANOVA was used to compare changes in relative s-IgA concentration and secretion rate before, during, and after URS episodes. Non-normally distributed data were log transformed to meet the requirements of ANOVA.

EBV DNA shedding frequency was calculated for each individual participant as the percentage of saliva samples that were positive for EBV DNA. The mean concentration of EBV DNA across all saliva samples provided by each individual participant was calculated. A Pearson correlation was carried out between EBV shedding frequency and s-IgA baseline scores, and also EBV shedding frequency and URS incidence. Un-paired samples t-test was used to assess the difference between URS and HEALTHY groups for individual shedding frequency, and also mean EBV DNA concentration.

Repeated measures ANOVA was used to compare the changes in concentration of EBV DNA in saliva provided three weeks before URS, during URS, and two weeks after URS episodes.

After grouping participants according to mean weekly training hours, un-paired samples t-tests were used to assess differences in healthy baseline values for s-IgA concentration and secretion rate, number of URs episodes, individual EBV

shedding frequency, and mean EBV DNA concentration between the groups of moderate and high training load.

All statistical analyses were carried out using SPSS (IBM SPSS Statistics for Windows, version 24.0, Armonk, NY:IBM Corp).

4.3 Results

100% of participants were seropositive EBV at the onset of the study.

4.3.1 URS

17 participants experienced at least one episode of URS during the monitoring period, with a total of 27 individual URS episodes reported. 13 participants did not experience any symptoms of URS during the 6-month monitoring period. Illness incidence on average was 1 ± 1 episode per participant with symptoms lasting for 6 ± 3 days.

4.3.2 EBV DNA

DNA was extracted from a total of 306 saliva samples, with EBV DNA detected in 124 samples (41%). Of the total 30 participants, all but one participant (in the URs group) (96%) had at least one sample that was positive for EBV DNA. One participant (HEALTHY group) had 100% of samples (8/8) positive for EBV DNA. All 306 saliva samples were positive for human DNA, which served as an endogenous control for the extraction process.

Of the total 306 saliva samples that underwent the DNA extraction and qPCR process, 204 were provided by 15 of the 17 participants in the URS group. Two

participants' samples were too small in volume for DNA extraction (at least 200 µl required), which reduced the total number of illness episodes for analysis of EBV DNA to 25 individual episodes. There was no correlation between shedding frequency and URI incidence ($P > 0.05$).

For the URS group, 70/204 extracted saliva samples were positive for the presence of EBV DNA (34%). The mean (± SD) for the individual shedding frequencies of the URS group was 32 ± 22%. One participant was experiencing symptoms at the start of the monitoring period, which reduced the number of URS episodes with pre-URS data for inclusion in ANOVA analysis to 24 individual URS episodes. EBV DNA was detected in saliva collected the week before and the week of URI for 46% and 33% of the 24 individual illness episodes (Figure 4.1).

For the HEALTHY group, 54/102 saliva samples analysed by qPCR were positive for EBV DNA (53%) (Figure 4.1). All participants in this group had at least one positive sample. Mean (SD) individual shedding frequency was 53 ± 35%.

There was no significant difference between URS and HEALTHY groups for individual EBV shedding frequency ($P = 0.14$) and mean EBV DNA concentration ($P = 0.44$).

The concentration of EBV DNA in saliva samples provided before, during and after URS episodes was highest at 3 and 2-weeks pre-URS, but there was no significant difference in EBV DNA concentration before, during, or after URI ($P < 0.05$) (figure 4.2).

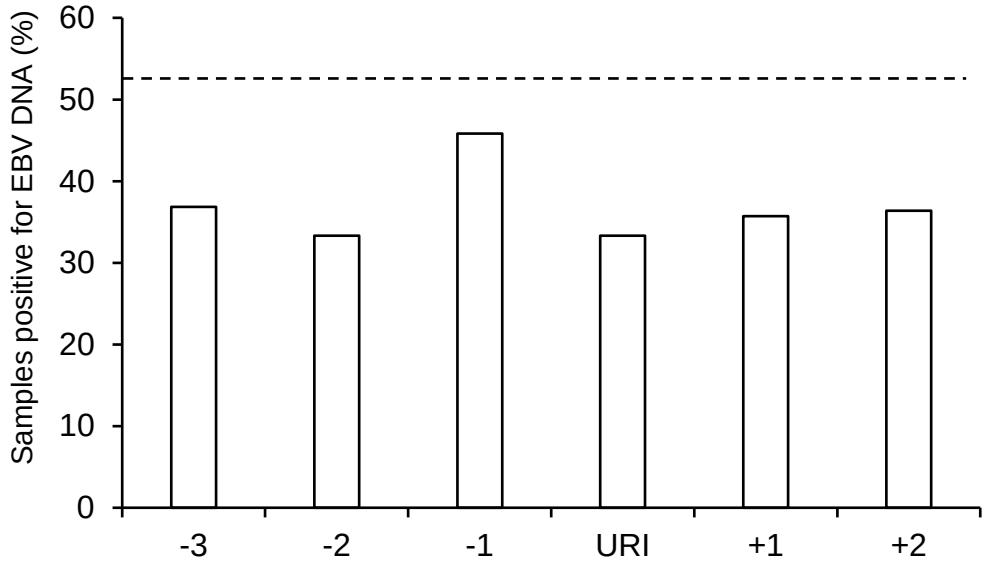

Figure 4.1 Percentage of total number of saliva samples containing EBV DNA for the three samples provided pre-URS, one during URS, and two-post URS (n = 24 URI episodes). Dashed black line indicates mean individual shedding frequency for HEALTHY group (53%).

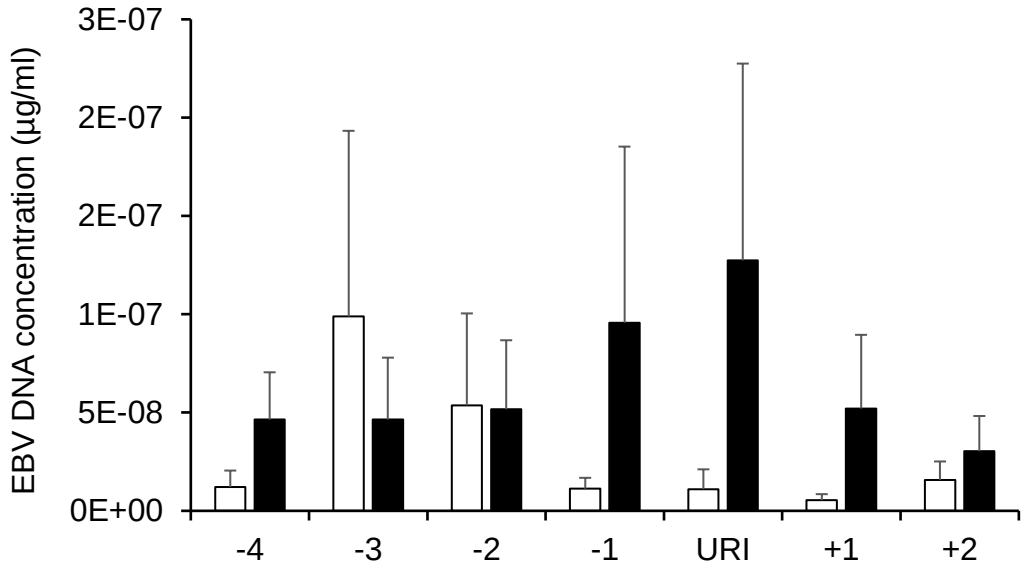

Figure 4.2 EBV DNA concentration for the four samples provided pre-URS, one during URS, and two post-URS. White bars represent URS group (n=24 episodes). Black bars represent the time matched period for the HEALTHY group (n=13 matched periods). Values are mean ± SEM (SEM used to improve clarity of the figure).

4.3.3 Secretory Immunoglobulin A

A total of 663 saliva samples were analysed for s-IgA. Median (IQR) s-IgA concentration for all participants was 363 (270-431) mg/L with a mean CV of 35%, and a between-subject CV of 49%. Mean s-IgA secretion rate was 119 ± 67 mg/min with a mean CV of 48%, and a between CV of 74%.

Participants in the HEALTHY group had significantly higher healthy baseline s-IgA concentration median (IQR) (410 (340-443) vs 297 (179-393) mg/L) and secretion rate (111 (94-178) vs 96 (57-123) mg/min) in comparison to the URS group ($P < 0.05$, Figure 4.3 A and B). When expressed as a percentage of healthy baseline levels, s-IgA concentration and secretion rate were significantly elevated for the two weeks after a URS episode in comparison to when URS was present ($P < 0.05$). There was no significant change in s-IgA levels in the weeks before URS ($P > 0.05$) (figure 4.4 A and B). There was no correlation between individual EBV shedding frequency and baseline s-IgA concentration or secretion rate ($P > 0.05$).

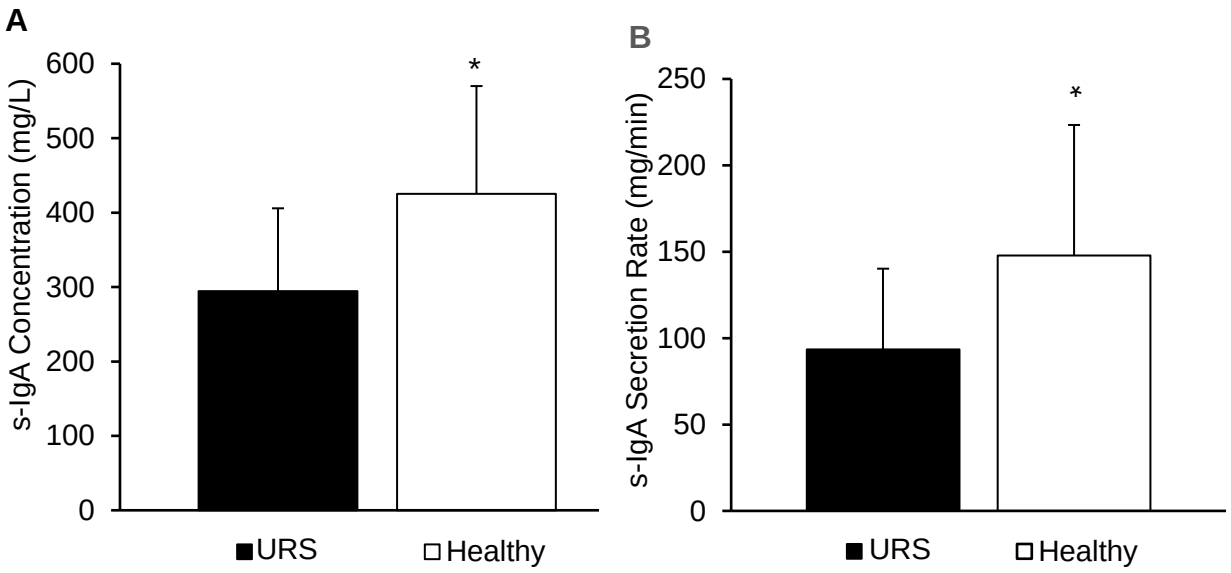

Figure 4.3 Mean healthy baseline s-IgA concentration (A) and secretion rate (B) for the URS group (black bars, n=17) and HEALTHY group (white bars, n=13). Values are mean (± SD). *Significantly greater than URI group ($P < 0.05$).

4.3.4 Training Load

16 participants were identified as engaging in a moderate volume of endurance training (8 ± 1 hours per week) and 14 were identified as high (13 ± 1 hours per week). For the moderate and high training load groups there was no difference in healthy baseline s-IgA concentration (344 (216-426) vs 370 (291-428) mg/L, $P > 0.05$) or secretion rate (103 (73-134) vs 110 (107-201) mg/L, $P > 0.05$), number of URS episodes (1 ± 1 vs 1 ± 1, $P > 0.05$), or individual EBV shedding frequency (35 ± 29 vs 50 ± 31 %, $P = 0.09$) between the two groups.

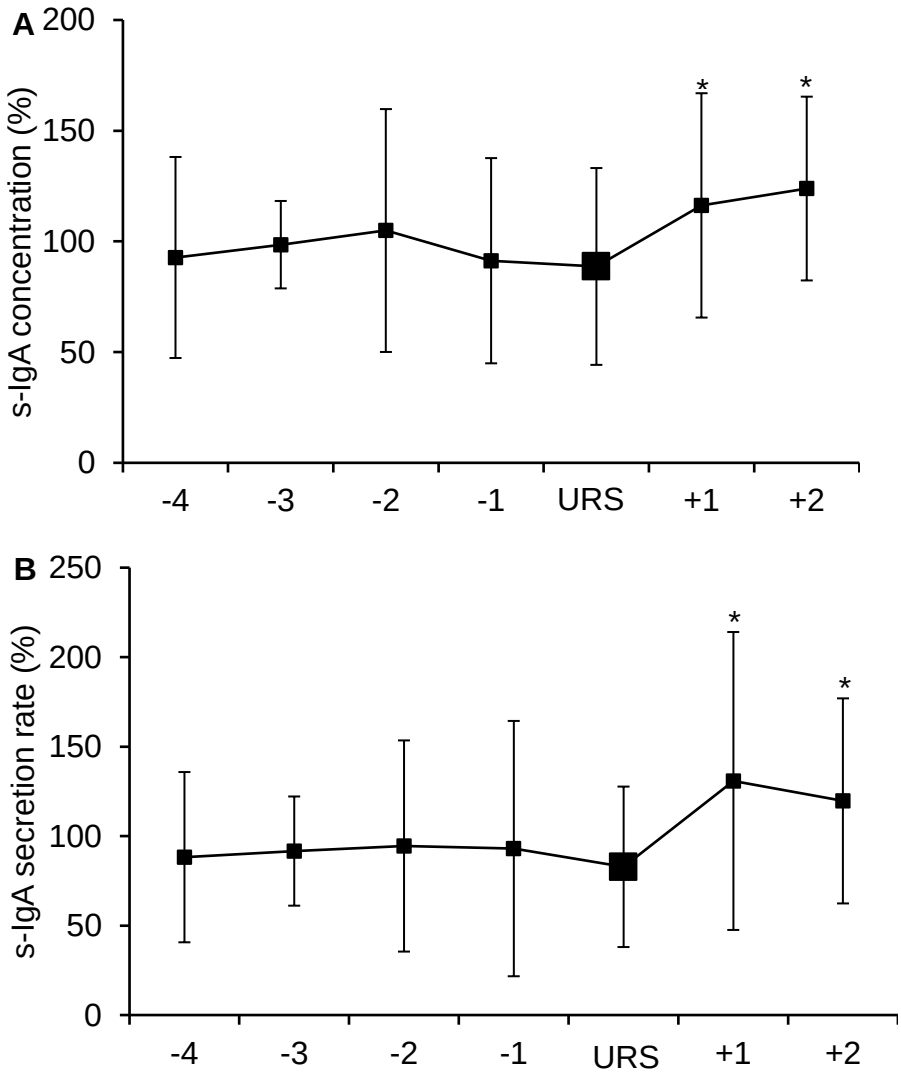

Figure 4.4 Relative s-IgA concentration (A) and secretion rate (B) for the four weeks pre-URS, during URS, and two weeks post-URS (n=26). Values are mean (± SD). *Significantly different to URS ($P < 0.05$).

4.4 Discussion

The results of this study suggest that EBV reactivation, and subsequent shedding of viral DNA into saliva, is highly variable between individuals and is not related to incidence of URS. This is not in agreement with the study hypothesis of increased frequency of EBV reactivation immediately before URS. Furthermore, for the present group of sub-elite endurance training adults, there is no clear relationship between changes in s-IgA levels and detection of EBV DNA, or incidence of URS.

Previous studies of elite athletes have suggested a positive relationship between the frequency of EBV positive saliva samples and URS incidence (Gleeson et al., 2002; Gleeson et al., 2017). Interestingly, for the present group of sub-elite endurance training adults, the mean viral shedding frequency was 21% higher (although not significantly higher, $P = 0.14$) for the group that did not report any URS over the 6-month monitoring period. Furthermore, EBV DNA was only detected in saliva during the seven days before URS or during URS for 30% and 45% of illness episodes respectively, which is lower than the overall mean shedding frequency for the HEALTHY group (53%). Therefore, there is no clear evidence from this study to suggest that increased frequency of viral shedding in sub-elite athletes is linked to URS.

Reactivation of EBV infected memory B-cells from the latent to the lytic life cycle results in infection of epithelial cells of the oropharynx, release of new viral cells directly into saliva, and a specific CD8+ T-cell response that is instigated by the expression of lytic genes occurring as the infected B-cells replicate (Rickinson & Moss, 1997). The inability of the immune system to control the actively replicating infected epithelial cells, identified by an increase in the amount of EBV DNA present in saliva, may be an *in vivo* marker of immunodepression. Previous

studies utilising *in vitro* methods have demonstrated a reduction in cell-mediated immunity after periods of intensified training. For example, an increase in weekly training load for 1-3 weeks has been shown to result in lowered levels of circulating type 1 T-cells in cyclists at rest (Lancaster et al., 2004), and reduced T-cell proliferation after acute exercise in runners (Verde et al., 1992). Therefore, it is plausible that heavier training loads may be associated with increased EBV shedding frequency and/or a higher concentration of viral DNA measured in saliva, as a reflection of the exercise-induced depression in T-cell function. In the present study, EBV shedding frequency was 15% higher in HIGH training load group, but this difference did not reach statistical significance ($P = 0.09$). There was also no difference in illness incidence or resting s-IgA levels between the two training load groups. No assessments of T-cell number or function were carried out alongside collection of saliva samples so it is not possible to examine any possible relationship between *in vivo* immune markers (EBV shedding frequency), cell mediated immune status, and training load in this study. The method for quantification of weekly training load used in the present study is also a limitation as it does not account for training intensity. Inclusion of a metric for training intensity such as recording session rating of perceived exertion (RPE) or pre-study identification of heart rate (HR) training zones for use with training intensity models such as the training impulse (TRIMP) method (Foster et al., 2001) may provide a more robust method for quantification of training load, and subsequent statistical analysis, in future studies.

The results of this study suggest that shedding frequency is highly variable between individuals with individual shedding frequency ranging from 0-100% across all participants, with at least one positive sample being provided by 96% of all participants. By comparison, in previous studies, 58-100% of seropositive study

participants provided at least one EBV positive saliva sample during a period of immune monitoring (Cox et al., 2004; Gleeson et al., 2002; Mehta et al., 2000; Payne et al., 1999). For this group of sub-elite athletes, 41% of all saliva samples were positive for EBV DNA. This is also higher than previous studies of elite athletes with viral shedding frequencies across all saliva samples reported in the range of 17-27% (Mehta et al., 2000; Pierson et al., 2005; Reid et al., 2004; Yamauchi et al., 2011). The majority of previous EBV research involving exercising populations has identified viral status (i.e. latent or lytic life cycle) by stating whether or not EBV DNA was detected in saliva. This form of qualitative data does not allow for further investigation into changes in viral load that may reflect the effectiveness of the specific cell-mediated response in controlling viral replication in the oropharynx. In the present study, quantitative measures were used to attempt to investigate changes in the concentration of EBV DNA in relation to URS incidence. EBV DNA concentration does appear to be elevated at three- and two-weeks before the appearance of symptoms. However, it is important to note that mean viral DNA concentration across all qPCR analysed saliva samples was not different between the URS and HEALTHY groups, which does not support previous suggestions of a positive relationship between EBV reactivation and URS (Gleeson et al., 2002). The low incidence of EBV positive saliva samples provided in the weeks before and during URS, has limited the ability to carry out statistical analysis on changes in viral load, but the low number of Pre-URS positive samples may in itself may indicate the absence of a meaningful relationship between EBV reactivation and URS. The high variability in shedding frequency that is consistent across the literature indicates the need to include quantitative measures of viral load to aid the future investigation into EBV reactivation and URS in elite athletes.

Healthy baseline values for s-IgA concentration and secretion rate were 30-40% lower for people who experienced at least one URS episode during the 6-month monitoring period. However, when s-IgA concentration and secretion rate were expressed as a percentage of calculated individual healthy baseline levels, the relative s-IgA values did not significantly decrease in the one-to-two weeks immediately before or during URS. This is not consistent with previous studies that have showed a strong relationship between decreasing levels of s-IgA and increased risk of imminent URI in athletes (Neville et al., 2008). Furthermore, this does not support previous findings of a relationship between lowered s-IgA and detection of EBV DNA that precedes URS as neither of these salivary markers showed agreement with previous findings (Gleeson et al., 2002; Yamouchi et al., 2011). Despite no evidence of lowered s-IgA preceding URS, there was a characteristic increase in s-IgA after illness that demonstrates a mucosal immune response to infection.

The healthy baseline values for s-IgA concentration and secretion rate for the URS and HEALTHY groups in the present study were noticeably higher than those previously reported for elite athletes (Cunniffe et al., 2011; Fahlman & Engles, 2005; Gleeson et al., 2002; Neville et al., 2008) and also the professional football players in Study 1 (with the latter analysed in the same lab with the same assay and equipment). However, the high within- and between-subject variability for resting s-IgA was in agreement with previous studies of elite athletes. This difference in resting s-IgA levels may in part be due to the difference in weekly training load between elite athletes and the present group of sub-elite endurance training adults (Svendsen et al., 2016) as completing 20-25 hours per week of swimming training has been shown to reduce s-IgA levels in apparently healthy elite athletes (Gleeson et al., 1999b). Similar to the findings of the present study,

previous research has also found no relationship between s-IgA levels and training load in non-elite endurance training individuals (Ihalainen et al., 2016), and when mean weekly training load was similar to the present study (7-10 hours and >11 hours per week identified as moderate and high) (Gleeson et al., 2011b). In a study of elite athletes Gleeson et al. (2017) identified a s-IgA concentration value of 60 mg/L as a threshold above which the risk of URS was considered to be low. This threshold value for elite athletes is five times smaller than the healthy baseline score for participants in the URS group. This possibly suggests that groups of sub-elite endurance training adults with lower training loads (compared to elite athletes) do not reach the same critical threshold for s-IgA level and risk of imminent URS, which in-turn possibly indicates that EBV reactivation is not a reliable tool to assess immune status in sub-elite endurance athletes. In order to fully investigate the utility of EBV reactivation as an *in vivo* tool to assess URS risk, future investigations of elite athletes should aim to include fully quantitative PCR alongside s-IgA analysis with sampling frequency kept as high as practically possible in order to investigate day-to-day fluctuations in viral load.

In summary, there is no evidence of a relationship between EBV DNA detection and URS incidence in this group of sub-elite endurance training adults. However, inclusion of quantitative changes in viral load in future work aiming to investigate the relationship between viral replication and URS in elite athletes may be useful. Baseline levels of s-IgA were lower for participants who experienced episodes of URS during the 6-month monitoring period, but there was no evidence of lowered s-IgA in the two weeks before URS or during URS.

Chapter 5. Summary of longitudinal monitoring of EBV reactivation, URS, and s-IgA in athletes

5.1 Introduction

Previous longitudinal studies of elite athletes have suggested a relationship between detection of EBV DNA, lowered s-IgA levels, and onset of upper respiratory symptoms (URS) (Gleeson et al., 2002; Yamouchi et al., 2011). However, the role of EBV as the causative agent in the appearance of URS has been questioned (Cox et al., 2004). The overall aim of studies 1 and 2 was to investigate the use of monitoring EBV reactivation as an *in vivo* marker of immune status and risk of imminent URS by examining the temporal relationship between EBV DNA detection and URS incidence alongside other mucosal immune markers that have previously been shown to be linked with URI (Neville et al., 2008). The data from these two longitudinal monitoring studies has been combined for further analysis into the occurrence of viral shedding and URS this chapter.

5.2 Methods

5.2.1 Participants

This chapter has pooled together the longitudinal data for EBV reactivation and URS incidence for a total of 36 participants across studies 1 and 2. This includes eight of the football players from study 1 (DNA extractions were only carried out on 8/16 players' saliva samples) and 28 of the sub-elite endurance athletes of study 2

(two participants' samples were too small in volume for the DNA extraction process).

Further analysis of s-IgA levels incorporates all participants from studies 1 and 2; 16 football players and 30 sub-elite endurance athletes (total n=46).

Participants were assigned to the URS group if they experienced at least one URS episode, or to the HEALTHY group if they remained free from URS throughout the monitoring period.

All participants were seropositive for previous infection with EBV.

5.2.2 Saliva analysis

All saliva samples included in the analyses for EBV DNA and s-IgA from studies 1 and 2 were included in the analysis for this chapter.

5.2.3 Statistical analysis

An un-paired t-test was used to examine differences between the URS and HEALTHY groups for individual shedding frequency, and EBV DNA concentration.

An un-paired t-test was used to examine the difference in healthy baseline s-IgA concentration and secretion rate (i.e. mean s-IgA levels of all weeks when no URS were present) between the whole group of football players of study 1 and the whole group of sub-elite endurance athletes of study 2. The same analysis was carried out to compare baseline s-IgA levels for the two individual HEALTHY groups from studies 1 and 2. A comparison of the URI groups from each study could not carried out due to having just two URI participants for study 1.

5.3 Results

5.3.1 Upper respiratory symptoms

When grouped together, 17/36 (47%) participants experienced at least one episode of URS and 19/36 (53%) remained healthy over a monitoring period of 4-6 months. The 17 participants in the URS group experienced a total of 26 individual illness episodes.

5.3.2 EBV DNA

A total of 376 saliva samples from studies 1 and 2 underwent the DNA extraction process, with an average of 10 (±3) samples provided per participant.

The overall mean individual shedding frequency of the HEALTHY group was similar to the URS group (49 vs 32%, $P = 0.15$) (table 5.1). Similarly, mean concentration of viral DNA across all saliva samples was similar between the HEALTHY and URS groups (1.60 vs 1.16 ng/µl $x10^{-7}$, $P = 0.60$) (table 5.1).

EBV DNA was detected in saliva in the week before URS were first reported for 12/26 episodes (46%), and when URS were present for 9/26 episodes (35%) (table 5.2).

Table 5.1 Individual shedding frequency and concentration (mean ± SD) of EBV DNA in saliva samples provided by participants in the URS and HEALTHY groups of studies 1 and 2.

	Shedding frequency (%)	EBV DNA concentration (ng/µl $x10^{-7}$)
All participants (n=36)	42 (29)	1.37 (0.44)
HEALTHY (n=19)	49 (32)	1.60 (0.81)
URI (n=17)	35 (24)	1.16 (0.54)

Table 5.2 Percentage of total number of saliva samples that were positive for EBV DNA provided three weeks before URS, during URS, and two weeks after URS for participants in the URS groups of studies 1 and 2 (n=26 URS episodes).

	-3	-2	-1	URS	+1	+2
Positive samples (%)	38	35	46	35	38	38

5.3.3 s-IgA

Healthy baseline levels of s-IgA concentration and secretion rate were significantly lower for the whole group of professional football players of study 1 in comparison to the whole group of sub-elite endurance athletes of study 2 ($P < 0.05$, table 5.3). Similarly, the baseline levels for s-IgA concentration and secretion rate for the HEALTHY group from study 1 were also lower than the HEATHY group from study 2 ($P < 0.05$, table 5.3).

Table 5.3 Baseline s-IgA concentration and secretion rate (median (IQR) and mean (± SD). Individual baseline scores for players 5 & 8 are reported as Study 1 URS data. * Indicates significantly different to Study 1 HEALTHY. # indicates significantly different to study 1 ALL.

	URS	HEALTHY	ALL
Study 1			
s-IgA Concentration (mg/L)	158 & 138	113 (98 - 126)	107 (76 - 150)
s-IgA Secretion rate (mg/min)	117 & 55	64 (39 - 67)	51 (30 - 78)
	(n=2)	(n=13)	(n=15)
Study 2			
s-IgA Concentration (mg/L)	297 (179-393)	410 (340-443)*	363 (270-431)#
s-IgA Secretion rate (mg/min)	96 (57-123)	111 (94-178)*	119 (± 67)#
	(n=19)	(n=11)	(n=30)

5.4 Discussion

The EBV and URS data from studies 1 and 2 presented in this chapter does not support previous suggestions of a meaningful relationship between detection of EBV DNA in saliva and incidence of URS in elite athletes (Gleeson et al., 2002; Yamouchi et al., 2011). The significant difference in s-IgA healthy baseline levels between the group of professional football players and sub-elite endurance athletes supports the J-shaped model for URI risk (Nieman, 1994).

Combination of the data from studies 1 and 2 has helped to overcome the statistical underpowering caused by a low URS incidence in study 1. For this combined data, EBV DNA was detected in saliva collected during the week before URS for 46% of individual URS episodes. This detection rate is lower than the rate of EBV shedding pre-URS reported in previous work from Gleeson et al. (2002), where the authors suggested a positive relationship between EBV shedding and URS occurrence. However, the work from Gleeson et al. (2002) does not include a large HEALTHY group for comparison, and is relatively underpowered with a total of 6/9 individual URS episodes being associated with EBV DNA detection (in comparison to the present analysis that includes 26 individual episodes of URS). It is important to note that the detection rate for EBV DNA in the weeks before and during URS (46% and 35%) does not exceed the whole group mean individual shedding frequency for the HEALTHY group (49%). Reactivation of EBV from the latent to lytic life cycle is essential for viral survival as the virus must be actively replicating in epithelial cells of the oropharynx in order to infect naïve B-cells in the already infected host as well as disseminate to a new seronegative host (Gandhi et al., 2015). Therefore, detection of EBV DNA in saliva collected at rest may not necessarily be an *in vivo* sign of immunosuppression and increased risk of

imminent URS if indeed viral reactivation is a natural process with memory B-cells intermittently reactivating and infecting epithelial cells that will subsequently shed viral DNA into saliva. If, however, EBV reactivation is an *in vivo* sign of immunosuppression it may be plausible to expect a high detection rate of EBV DNA in saliva for groups of illness prone athletes, which was not the case in a previous study of athletes experiencing recurrent URTI where just 22% of athletes provided a positive saliva sample (Reid et al., 2004). The results of the first two studies of this thesis therefore suggest that “one off” saliva samples analysed for presence of EBV DNA should be interpreted with caution. The detection rate of EBV DNA in saliva is similar between periods of URS and symptom-free periods, therefore suggesting that a positive saliva sample does not indicate an increased risk of imminent URS or a state of immunodepression.

The use of fully quantitative qPCR has permitted calculation of viral DNA concentration. While EBV shedding *per se* may not be an in vivo marker of immunosuppression, the amount of DNA that is present in saliva could provide an indication as to the degree of viral reactivation and/or the ability of CD8+ cells to control the replicating viral cells in the oropharynx. However, the pooled data shows no significant difference in EBV DNA concentration between the HEALTHY and URS groups and therefore no support to previous suggestions that EBV reactivation is linked to URS incidence. Future studies of EBV reactivation may want to include fully quantitative qPCR in order to examine any relationship between exercise training load and EBV reactivation. The specific CD8+ T-cells that are responsible for controlling lytic activity may at times experience depressed cell function (particularly during periods of heavy training) resulting in a loss of control over actively replicating viral cells and a subsequent increase in the concentration of EBV DNA in saliva.

The healthy baseline s-IgA levels for the whole group of professional football players of study 1 were found to be significantly lower than the whole group of sub-elite participants of study. Taking the results of s-IgA baseline comparisons in isolation, this would appear to reflect the J-shaped model for illness risk (previously discussed in chapter 1; Nieman, 1994) as the higher-level athletes had lower resting levels of a key mucosal immune marker that would suggest a higher risk of URI (Neville et al., 2008). The group of elite athletes studied in this thesis did however experience fewer URS episodes, but they were monitored over a shorter period of four months in comparison to the six-month monitoring period for the sub-elite athletes. It is possible that more URS episodes may well have occurred in the winter months that followed the cessation of monitoring (January and February). The notable difference in s-IgA levels between the groups of professional football players and sub-elite endurance athletes highlights a key point for consideration in immune monitoring. If indeed a relationship between s-IgA and EBV reactivation does exist (as proposed by Gleeson eta l., 2002 and Yamouchi et al. 2011), it should perhaps be further investigated in large groups of elite athletes that are experiencing similarly high training loads and therefore experiencing greater changes in levels of mucosal immune markers. Furthermore, the large difference in healthy resting values between elite and sub-elite athletes highlights the need to clearly define the training status of research participants in future immune monitoring studies.

In summary, the detection rate of EBV DNA in saliva does not appear to differ between periods of URS (week before and when URS are present) and URS-free periods. Therefore, monitoring saliva samples for evidence of EBV reactivation may not be a reliable method for assessing immune status in elite or sub-elite athletes.

Chapter 6. Study 3 – Lytic reactivation of Epstein-Barr Virus does not occur after a single bout of prolonged cycling in well trained males

Abstract

Introduction: Prolonged exercise can cause a transient immune depression for up to 24 hours post exercise. However, it is currently unknown what effect this has on the control and subsequent reactivation of Epstein-Barr Virus (EBV) in the hours following a single bout of endurance exercise. **Aim:** To investigate the acute effect of prolonged cycling on EBV reactivation and other immune markers in blood and saliva up to 44 h post exercise. **Methods:** In a randomised crossover design ten club level male cyclists (mean ± SD: aged 31 ± 8 yrs; VO_2max 57.3 ± 10.0 ml/kg/min) completed 2.5 h of cycling at 20%Δ (20% of the difference between power at lactate threshold and power at maximal aerobic power added to power output at lactate threshold) and 2.5 h seated rest after an overnight fast. Unstimulated saliva samples were provided upon waking on the morning of each trial as well as the two following mornings. Unstimulated saliva and venous blood samples were collected immediately pre, post, and 1 h post exercise/rest. Saliva was analysed for s-IgA concentration and secretion rate (ELISA) and EBV DNA concentration (qPCR). Venous blood samples were analysed for EBV serostatus (ELISA) and cell counts for total leukocytes, neutrophils and lymphocytes. **Results:** 100% of participants were EBV seropositive. EBV DNA was detected in saliva of seven participants for at least one time point during the study period. Over the course of the exercise trial, 40% (4/10) of participants provided EBV positive saliva samples at both pre and post-exercise time points. There was no significant change in viral load during the exercise ($P > 0.05$) and rest trials ($P > 0.05$). Blood neutrophil count significantly increased pre-to-post-exercise (2.6 ± 0.5 to 7.5 ± 3.6 x 10^9 cells/L, $P<0.05$) and remained elevated at 1 h post (7.7 ± 3.0 x 10^9 cells/L, $P<0.05$). There were no significant main effects for time, trial, and time x trial interaction for s-IgA concentration ($P > 0.05$) or secretion rate ($P > 0.05$). **Conclusion:** Detection of EBV DNA in saliva did not change after acute exercise and therefore may not be a useful *in vivo* marker for monitoring short term changes in immune function post-exercise. Quantitative analysis of EBV DNA in saliva should be included in future investigations.

6.1 Introduction

Previous studies that have utilised *in vitro* immune markers before and after a single bout of acute prolonged exercise have provided evidence of suppressed T-cell mediated immunity (Lancaster et al., 2005) and highlighted the reduction in anti-viral immune defences post-exercise (Peake et al., 2017; Walsh et al., 2011), which seem to increase the risk of experiencing URS (Nieman, 1994; Pederson, Rhode & Ostrowski, 1998). However, the clinical relevance and utility of *in vitro* methods has been questioned (Walsh et al., 2011) and Albers et al. (2005) highlighted the importance of using *in vivo* measures to assess immune status as this represents the whole integrated immune response to exercise rather than function of isolated components (i.e. leukocytes).

In vivo methods, such as the measurement of cutaneous responses to application of a novel antigen (diphenylcyclopropenone, DPCP), represent a more clinically relevant method for examining T-cell-mediated responses to antigenic challenge following exercise. Previous research has consistently shown that prolonged, moderate intensity exercise reduces the induction and elicitation of *in vivo* immunity via T-cell-mediated responses that are independent of the stress hormone response to prolonged exercise (Davison et al., 2016; Diment et al., 2015; Harper Smith et al., 2011). This particular method of examining immune status *in vivo* is a robust technique that does not require invasive procedures such as venous blood sampling. However, this method is not always practical in a research or an applied environment. Researchers are limited to a between-subject study design when investigating the induction of a new immune response because naïve participants can only experience the first sensitisation once. Within-subject designs are possible, but researchers must first sensitise all participants over a

period of 4-8 weeks and the subsequent investigation is limited to the elicitation of existing immunity, which has been shown to be less sensitive to physical exercise stress (Harper Smith et al. (2011). In an applied sport science setting, contact hypersensitivity may not be practical as the reaction at the skin surface to the antigen (DPCP) can cause discomfort (swelling and possible blistering), and measurements of oedema and erythema require individuals to return to the laboratory at 24 and 48 hours after DPCP application. An *in vivo* immune marker that allows for a more feasible within-subject design is desirable to allow intervention studies such as nutritional or training interventions to be undertaken.

Monitoring the reactivation of Epstein-Barr virus (EBV), measured via collection of saliva samples, may provide a less invasive and time consuming (on the part of the participant) option for investigating *in vivo* immune function following exercise. There is currently limited evidence on the acute time course of EBV reactivation and appearance of EBV DNA in saliva after acute exercise as the only previous study to examine acute exercise and EBV reactivation (Gleeson et al., 2017) collected saliva samples at just the immediately post and 24-hour post-exercise timepoints (i.e. no samples were collected in the hours between exercise cessation and 24 hours post). Furthermore, the study from Gleeson et al. (2017) was limited to reporting qualitative results of EBV DNA detection, which does not allow for investigation into any possible changes in viral load after acute exercise. The clinical relevance of detecting EBV DNA in saliva at rest and after exercise is not fully understood. This has implications for future research on the influence of acute or short-term nutritional interventions and manipulations on immune status and URI in athletes. Therefore, the aim of this study was to investigate the acute changes in EBV shedding after a single bout of prolonged exercise. It was

hypothesised that the concentration of EBV DNA in saliva samples collected the morning after exercise would be higher than pre-exercise levels.

6.2 Methods

6.2.1 Participants

Ten male cyclists volunteered to participate in this study (aged 31 ± 8 years, body mass 73.4 ± 9.3 kg, maximal oxygen uptake, $\dot{V}O_{2max}$, 57.3 ± 10.0 ml/kg/min; mean ± SD). All participants were healthy, non-smoking, and had at least three years experience of endurance cycling. All participants were free from upper respiratory illness symptoms for at least 14 days before performing any of the preliminary or experimental trials.

6.2.2 Preliminary testing

All participants first performed preliminary testing in order to determine cycling power output (W) at lactate threshold and at maximal oxygen uptake ($\dot{V}O_{2max}$) on an electronically braked cycle ergometer (Excalibur Sport, Lode, Groningen, the Netherlands). In order to determine lactate threshold, participants performed a sub-maximal continuous incremental cycling test starting at 100-150 W with 25-30 W increments (depending on fitness) every four minutes for 6-7 stages. During the fourth minute of each stage, finger-tip blood samples were taken for determination of blood lactate concentration (Biosen C-Line, EKF Diagnostic, London, UK). A rating of perceived exertion (RPE) was recorded at the end of every 4-minute stage (Borg, 1982). Heart rate (HR) (Polar Electro, Kempele, Finland), and breath-by-breath gas exchange (MetaLyser 3BR2, Cortex Biophysik, GmbH, Leipzig,

Germany) were recorded continuously throughout the test. The gas analysis system was calibrated prior to use according to the manufacturer's guidelines using a three-litre syringe (Hans Rudolf Inc, Kansas, USA) and a calibration gas of known composition.

After at least 15 minutes of recovery, $\dot{V}O_{2max}$ and maximal aerobic power (MAP) were determined via a maximal incremental cycling test starting at 100 W with a ramp rate of 30 W/min. The test was terminated when participants could no longer hold the required power output for more than 10 s or when participants reached volitional exhaustion. Verbal encouragement was provided throughout the test for all participants. HR and $\dot{V}O_2$ were again measured throughout the test. $\dot{V}O_{2max}$ and MAP were taken as the highest 30 s average during the test. The work rate required to elicit 20% Δ was calculated by adding the power output at lactate threshold to 20% of the difference between power at lactate threshold and MAP. This method ensures the same relative exercise intensity (and hence physiological stress) between participants during the prolonged cycling trials (Lansley et al., 2011).

6.2.3 Familiarisation and Experimental trials

Approximately seven days before the first experimental trial, all participants first completed a full familiarisation trial of 2.5 hours cycling at 20% Δ. For cycling trials, HR was monitored throughout, and $\dot{V}O_2$ and RPE were monitored every 30 minutes. Power output was adjusted when necessary so that participants were cycling at the required work rate. Following familiarisation, the starting power output of the main exercise trial was adjusted where necessary so that participants would be able to complete 2.5 h of cycling in the experimental trial.

Experimental trials of 2.5 h of cycling at 20% Δ and 2.5 h seated rest were then completed in a randomised crossover design. All trials were performed in a fasted state starting between 7:00 and 8:00 am, and were separated by at least five days. Participants were allowed to drink water freely throughout all trials. Participants were asked to abstain from strenuous exercise for 24 h pre and 48 h post familiarisation and experimental trials, and also to complete a food diary for the 24 h preceding the familiarisation trial and then to replicate this as closely as possible before the two main experimental trials.

6.2.4 Blood sampling and analytical methods

Whole blood samples were collected from the antecubital vein into one K_3EDTA coated vacutainer and one serum vacutainer (Becton Dickinson, UK) pre, post, and 1 h post-trial. Blood collected into K_3EDTA vacutainers was used to obtain differential leukocyte counts including neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts (Hemocue® WBC DIff system, Hemocue, Angleholm, Sweden). EBV serostatus of each participant was determined according to the methods outlined in chapter 2 from blood samples collected into serum vacutainers at the start of the study.

6.2.5 Saliva collection and analytical methods

Timed, unstimulated saliva samples were provided upon waking on the morning of each trial (0 h), and then at pre, post, 1 h post exercise/rest, and upon waking for the two mornings following each trial (~20 h and 44 h post).

All saliva samples collected during the experimental trials were analysed for s-IgA concentration and secretion rate, EBV DNA concentration, and presence of human DNA as described in Chapter 2.

6.2.6 Illness reports and criteria for URS

Participants recorded any URS experienced from 14 days before the first day of preliminary testing up to seven days after the second experimental trial using a self-report questionnaire as described in chapter 2.

6.2.7 Statistical analysis

Data are expressed as mean ± SD, unless otherwise stated, with the level of significance set at $P < 0.05$. All statistical analyses were carried out using SPSS (IBM SPSS Statistics for Windows, version 24.0, Armonk, NY:IBM Corp). Repeated measures of blood leukocyte, neutrophil, and lymphocyte counts, and also s-IgA concentration and secretion rate were compared within and between trials using 2-way repeated measures ANOVA. Post hoc paired t-tests with Bonferroni correction applied were used, where necessary, to follow up any main effects identified in the ANOVAs. Non-normally distributed data were normalised using log transformation and displayed as the median (IQR). Wilcoxon signed-rank tests were used to analyse changes in EBV DNA concentration.

6.3 Results

All ten participants completed the full 2.5-hour exercise trial. Physiological responses and RPE scores are shown in table 6.1.

6.3.1 WBC Counts

There were significant main effects for ANOVA comparisons for time, trial, and time x trial interaction for leukocyte count and neutrophil count ($P < 0.05$) (Table 6.2). For the exercise trial, post-hoc analysis revealed a significant increase in

leukocyte count and neutrophil count immediately post ($P < 0.01$) and 1 h post-exercise ($P < 0.01$) in comparison to pre-exercise. There was no effect for time or trial for lymphocyte count ($P > 0.05$).

Table 6.1 Physiological responses and RPE scores during exercise trial. Values are mean (± SD). (n=10).

	0.5 h	1 h	1.5 h	2 h	2.5 h
HR (bpm)	131 ± 5	134 ± 6	134 ± 5	136 ± 4	141 ± 5
VO_2 (L/min)	2.5 ± 0.3	2.6 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	2.7 ± 0.2
RPE	11 ± 1	11 ± 1	12 ± 1	13 ± 1	13 ± 1

Table 6.2 Differential leukocyte counts. Values are mean (± SD). (n=10).

	Pre	Post	1 h Post
Leukocyte count ($x10^9L$)			
Exercise	4.8 (1.1)	10.3 (4.4)*	10.2 (3.6)*
Rest	3.9 (0.6)	4.5 (1.2)	4.7 (1.2)
Neutrophil count ($x10^9L$)			
Exercise	2.6 (0.5)	7.5 (3.6)*	7.7 (3.1)*
Rest	1.9 (0.2)	2.3 (0.6	2.3 (0.5)
Lymphocyte count ($x10^9L$)			
Exercise	1.9 (0.6)	2.3 (0.9)	1.8 (0.7
Rest	1.7 (0.4)	1.9 (0.6)	2.1 (0.7)

*Significantly different to Pre-exercise, $P < 0.05$.

6.3.2 Salivary Immunoglobulin A

There were no significant main effects for ANOVA comparisons for time, trial, and time x trial interaction for s-IgA concentration ($P > 0.05$) or secretion rate ($P > 0.05$) (Figure 6.1).

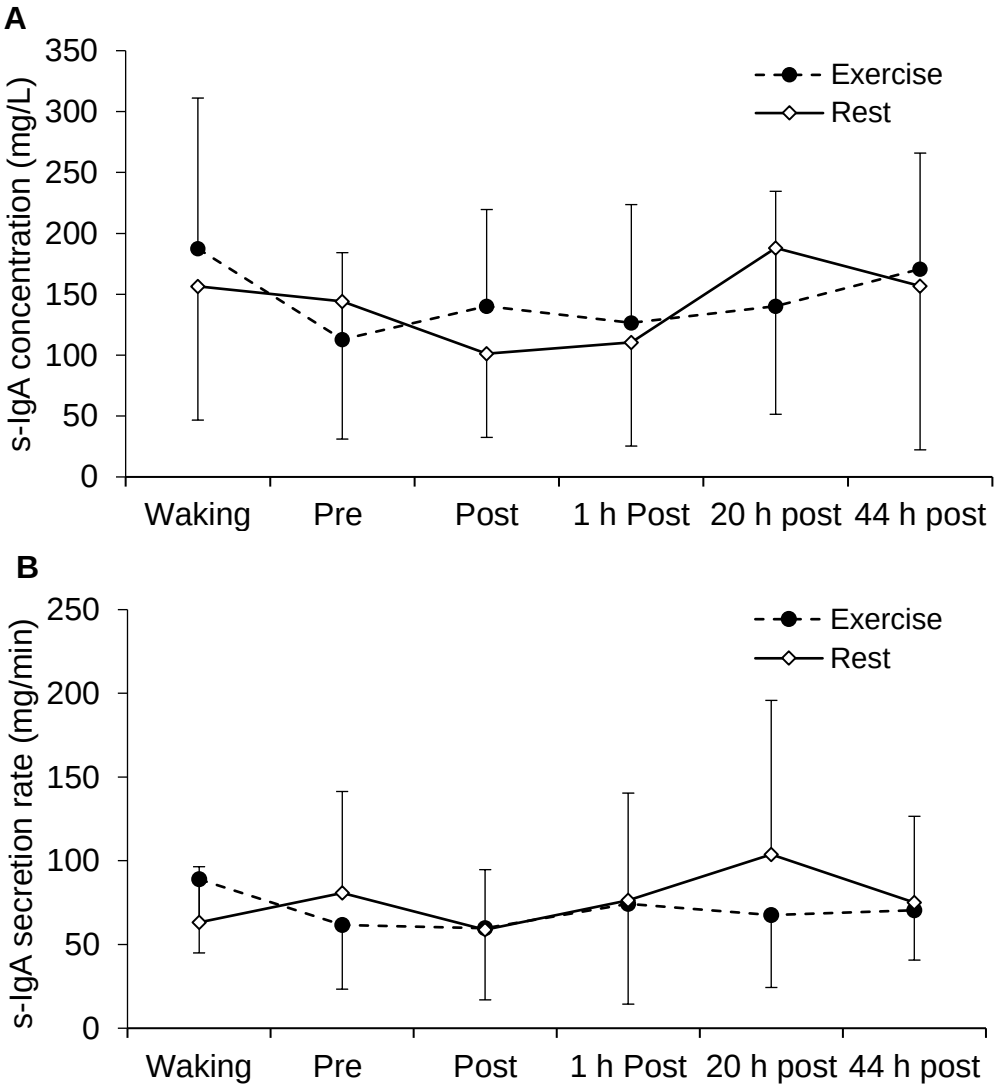

Figure 6.1 Salivary IgA concentration (A) and secretion rate (B) for the experimental trials. Values are mean (± SD), (n=10).

6.3.3 Epstein-Barr Virus

10/10 participants were seropositive for previous infection with EBV. Over the two experimental trials, seven participants had at least one saliva sample that was positive for EBV DNA (table 5.3). Individual viral shedding frequency ranged from 0-90%. 100% of saliva samples were positive for human DNA.

Over the course of the exercise trial, four participants provided EBV positive saliva samples at both pre- and post-exercise time points (table 6.3). Two participants

switched from negative EBV samples pre-exercise, to positive in the post-exercise time period up to 44 h post. Four participants remained negative for EBV DNA throughout the exercise trial. There was no significant change in EBV DNA concentration during the exercise (P = 0.46) and rest trials (P = 0.50) (figure 6.2A). Similarly, EBV DNA secretion rate also showed no significant change during the exercise (P = 0.48) and rest trials (P = 0.75) (figure 5.2B).

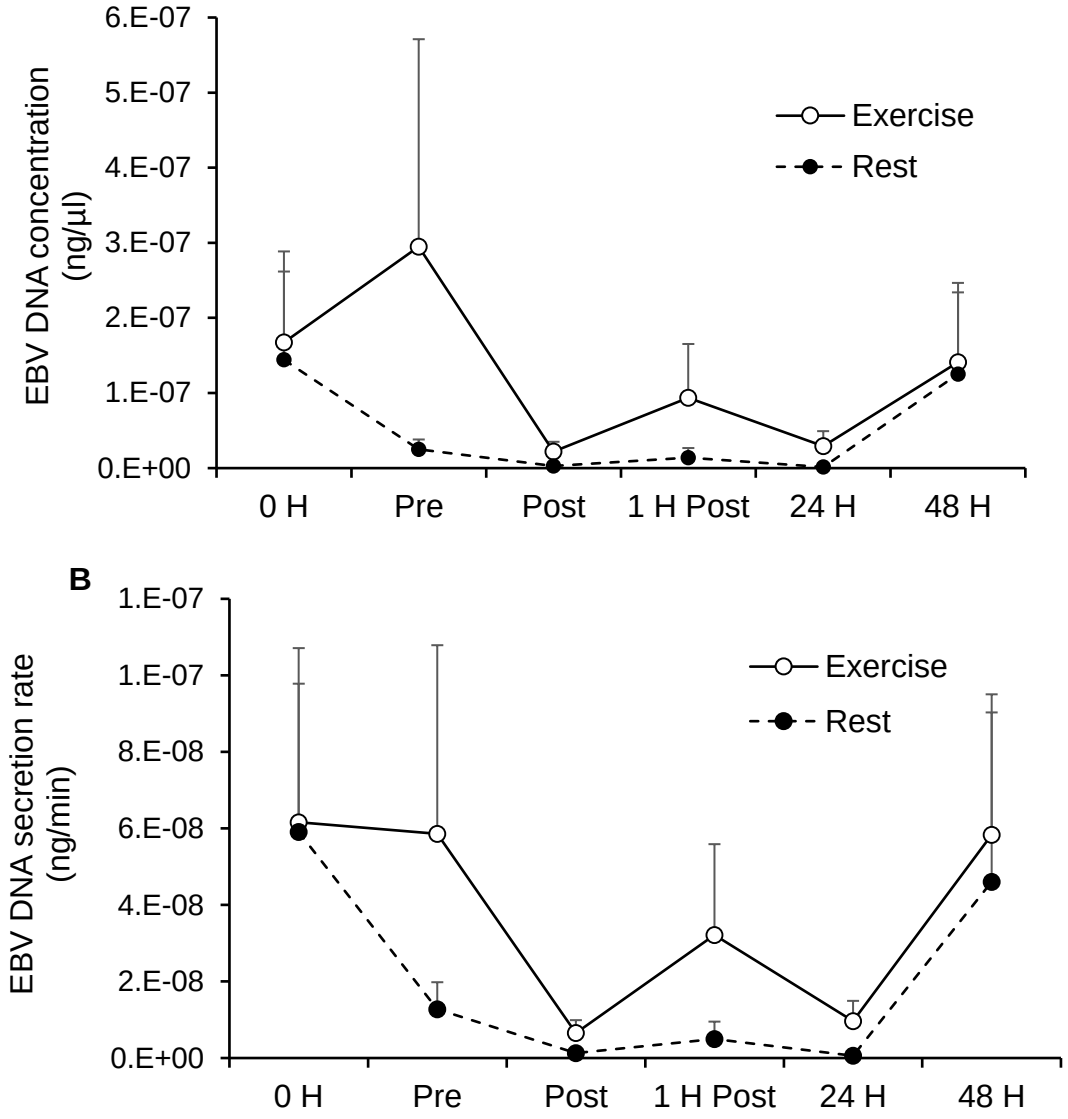

Figure 5.2 EBV DNA concentration (A) and secretion rate (B) throughout familiarisation, exercise and rest trials (n=10). Values are mean (± SEM) (SEM used to improve clarity of the figure).

Table 6.3 EBV serostatus at the start of the study, and detection of EBV DNA in saliva throughout the experimental trials. “No data” indicates timepoints when a saliva sample was not collected.

ID	EBV Serostatus	EBV DNA detected in saliva - Exercise trial						EBV DNA detected in saliva - Rest trial					
		Waking	Pre	Post	1 h Post	20 h Post	44 h Post	Waking	Pre	Post	1 h Post	20 h Post	44 h Post
1	Positive	-	-	-	-	-	Positive	Positive	Positive	-	-	-	-
2	Positive	Positive	Positive	Positive	Positive	-	Positive	Positive	Positive	Positive		Positive	Positive
3	Positive	No data	-	-	-	Positive	-	-	-	-	-	-	-
4	Positive	-	-	-	-	-	-	-	-	-	-	-	-
5	Positive	-	-	-	-	-	-	-	-	-	-	-	-
6	Positive	-	-	-	-	-	-	-	-	-	-	-	-
7	Positive	Positive	-	-	-	Positive	-	Positive	-	-	-	-	-
8	Positive	-	-	No data	-	-	-	-	-	Positive	Positive	-	-
9	Positive	Positive	Positive	Positive	Positive	Positive	Positive	No data	Positive	Positive	Positive	-	Positive
10	Positive	Positive	Positive	Positive	Positive	Positive	-	-	Positive	-	-	-	-

6.3.4 Upper respiratory symptoms

All participants were free from URS during the 14 days before the familiarisation trial through to the seven days after the second experimental trial.

6.4 Discussion

The objective of this study was to investigate the acute change in shedding of EBV DNA into saliva after a single bout of intense and prolonged cycling. Despite evidence of a significant stress response to the exercise bout (leukocyte trafficking) there was no evidence of an increase in viral shedding or acute reactivation of EBV after prolonged cycling exercise in healthy trained cyclists, which is not in agreement with the study hypothesis. Previous studies utilising in vitro techniques have identified a transient change to the number and function of circulating cells of the innate and acquired immune system, with recovery to pre-exercise levels occurring within 24 hours post-exercise (Peake et al., 2017; Walsh et al., 2011).

The process of EBV lytic reactivation *in vitro* has been well documented (Tsurumi, Fujita & Kudoh, 2005) and the biological reason for lytic gene expression is known to be essential for viral survival through infection of naïve B-cells in the already infected host as well as dissemination to a new seronegative host (Gandhi et al., 2015). However, the stimulus for the switch from latent to lytic gene expression in the EBV infected memory B-cells *in vitro* is not precisely understood (Odumade, Hogquist & Balfour, 2011). Previous longitudinal research of EBV reactivation has suggested that lytic reactivation may be due to physiological and/or psychological stress (Mehta et al., 2000). There is evidence of elevated levels of urinary stress

hormones (cortisol, adrenaline and noradrenaline) being associated with EBV reactivation in astronauts after spaceflight (Stowe et al., 2000; Stowe, Pierson & Barrett, 2001) with the suggestion that the psychological and physiological stress of spaceflight and isolation caused a down regulation in cellular immunity that lead to the observed viral reactivation. However, these findings were based on a relatively small number of sampling time points and no association with plasma stress hormone levels was observed. Furthermore, the detection of EBV DNA in saliva samples collected over a relatively long period of time with such infrequent sampling time points may be due to the natural variation that occurs within individuals over a period of several months (Hadinoto et al., 2009). In the present study, the acute physical stress of 2.5 hours fasted cycling resulted in two (20%) participants showing evidence of lytic reactivation from pre-to-post-exercise with a single positive saliva sample provided at 20 h (ID 3) and 44 h (ID 1) post-exercise. Four (40%) participants remained negative, and four (40%) participants provided positive saliva samples at both pre and post-exercise time points. Reactivation of latent B-cells in this particular *in vivo* model *per se* may not be a sign of immunodepression (i.e. participants 1 and 3), if indeed the switch from the latent to lytic life cycle is a continual viral process that is essential for viral survival. Instead, the inability of EBV specific CD8+ T-cells to control the actively replicating infected epithelial cells in the oropharynx after prolonged exercise may be an indicator of depressed cell mediated immunity *in vivo*. However, levels of EBV DNA concentration and secretion rate did not significantly change from pre-to-post-exercise in the four participants that were shedding viral DNA through the exercise trial period.

Viral shedding has previously been shown to be relatively stable (i.e. shedding or not shedding) over a short period of time (several hours and days) in healthy

seropositive individuals at rest (Hadinoto et al., 2009). The results of the present study seem to reflect this conclusion as 80% of participants were either shedding or not shedding over the period of several days. It is not possible to determine whether or not an individual is shedding at any given time without analysis of a saliva sample. This ultimately presents a barrier to the use of EBV reactivation as an outcome measure in future studies as researchers will not know whether their study participants are shedding or not shedding until the point of saliva analysis. In this study, the relatively low number of participants actively shedding viral DNA and the overall high variability in individual shedding frequency has limited the statistical comparisons that can be performed on this data and questions the use of EBV reactivation as an outcome measure in future studies of acute exercise.

If EBV reactivation is indeed stimulated by a stress hormone induced reduction in T-cell function, as suggested by Stowe et al. (2000) and also by Stowe, Pierson & Barrett (2001), examination of EBV reactivation after more strenuous exercise bouts (repeated bouts, intensified training periods etc) may provide insight into acute exercise and lytic reactivation. Indeed, the stress hormone response to repeated bouts of exercise performed on the same day has been shown to be elevated after the second exercise bout (Ronsen et al., 2001) and T-cell function is known to decrease after an intensified training period (Hoffman-Goetz et al., 1990; Lancaster et al., 2004). Previous exercise immunology research using *in vitro* methods has identified the production of exercise-induced stress hormones (specifically cortisol) as the key mechanism responsible for reduced production of cytokines and decreased concentration of lymphocytes in peripheral circulation (Lancaster et al., 2005). However, conversely, when using the DPCP model of CHS to assess post-exercise immune function there was no evidence of stress hormones being linked to *in vivo* immune responses to exercise (Davison et al.,

2016; Diment et al., 2015; Harper Smith et al., 2011). A limitation of the present study is the lack of measurements of stress hormones and catecholamines, and *in vitro* measures of immune cell function (e.g. T-cell cytokine production) carried out alongside the *in vivo* assessments (Albers et al., 2013). The significant neutrophilia that was observed post and one-hour post-exercise has been well documented as a sign of immune stress caused by exercise-induced elevations in plasma cortisol (Walsh et al., 2011), but this does not necessarily infer that T-cell function would also be significantly reduced. Additionally, leukocyte numbers and functions can continue to fluctuate between 1-hour and 24-hours post exercise (Gleeson, Bishop & Walsh, 2013; Walsh et al., 2011). Therefore, the inclusion of additional post exercise sampling time points (not limited to post and 1-hour post) would also be beneficial to future investigations.

Only one study has previously examined EBV reactivation in an acute exercise setting. Gleeson et al. (2017) analysed saliva samples collected immediately pre and post, and 24-hours post two separate bouts of exercise each lasting 60 minutes (one continuous at 65% $\dot{V}O_{2max}$, and one high intensity interval bout). In this instance, results were limited to classifying samples as either positive or negative for the presence of EBV DNA, which limited the analysis to identification of acute reactivation (i.e. switch from latent to lytic gene expression, or increase in viral load). Across the two exercise bouts, there were 11 cases of participants being positive for EBV DNA at both the immediately pre- and post-exercise timepoints. The inclusion of quantitative measures of viral load may have permitted investigation of changes in T-cell function *in vivo*, particularly as some of the participants in this particular study had been identified as illness prone and may therefore have been more susceptible to exercise-induced immunodepression. Fully quantitative qPCR methods are essential to the future

investigation of EBV reactivation and/or T-cell control of viral cells that are already shedding DNA into saliva at the onset of an acute exercise challenge.

There was no evidence of the transient depression of s-IgA concentration or secretion rate that typically occurs after a single bout of prolonged and strenuous exercise (Gleeson & Pyne, 2000; Walsh et al., 2011). All participants were healthy and free from URS for a period of 5-6 weeks (14 days before familiarisation trial through to 7 days after the second experimental trial), therefore it is unsurprising that none of the sampling timepoints that occurred upon waking (0, 20, 44 h) showed evidence of depressed s-IgA levels close to the threshold of 40 mg/L that has previously been suggested as a critical threshold for URI risk (Gleeson et al., 1999a; Gleeson et al., 2017). The fact that s-IgA levels were not depressed in the hour immediately after exercise may be due to the intensity (and/or duration) of the exercise bout. Despite being prolonged and fasted, the exercise bout being fixed at an intensity of 20%Δ was challenging (as shown by lymphocyte trafficking) but not sufficient to induce a significant decline in markers of mucosal immunity. Previous longitudinal research has suggested a link between lowered levels of s-IgA, shedding of EBV DNA, and occurrence of URS (Gleeson et al., 2002). In order for this relationship to be examined in an acute exercise setting the exercise bout may need to be at a higher intensity, or involve repeated bouts. As previously discussed, repeated exercise bouts can amplify the post-exercise decrease in immune function (Lancaster et al., 2004). However, it is important to note that the shape of the response of change in EBV DNA concentration from pre to post-exercise seems to reflect the response of s-IgA over the same time period. The absence of an inverse relationship between s-IgA and EBV DNA possibly suggests that s-IgA does not interfere with transport of new viral cells into saliva. Hadinoto et al. (2009) previously demonstrated that shedding of new viral cells into

saliva is due to activity of the EBV infected epithelial cells of the oropharynx, and that saliva is rapidly replenished with viral cells after rinsing of the mouth and swallowing.

A single bout of prolonged exercise can result in depressed leukocyte function *in vitro* (Walsh et al., 2011) and a reduced immune response to antigen challenge *in vivo* (Harper-Smith et al., 2011). The high variability of viral shedding frequency observed in the present study has been a major limiting factor in the ability to fully assess the change in levels of EBV DNA concentration and/or secretion rate after the acute exercise bout. It is important to note that all ten participants were free from URS for two weeks before and seven days after each trial, indicating that such a large range in shedding frequency can occur within healthy individuals. It is possible that EBV reactivation is too variable between individuals, and ultimately too unreliable, to be used as a tool to assess post-exercise immune function *in vivo*. Future investigations should aim to include measures of *in vitro* T-cell function and levels of stress hormones to aid the understanding of the possible cause of lytic reactivation and/or inability of CD8+ T-cells to control virus output by the infected epithelial cells of the oropharynx. More strenuous exercise bouts/repeated bouts may elicit a greater physiological stress, which may induce greater immunodepression for examination of EBV reactivation.

Chapter 7. General Discussion

7.1 Discussion

The main findings from the two longitudinal studies in this thesis suggest that reactivation of EBV, identified through either qualitative (positive detection of EBV DNA in saliva) or quantitative methods (concentration or secretion rate of EBV DNA in saliva), is not related to URS incidence or other mucosal immune markers of URS risk (s-IgA) as detection of EBV DNA in saliva is equally likely between periods of no URS and imminent/present URS. There is no clear evidence of acute reactivation following prolonged exercise (study 3) from the latent to lytic viral life cycle. Furthermore, the high variability in individual shedding frequency seen across all three experimental studies may indicate that EBV reactivation is too unreliable to be used as a marker of *in vivo* immune status in a longitudinal setting and/or an outcome measure in an acute exercise setting.

For the level of athlete used in study 2 (sub-elite endurance athletes), monitoring saliva for evidence of EBV reactivation alongside changes in s-IgA levels does not appear to be a useful model for assessing immune status and risk of imminent URS. In this instance, there was no evidence of increased detection rate of EBV DNA in saliva for the weeks before or during URS, no significant decrease in s-IgA concentration or secretion rate in the weeks leading up to URS, and training load was not associated with either lower resting s-IgA levels nor increased shedding of EBV DNA. However, in study 1, when elite athletes were monitored (professional football players) there was evidence of a relationship between s-IgA levels and competitive match load, as well as a decrease in s-IgA levels that preceded URS. Unfortunately, low incidence of URI (n=2) limited full analysis of s-IgA, EBV DNA

detection, and URS risk. For future studies of elite athletes, identification of EBV serostatus appears to be possible through analysis of saliva samples for the presence of latent EBV DNA (specifically *Bam*HI fragments), which negates the need to collect a blood sample.

Unique to the results of this thesis, 100% of participants that completed all 3 studies (n=44) were seropositive. Previous studies of athletic populations have reported seropositivity in the region of 75-85% (Cox et al., 2004; Gleeson et al., 2002; He et al., 2013), although higher values are not unheard of as more recently a study of endurance athletes from Gleeson et al. (2017) reported whole group seropositivity of 94% (15/16 athletes).

Previous investigations into the use of *in vivo* markers of immune status in an acute exercise setting have demonstrated the utility and robust nature of the cutaneous application of a novel antigen and subsequent measurement of the immune response at the skin surface (typically DPCP) (Davison et al., 2016; Diment et al., 2015; Harper-Smith et al., 2011). Given the greater clinical relevance of *in vivo* markers in comparison to the well-researched *in vitro* markers (Albers et al., 2005; Albers et al., 2013; Walsh et al., 2011), further research into EBV reactivation as a potential tool for monitoring immune status at rest may be of interest to researchers and elite sport coaches/practitioners alike due to the relative ease of saliva collection as opposed to the methods associated with DPCP measurements (e.g. discomfort due to skin swelling).

7.2 Practical applications

Until further research involving large groups of elite level athletes has been carried out, monitoring of EBV reactivation in an applied sport science setting should interpret “one off” saliva samples with caution. The pooled data presented in chapter 5 demonstrates the equal likelihood of providing a positive sample in the weeks before URS compared to periods of no imminent URS.

As shown in this thesis, elite and sub-elite athletes appear to have very different healthy baseline levels for s-IgA. It is therefore important to identify the training level of an individual in an applied sport science setting before interpreting the level of risk of URS and/or mucosal immune status from a saliva sample (or a series of saliva samples). Furthermore, future immune monitoring research should clearly state the training level of study participants and consider the upper and lower thresholds for inclusion/exclusion criteria so that study results can be appropriately interpreted according to the training level of the study participants.

7.3 Limitations of the thesis

The main limitations across this thesis are incidence of URS and participant numbers. These two limitations are commonly recurring factors within sport and exercise immunology research. Ultimately, rate of URS incidence is an uncontrollable factor and was a strong limitation to study 1. Recruitment of participants to studies 2 and 3 proved to be a challenge, which limited the participant numbers in this case. The addition of non-exercising control groups to studies 1 and 2 may have provided a useful comparison when examining the role

of training load. Finally, the use of self-report questionnaires for collection of illness data is not a fully reliable and robust method for determining whether or not URS are of infectious origin. Laboratory confirmation of infection would have been closer to a gold-standard method, but this method was not available for this thesis.

7.4 Future directions

For studies 2 and 3, all saliva samples were analysed for the presence of human DNA as an internal extraction control for the qPCR assay. This was an essential step in the methods as any samples that were identified as being negative for human DNA could be re-examined by returning to the original saliva sample to repeat the DNA extraction process. However, this thesis did not include a method that would account for the purity of the DNA present in the extracted samples. This may be important when conducting qPCR on samples that contain relatively small amounts of the template DNA (i.e. BALF5 gene) in the original sample as the purity of the template DNA that is obtained from the DNA extraction process is known to influence the efficacy of the subsequent qPCR reaction (Chidley, 2018). The results of study 1 demonstrate that DNA from the *Bam*HI gene is continuously detected in saliva even when the lytic gene (BALF5) was not detected. Therefore, the *Bam*HI gene could be used as an EBV specific DNA extraction control in future studies of EBV reactivation. Normalising the results of BALF5 DNA concentration and/or secretion rate against the values for *Bam*HI may provide an alternative method for assessing the change in viral load within an individual's saliva samples, as the purity of the extracted saliva samples has been accounted for with this EBV specific control step. In this thesis, the qPCR assay for *Bam*HI did not include a

standard curve as at that time we had not produced the necessary positive control required for a standard curve for this specific gene, and therefore the amount of latent DNA in the saliva samples could not be quantified. If future investigations were to include a standard curve, the concentration of *Bam*HI DNA in saliva could possibly be used to normalise the concentration of BALF5 DNA in an attempt to account for DNA purity in the extracted samples.

As discussed in Chapter 6 (Study 3), the acute response of EBV reactivation as either lytic reactivation and/or loss of T-cell control over viral cells that are actively replicating at the onset of exercise may need to be investigated in an exercise scenario that involves repeated bouts/intensified periods of exercise in order to induce a greater degree of immunodepression. Inclusion of in vitro measures of T-cell function may provide further insight into any relationship between changes in T cell function from pre-to-post-exercise and changes in EBV shedding for people who are actively shedding viral DNA into saliva at the onset of exercise (i.e. in the lytic life cycle).

Given the notable difference in s-IgA levels between the participants of study 2 and study 1, it would appear that salivary markers of immune status exhibit a different profile in the weeks before URS according to the competitive level/training status of the individual. The findings from this thesis should therefore not be taken as conclusive evidence that EBV reactivation is not linked to URS incidence, and is not a useful *in vivo* marker of immune status, for groups of elite athletes. More work with large sample sizes of elite populations is required to fully understand any links between EBV lytic DNA shedding and URS incidence in those populations. This particular area of research into immune monitoring may indeed be welcomed by professional athletes and coaches due to the non-invasive and

relatively convenient nature of saliva collection in comparison to venous blood sampling and cutaneous *in vivo* techniques.

7.5 Conclusion

In healthy elite and sub-elite athletes, the detection of EBV DNA in saliva could simply reflect the natural process of latent B-cell reactivation and infection of epithelial cells in the oropharynx. Therefore, detection of EBV DNA in saliva may not necessarily be an *in vivo* sign of immunodepression and increased risk of imminent URS.

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Appendix A

The following questions relate to any symptoms of illness **experienced today**. If a **symptom is present**, please place **a tick** in the box and rate as: ***Light (L), Moderate (M), or Severe (S)***. If a symptom is not present, please leave blank.

Symptoms	***Day 1***		***Day 2***		***Day 3***		***Day 4***		***Day 5***		***Day 6***		***Day 7***	
	Tick if present	*Severity*	*Tick if present*	*Severity*	*Tick if present*	*Severity*	*Tick if present*	*Severity*	*Tick if present*	*Severity*	*Tick if present*	*Severity*	*Tick if present*	*Severity*
Fever														
Persistent muscle soreness or tenderness (more than 8 hours)														
Sore, painful throat														
Catarrh in the throat														
Runny nose														
Cough														
Repetitive sneezing														
Joint aches and pains														
Weakness/fatigue														
Headache														
Loss of sleep														
Inability to train/compete Light (L) = normal training maintained, Moderate (M) = training reduced, Severe (S) = training discontinued														

Have you taken any over-the-counter medication this week to alleviate respiratory illness or gastrointestinal discomfort symptoms? **Yes o** **No o**
If yes, name of medication……
Have you been to see you doctor about your illness symptoms this week? **Yes o** **No o**
If yes, have you taken any prescribed medication this week? **Yes o** **No o** If yes, name of medication ……