Factors Influencing

Upper Respiratory Tract Illness Incidence In Athletes:

The Important Role Of Vitamin D

By

Cheng-Shiun He

A Doctoral Thesis

Submitted in partial fulfillment of the requirements for the award of

Doctor of Philosophy of Loughborough University

January 2015

© by Cheng-Shiun He 2015
Contents
General abstract ................................................................. i
Acknowledgements .......................................................... iii
Publications ........................................................................ iv
List of Tables ...................................................................... vi
List of Figures ..................................................................... vii
Abbreviations ..................................................................... viii
Chapter 1: Literature review ................................................. 1
1.1 Exercise and upper respiratory tract illness .................. 2
1.2 Factors influencing infection risk in athletes ................. 3
1.3 Sex differences in upper respiratory illness symptoms prevalence ........ 5
1.4 Cytomegalovirus and Epstein-Barr virus ....................... 7
1.5 The influence of the chronic latency of CMV and EBV on the immune system ........ 9
1.6 Vitamin D synthesis and metabolism ......................... 11
1.7 Vitamin D measurements ........................................ 13
1.8 Vitamin D status in athletes ..................................... 15
1.9 Vitamin D and the immune system ............................ 18
1.10 Vitamin D and upper respiratory tract illness ............. 21
1.11 Aims of thesis ........................................................... 23
Chapter 2: General methods ................................................. 25
2.1 Participant recruitment ..................................................... 26
2.2 Saliva and blood samples collection ........................... 26
2.3 Saliva analysis ............................................................... 27
2.4 Plasma analysis ............................................................. 28
2.5 Antigen-stimulated cytokine production ..................... 29
Chapter 3: Factors influencing upper respiratory tract illness incidence in athletes .......... 30
3.1 Abstract ................................................................... 31
3.2 Introduction ................................................................ 32
3.3 Methods .................................................................... 35
3.4 Results ...................................................................... 40
  3.4.1 The effects of sex differences ................................... 44
  3.4.2 The effects of CMV/EBV serostatus ......................... 48
  3.4.3 The effects of vitamin D status ............................... 51
General abstract

Firstly, the aims of the study were to investigate the influences of various factors, sex differences, Cytomegalovirus/Epstein-Barr virus (CMV/EBV) serostatus and vitamin D concentrations on respiratory illness incidence and immune function during the winter months in a student cohort of endurance athletes. In Chapter 3, the findings of the study concur with recent reports of illness incidence at major competitive games which indicate that female athletes may be more susceptible than their male counterparts to upper respiratory tract illness (URTI) symptoms and that lower oral-respiratory mucosal immunity may, in part, account for this. It was also found that previous coinfection with CMV and EBV might promote protective immune surveillance to lower the risk of URTI. In addition, it can be concluded that athletes with low plasma vitamin D concentrations may have a higher risk of URTI and suffer more severe symptoms when URTI is present. This may be due to impaired mucosal and systemic immunity as secretory immunoglobulin A (SIgA) secretion, cathelicidin levels and antigen-stimulated pro-inflammatory cytokine production appear to be increased by vitamin D-dependent mechanisms.

A series of follow-up studies were also conducted to examine the effect of vitamin D on mucosal and systemic immunity in athletes. In Chapter 4, it was reported that the influence of vitamin D on circulating cytokines might be different in athletes compared with non-athletes and that both pro-inflammatory and anti-inflammatory cytokine production by multi-antigen stimulated whole blood culture were not influenced by 1,25-dihydroxy vitamin D (1, 25(OH)₂D) concentrations within the normal healthy range. In Chapter 5, it was found that 5000 IU of vitamin D₃ supplementation daily appears to have a beneficial effect in up-regulating the expression of SIgA and cathelicidin in athletes during a winter training period. Nevertheless, the findings reported in Chapter 6 showed that there were no significant effects of vitamin D status and a 4-week period of daily high does vitamin D₃ supplementation on
salivary antimicrobial protein (AMP) responses to prolonged exercise. In conclusion, a series of studies in this thesis have demonstrated the influence of various factors (sex differences, CMV/EBV serostatus and vitamin D concentrations) on susceptibility to URTI among athletes. Moreover, it was suggested that vitamin D₃ supplementation could have a positive effect on immune function and lead to decreased incidence of respiratory infections.

Key Words: URTI, Sex difference, Cytomegalovirus, Epstein-Barr virus, Vitamin D, Salivary flow rate, Antimicrobial protein
Acknowledgements

There are a number of people I would like to thank who have made this PhD thesis possible. First and foremost I would like to thank my supervisor, Professor Mike Gleeson. Thanks for the opportunities that he has given to me, the skills in and out of the laboratory, and for showing me the attitude that is required to succeed as an academic researcher. Moreover, I did enjoy playing tennis with him even though I didn’t win a set from him. I am very honoured to have worked with him. I would like to extend thank to Dr Phil Watson, who have provided advice at the annual review meetings of my PhD. Thanks also to Dr Nicolette Bishop and Dr Graeme Close for their insightful suggested amendments that have improved the thesis.

I would like to thank my colleagues, Dr Marta Oliveira, Siobhan Svendsen, Dr Christof Leicht, Maurice Dungey and Dr Sophie Killer, for their immediate support in the lab. I thank them for lending a helping hand in some of the studies. Thanks to Dr Ayu Muhamad and Michal Handzlik for their sample collection for some of the studies. Thanks to Dr Jon Tang and Professor William Fraser for the plasma vitamin D LC-MS/MS analysis. Thanks also to Dr James Carter and Dr Ian Rollo for their support in the GSSI lab.

I would like to thank the MSc and BSc students who helped with data collection for this thesis. Also to all of the participants who took part in the studies. Without their time and dedication this work would not have been possible. I truly appreciate their efforts. I would also like to extend a huge thank to the Loughborough University volleyball team that they brought me unforgettable memory in my PhD life.

Finally, I would like to thank my family, especially my sister, Kuan-Yi Ho, for their support throughout my studies. My special appreciations to Professor Shih-Hua Fang and Professor Jerry Li. Without their help and encouragement, this work would not have been possible.
Publications


CS He, WD Fraser, M Gleeson (2014) Influence of vitamin D metabolites on plasma cytokine concentrations in endurance sport athletes and on multi antigen stimulated cytokine production by whole blood and peripheral blood mononuclear cell cultures, ISRN Nutrition, 2014, 820524.


Conference proceedings


CS He, M Handzlik, WD Fraser, A Muhamad, H Preston, A Richardson, M Gleeson (2013) Influence of vitamin D status on respiratory infection incidence and immune function during 4 months of winter training in endurance sport athletes, International Journal of Exercise Science: Conference Proceedings, Vol. 10: Iss. 1, Article 75.

Presentations

M Gleeson, CS He, E Tanqueray, J Rudland-Thomas, K Brown, S Renwick (2014) The effect of chronic vitamin D₃ supplementation on antimicrobial peptides and proteins in athletes. The Annual BASES Conference, St George’s Park, Burton, UK. (Oral)

CS He, M Handzlik, A Muhamad, M Gleeson (2013) Influence of CMV/EBV serostatus on respiratory infection incidence during 4 months of winter training in a student cohort of endurance athletes. The 11th International Society of Exercise and Immunology Conference, Newcastle, Australia. (Poster)

CS He, M Handzlik, WD Fraser, A Muhamad, H Preston, A Richardson, M Gleeson (2013) Influence of vitamin D status on respiratory infection incidence and immune function during 4 months of winter training in endurance sport athletes. The 11th International Society of Exercise and Immunology Conference, Newcastle, Australia. (Poster)
List of Tables

Table 3.1 Anthropometric, training and haematological variables in male and female athletes

Table 3.2 Salivary variables in male and female athletes

Table 3.3 Illness incidence among athletes at major competitive events

Table 3.4 Infection symptom incidence in CMV positive and negative subjects

Table 3.5 Infection symptom incidence in EBV positive and negative subjects.

Table 3.6 Infection symptom incidence in CMV+EBV+ and CMV-EBV- subjects.

Table 3.7 Blood total and differential leukocyte counts

Table 3.8 Infection symptom incidence among different vitamin D status groups

Table 3.9 Salivary concentrations and secretion rates of AMP

Table 3.10 Blood total and differential leukocyte counts

Table 3.11 The antigen-stimulated cytokines production by whole blood culture

Table 4.1 Physical characteristics of athletes with high and low vitamin D status

Table 4.2 Circulating cytokine concentrations

Table 4.3 The correlation between circulating cytokine and plasma 25(OH)D concentrations

Table 4.4 Comparison of the whole blood and PBMC culture

Table 5.1 Anthropometric, training and haematological variables

Table 5.2 Plasma total 25(OH)D and cathelicidin concentrations

Table 5.3 Salivary flow rate and AMP concentration
Table 6.1 Physiological variables in the high-level and low-level vitamin D groups

Table 6.2 Salivary variables during exercise

Table 6.3 Physiological variables in the pre-intervention and post-intervention trials

Table 6.4 Salivary variables during prolonged exercise
List of Figures

Figure 1.1 Factors affecting immune function

Figure 3.1 Training loads in MET-h/week over the 16-week study period

Figure 3.2 Percentage of the cohort reporting URTI episode

Figure 3.3 Changes in salivary secretion rates over time

Figure 3.4 Percentage of the cohort reporting URTI episode

Figure 4.1 The antigen-stimulated cytokines production by whole blood culture

Figure 4.2 The antigen-stimulated cytokines production by PBMC culture

Figure 5.1 The percentage change of plasma cathelicidin concentration

Figure 5.2 Changes in salivary secretion rates

Figure 7.1 Overview of the experimental studies
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 25(OH)$_2$D</td>
<td>1,25-dihydroxy vitamin D</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25 hydroxy vitamin D</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial protein</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DEQAS</td>
<td>Vitamin D external quality assessment scheme</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HNP</td>
<td>Human neutrophil peptides</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPAQ</td>
<td>International Physical Activity Questionnaire</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>K₂EDTA</td>
<td>Potassium ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MET</td>
<td>Metabolic equivalent</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction mode</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PLC-γ1</td>
<td>Phospholipase C-gamma 1</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RTI</td>
<td>Respiratory tract infection</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SIgA</td>
<td>Secretory immunoglobulin A</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell antigen receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>URTI</td>
<td>Upper respiratory tract illness</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet radiation</td>
</tr>
<tr>
<td>VDBP</td>
<td>Vitamin D-binding protein</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D response elements</td>
</tr>
</tbody>
</table>
Chapter 1:

Literature review
1.1 Exercise and upper respiratory tract illness

URTI is the most common form of infectious illness in athletes undertaking prolonged intense exercise and may decrease the quality of training and hinder performance during competition. The relationship between exercise and susceptibility to infection has been modelled in the form of a 'J' shaped curve (Nieman, 1994). This model suggests that while engaging in moderate activity may enhance immune function above sedentary levels, excessive amounts of prolonged high-intensity exercise may depress immune function, resulting in an elevated risk of URTI. Prolonged bouts of strenuous exercise have a temporary negative impact on various aspects of immune function. Both aspects of innate immunity including neutrophil chemotaxis, phagocytosis, degranulation, and oxidative burst activity; and natural killer (NK) cell cytotoxic activity and acquired immunity including toll-like receptors (TLRs) expression and antigen presentation by monocytes or macrophages; T lymphocyte cytokine production and proliferation, immunoglobulin production by B lymphocytes are depressed by prolonged exercise (Gleeson & Walsh, 2012). Post-exercise immune function depression is most pronounced when the exercise is continuous, prolonged (longer than 1.5 h), of moderate to high intensity (55–75% of VO₂ max), and performed without food intake (Gleeson, 2007). The increases in circulating stress hormones including adrenaline and cortisol, alterations in the pro-inflammatory and anti-inflammatory cytokine balance and increased free radicals are thought to cause the depression of immune function after prolonged exercise (Gleeson & Walsh, 2012). Furthermore, several epidemiological studies have showed that there is an increased incidence of URTI among highly trained athletes, compared with low or moderate exercising groups (Gleeson, Bishop, Oliveira, & Tauler, 2011; Nieman, 1994; Peters, Goetzsche, Grobbelaar, & Noakes, 1993).
1.2 Factors influencing infection risk in athletes

Within the general healthy human population there is a range of immuno-competency due to genetic differences, sex, age, nutritional status and lifestyle habits (Figure 1.1). Prolonged strenuous exercise has been associated with a transient depression of numerous aspects of immune function (Gleeson, 2006; Gleeson, 2007; Walsh et al., 2011) and a heavy schedule of training and competition can lead to immune impairment in athletes. This is associated with an apparent increased susceptibility to URTI symptoms (Fahlman & Engels, 2005; Gleeson et al., 1999; Heath, Macera, & Nieman, 1992; Neville, Gleeson, & Folland, 2008; Nieman, Johanssen, Lee, & Arabatzis, 1990; Peters & Bateman, 1983). Although several studies have now demonstrated a non-infectious cause of these symptoms in many cases (for example, airway inflammation caused by high ventilation rates or allergy) (Cox, Pyne, Saunders, Callister, & Gleeson, 2007; Spence et al., 2007), infectious causes of URTI appear to be
mainly viral in nature. However, it is not clear what aspects of immune dysfunction are mostly responsible for the apparent greater incidence of infectious URTI in athletes compared with non-athletes. Furthermore, while it is clear that some athletes are more illness-prone than others, the influence of various extraneous factors (e.g. vitamin D status, prior infection history including CMV and EBV serostatus) on susceptibility to infection among athletes has not been established.

Several risk factors have been identified for URTI in athletes including low salivary SIgA concentration (Gleeson et al., 1999; Neville et al., 2008) or secretion rate (Fahlman & Engels, 2005; Gleeson et al., 2012), high anti-inflammatory cytokine response to antigen challenge (Gleeson et al., 2012; Gleeson, Bishop, Oliveira, & Tauler, 2011) and latent viral shedding of EBV (Gleeson et al., 2002). While the salivary SIgA response to acute exercise is variable, prolonged bouts of exercise are commonly reported to result in decreased salivary SIgA secretion (Bishop & Gleeson, 2009). In addition, previous studies have shown an inverse relationship between salivary SIgA values and URTI prevalence. For example, low salivary SIgA values have been reported to be associated with increased incidence of URTI in athletes (Fahlman & Engels, 2005; Gleeson et al., 2012; Neville et al., 2008). With regard to anti-inflammatory cytokine response, Gleeson et al. (2013) demonstrated that the increased risk of URTI in individuals with high levels of physical activity might be related to an elevated anti-inflammatory cytokine response to antigen challenge. Especially, the production of interleukin (IL)-10 was positively correlated with the number of weeks with URTI symptoms (Gleeson et al., 2012).

Other possible factors that might predispose highly trained athletes to more frequent infection are sex differences, previous infection with CMV and/or EBV and low vitamin D status. The sex of the individual affects immune function. In females, oestrogens and progesterone modulate immune function (Paavonen, 1994) and thus immunity is influenced by the
menstrual cycle and pregnancy (Haus & Smolensky, 1999). Consequently, sex-based differences in responses to infection, trauma and sepsis are evident (Beery, 2003). In addition, another possible risk factor is previous infection with CMV and/or EBV. Both CMV and EBV are the members of the human herpes virus group and they persist in the body in latent form for a long time after primary infection and can be become reactivated when immune function is depressed. It has been shown that the chronic latency of both viruses has an influence on the immune system (Chang & Barry, 2010; Gleeson et al., 2002; Slobedman, Barry, Spencer, Avdic, & Abendroth, 2009). There is some new evidence that previous infection with CMV or EBV has a potent influence on the cellular immune responses to exercise (Simpson, 2011; Turner et al., 2010). Furthermore, It has only recently been recognised that vitamin D plays an important role in up-regulating immunity (Kamen & Tangpricha, 2010). Several recent studies have found an inverse association between vitamin D status and respiratory infection incidence in young and elderly adults (Berry, Hesketh, Power, & Hypponen, 2011; Ginde, Mansbach, & Camargo, 2009; Laaksi et al., 2007; Sabetta et al., 2010). The incidence of respiratory illnesses is generally higher in athletes (Gleeson, 2007) and low vitamin D status could be a contributing factor as vitamin D insufficiency has been reported to be common in athletes (Larson-Meyer & Willis, 2010) especially if exposure to natural sunlight is limited.

1.3 Sex differences in upper respiratory illness symptoms prevalence

Within the general population women are generally more resistant to viral infections and tend to have more autoimmune diseases than men (Beery, 2003). In a review of the literature on respiratory tract infections (RTIs) (Falagas, Mourtzoukou, & Vardakas, 2007) in which data from 84 studies was extracted it was concluded that males are more susceptible than females
to most types of RTIs in all age groups (children, adolescents, adults and the elderly). Physiological, lifestyle, behavioural and socioeconomic differences between males and females may explain the observed findings and the involvement of sex hormones in the regulation of immune function may also contribute to the reported sex differences in the incidence and severity of the various types of RTIs, especially in adults and adolescents (Falagas et al., 2007). Oestrogens are generally immune enhancing, whereas progesterone and androgens, including testosterone, exert suppressive effects on both humoral and cellular immune responses. Females have higher levels of plasma immunoglobulin M (IgM) than men and exhibit more vigorous responses to exogenous antigens, indicating a higher level of humoral immunity in females than in males (Bouman, Schipper, Heineman, & Faas, 2004). In females, there is increased expression of some cytokines in peripheral blood and vaginal fluids during the follicular phase of the menstrual cycle and with use of hormonal contraceptives (Brabin, 2002). In the luteal phase of the menstrual cycle, blood leukocyte counts are higher than in the follicular phase (Faas et al., 2000), mononuclear cell expression of the heterodimeric transcription factor 1 (a key regulator of the innate immune response) is lower (Schaible, Boehringer, Callau, Niess, & Simon, 2009), and the immune response is shifted towards a T helper (Th) 2-type response (Faas et al., 2000). Thus, in the general population, there are differences in some aspects of immune function between men and women that appear to result in women getting fewer viral infections, including those affecting the respiratory tract.

In addition to these sex differences in resting conditions, several studies have documented sex differences in some aspects of the immunological response to exercise (Gillum, Kuennen, Schneider, & Moseley, 2011; Timmons, Hamadeh, Devries, & Tarnopolsky, 2005; Timmons, Tarnopolsky, Snider, & Bar-Or, 2006) including larger post-exercise increases in circulating lymphocytes and NK cells in females. The expression of pro-inflammatory and anti-
inflammatory genes in response to exercise is also influenced by the menstrual cycle and there are distinct differences in gene expression between women in the luteal phase and men (Northoff et al., 2008). Prolonged strenuous exercise has been associated with a transient depression of immune function (Gleeson, 2007; Gleeson et al., 2013; Walsh et al., 2011) and a heavy schedule of training and competition can lead to immune impairment in both male and female athletes (Walsh et al., 2011). This is associated with an increased susceptibility to URTI symptoms ((Fahlman & Engels, 2005; Gleeson, 2006; Gleeson et al., 1999; Heath et al., 1992; Nieman et al., 1990; Peters & Bateman, 1983; Walsh et al., 2011) and several studies suggest that reduced secretion of salivary SIgA and possibly other mucosal antimicrobial proteins may be an important causal factor (Fahlman & Engels, 2005; Gleeson et al., 2012; Gleeson et al., 2013; Gleeson et al., 1999; Neville et al., 2008; Walsh et al., 2011). However, it is not clear whether any substantial sex differences exist in any aspect of oral-respiratory mucosal immune function in an athletic population or whether any such differences affect URTI risk. In contrast to what has been reported for the general population, some recent reports of illness rates among athletes attending large competitive events (e.g. winter and summer Olympic games, athletic and aquatic sport world championships) suggest that URTI episodes may actually be more prevalent in the women than the men (Alonso et al., 2012; Alonso et al., 2010; Edouard, Depiesse, Hertert, Branco, & Alonso, 2013; Engebretsen et al., 2013; Engebretsen et al., 2010; Mountjoy et al., 2010; Ruedl et al., 2012).

### 1.4 Cytomegalovirus and Epstein-Barr virus

Both CMV and EBV, the members of the human herpes virus family of deoxyribonucleic acid (DNA) viruses, are predominantly associated with asymptomatic infections but may present as infectious mononucleosis in a proportion of cases. They persist in the body in
latent form for a long time after primary infection and can be become reactivated when immune function is depressed (Wang, Yang, Wei, Huang, & Zhao, 2010). Children and young adults are susceptible populations at high risk of CMV and EBV infection. While the majority of young children have asymptomatic CMV and EBV infections early in life in developing countries, the primary EBV infection is often delayed, sometimes until puberty, in industrialized countries (Nilsson et al., 2009). The primary CMV infections usually result from contact with infected body fluids, transfusion of blood products or transplantation of solid organs from CMV seropositive individuals (Bale, 2012). Also, primary EBV infection often occurs through direction contact with infected oral secretions (Savard & Gosselin, 2006). Furthermore, EBV infection in 50-70% of cases produces symptoms of infectious mononucleosis characterized by fever, pharyngitis and lymphadenopathy in childhood and primary CMV infection might cause approximately 5-10% of cases of mononucleosis syndrome and will manifest symptoms almost indistinguishable from those of EBV-induced mononucleosis (Bale, 2012). Moreover, Bigley et al. (2012) found that a greater percentage of the CMV seropositive adults were EBV seropositive compared to those who were CMV seronegative. Thus, the coinfection with these two viruses occurs occasionally in children and adults as CMV and EBV have so much in common (Bigley et al., 2012; Wang et al., 2010).

A large proportion of the adult western population has been reported to have had a previous infection with CMV or EBV. Bate et al. (2010) found that approximately 50% of individuals aged 6-49 years in the general population of the USA were CMV seropositive. Likewise, Lubeck et al. (2010) indicated that approximately 60% of the general population in the Germany had experienced a previous infection with CMV. Nevertheless, to our knowledge, there have been no studies examining CMV serostatus in a large number of athletes. On the other hand, the high proportion of previous EBV infection in general population and athletes has been reported in several studies. Pottgiesser et al. (2006) found that approximately 83%
of 200 healthy university students were EBV positive. In addition, it has been showed that 79% of 14 elite swimmers in Australia and 80% of 202 endurance athletes in Germany had previous infection with EBV (Gleeson et al., 2002; Pottgiesser et al., 2006). Given that a large proportion of athletes appear to have had a previous infection with CMV or EBV, it is important to clarify whether previous CMV or EBV infection (or both) has an important influence on the risk of URTI in athletes.

1.5 The influence of the chronic latency of CMV and EBV on the immune system

The successful coexistence of CMV and EBV with their hosts requires a variety of mechanisms of antiviral immune evasion and it has been shown that the chronic latency of both viruses has an influence on the immune system. Positive CMV serostatus is identified by the presence of IgG antibodies to CMV in plasma and although CMV does not cause overt symptoms of illness in itself, it can cause a general suppression of immune function via its production of a viral homologue of human IL-10 which might down-regulate the antigen presentation capacity of dendritic cells and macrophages as a strategy to limit host immune recognition (Chang & Barry, 2010; Slobedman et al., 2009). Thus, when immunity is depressed (e.g. during hard training) the latent CMV could become reactivated and might produce a more severe immunodepression making the individual more susceptible to infection. This possibility has been suggested but not yet confirmed in athletes. Likewise, EBV infection also produces a viral homologue of human IL-10 as a strategy to escape from immune detection (Slobedman et al., 2009) and could have similar effects to latent CMV reactivation. One small scale study with athletes has found that latent viral shedding of EBV in saliva during intensive training might be a factor in increasing susceptibility to other infections such as influenza and the common cold (Gleeson et al., 2002).
On the other hand, previous infection with CMV causes a substantial and sustained accumulation of late-differentiated effector-memory cluster of differentiation (CD)8 T cells. For CMV infection, it has been reported that the phenotype of virus-specific CD8 T cells changes over time from activated T cell in the early phase to resting vigilant effector-memory T cell during latency (van Leeuwen, ten Berge, & van Lier, 2007). In contrast, EBV-specific CD8 T cells in healthy virus carriers mostly have an early memory T-cell phenotype (Callan, 2003). Furthermore, repeated periods of rest and reactivation from CMV or EBV latency may provide effective T-cell boosts and play a role in maintaining a robust and functional virus-specific T cell population (Wherry & Ahmed, 2004). Effector-memory CD8 T cells possessing the ability to rapidly produce cytokines, such as interferon (IFN)-γ and tumour necrosis factor (TNF)-α, are an important component of protective immunity against viral infections (Wherry & Ahmed, 2004). It has been reported that latent CMV infection appears to amplify memory CD8 T cell mobilisation and egress in response to exercise (Turner et al., 2010). The amplification of this response may promote protective immune surveillance, and thereby reduce risk of infection. It could be especially protective against infection in the post-exercise period when several other aspects of immune function are depressed.

To our knowledge, there have been no the large-scale studies carried out to date to examine whether previous CMV and EBV infection modifies the incidence of URTI although it has been demonstrated that the chronic latency of both viruses has an influence on the immune system in several studies. The chronic latency of CMV and EBV might promote protective immune surveillance or alternatively become reactivated to produce a more severe immunodepression. Further research is still warranted to understand the influence of previous CMV or EBV infection on the risk of URTI incidence in athletes.
1.6 Vitamin D synthesis and metabolism

In humans, vitamin D can be obtained either from dietary sources or the epidermal layer of the skin via exposure to sunlight. Two forms of vitamin D can be obtained from dietary sources: vitamin D\(_3\) (cholecalciferol) and vitamin D\(_2\) (ergocalciferol). While vitamin D\(_3\) is found in food from animal origin, such as cod-liver oil, salmon and egg yolk, vitamin D\(_2\) is present in some plants and fungi. The vitamin D production from ultraviolet-mediated conversion of 7-dehydrocholesterol in the plasma membrane of skin cells provides 80-100\% of body requirements (Lanteri, Lombardi, Colombini, & Banfi, 2013). This process is extraordinarily rapid and the production of vitamin D in the skin after only a few minutes of appropriate sunlight easily exceeds dietary sources. In the skin, ultraviolet radiation (wavelength of 290-315 nm) promotes photolytic cleavage of 7-dehydrocholesterol into pre-vitamin D\(_3\), which is subsequently converted into vitamin D\(_3\) by a spontaneous thermal isomerization. Newly synthesized vitamin D and its metabolites are bound to vitamin D-binding protein (VDBP) for systemic transport.

Vitamin D needs to be hydroxylated twice to achieve the biologically active form, 1, 25(OH)\(_2\)D. The endogenously synthesised vitamin D\(_3\) and diet-derived D\(_2\) and D\(_3\) must first be hydroxylated in the liver into 25-hydroxy vitamin D, 25(OH)D, at the carbon 25-position by an enzyme called 25-hydroxylase. 25(OH)D is the main storage form, which can be stored in adipose tissue, and the major circulating metabolite of vitamin D, with a half-life of 2-3 weeks. Therefore, the plasma concentration of 25(OH)D is considered to be the primary indicator of vitamin D status (Baeke, Takiishi, Korf, Gysemans, & Mathieu, 2010). Plasma 25(OH)D values commonly accepted as the reference range are as follows (Heaney et al., 2011; IoM, 2011). In healthy humans, 25(OH)D plasma levels >120 nmol/L are suggested as optimal vitamin D concentrations and levels from 50 to 120 nmol/L are defined as adequate. Plasma levels of 25(OH)D <50 nmol/L are proposed to define inadequate vitamin D
concentrations and values <30 nmol/L represent vitamin D deficiency. In the second hydroxylation, 25(OH)D is converted in the kidney to the biologically active form, 1, 25(OH)₂D, by 1-α-hydroxylase, an enzyme which is stimulated by parathyroid hormone (PTH) when serum calcium and phosphate concentrations fall below the physiological range. 1, 25(OH)₂D, is released into the circulation from the kidney which is considered as a vital endocrine source of hormone. Normal concentrations of circulating 1, 25(OH)₂D are approximately 50-250 pmol/L, about 1000 times lower than its precursor, 25(OH)D; the plasma half-life of 1, 25(OH)₂D is 4-6 hours. Some cells other than kidney, such as T cells, B cells, macrophages and dendritic cells, also express 1-α-hydroxylase and have the enzymatic machinery to convert 25(OH)D to 1, 25(OH)₂D, in non-renal compartments (Aranow, 2011). Importantly, 1, 25(OH)₂D limits its own activity in a negative feedback loop by inducing 24-hydroxylase, which converts 1, 25(OH)₂D into a biologically inactive metabolite. In addition, 1, 25(OH)₂D also inhibits the hydroxylation of renal 1-α-hydroxylase. Therefore, this negative feedback loop prevents excessive vitamin D activity.

1, 25(OH)₂D exerts its functions by binding to the vitamin D receptor (VDR), which is a nuclear receptor and ligand-activated transcription factor. The VDR is a member of the superfamily of nuclear hormone receptors and it is composed of an α-helical ligand-binding domain and a highly conserved DNA binding domain. High-affinity binding of 1, 25(OH)₂D to the α-helical ligand-binding domain of VDR activates transcription by heterodimerization with the retinoid X receptor (RXR), which is essential for the high-affinity DNA binding to cognate vitamin D response elements (VDRE). The 1, 25(OH)₂D-VDR-RXR heterodimer translocates to the nucleus where it binds VDRE located in the regulatory regions of 1, 25(OH)₂D target genes and then induces expression of the vitamin D responsive genes (Aranow, 2011).
Typically, the classic function of vitamin D is to improve intestinal absorption of calcium by regulating several calcium transport proteins in the small intestine, and stimulating osteoclast differentiation to promote calcium homeostasis and bone health. However, many tissues other than kidney, including brain, lung, muscle, skin and adipose tissue, possess the 1-α-hydroxylase and VDR (Baeke et al., 2010). Those cells with the 1-α-hydroxylase and VDR are able to produce the biologically active form of vitamin D, 1, 25(OH)₂D, from circulating 25(OH)D. It is important to note that the extra-renal 1-α-hydroxylase differs from the renal 1-α-hydroxylase as it is not regulated by PTH, calcium and phosphate (Wu, Ren, Nguyen, Adams, & Hewison, 2007). Instead, the production of 1, 25(OH)₂D in the extra-renal cells is dependent on circulating levels of 25(OH)D.

1.7 Vitamin D measurements

Measurement of plasma or serum 25(OH)D concentration is widely used in clinical practice and research reports to assess vitamin D status as 25(OH)D is the major circulating metabolite of vitamin D and seems to be “solid as a rock” in whole blood at room temperature. It has been demonstrated that 25(OH)D in whole blood, serum or plasma is stable at room temperature or when stored at -20°C and is unaffected by multiple freeze-thaw cycles (Agborsangaya et al., 2010; Antoniucci, Black, & Sellmeyer, 2005; Wielders & Wijnberg, 2009). Long term storage, at least three years, of serum samples at -20°C does not affect serum 25(OH)D concentrations (Agborsangaya et al., 2010). Furthermore, 25(OH)D concentrations in serum samples that have been thawed and refrozen up to four times are still reliable (Antoniucci et al., 2005).

Plasma or serum 25(OH)D concentration can be measured by competitive protein binding assay, immunoassay, high pressure liquid chromatography (HPLC) and liquid
Chapter 1  

Literature review

Chromatography-tandem mass spectrometry (LC-MS/MS) (Fraser & Milan, 2013). The LC-MS/MS method is generally considered to be the gold standard method for the measurement of plasma or serum 25(OH)D levels because isotope dilution LC-MS/MS method can simultaneously and accurately quantitate both 25(OH)D$_2$ and 25(OH)D$_3$ (Wallace, Gibson, de la Hunty, Lamberg-Allardt, & Ashwell, 2010; Zerwekh, 2008). Furthermore, both 25(OH)D$_2$ and 25(OH)D$_3$ can be extracted from plasma samples using isolute C18 solid phase extraction cartridges in the LC-MS/MS assay. However, as the LC-MS/MS method requires expensive equipment, large plasma sample volume and specialised staff, the commercial enzyme-linked immunosorbent assays (ELISAs) are the most popular methods for measurement of plasma 25(OH)D concentration (Fraser & Milan, 2013; Wallace et al., 2010). Current 25(OH)D ELISAs employ polyclonal or monoclonal antibodies that bind specifically to human 25(OH)D. Nevertheless, the competition between the 25(OH)D specific antibodies and VDBP in plasma samples makes these assays difficult to control and may lead to poor agreement with the LC-MS/MS assay (Farrell et al., 2012; Herrmann et al., 2010). The plasma 25(OH)D concentration cannot be measured accurately unless it is released from VDBP and the strong protein binding of 25(OH)D requires the employment of suitable conditions to release 25(OH)D from VDBP (Fraser & Milan, 2013; Wallace et al., 2010). In addition, most commercial immunoassays cannot measure the concentration of 25(OH)D$_2$ and 25(OH)D$_3$ independently. It has been reported that there was a underestimation of plasma 25(OH)D$_2$ concentration in several commercial immunoassays which resulted in marked variations of the total plasma 25(OH)D levels (Fraser & Milan, 2013; Wallace et al., 2010). Given the recent explosion of interest in vitamin D, it seems to be necessary establish which of the commercial ELISAs for plasma or serum 25(OH)D are the most reliable. The obvious potential advantages of these methods are their relative ease of use, low cost and high throughput using small plasma sample volumes.
Chapter 1

1.8 Vitamin D status in athletes

Recently, in a systematic literature review using the Medline and EMBASE databases including 195 studies conducted in 44 countries, it has been indicated that there were 37.3 % of the studies reporting mean plasma 25(OH)D values below 50 nmol/L (Hilger et al., 2014). Despite the wide number of studies that have documented a high prevalence of vitamin D insufficiency in the general population worldwide, there are only a handful of reports about the vitamin D status of athletes. A summary of the current literature on vitamin D status in athletes is shown in Table 1.1.

Overall, vitamin D insufficiency has been reported to be common in athletes especially if exposure to natural sunlight is limited (e.g. when training in the winter months or when training mostly indoors). The reason for the vitamin D insufficiency in athletes might be the inadequate endogenous synthesis from the insufficient ultraviolet radiation (UV) exposure, rather than the inadequate daily vitamin D intake (Larson-Meyer & Willis, 2010). The vitamin D production in the skin from sufficient UV exposure provides 80-100 % of body requirements (Lanteri et al., 2013). Because UV radiation of appropriate wavelength needed to produce vitamin D in the skin is absent or drastically reduced during the winter months, athletes need to rely more on the diet and their stores of vitamin D that were accumulated during the summer months. The lack of significant amounts of vitamin D in the diet causes serum 25(OH)D levels to decrease precipitously and reach its nadir in the winter. In addition, athletes with high concentrations of melanin in their skin need up to 10 times longer UV exposure to produce the same amount of 25(OH)D than do fair-skinned ones as cutaneous melanin acts as an effective sunscreen (Hamilton et al., 2010). Therefore, athletes training in the winter months, training indoors, dark-skinned individuals, those who excessively use
high-factor sunscreen, and those living at high latitudes, are all at risk for vitamin D insufficiency.
Table 1.1 Vitamin D status in athletes

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Location (latitude)</th>
<th>Number</th>
<th>Age (years)</th>
<th>Season</th>
<th>25(OH)Vitamin D concentration (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bescos Garcia et al., 2011</td>
<td>Professional basketball players</td>
<td>Barcelona, Spain (41°N)</td>
<td>21</td>
<td>25</td>
<td>Winter</td>
<td>57 % &lt; 50</td>
</tr>
<tr>
<td>Close et al., 2013</td>
<td>Professional UK athletes</td>
<td>Liverpool, UK (53°N)</td>
<td>61</td>
<td>18 - 27</td>
<td>Winter</td>
<td>62 % &lt; 50</td>
</tr>
<tr>
<td>Close et al., 2013</td>
<td>UK club athletes</td>
<td>Liverpool, UK (53°N)</td>
<td>30</td>
<td>20 - 24</td>
<td>Winter</td>
<td>57 % &lt; 30</td>
</tr>
<tr>
<td>Hamilton et al., 2010</td>
<td>Middle-eastern sportmen</td>
<td>Doha, Qatar (25.4°N)</td>
<td>93</td>
<td>13 – 45</td>
<td>Summer</td>
<td>91 % &lt; 50</td>
</tr>
<tr>
<td>Hamilton et al., 2014</td>
<td>Professional Qatar based footballers</td>
<td>Doha, Qatar (25.4°N)</td>
<td>342</td>
<td>16 - 33</td>
<td>Summer</td>
<td>55.5 % &lt; 50</td>
</tr>
<tr>
<td>Lovell, 2008</td>
<td>Australian female gymnasts</td>
<td>Bruce, Australia (35.3°S)</td>
<td>18</td>
<td>10 - 17</td>
<td>Autumn</td>
<td>33 % &lt; 50</td>
</tr>
<tr>
<td>Maroon et al., 2015</td>
<td>National football league players</td>
<td>Pittsburgh, USA (40.4°N)</td>
<td>80</td>
<td>22 - 37</td>
<td>Winter</td>
<td>26 % &lt; 50</td>
</tr>
<tr>
<td>Morton et al., 2012</td>
<td>Elite soccer players</td>
<td>Liverpool, UK (53°N)</td>
<td>20</td>
<td>24</td>
<td>Winter</td>
<td>65 % &lt; 50</td>
</tr>
<tr>
<td>Storlie et al., 2011</td>
<td>Collegiate athletes</td>
<td>Washington, US (46.9°N)</td>
<td>27</td>
<td>18 - 33</td>
<td>Autumn</td>
<td>3.7 % &lt; 50</td>
</tr>
</tbody>
</table>

Data are presented as mean or range.
1.9 Vitamin D and the immune system

The discovery of VDR in almost all immune cells, including T lymphocytes, B lymphocytes, neutrophils and antigen presenting cells, such as macrophages and dendritic cells prompted the idea that vitamin D could have a vital role in the regulation of immune responses (Baeke et al., 2010). These immune cells also express the vitamin D-activating enzyme, 1-α-hydroxylase, and thus possess the ability to convert 25(OH)D to 1, 25(OH)₂D, which is regulated by circulating levels of 25(OH)D or induced by activation of specific toll-like TLRs (Bikle, 2009) which act as detectors of pathogens. Thus, 1, 25(OH)₂D could play important roles in both innate and adaptive immune responses.

It has been demonstrated that 1, 25(OH)₂D is a vital mediator of innate immune responses, enhancing the antimicrobial properties of immune cells such as monocytes and macrophages (Bikle, 2009). 1, 25(OH)₂D is a key link between TLR activation and antimicrobial responses in innate immunity. Following activation of the TLR signalling cascade in the presence of microbes, 1, 25(OH)₂D has a vital role in up-regulating the production of AMPs, such as cathelicidin and β-defensin (Liu et al., 2006). Both macrophage and epithelial cells, possessing the 1-α-hydroxylase and VDR, are capable of responding to and producing 1, 25(OH)₂D. The biologically active form, 1, 25(OH)₂D, could induce expression of the vitamin D responsive genes to enhance the production of cathelicidin and β-defensin by binding to VDRs (Wang et al., 2004). The stimulation of TLRs by an antimicrobial peptide in macrophages or by wounding the epidermis in keratinocytes results in increased expression of the 1-α-hydroxylase enzyme, which up-regulates the production of 1, 25(OH)₂D to stimulate the expression of cathelicidin in the presence of adequate 25(OH)D (Liu et al., 2006). 25(OH)D, the major circulating form used to determine vitamin D status, is an essential factor for the local production of 1, 25(OH)₂D to up-regulate cathelicidin production.
in the skin and in macrophages. While 1, 25(OH)₂D alone is sufficient for the strong induction of cathelicidin expression, the combination of IL-1β and 1, 25(OH)₂D is required for the strong induction of β-defensin. 1, 25(OH)₂D can double the induction of β-defensin production by IL-1β signalling which stimulates the NF-κB transcription factor function (Liu et al., 2009). These AMPs have a broad range of activities against microorganisms and may be involved in the direct inactivation of viruses through membrane destabilization (Kamen & Tangpricha, 2010). They are produced by epithelial cells and macrophages and in the lungs are secreted into the biofilm covering the inner surface of the airways, thereby creating a barrier that is chemically lethal to microbes. In addition, 1, 25(OH)₂D enhances the effectiveness of monocytes and macrophages in killing microbes by enhancing the generation of reactive oxygen species and the expression of inducible nitric oxide synthase in these phagocytic cells (Sly, Lopez, Nauseef, & Reiner, 2001).

In contrast with the innate immune responses, most of the reported actions of vitamin D on adaptive immunity are focused on suppressive mechanisms, which could be beneficial for autoimmune disorders. It has been demonstrated that 1, 25(OH)₂D can suppress B cell proliferation and immunoglobulin production and inhibit the differentiation of B cell precursors into plasma cells, which highlights a potential role for vitamin D in B cell related disorders (Chen, Sims, Chen, Gu, & Lipsky, 2007). Furthermore, 1, 25(OH)₂D could inhibit T cell proliferation and also influence the phenotype of T cells, in particular through the suppression of Th1 cells which are associated with cellular immunity (Lemire, Archer, Beck, & Spiegelberg, 1995). In contrast, 1, 25(OH)₂D can enhance cytokine production by Th2 cells which are associated with humoral immunity (Boonstra et al., 2001). Thus, vitamin D could help limit the tissue damage associated with excessive Th1 cellular immunity by shifting the balance to a Th2 cell phenotype. Recently, the impact of 1, 25(OH)₂D on Th17 cells, which are linked to inflammatory tissue damage, has been discovered. It appears that 1,
25(OH)₂D can suppress the development of Th17 cells and inhibit the production of cytokines by Th17 cells (Chang, Chung, & Dong, 2010). In addition, it has been showed that treatment of naive CD4 T cells with 1, 25(OH)₂D potently induces the development of regulatory T cells (Treg) which are capable of producing cytokines that block Th1 development (Gorman et al., 2007). Overall, vitamin D acts to maintain a balance between inflammatory Th1/Th17 cells and immunosuppressive Th2/Treg cells to temper inflammation and tissue damage (Hewison, 2012).

On the other hand, recent studies have indicated that vitamin D is essential in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes (Kongsbak, Levring, Geisler, & von Essen, 2013; von Essen et al., 2010) leading to an activation of the cellular immune response in response to pathogen exposure. Naive human T cells have very low expression of phospholipase C-gamma 1 (PLC-γ1) (a key signaling protein downstream of many extracellular stimuli) and this is associated with low T cell antigen receptor (TCR) responsiveness in naive T cells. However, TCR triggering leads to a large upregulation of PLC-γ1 expression, which correlates with greater TCR responsiveness. Induction of PLC-γ1 is dependent on vitamin D and expression of the VDR. Naive T cells do not express the VDR, but VDR expression is induced by TCR signaling via the alternative mitogen-activated protein kinase p38 pathway. Thus, initial TCR signaling via p38 leads to successive induction of VDR and PLC-γ1, which are required for subsequent classical TCR signaling and T cell activation. These findings indicate that vitamin D is crucial for the activation of the acquired immune system and therefore very important for the effective clearance of viral infections. Further studies are still warranted to understand the mechanism of vitamin D on adaptive immunity.
1.10 Vitamin D and upper respiratory tract illness

Several recent studies have found a negative association between vitamin D status and respiratory infection incidence in young and elderly adults (Berry et al., 2011; Ginde et al., 2009; Laaksi et al., 2007; Sabetta et al., 2010). In the Third National Health and Nutrition Examination Survey involving 18,883 participants (Ginde et al., 2009), it was found that individuals with plasma 25(OH)D concentration less than 25 nmol/L had a significantly higher risk of respiratory infections (24%) than those with 25(OH)D levels of 25-74 nmol/L (20%) and levels higher than 75 nmol/L (17%). Moreover, Ginde et al. (2009) demonstrated that an inverse association between vitamin D status and respiratory infections was present throughout the whole year. In addition, a population-based study on 6,789 British adults demonstrated that there were seasonal patterns both in plasma 25(OH)D concentrations and in the prevalence of URTIs, including in the winter months, that there was a 7% reduction in the risk of respiratory infections for each 10 nmol/L increase in plasma 25(OH)D (Berry et al., 2011). Furthermore, a Finnish study involving 800 participants has reported that young male Finnish soldiers with plasma 25(OH)D concentration less than 40 nmol/L had significantly more days of absence from duty (median: 4 days) due to respiratory infections during the 6-month study period than soldiers with plasma 25(OH)D concentration more than 40 nmol/L (median: 2 days) (Laaksi et al., 2007). Also, an observational study involving 198 healthy adults has shown that subjects with 25(OH)D concentrations less than 95 nmol/L had a significant two-fold increase in the risk of developing URTI and a longer duration of illness (median: 9 days) compared with those whose 25(OH)D concentrations was higher than 95 nmol/L (median: 2 days) (Sabetta et al., 2010). Therefore, vitamin D might play an important role in preventing URTIs and reducing both the severity and duration of URTI symptoms.

There are some randomised placebo-controlled and double-blind trials of vitamin D supplementation for the prevention of URTIs in children and adults (Aloia, Talwar, Pollack,
& Yeh, 2005; Camargo et al., 2012; Laaksi et al., 2010; Li-Ng et al., 2009; Murdoch et al., 2012; Urashima et al., 2010). A 3-year randomised controlled trial with 208 healthy post-menopausal African American women who were given 800 IU vitamin D daily or placebo for 2 years, followed by 2000 IU vitamin D daily or placebo for one year demonstrated that there were less self-reported URTI symptoms in the vitamin D supplemented group compared with the placebo group. There were 9 reports of URTI symptoms in the vitamin D group and 30 reports in the placebo group during the study period (Aloia et al., 2005). However, a follow-up study by the same research group involving 162 healthy adults given 2000 IU vitamin D or placebo daily for 12 weeks during the winter months showed no difference in the incidence (vitamin D: 48 URTIs; placebo: 50 URTIs) and the duration of URTI symptoms (vitamin D: 5.4 days; placebo: 5.3 days) between the vitamin D group and the placebo group (Li-Ng et al., 2009). Nevertheless, it was noted that serum 25(OH)D levels increased significantly from 63.0 to 88.5 nmol/L in the intervention group after 12 weeks of vitamin D supplementation. In another placebo-controlled study involving 164 Finnish men who received vitamin D (400 IU/day) or placebo for 6 months during winter, Laaksi et al. (2010) found that the number of days absent from duty owing to respiratory tract infection was slightly lower (vitamin D: 2.2 days; placebo: 3.0 days) and the proportion of subjects without any days absent was significantly higher (vitamin D: 51.3 %; placebo: 35.7 %) in the vitamin D supplemented group compared with the control group. Furthermore, a randomised placebo-controlled trial of daily vitamin D supplementation in 334 Japanese schoolchildren in the winter months demonstrated that individuals who were provided with vitamin D supplementation (1200 IU/day) had a significantly lower risk of type A influenza infections (10.8%) than those not taking any vitamin D supplement (18.6%) during the 4-month study period (Urashima et al., 2010). In addition, there was a vitamin D supplement study in Mongolian schoolchildren as well. The randomised placebo-controlled trial of daily fortified
milk ingestion with 300 IU of vitamin D in 247 Mongolian schoolchildren with vitamin D deficiency (median 25(OH)D: 17.5 nmol/L) in winter found that children receiving the vitamin D supplement reported significantly fewer acute respiratory infections during the 3-month study period, compared with controls (vitamin D: 0.8; placebo: 0.4) (Camargo et al., 2012). In contrast, Murdoch et al. (2012) conducted a high dose vitamin D supplementation study involving 322 healthy adults in New Zealand and reported that a monthly dose of 100,000 IU of vitamin D in healthy adults did not significantly reduce the incidence and severity of URTIs during the 18-month study period.

In conclusion, there have been mixed findings for randomised trials of vitamin D supplementation in the prevention of URTIs in children and adults although a negative association between vitamin D status and respiratory infection incidence was commonly presented in the observational studies. This discrepancy might be due to the varied baseline plasma 25(OH)D levels of participants, the different dose and duration of vitamin D supplementation, different populations, or the varied methods to define URTIs. Further studies are still warranted to investigate the preventive effect of vitamin D supplementation on respiratory tract infections in different populations, particularly those that may be more at risk of URTIs due to occupational or lifestyle stress (e.g. athletes and military personnel).

1.11 Aims of thesis

This thesis investigated the influence of various possible factors (sex differences, CMV/EBV serostatus and vitamin D status) on respiratory illness and immune function during a winter training period in athletes. Also, a series of follow-up studies were conducted to examine the effect of vitamin D on mucosal and systemic immunity in athletes in this thesis.
In Chapter 3, we conducted a study on a large cohort of endurance athletes who completed daily URTI symptom diaries using validated questionnaires to determine the influence of several possible factors on mucosal and systemic immunity as well as the incidence, severity and duration of URTI episodes during a winter training period. Chapter 4 describes a study that determined the influence of plasma 25(OH)D concentrations on circulating plasma cytokine concentrations in athletes and the effects of different doses of 1, 25(OH)₂D₃ on the *in vitro* multi-antigen stimulated cytokine production by whole blood and PBMC cultures. In Chapter 5, the purpose of the study was to examine the effect of vitamin D₃ supplementation over the winter months (from December to February) on the resting plasma cathelicidin concentration and the salivary concentrations and secretion rates of SIgA, cathelicidin, lactoferrin and lysozyme in a population of individuals engaged in regular sport training. Chapter 6 describes a preliminary study that investigated the influence of vitamin D status and a short term period of daily high dose vitamin D₃ supplementation on mucosal immunity in response to prolonged, moderate intensity exercise.
Chapter 2:

General methods
Several studies in this thesis required the use of similar methods. To avoid repetition, methods used in more than one of the experimental chapters are described in this chapter and will only be described in brief in the following experimental chapters.

### 2.1 Participant recruitment

The majority of participants were recruited from Loughborough University. Subjects were enrolled after having fulfilled all inclusion criteria, and presenting none of the exclusion criteria (determined by both questionnaire and interview). Subjects could be included if they were currently healthy, had been involved in endurance training for at least 2 years, engaged in at least 3 sessions and at least 3 h of total moderate/high-intensity training time per week and were between 18-40 years of age. Subjects representing one or more of the following criteria were excluded from participation: smoking or use of any medication, suffering from or had a history of cardiac, hepatic, renal, pulmonary neurological, gastrointestinal, haematological or psychiatric illness.

### 2.2 Saliva and blood samples collection

Participants came to the laboratory in the morning after an overnight fast. Subjects then sat quietly for 10 min before providing an unstimulated saliva sample by passive dribble into a pre-weighed sterile collection tube for a timed period (usually 2 min; longer was allowed if the volume of saliva collected after 2 min was insufficient). After centrifugation for 2 min at 5000 g to remove cells and insoluble matter, saliva samples were stored frozen at -80°C prior to analysis. Subsequently, a resting venous blood sample (12 mL) was obtained by venepuncture from an antecubital forearm vein into two vacutainer tubes (Becton Dickinson, Oxford, UK) containing K$_3$EDTA and lithium heparin. Haematological analysis was
immediately carried out on the EDTA sample (including haemoglobin, haematocrit and total and differential leukocyte counts) using an automated cell-counter (Ac.T™5diff haematology analyser, Beckman Coulter, High Wycombe, UK). Subjects had to have normal haematology to be included in the study. The remaining EDTA blood was centrifuged for 10 min at 1500 g and 4°C and the plasma stored at -80 °C prior to analysis. Heparinised blood was used immediately for the measurement of antigen-stimulated cytokine production.

2.3 Saliva analysis

Saliva samples were analysed in duplicate for all the salivary AMPs. The saliva volume collected was estimated by weighing and the saliva flow rate was calculated. Saliva samples with 5X dilution were analysed for SIgA concentration using an ELISA kit (Salimetrics, Philadelphia, USA) and cathelicidin concentration using a commercially available ELISA kit (Hycult Biotech, Uden, The Netherlands). Salivary lactoferrin and lysozyme were analysed using commercially available ELISA kits (Calbiochem, USA and Biomedical Technologies, USA, respectively) with 500X dilution. Salivary HNP1-3 was analysed using commercially available ELISA kits (Hycult Biotech, Uden, Netherlands) with 1000X dilution. Secretion rates for each of the salivary AMPs were calculated as the multiple of the saliva flow rate and AMP concentration. All saliva assays were carried out in duplicate. The intra-assay CVs for all the assays were <4% across a working range of 2.5-600 mg/L for SIgA, 0.1–100 μg/L for cathelicidin, 1.6-100 μg/L for lactoferrin, 0.78-50 μg/L for lysozyme and 0-10000 ng/L for HNP1-3.


Chapter 2

General methods

2.4 Plasma analysis

EDTA plasma was assayed for IgG antibodies to CMV and EBV using commercially available ELISA kits (Biocheck Inc., CA, USA and NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany, respectively) according to the manufacturers’ instructions. The intra-assay CVs for CMV and EBV assays were <3%. Plasma with 20X dilution was assayed in duplicate for cathelicidin concentration using a commercially available ELISA kit (Hycult Biotech, Uden, Netherlands) according to the manufacturer’s instructions. The intra-assay CV for cathelicidin assay was <3% across a working range of 0.1–100 μg/L.

Vitamin D occurs in two forms: cholecalciferol (D₃) which is formed by the action of UV light on the skin and ergocalciferol (D₂) from plant food sources. The best measure of vitamin D concentration is considered to be the sum of the 25-hydroxy metabolites of D₂ and D₃ (25(OH)D₂ and 25(OH)D₃) and the best way of measuring these is considered to be the LC-MS/MS method (Wallace et al., 2010). EDTA plasma samples were analysed for 25(OH)D₃ and 25(OH)D₂ with a LC-MS/MS (Waters Acuity, Manchester, UK) after a maximum of 10 months in storage with no previous freeze-thaw cycles as described previously (Tolppanen, Sayers, Fraser, & Lawlor, 2012). Briefly, 25(OH)D₂, 25(OH)D₃ and deuterated internal standard were extracted from plasma samples, following protein precipitation, using Isolute C18 solid phase extraction cartridges. Potential interfering compounds were removed by initial elution with 50% methanol followed by elution of the vitamins using 10% tetrahydrofuran in acetonitrile. Dried extracts were reconstituted prior to injection into a high performance liquid chromatography tandem mass spectrometer in the multiple reaction mode (MRM). The MRM transitions (m/z) used were 413.2 > 395.3, 401.1 > 383.3 and 407.5 > 107.2 for 25(OH)D₂, 25(OH)D₃ and hexa-deuterated(OH)D₃ (internal standard), respectively. Intra-assay CVs were <10% across a working range of 2.5–624 nmol/L for both 25(OH)D₃ and 25(OH)D₂. Measurements were performed in a laboratory meeting the performance...
target set by the Vitamin D External Quality Assessment Scheme (DEQAS) Advisory Panel for 25(OH)D assays.

2.5 Antigen-stimulated cytokine production

Stimulated whole blood culture production of cytokines (IFN-γ, TNF-α, IL-1β, IL-2, IL-6 and IL-10) was determined as described previously (Gleeson et al., 2012). The stimulant was a commercially available multi-antigen vaccine (PediaCel Vaccine, Sanofi Pasteur, UK) containing diphtheria, tetanus, acellular pertussis, poliomyelitis and haemophilus influenzae type b antigens. Briefly, 0.25 mL of heparinized whole blood was added to 0.75 mL of RPMI 1640 medium (Sigma Chemicals, Poole, UK) with an added 40 µL of PediaCel vaccine cocktail (Sanofi Pasteur msd Limited, Maidenhead, UK) at a dilution of 1:100, before being incubated at 37°C and 5% CO₂ for 24 h. The stimulant dilution of 1:100 used in this study was based on a separate experiment (unpublished data), which established the dose–response curve for the measured cytokines over the dilution range of 1:100–1:20 000. Samples were then centrifuged at 15000 rpm for 4 min at 4°C, following which the supernatant fluid was harvested and stored at -80°C prior to analysis of cytokine concentrations using an Evidence Investigator System using the high sensitivity cytokine biochip array EV3513 (Randox, County Antrim, UK). The intra-assay CV for all measured cytokines was less than 5.0%. 


Chapter 3: Factors influencing upper respiratory tract illness incidence in athletes
3.1 Abstract

The purposes of the study were to determine the influence of possible factors, sex differences, CMV/EBV serostatus and vitamin D status, on mucosal and systemic immunity as well as the incidence, severity and duration of URTI episodes in endurance athletes during a winter training period. During a 4-month winter training period, we conducted a study on a large cohort of endurance athletes including 236 subjects (166 males, 70 females) who completed daily URTI symptom diaries and reported weekly training loads using validated questionnaires. We also collected blood samples from these athletes at the start and end of the study and saliva samples at the start and at 4-weekly intervals. The findings of this study concur with recent reports of illness incidence at major competitive games indicating that female athletes may be more susceptible than their male counterparts to URTI and that lower oral-respiratory mucosal immunity (i.e. lower secretion rates of mucosal AMPs), may account for this in part. Moreover, it has been indicated in the present study that athletes who had experienced prior infection with both CMV and EBV had fewer URTI episodes and fewer symptom days than those with negative serostatus for both CMV and EBV. In addition, our study suggested that low vitamin D status could be an important determinant of URTI risk in endurance athletes. Athletes with low vitamin D concentrations may have a higher risk of URTI and suffer more severe symptoms when URTI is present. This may be due to impaired mucosal and systemic immunity as salivary SIgA secretion, cathelicidin levels and antigen-stimulated pro-inflammatory cytokine production appear to be increased by vitamin D-dependent mechanisms.
3.2 Introduction

URTIs are the most common infectious illness in athletes undertaking prolonged intense exercise and may decrease the quality of training and hinder performance during competition. The relationship between exercise and susceptibility to infection has been modelled in the form of a 'J' shaped curve (Nieman, 1994). This model suggests that while engaging in moderate activity may enhance immune function above sedentary levels, excessive amounts of prolonged high-intensity exercise may depress immune function, resulting in an elevated risk of URTI. Several epidemiological studies have showed that there is an increased incidence of URTI among highly trained athletes, compared with low or moderate exercising groups (Gleeson et al., 2013; Nieman, 1994; Peters et al., 1993).

Several risk factors have been identified for URTI in athletes including low salivary SIgA concentration (Gleeson et al., 1999; Neville et al., 2008) or secretion rate (Fahlman & Engels, 2005; Gleeson et al., 2012), high anti-inflammatory cytokine response to antigen challenge (Gleeson et al., 2012; Gleeson, Bishop, Oliveira, & Tauler, 2011) and latent viral shedding of EBV (Gleeson et al., 2002). Furthermore, other possible factors that might predispose highly trained athletes to more frequent infection are sex differences, previous infection with CMV and/or EBV and low vitamin D status.

The sex of the individual affects immune function. In females, oestrogens and progesterone modulate immune function (Paavonen, 1994) and thus immunity is influenced by the menstrual cycle and pregnancy (Haus & Smolensky, 1999). Consequently, sex-based differences in responses to infection, trauma and sepsis are evident (Beery, 2003). Within the general population, women are generally more resistant to viral infections and tend to have more autoimmune diseases than men (Beery, 2003). In contrast to what has been reported for
the general population, some recent reports of illness rates among athletes attending large competitive events (e.g. winter and summer Olympic games, athletic and aquatic sport world championships) suggest that URTI episodes may actually be more prevalent in the women than the men (Alonso et al., 2012; Alonso et al., 2010; Edouard, Depiesse, Hertert, Branco, & Alonso, 2013; Engebretsen et al., 2013; Engebretsen et al., 2010; Mountjoy et al., 2010; Ruedl et al., 2012). However, it is not clear whether any substantial sex differences exist in any aspect of mucosal immune function in an athletic population or whether any such differences affect URTI risk.

Both CMV and EBV are the members of the human herpes virus group and they persist in the body in latent form for a long time after primary infection and can be become reactivated when immune function is depressed. It has been shown that the chronic latency of both viruses has an influence on the immune system (Chang & Barry, 2010; Liu et al., 1997). Positive CMV serostatus (identified by the presence of IgG antibodies in plasma) is common among adults and although CMV does not cause overt symptoms of illness in itself, it can cause a general suppression of immune function via its production of a viral homologue of human IL-10 which is a potent inhibitory cytokine (Chang & Barry, 2010). Likewise, EBV infection, which in 50-70% of cases produces symptoms of infectious mononucleosis (glandular fever) (Niederman, Evans, Subrahmanyan, & McCollum, 1970) also produces a viral homologue of human IL-10 (Liu et al., 1997) and could have similar effects to CMV in increasing susceptibility to other infections such as influenza and the common cold. Thus, when immunity is depressed (e.g. during hard training) the latent CMV and/or EBV can become reactivated and may produce a more severe immunodepression making the individual more susceptible to infection. However, this possibility has been suggested but not yet confirmed in athletes.
It has only recently been recognised that vitamin D plays an important role in up-regulating immunity (Kamen & Tangpricha, 2010). Vitamin D is a key link between TLR activation and antimicrobial responses in innate immunity. Vitamin D has a vital role in up-regulating the expression of AMPs, such as cathelicidin and β-defensin (Liu et al., 2006). These AMPs have a broad range of activities against microorganisms and may be involved in the direct inactivation of viruses (Kamen & Tangpricha, 2010). The biologically active form of vitamin D, 1,25(OH)₂D, activates the genes for cathelicidin synthesis and enhances the effectiveness of monocytes and macrophages in killing microbes by enhancing the oxidative burst potential of these phagocytic cells (Schwalfenberg, 2011). Furthermore, vitamin D has been shown to be essential in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes (von Essen et al., 2010). Vitamin D may also influence cytokine production during periods of infection (Cannell, Zasloff, Garland, Scragg, & Giovannucci, 2008). In addition, several recent studies have found a negative association between vitamin D status and respiratory illness incidence in young and elderly adults (Berry et al., 2011; Ginde et al., 2009; Laaksi et al., 2007) and the incidence of respiratory illnesses is generally higher in athletes (Gleeson, 2006). Therefore, low vitamin D status could be a contributing factor of developing URTI as vitamin D insufficiency has been reported to be common in athletes (Larson-Meyer & Willis, 2010) especially if exposure to natural sunlight is limited (e.g. when training in the winter months or when training mostly indoors).

During a 4-month winter training period, we conducted a study on a large cohort of endurance athletes who completed daily URTI symptom diaries and reported weekly training loads using validated questionnaires. We also collected blood samples from these athletes at
the start and end of the study and saliva samples at the start and at 4-weekly intervals. The aims of the study were to determine the influence of possible factors, sex differences, CMV/EBV serostatus and vitamin D status, on mucosal and systemic immunity as well as the incidence, severity and duration of URTI episodes in endurance athletes during a winter training period. Our hypothesis was that mucosal immune variables would be lower in female athletes and that this might be associated with a higher prevalence of URTI episodes. We also hypothesized that athletes who had previous infection with CMV and/or EBV were predisposed to increased susceptibility to URTI. In addition, it has been hypothesized that athletes with low vitamin D status may have a higher risk of URTI and suffer more severe symptoms when URTI is present.

3.3 Methods

Subjects

Two hundred and sixty seven subjects (184 males, 83 females) who were engaged in regular sports training (predominantly endurance-based activities such as running, cycling, swimming, triathlon, team games and racquet sports) volunteered to participate in the study. Subjects ranged from recreationally active to Olympic triathletes and their self-reported training loads averaged 10 h/week. Subjects were required to complete a comprehensive health-screening questionnaire prior to starting the study and had not taken any regular medication or antibiotics in the 3 months prior to the study. All subjects were fully informed about the rationale for the study and of all experimental procedures to be undertaken. Subjects provided written consent to participate in the study, which had earlier received the approval of Loughborough University ethical advisory committee. The details of inclusion and exclusion criteria were described in Chapter 2.
A total of 267 healthy individuals were recruited as subjects from Loughborough University, UK (latitude 53°N) during November 2011 with the mean age of the study cohort at recruitment being 21 ± 3 years (mean ± SD). For the first visit to the laboratory, subjects arrived in the morning at 08:30-10:30 following an overnight fast of approximately 12 h and their body mass and height were recorded. Information about the study was given to them and they then signed an informed consent form. Saliva and blood samples were collected as described in Chapter 2.

**Study protocol**

During the 4-month study period subjects were requested to continue with their normal training programs. Subjects completed a validated health (URTI symptoms) questionnaire (Jackson et al., 1958; Appendix A) on a daily basis. Subjects were not required to abstain from medication when they were suffering from illness symptoms but they were required, on a weekly basis, to report any unprescribed medications taken, visits to the doctor or any prescribed medications.

The illness symptoms listed on the questionnaire were: sneezing, headache, malaise, nasal discharge, nasal obstruction, sore throat, cough, ear ache, hoarseness, fever, chilliness and joint aches and pains. The non-numerical severity ratings of mild, moderate and severe of severity of symptoms were scored as 1, 2 or 3, respectively to provide a quantitative means of data analysis and the total symptom score for every subject each day was calculated as a sum of multiplied numbers of symptoms experienced by the numerical severity ratings. A URTI
was deemed present when (i) total symptom score was ≥15 on any two consecutive days and 
(ii) when a subject positively indicated suffering a common cold on ≥ 3 days according to 
Jackson et al. (1958). Subjects were also asked to rate the impact of illness symptoms on their 
ability to train (above normal, at the same level, below normal or training stopped). The total 
number of URTI symptom days was also determined as the number of days with a symptom 
score of ≥5 according to Predy et al. (2005).

Subjects were also asked to fill in a standard short form of IPAQ 
(http://www.ipaq.ki.se/downloads.htm) at weekly intervals, thus providing a quantitative 
information on training loads in MET-h/week (Craig et al., 2003; Appendix B). Subjects 
attended the laboratory every 4 weeks following an overnight fast. Subjects were required to 
abstain from any strenuous physical activity for 24 h before coming to the laboratory. During 
these visits body mass was recorded and an unstimulated saliva sample was collected. 
Venous blood samples were collected only at the start and end of the study period.

Saliva analysis

The saliva volume collected and flow rate were estimated as described in Chapter 2. Saliva 
samples were analysed for SIgA, α-amylase, lactoferrin and lysozyme as described in 
Chapter 2. Secretion rates for each of the salivary AMPs were calculated as described in 
Chapter 2. Values obtained from the 5 visits at 4-week intervals were averaged for each 
subject.
**Plasma analysis**

EDTA plasma samples were assayed for IgG antibodies to CMV and EBV, cathelicidin concentrations and vitamin D concentrations as described in Chapter 2.

**Antigen-stimulated cytokine production**

Stimulated whole blood culture production of cytokines (IFN-γ, TNF-α, IL-1β, IL-2, IL-6 and IL-10) was determined as described in Chapter 2. The analysis of cytokine concentrations was also described in Chapter 2. The measured cytokine concentrations for the monocyte-derived cytokines (TNF-α and IL-1β) and lymphocyte-derived cytokines (IL-2 and IFN-γ) were divided by the monocyte and lymphocyte counts, respectively to give cytokine production per $10^6$ cells. Although the production of TNF-α is divided by monocyte counts (the main source), it can be also produced by other cell types, such as neutrophils, NK cells and CD4+ T cells. IL-6 and IL-10 are produced by both monocytes and lymphocytes, so it is not appropriate to normalise the production of these two cytokines by cell subset counts.

**Statistical analysis**

The Shapiro-Wilk test was used to determine if data sets were normally distributed. Data are presented as mean (±SD) for data sets that were normally distributed; for data sets that were not normally distributed, the median and IQR are shown. The accepted level of significance was P<0.05.
Chapter 3  
Factors influencing respiratory illness incidence in athletes

The difference in proportion of subjects who presented with symptoms of infection during the trial between the males and females was assessed by a Chi-squared test. Self-reported training load (h/week), average IPAQ scores (MET-h/week), anthropometric and haematological variables (including blood leukocyte, neutrophil, monocyte and lymphocyte counts) were compared between males and females using unpaired t tests for normally distributed data. Changes in training load over time for both sexes were evaluated by 2-way ANOVA with post hoc Bonferroni t tests to locate differences from week 1. The salivary AMP concentrations and secretion rates were compared between males and females using nonparametric Mann-Whitney tests for data that were not normally distributed. The changes in the secretion rates of salivary AMPs over the 16 weeks of the study in males and females were assessed by non-parametric Friedman tests with post hoc Dunn’s tests to compare values at weeks 4, 8, 12 and 16 with baseline within sex. Differences between sexes at specific sampling timepoints were examined using Mann Whitney tests.

The difference in proportion of subjects who presented with symptoms of infection during the trial between CMV (and EBV) positive and negative groups was compared by chi-squared test. Differences in mean training loads between CMV (and EBV) positive and negative groups were compared by independent t-test. Differences in the total number of URTI episodes and symptom days, the mean symptom-severity score and the mean duration of subjects with infection symptoms between CMV (and EBV) positive and negative serostatus were assessed with Mann-Whitney test. A similar analysis was applied for CMV-EBV combined serostatus. Mann-Whitney test was also used to examine differences in the blood leukocyte counts between CMV-EBV combined positive and negative serostatus.
The Kruskal-Wallis test (nonparametric equivalent of one-way ANOVA) with post-hoc Mann-Whitney test was used to examine differences in the salivary variables, blood leukocyte counts and the total number of URTI episodes and symptom days among groups classified by vitamin D status using the following ranges for plasma total 25(OH)D: 12-30 nmol/L (deficient); 30-50 nmol/L (inadequate), 50-120 nmol/L (adequate) and >120 nmol/L (suggested as optimal) (Heaney et al., 2011; IoM, 2011). For subjects with URTI symptoms, the symptom-severity score and the duration of URTI episodes among the 4 groups were also assessed using the Kruskal-Wallis test with post-hoc Mann-Whitney test. The plasma total 25(OH)D concentrations were compared between male and female subjects, and indoor and outdoor training locations using the Mann-Whitney test. The total plasma 25(OH)D concentrations at the start and the end of the study were compared by Wilcoxon signed-rank test. The difference in proportion of subjects who presented with symptoms of URTI during the trial between the vitamin D optimal and deficient groups was assessed by the chi-squared test. Correlation between the number of URTI episodes and the plasma 25(OH)D concentration as well as the plasma cathelicidin and 25(OH)D concentration was done using Spearman's rank correlation coefficient. One-way ANOVA with post-hoc Bonferroni test was used to examine differences in the plasma cathelicidin among high, middle and low level vitamin D status groups. Differences in antigen-stimulated cytokine production between high and low vitamin D status groups were compared with the Mann-Whitney test.

3.4 Results

Adherence to the study

Of the 267 subjects, 239 subjects completed the full 16 weeks of the study. Reasons for dropout included overseas travel, injury or persistent non-respiratory illness (preventing them
from performing training) or due to undisclosed reasons. 236 subjects (166 males, 70 females) provided sufficient blood for both CMV/EBV serostatus analysis and routine haematology on 2 occasions as well as and sufficient saliva for analysis of AMPs on all 5 occasions. Among the females 96% reported having regular periods and 40% were taking oral contraceptives. In addition, plasma samples were analysed for total 25(OH)D from 225 subjects because there were 11 subjects having insufficient plasma volume for this analysis. At the end of the study blood samples with sufficient volume for analysis were obtained from 181 subjects.

**Baseline characteristics and physical activity levels**

The baseline characteristics between males and females are as shown in Table 3.1. There was no significant difference in age between males and females (Table 3.1) but males were taller, heavier and had higher body mass index (BMI) than females. Males had higher red blood cell (RBC) count, haematocrit and haemoglobin concentration than females. Total blood leukocyte, monocyte and lymphocyte counts were not different between the sexes but females had higher circulating numbers of neutrophils than males (P = 0.040).
Self-reported weekly training loads (mean ± SD) based on information gathered in the pre-screening questionnaire were similar in males and females (both 10 ± 3 h/week, P = 0.857). Analysis of the IPAQ questionnaires indicated that the weekly training loads were relatively stable within both sexes over the 16 weeks of the study (Figure 3.1) although there was a significant main effect of time with training load falling below week 1 values in weeks 6-11. There was no significant effect of sex and no significant sex x time interaction. Training loads were, on average, about 66 MET-h/week which is equivalent to about 11 h of moderate-vigorous activity per week. The training loads averaged over the whole 16-week study period were not significantly different between males and females (Table 3.1).
Figure 3.1 Training loads in MET·h/week over the 16-week study period for men (n=186) and women (n=70) who completed the study. Data are mean ± SD. There was a main effect of time (P<0.001) and the location of significant differences from week 1 are indicated as follows: * P<0.05, ** P<0.01. There was no significant effect of sex and no significant sex x time interaction.

Infection symptom incidence and its impact on training loads

Analysis of the URTI symptom questionnaires indicated that 4.0 ± 1.6% of the cohort experienced an URTI episode each week (Figure 3.2); 136 subjects did not experience a single URTI episode during the study period whereas 103 subjects experienced at least one URTI episode during the study period. The proportion of subjects whose training was negatively affected when URTI was present was 0.70 and when URTI was present subjects reduced their weekly training load by an average of 24%.
3.4.1 The effects of sex differences

Salivary variables

When averaged over the 5 sampling occasions, saliva flow rates and the secretion rates of lactoferrin, lysozyme and amylase (Table 3.2) were significantly higher in males than females. Concentrations of SIgA and amylase were not different between the sexes whereas lactoferrin (P=0.021) and lysozyme (P=0.033) concentrations were significantly higher in males. While there were significant effects of time for the secretion rates of lactoferrin, lysozyme, amylase and SIgA in males, there were significant effects of time only for amylase and SIgA secretion rates in females (Figure 3.3). The changes over time followed the same pattern for both sexes.

URTI incidence and severity and duration of URTI

Females tended to be more susceptible to URTI than males: The proportion of males and females who experienced one or more URTI episodes during the study period was 40% of all
males and 52% of all females (P=0.083, chi-squared test) and the average number of URTI episodes was 0.6 ± 0.8 in males and 0.8 ± 1.0 in females (P=0.103). The number of URTI days was significantly higher in females (males: 4.7 ± 5.0 days vs females: 6.8 ± 7.1 days, P=0.016). When an URTI episode was present, the mean total symptom severity score was not significantly different between sexes (males: 90 ± 67 vs females: 106 ± 77, P=0.312) but the mean duration of symptoms was significantly longer in females (males: 11.6 ± 6.8 days vs females: 15.5 ± 9.3 days, P=0.024).

Only 17% of subjects reported that they took some medication when suffering from an URTI episode and only 4% reported that they visited their doctor and none were given prescription drugs. The study results were not corrected for this (i.e. all URTI episodes were included in the analysis).

<table>
<thead>
<tr>
<th>Table 3.2 Salivary variables in male and female athletes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
</tr>
<tr>
<td>Saliva flow rate (µL/min)</td>
</tr>
<tr>
<td>SIgA concentration (ng/L)</td>
</tr>
<tr>
<td>SIgA secretion rate (µg/min)</td>
</tr>
<tr>
<td>Lysozyme concentration (µg/L)</td>
</tr>
<tr>
<td>Lysozyme secretion rate (ng/min)</td>
</tr>
<tr>
<td>Lactoferrin concentration (µg/L)</td>
</tr>
<tr>
<td>Lactoferrin secretion rate (ng/min)</td>
</tr>
<tr>
<td>Amylase activity (U/mL)</td>
</tr>
<tr>
<td>Amylase secretion rate (U/min)</td>
</tr>
</tbody>
</table>

Values are expressed as median and interquartile range. P value is from Mann-Whitney U test.
Figure 3.3 Changes in salivary secretion rates over time for (A) Lactoferrin, (B) Lysozyme, (C) Amylase and (D) SIgA. Data are median and interquartile range. Significantly different from baseline sample within sex: † P<0.05, ‡ P<0.01 (Dunn’s post hoc test applied when Friedman test P<0.05). Significant difference between males and females at specific timepoint: * P<0.05, ** P<0.01 (Mann Whitney test).
Table 3.3 Illness incidence among athletes at major competitive events lasting 2-3 weeks.

<table>
<thead>
<tr>
<th>Games</th>
<th>Season</th>
<th>Athletes (n)</th>
<th>Males (n)</th>
<th>Females (n)</th>
<th>Illness in all athletes (%)</th>
<th>Illness in males (%)</th>
<th>Illness in females (%)</th>
<th>Respiratory (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olympics 2012</td>
<td>Summer</td>
<td>10568</td>
<td>5892</td>
<td>4676</td>
<td>7.2</td>
<td>5.3</td>
<td>8.6</td>
<td>41</td>
</tr>
<tr>
<td>Youth Olympics 2012</td>
<td>Winter</td>
<td>1021</td>
<td>562</td>
<td>459</td>
<td>8.4</td>
<td>6</td>
<td>11</td>
<td>61</td>
</tr>
<tr>
<td>IAAF 2011</td>
<td>Summer</td>
<td>1851</td>
<td>971</td>
<td>880</td>
<td>6.8</td>
<td>7.1</td>
<td>7.7</td>
<td>39</td>
</tr>
<tr>
<td>Olympics 2010</td>
<td>Winter</td>
<td>2567</td>
<td>1522</td>
<td>1045</td>
<td>7.2</td>
<td>5.2</td>
<td>8.7</td>
<td>63</td>
</tr>
<tr>
<td>IAAF 2009</td>
<td>Summer</td>
<td>1979</td>
<td>1082</td>
<td>897</td>
<td>6.8</td>
<td>5.6</td>
<td>8.4</td>
<td>36</td>
</tr>
<tr>
<td>FINA 2009</td>
<td>Summer</td>
<td>2318</td>
<td>1306</td>
<td>1012</td>
<td>6.6</td>
<td>5.1</td>
<td>7.9</td>
<td>50</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>20304</td>
<td>11335</td>
<td>8969</td>
<td>7.2 ± 0.6</td>
<td>5.7 ± 0.8</td>
<td>8.7 ± 1.2*</td>
<td>48 ± 12</td>
</tr>
</tbody>
</table>

IAAF: International Association of Athletics Federations; FINA: Federation Internationale de Natation; n = number of registered athletes.

* Significant difference in mean illness rates between male and female athletes (P < 0.05).
3.4.2 The effects of CMV/EBV serostatus

**CMV serostatus and infection incidence, severity and duration**

Twenty five percent of the subject cohort were CMV positive with a similar proportion in males (24%) and females (26%). There was no difference in the mean training load between CMV positive and negative groups (positive: 64.5 ± 29.9 MET-h/week, negative: 67.6 ± 31.1 MET-h/week; p=0.505). There was no difference in the proportion of subjects who presented with symptoms of infection between CMV positive and negative groups (positive 0.38, negative 0.44; P=0.349). The total number of URTI symptom days in the CMV negative group was significantly higher than the CMV positive group (Table 3.4). There was no significant difference for the total number of URTI episodes and there were no significant differences in symptom-severity score or the duration of episodes between subjects with CMV positive and negative serostatus.

| Table 3.4 Infection symptom incidence in CMV positive and negative subjects. |
|---------------------------------|-----------------|-----------------|---|
|                                | CMV-positive    | CMV-negative    | P  |
|                                | n = 58          | n = 178         |    |
| Number of episodes             |                 |                 |    |
| Mean ± SD                      | 0.5 ± 0.7       | 0.7 ± 0.9       |    |
| Median (95 % CI)               | 0 (0.3-0.7)     | 0 (0.6-0.8)     | 0.107 |
| Number of symptom days         |                 |                 |    |
| Mean ± SD                      | 4.1 ± 5.4       | 5.8 ± 6.2       |    |
| Median (95 % CI)               | 2 (2.7-5.6)     | 4 (4.9-6.8)     | 0.033 |
| Symptom-severity score         |                 |                 |    |
| Mean ± SD                      | 66.3 ± 46.0     | 65.2 ± 36.9     |    |
| Median (95 % CI)               | 60 (45.9-86.7)  | 53 (56.9-73.4)  | 0.817 |
| Episode duration (days)        |                 |                 |    |
| Mean ± SD                      | 10.6 ± 5.7      | 8.6 ± 4.5       |    |
| Median (95 % CI)               | 9 (8.1-13.1)    | 8 (7.6-9.6)     | 0.082 |

Also shown are the severity score and duration of URTI episodes. Data are shown as mean ± SD and also as median and 95 % confidence intervals (CI). The P values refer to outcomes of Mann-Whitney U tests on nonparametric data.
Chapter 3  Factors influencing respiratory illness incidence in athletes

**EBV serostatus and infection incidence, severity and duration**

Eighty four percent of the subject cohort were EBV positive with a similar proportion in males (84%) and females (83%). There was no difference in the mean training load between EBV positive and negative groups (positive: 68.4 ± 31.1 MET-h/week, negative: 59.1 ± 28.3 MET-h/week; p=0.085). There was no difference in the proportion of subjects who presented with symptoms of infection between EBV positive and negative groups (positive 0.41, negative 0.51; P=0.266). There were no significant differences for the number of URTI episodes, number of symptom days, the symptom-severity score or the duration of episodes between subjects with EBV positive and negative serostatus (Table 3.5).

| Table 3.5 Infection symptom incidence in EBV positive and negative subjects. |
|-------------------------------------------------|-----------------|-----------------|---|
|                                                | EBV-positive    | EBV-negative    | P  |
| Number of episodes                             | n = 197         | n = 39          |    |
| Mean ± SD                                      | 0.6 ± 0.8       | 0.9 ± 1.0       |    |
| Median (95 % CI)                               | 0 (0.5-0.7)     | 1 (0.5-1.2)     | 0.132|
| Number of symptom days                         | n = 82          | n = 20          |    |
| Mean ± SD                                      | 5.1 ± 5.9       | 6.9 ± 6.8       |    |
| Median (95 % CI)                               | 4 (4.3-5.9)     | 6 (4.7-9.1)     | 0.133|
| Symptom-severity score                         | n = 82          | n = 20          |    |
| Mean ± SD                                      | 65.0 ± 40.3     | 67.3 ± 32.9     |    |
| Median (95 % CI)                               | 55 (56.1-73.8)  | 62 (51.9-82.7)  | 0.404|
| Episode duration (days)                        | n = 82          | n = 20          |    |
| Mean ± SD                                      | 9.0 ± 5.0       | 9.1 ± 4.4       |    |
| Median (95 % CI)                               | 8 (7.9-10.1)    | 7 (7.1-11.2)    | 0.859|

Also shown are the mean severity score and duration of URTI episodes. Data are shown as mean ± SD and also as median and 95 % confidence intervals (CI). The P values refer to outcomes of Mann-Whitney U tests on nonparametric data.
**CMV-EBV combined serostatus and infection incidence, severity and duration**

Twenty one percent of the subject cohort were both CMV and EBV positive (CMV+EBV+) with a similar proportion in males (19%) and females (21%), whereas 13% of the subject cohort had no prior CMV or EBV infection (CMV-EBV-). There was no difference in the mean training load between the CMV+EBV+ and CMV-EBV- groups (positive: 61.9 ± 27.8 MET-h/week, negative: 53.5 ± 22.5 MET-h/week; P=0.161). There was no difference in the proportion of subjects who presented with symptoms of infection between the CMV+EBV+ and CMV-EBV- groups (positives 0.38, negatives 0.55; P=0.138). However, subjects with prior infection with both CMV and EBV had fewer URTI episodes and fewer URTI symptom days than subjects with no prior history of CMV and EBV infection (Table 3.6). The symptom-severity score and the duration of episodes were not significantly different between subjects in the CMV+EBV+ and CMV-EBV- groups.

<table>
<thead>
<tr>
<th></th>
<th>CMV+EBV+</th>
<th>CMV-EBV-</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of episodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.5 ± 0.8</td>
<td>1.0 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Median (95 % CI)</td>
<td>0 (0.3-0.7)</td>
<td>1 (0.6-1.4)</td>
<td>0.040</td>
</tr>
<tr>
<td>Number of symptom days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>4.3 ± 5.6</td>
<td>7.9 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>Median (95 % CI)</td>
<td>2 (2.7-5.9)</td>
<td>8 (5.3-10.4)</td>
<td>0.010</td>
</tr>
<tr>
<td>Symptom-severity score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>66.8 ± 49.4</td>
<td>68.0 ± 35.3</td>
<td></td>
</tr>
<tr>
<td>Median (95 % CI)</td>
<td>59 (43.0-90.6)</td>
<td>55 (49.8-86.1)</td>
<td>0.763</td>
</tr>
<tr>
<td>Episode duration (days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>10.0 ± 5.9</td>
<td>8.2 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Median (95 % CI)</td>
<td>8 (7.2-12.9)</td>
<td>7 (6.2-10.3)</td>
<td>0.299</td>
</tr>
</tbody>
</table>

Also shown are the severity score and duration of URTI episodes. Data are shown as mean ± SD and also as median and 95 % confidence intervals (CI). The P values refer to outcomes of Mann-Whitney U tests on nonparametric data.
Blood total and differential leukocyte counts in CMV+EBV+ and CMV-EBV- subjects

The circulating number of lymphocytes in the CMV+EBV+ group was significantly higher than the CMV-EBV- group. However, there was no difference in the circulating numbers of total leukocytes, neutrophils or monocytes between the CMV+EBV+ and CMV-EBV- groups (Table 3.7).

<table>
<thead>
<tr>
<th></th>
<th>CMV+EBV+</th>
<th>CMV-EBV-</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (x10⁹ cells/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.4 ± 1.5</td>
<td>5.9 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Median (95 % CI)</td>
<td>6.0 (5.9-6.8)</td>
<td>6.1 (5.4-6.3)</td>
<td>0.593</td>
</tr>
<tr>
<td>Neutrophils (x10⁹ cells/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>3.1 ± 1.2</td>
<td>3.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Median (95 % CI)</td>
<td>2.8 (2.8-3.5)</td>
<td>3.0 (2.6-3.3)</td>
<td>0.888</td>
</tr>
<tr>
<td>Lymphocytes (x10⁹ cells/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2.4 ± 0.7</td>
<td>2.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Median (95 % CI)</td>
<td>2.3 (2.2-2.5)</td>
<td>2.0 (1.8-2.2)</td>
<td>0.029</td>
</tr>
<tr>
<td>Monocytes (x10⁹ cells/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Median (95 % CI)</td>
<td>0.6 (0.6-0.7)</td>
<td>0.6 (0.6-0.7)</td>
<td>0.574</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD and also as median and 95 % confidence intervals (CI). The P values refer to outcomes of Mann-Whitney U tests on nonparametric data.

3.4.2 The effects of vitamin D status

**Vitamin D concentrations**

Plasma 25(OH)D₂ was below the detection limit (2.5 nmol/L) in 57% of subjects and the average plasma 25(OH)D₂ concentration was only 4.4 nmol/L (median, 0; IQR, 0-9 nmol/L). The median (IQR) plasma 25(OH)D₃ concentration at the start of the study was 53 (40-66) nmol/L and the median total 25(OH)D concentration was 57 (44-71) nmol/L. The number (and percentage) of subjects classed as optimal, adequate, inadequate and deficient was 11
Factors influencing respiratory illness incidence in athletes

(5%), 128 (57%), 68 (30%) and 18 (8%). Plasma 25(OH)D concentration was not significantly different (p=0.478) between males (N=157; median, 56; IQR, 43-69 nmol/l) and females (N=68; median, 58; IQR, 45-72 nmol/L). Plasma 25(OH)D concentration was not significantly different (P = 0.120) between indoor (N=50; median, 64; IQR, 46-73 nmol/L) and outdoor sports (N=175; median, 55; IQR, 43-69 nmol/L). The total plasma 25(OH)D concentration at the end of the study (N=181; median, 47; IQR, 35-68 nmol/L) was significantly lower (P=0.003) than that at the start of the study. At the start of the 4-month study period 38% of athletes had insufficient or deficient plasma 25(OH)D values (< 50 nmol/L) and by the end of the study 55% of athletes had plasma 25(OH)D values of less than 50 nmol/L.

Vitamin D status and URTI incidence, severity and duration

The proportion of subjects in the optimal vitamin D concentration group who experienced one or more URTI episodes during the trial was significantly lower than for the vitamin D deficient group (optimal 27%, deficient 67%; P=0.039). There was a significant difference for URTI symptom days among the four vitamin D status groups and the total number of URTI symptom days in the deficient group was significantly higher than the other groups (Table 4.1). Vitamin D concentration tended to influence prevalence of URTI episodes but this fell just short of statistical significance (P=0.061) and there was a tendency for the deficient group to have more episodes (Table 3.8). For subjects who experienced one or more URTI episodes, there was a significant difference in the median symptom-severity score per URTI episode among the four groups but no significant difference in the median duration of episodes (Table 3.8) although there was a tendency for episodes to be longer with low vitamin D concentration. The median symptom-severity score in the deficient group was significantly higher than the other groups.
Table 3.8 Infection symptom incidence among different vitamin D status groups. Also shown are the mean severity score and duration of URTI episodes.

<table>
<thead>
<tr>
<th></th>
<th>Optimal N = 11</th>
<th>Adequate N = 128</th>
<th>Inadequate N = 68</th>
<th>Deficient N = 18</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INCIDENCE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of episodes</td>
<td>0 (0-1) *</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0.062</td>
</tr>
<tr>
<td>Number of symptom days</td>
<td>1 (0-6)*</td>
<td>4 (0-8)*</td>
<td>4 (1-8)*</td>
<td>9 (3-17)</td>
<td>0.040</td>
</tr>
<tr>
<td><strong>WHEN URTI PRESENT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptom severity score</td>
<td>43 (38-52)*</td>
<td>47 (40-69)*</td>
<td>62 (46-74)*</td>
<td>102 (67-199)</td>
<td>0.013</td>
</tr>
<tr>
<td>Duration (days)</td>
<td>5 (5-7)*</td>
<td>8 (6-9)*</td>
<td>8 (5-14)*</td>
<td>13 (10-17)</td>
<td>0.059</td>
</tr>
</tbody>
</table>

Data are median and IQR. P value from Kruskal-Wallis test is shown in right hand column.
* Significantly different from Deficient (Mann-Whitney U test).
Chapter 3  Factors influencing respiratory illness incidence in athletes

**Plasma cathelicidin concentration**

After vitamin D concentration analysis of baseline samples, we also analyzed the initial plasma cathelicidin concentration from a subset of 80 subjects with random selections of high-level (N=26; median, 111; IQR, 96-138 nmol/L), mid-level (N=27; median, 50; IQR, 33-52 nmol/L) and low-level (N=27; median, 26; IQR, 23-28 nmol/L) 25(OH)D concentration. The plasma cathelicidin concentrations were 32.2 ± 11.9, 27.7±10.6 and 24.5±7.5 ng/ml in the high, mid and low level vitamin D status groups, respectively. There was a significant influence of vitamin D status on the plasma cathelicidin concentration (P=0.027). The plasma cathelicidin concentration in the high-level vitamin D status group was significantly higher than in the low-level group (P=0.023). In addition, there was a positive correlation between the plasma 25(OH)D and cathelicidin concentrations (r = 0.234, P=0.036).

**Salivary variables**

There was a significant difference in SIgA secretion rate among the four vitamin D groups (Figure 3.4) but no significant difference for the other salivary AMPs (Table 3.9). The SIgA secretion rate in the optimal vitamin D status group was significantly higher than in the other groups. The SIgA concentration tended to be lowest in the deficient group.
Figure 3.4 Influence of vitamin D status on the saliva IgA secretion rate. Median values for each group are indicated by the horizontal lines. Kruskal-Wallis test indicated a significant influence of vitamin D status on IgA secretion rate (P=0.018). Saliva IgA secretion rate was significantly higher in the Optimal group than in the other groups (P < 0.05).
Table 3.9 Salivary concentrations and secretion rates of AMPs among different vitamin D status groups.

<table>
<thead>
<tr>
<th></th>
<th>Optimal N = 11</th>
<th>Adequate N = 128</th>
<th>Inadequate N = 68</th>
<th>Deficient N = 18</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactoferrin concentration (ng/mL)</td>
<td>2006 (1413-3184)</td>
<td>2187 (1546-3184)</td>
<td>2491 (1606-3633)</td>
<td>2419 (1624-3001)</td>
<td>0.948</td>
</tr>
<tr>
<td>Lactoferrin SR (ng/min)</td>
<td>801 (587-1229)</td>
<td>756 (527-1151)</td>
<td>771 (367-1143)</td>
<td>872 (554-1645)</td>
<td>0.568</td>
</tr>
<tr>
<td>Lysozyme concentration (µg/mL)</td>
<td>1524 (641-2595)</td>
<td>1524 (926-2808)</td>
<td>1701 (1102-2693)</td>
<td>1361 (883-2437)</td>
<td>0.687</td>
</tr>
<tr>
<td>Lysozyme SR (µg/min)</td>
<td>435 (314-906)</td>
<td>517 (315-872)</td>
<td>504 (315-972)</td>
<td>609 (331-837)</td>
<td>0.970</td>
</tr>
<tr>
<td>SIgA concentration (µg/mL)</td>
<td>91 (82-130)</td>
<td>66 (49-100)</td>
<td>76 (46-11)</td>
<td>59 (46-78)*</td>
<td>0.103</td>
</tr>
<tr>
<td>SIgA SR (µg/min)</td>
<td>38.7 (30.3-48.6)</td>
<td>22.9 (14.2-36.6)*</td>
<td>19.5 (12.7-32.3)*</td>
<td>23.6 (14.8-32.9)*</td>
<td>0.018</td>
</tr>
<tr>
<td>Amylase activity (U/L)</td>
<td>131 (60-213)</td>
<td>142 (73-233)</td>
<td>131 (74-202)</td>
<td>121 (64-222)</td>
<td>0.839</td>
</tr>
</tbody>
</table>

Data are median and IQR. P value from Kruskal-Wallis test is shown in right hand column. SR = secretion rate.
* Significantly different from Optimal (Mann-Whitney U test).
Chapter 3  
Factors influencing respiratory illness incidence in athletes

**Blood leukocyte counts**

Based on analysis of blood samples collected at the start of the study there was no influence of vitamin D status on circulating numbers of total leukocytes, neutrophils, lymphocytes or monocytes (Table 3.10).

**Antigen-stimulated cytokine production**

After vitamin D concentration analysis of samples collected at the end of the study, we also measured antigen-stimulated cytokine production in a subset of 48 subjects with random selections of high-level (N=24; median, 101; IQR, 89-116 nmol/L) and low-level (N=24; median, 25; IQR, 20-26 nmol/L) 25(OH)D concentration (Table 3.11). Both blood monocyte and lymphocyte counts were not significantly different in the subset of subjects for whom antigen-stimulated cytokine production was determined. However, production of both monocyte-derived cytokines, TNF-α and IL-1β, were significantly lower in the low-level vitamin D subjects compared with those with high vitamin D status. Production of the lymphocyte-derived pro-inflammatory cytokine, IFN-γ, was significantly lower in the low-level vitamin D subjects compared with those with high vitamin D status. Moreover, production of IL-6 was also significantly lower in the low-level vitamin D subjects. The antigen-stimulated production of IL-10 was not significantly different between the high and low vitamin D status groups.
Table 3.10 Blood total and differential leukocyte counts among different vitamin D status groups at the start of the study.

<table>
<thead>
<tr>
<th></th>
<th>Optimal N = 11</th>
<th>Adequate N = 128</th>
<th>Inadequate N = 68</th>
<th>Deficient N = 18</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (x10^9 cells/L)</td>
<td>5.9 (5.3-6.6)</td>
<td>5.9 (5.2-6.9)</td>
<td>5.9 (5.2-6.9)</td>
<td>6.5 (5.8-7.8)</td>
<td>0.495</td>
</tr>
<tr>
<td>Neutrophils (x10^9 cells/L)</td>
<td>2.8 (2.3-3.7)</td>
<td>2.9 (2.4-3.9)</td>
<td>3.0 (2.3-3.5)</td>
<td>3.4 (2.9-4.2)</td>
<td>0.352</td>
</tr>
<tr>
<td>Lymphocytes (x10^9 cells/L)</td>
<td>1.9 (1.7-2.3)</td>
<td>2.1 (1.7-2.3)</td>
<td>2.1 (1.8-2.4)</td>
<td>1.9 (1.8-2.0)</td>
<td>0.555</td>
</tr>
<tr>
<td>Monocytes (x10^9 cells/L)</td>
<td>0.5 (0.4-0.6)</td>
<td>0.6 (0.5-0.7)</td>
<td>0.6 (0.5-0.7)</td>
<td>0.6 (0.6-0.8)</td>
<td>0.136</td>
</tr>
</tbody>
</table>

Data are median and IQR. P value from Kruskal-Wallis test is shown in right hand column.
Table 3.11 Antigen-stimulated cytokine production in the high and low level vitamin D groups

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>High-level (n=24)</th>
<th>Low-level (n=24)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>153.6 (73.1-398.6)</td>
<td>50.7 (23.9-83.1)</td>
<td>0.012</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>4.0 (2.4-8.8)</td>
<td>2.8 (2.0-6.0)</td>
<td>0.164</td>
</tr>
<tr>
<td>IL-2 (pg/10^6 Ly)</td>
<td>21.8 (9.6-46.0)</td>
<td>13.4 (4.8-26.7)</td>
<td>0.156</td>
</tr>
<tr>
<td>IFN-γ (pg/10^6 Ly)</td>
<td>9.3 (5.0-16.1)</td>
<td>3.8 (2.4-6.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>TNF-α (pg/10^6 Mo)</td>
<td>34.8 (20.8-46.9)</td>
<td>13.7 (6.2-21.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-1β (pg/10^6 Mo)</td>
<td>18.6 (11.3-28.0)</td>
<td>10.1 (6.7-13.6)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Data are median and IQR. Ly = lymphocytes; Mo = monocytes. P value from Mann-Whitney U test is shown in right hand column.
3.5 Discussion

The aims of this research were to determine the various possible factors affecting illness susceptibility in athletes. The main findings of the present study were that female athletes may be more susceptible than their male counterparts to URTI during winter training periods. Moreover, athletes who had experienced prior infection with both CMV and EBV may have fewer URTI episodes than those with negative serostatus for both CMV and EBV. In addition, athletes with low vitamin D status may have a higher risk of URTI and suffer more severe symptoms when URTI is present.

3.5.1 The effects of sex differences

Low salivary SIgA concentration or secretion rate has been identified as a risk factor for development of URTI in physically active individuals (Fahlman & Engels, 2005; Gleeson et al., 2012; Gleeson et al., 1999; Gleeson et al., 2002; Neville et al., 2008). In the present study, we found that female athletes tended to have lower salivary SIgA secretion rates than male athletes during a 16-week winter training period although this difference was not statistically significant. It has been suggested that salivary SIgA levels are a surrogate marker of host protection and the suppression of salivary SIgA after prolonged exercise or heavy training is itself a probable consequence of altered T lymphocyte function (Clancy et al., 2006). Females generally have lower unstimulated saliva flow rates than males (Percival, Challacombe, & Marsh, 1994), whereas SIgA concentration in unstimulated saliva has been reported to be unaffected by sex among relatively large cohorts of healthy young adults (Kugler, Hess, & Haake, 1992; van Anders, 2010; Van Anders, 2010). A previous smaller scale study reported lower salivary SIgA concentration and secretion rate in females (n=34) than in males (n=46) among a cohort of student athletes (Gleeson, Bishop, Oliveira, McCauley, & Tauler, 2011). Other small scale studies on elite swimmers have also reported lower SIgA concentrations in
females compared with males (n=11 females, n=15 males (Gleeson et al., 2012); n= 5 females, n=7 males (Allgrove, Gomes, Hough, & Gleeson, 2008)), as has a small scale study of recreational cyclists (n=8 females, n=8 males (Allgrove, Geneen, Latif, & Gleeson, 2009)); but, to our knowledge, our investigation is the first large scale study to report a sex difference in salivary AMP secretion rates in athletes from a range of endurance-based sports.

In the present study, it was observed that the female athletes had lower saliva amylase secretion rate than male athletes. Amylase, an enzyme that breaks down starch into maltose, is important to host defence in oral-respiratory mucosal immunity by inhibiting the adherence and growth of certain bacteria (Allgrove et al., 2008). The lower salivary amylase secretion rate in females could be at least partly due to lower circulating adrenaline levels that are observed both at rest (Weitz, Elam, Born, Fehm, & Dodt, 2001) and after exercise (Ruby et al., 1997) in females when oestrogen levels are high due to exogenous ovarian hormone administration. Both increased sympathetic nervous activity (Thoma, Kirschbaum, Wolf, & Rohleder, 2012), and elevated plasma adrenaline and noradrenaline (Chatterton, Vogelsong, Lu, Ellman, & Hudgens, 1996) are known to increase salivary amylase secretion. However, it has been indicated that sympathetic nervous activity may not be responsible for the changes in salivary amylase secretion (Leicht et al., 2011) and there were only 40% of females in the study using the oral contraceptive.

In addition, we also found that the secretion rates of lysozyme and lactoferrin were significantly lower in female athletes than male athletes. Lysozyme and lactoferrin play important roles in oral-respiratory mucosal immunity against pathogen infection: Lactoferrin possesses the ability to sequester iron, bind to bacteria, and has antimicrobial activities that act in synergy with SIgA and lysozyme (Ellison, Giehl, & LaForce, 1988; Legrand, Elass,
Pierce, & Mazurier, 2004). Therefore, it is possible that the lower secretion rates of amylase, lysozyme and lactoferrin might leave female athletes at greater risk of contracting RTIs during winter training periods.

Of course, other factors could also account for differences in infection risk between the sexes. For example, in the general population, women have been reported to have fewer blood monocytes and NK cells, but more CD4+ cells and more neutrophils than men (Bouman et al., 2004; Willemsen, Carroll, Ring, & Drayson, 2002) and women appear to suffer from fewer viral infections including RTIs than men (Beery, 2003). The present study also found athletic females to have higher blood neutrophil counts than their male counterparts but it was the females who appeared to be more susceptible to URTI than men. It is possible that the same training load could have a greater depressive effect on humoral and systemic immunity (e.g. lower secretion rates of mucosal AMPs and fewer numbers of circulating B cells and NK cells) for women (Gleeson, Bishop, Oliveira, McCauley et al., 2011) than for men (that is not evident in the normal, more sedentary population) but this possibility needs to be resolved by future research. Such an effect may be responsible for the reversal of the usual situation of more effective immune function in females into the opposite situation in athletes. Our data support the notion that URTI are more prevalent in female athletes than their male counterparts when they engage in similarly high training loads. In recent years medical and sport science support personnel have collected data on rates of injuries and illnesses in large cohorts of athletes attending (and intending to compete in) large competitive events lasting 2-3 weeks (e.g. winter and summer Olympic games, athletic and aquatic sport world championships). The findings from these studies are summarised in Table 2.3 and suggest that illness episodes actually are more prevalent in the women than the men (Alonso et al., 2012; Alonso et al., 2010; Edouard et al., 2013; Engebretsen et al., 2013; Engebretsen et al.,
2010; Mountjoy et al., 2010; Ruedl et al., 2012). About half of the illnesses reported at these events were URTI episodes. Other epidemiological studies on physically active (Nieman, Henson, Austin, & Sha, 2011) and athletic (Heath et al., 1991; Konig, Grathwohl, Weinstock, Northoff, & Berg, 2000) men and women also suggest that URTI are more prevalent in the females.

A limitation of the present study is that the phase of the menstrual cycle (when blood and saliva samples were taken) was not determined but we did establish that 40% of the females were taking oral contraceptives. It is possible that the high training loads of some of the female endurance athletes in our study could have caused them to be amenorrhoeic and one would expect that this would make their immune variables more similar to that of men. However, according to the health screen questionnaire used at the start of the study 96% of the females reported that they had regular periods so it seems likely that during the study period very few of the females were amenorrhoeic. This aside, menstrual cycle phase was not found to affect resting saliva SIgA responses in endurance trained female athletes (Burrows, Bird, & Bishop, 2002). Another limitation is that we did not attempt to distinguish between symptoms of an infectious/illness nature vs inflammation throughout the 16 weeks of the study design. However, nearly all studies to date have used self-reported symptoms rather than pathogen identification in studies involving athletes and URTI incidence. We used the validated Jackson score questionnaire which is a conservative instrument requiring a substantial symptom score criterion threshold to define a RTI episode (Jackson et al., 1958). Given that the average duration of URTI episodes in our study was 13 days, it is very likely that the vast majority of episodes were caused by infections as typically inflammation/allergy symptom episodes generally only last 1-3 days according to Walsh et al. (2011).
Chapter 3 Factors influencing respiratory illness incidence in athletes

The major strengths of our study are that we used a validated questionnaire (Jackson et al., 1958) to determine URTI episodes and measured saliva AMPs on 5 occasions at monthly intervals to better establish the average values for each individual. The average training loads of the athlete cohort were generally high and were not different between males and females. Our study population was a group of university athletes on a single campus site so that environment and pathogen exposure were likely to have been similar for all subjects.

3.5.2 The effects of CMV/EBV serostatus

Contrary to our original hypothesis athletes who had previous infection with CMV or EBV were not predisposed to increased susceptibility to URTI. The data from our study show that there was no difference in the proportion of subjects who presented with symptoms of infection during the 4-month study period between CMV/EBV positive and negative groups. Although CMV or EBV can cause a general suppression of immune function via the production of a viral homologue of human IL-10 which is a potent inhibitory cytokine (Chang & Barry, 2010; Liu et al., 1997), this does not seem to be an important influence on URTI risk. However, a previous study has reported a significant association between prior infection with EBV and upper respiratory symptoms (Gleeson et al., 2002) in elite swimmers. Gleeson et al. (2002) indicated that while 9 of 11 EBV seropositive athletes had upper respiratory symptoms during a 30-day intensive training period, there were no reported upper respiratory symptoms in 3 EBV seronegative athletes. The reason for the different result from our study may be due to the relatively small numbers of athletes in their study. More investigations are warranted to clarify the relationship between previous CMV and EBV infection and the incidence of URTI.
In fact, we found that athletes with previous CMV infection had fewer URTI symptom days during the 4-month study period than those with no previous infection. Furthermore, athletes who had experienced prior infection with both CMV and EBV had fewer URTI episodes and fewer symptom days than those with negative serostatus for both CMV and EBV despite having similar mean training loads. The reasons for this are still unclear but could be related to the altered T cytotoxic cell response to exercise in individuals with positive CMV serostatus (Turner et al., 2010). Latent CMV infection appears to amplify memory CD8 T-lymphocyte mobilisation and egress in response to exercise. Memory CD8 T cells possessing the ability to rapidly produce cytokines, such as IFN-γ and tumor necrosis factor alpha, are an important component of protective immunity against viral infections (Wherry & Ahmed, 2004). The amplification of this response may promote protective immune surveillance, and thereby reduce risk of infection. It could be especially protective against infection in the post-exercise period when several other aspects of immune function such as neutrophil oxidative burst, macrophage antigen presentation, T lymphocyte cytokine production and proliferation are depressed (Gleeson & Walsh, 2012). In the present study, it was also shown that the circulating numbers of lymphocytes at rest are significantly higher in athletes with CMV-EBV combined positive serostatus than those with negative serostatus which might also provide a higher level of immunosurveillance. Further research is still needed to understand the influence of previous CMV or EBV infection on the immune system.

On the basis of the present data, we found that 25% of athletes had a previous CMV infection, 84% of athletes had a previous EBV infection and 21% of athletes were both CMV and EBV positive. The proportion of subjects with previous CMV infection in the present study is lower than previously reported in the general population; Bate et al. (2010) found that approximately 50% individuals aged 6-49 years in the general population of the USA were
CMV positive and that CMV seropositivity was independently associated with older age, female sex, foreign birthplace and high household crowding. Nevertheless, the high proportion of previous EBV infection in our study is similar to other studies on young adults (Gleeson et al., 2002; Pottgiesser et al., 2006). Given that large proportion of athletes appear to have had a previous infection with CMV or EBV, it was important for further studies to clarify whether previous CMV or EBV infection (or both) has an important influence on the risk of URTI.

3.5.3 The effects of vitamin D status

According to the findings from the present study, it seems likely that vitamin D status has an influence on URTI symptom incidence. We found that a higher proportion of subjects (67%) in the vitamin D deficient status group experienced one or more URTI episodes during the 4-month study period than in the optimal vitamin D group (27%). Recent studies have also shown an inverse association between vitamin D status and respiratory infection incidence in young and elderly adults (Berry et al., 2011; Ginde et al., 2009). In the Third National Health and Nutrition Examination Survey involving 18,883 participants (Ginde et al., 2009), it was found that individuals with plasma 25(OH)D concentration less than 25 nmol/L had a significantly higher risk of respiratory infections (24%) than those with 25(OH)D levels higher than 75 nmol/L (17%). In addition, a population-based study on 6,789 British adults demonstrated that plasma 25(OH)D concentrations are inversely associated with recent URTI (Berry et al., 2011). There was a 7% reduction in the risk of respiratory infections for each 10 nmol/L increase in plasma 25(OH)D.

Furthermore, we also found that the total number of URTI symptom days in the vitamin D deficient group was significantly higher than in the other groups. There was a similar result in
a Finnish study in which young male Finnish soldiers with plasma 25(OH)D concentration less than 40 nmol/L had more days of absence from duty due to respiratory infections during the 6-month study period than soldiers with plasma 25(OH)D concentration more than 40 nmol/L (Laaksi et al., 2007). In addition, an observational study has shown that adults with 25(OH)D status less than 95 nmol/L had a longer duration of illness compared with those whose 25(OH)D status was higher than 95 nmol/L (Sabetta et al., 2010). In the present study, it was also shown that there was a tendency for episodes to be longer with low vitamin D status. Moreover, we found that subjects with plasma 25(OH)D higher than 30 nmol/L had less severe symptoms during URTI episodes compared with those having plasma 25(OH)D below 30 nmol/L. Thus, vitamin D might play a role in reducing both the severity and duration of URTI symptoms.

There is a plausible mechanism for the inverse association between plasma 25(OH)D status and risk/severity of URTI episodes: vitamin D is a key link between TLR activation and antimicrobial responses in innate immunity. Following activation of the TLR signalling cascade in the presence of microbes, vitamin D has a vital role in up-regulating the production of AMPs, such as cathelicidin and β-defensin (Liu et al., 2006). These AMPs are produced by both epithelial cells and macrophages and have a broad range of activities against microorganisms including the direct inactivation of viruses (Kamen & Tangpricha, 2010). They are secreted into the biofilm covering the epithelial surface, thereby creating a barrier that is chemically lethal to microbes. One of these AMPs, cathelicidin, enhances the microbicidal capability of monocytes and macrophages by increasing the oxidative burst potential of these phagocytic cells (Schwalfenberg, 2011) and has a defined vitamin D-dependent mechanism. Pathogenic antigens interact with TLRs on the epithelial cells and macrophages to upregulate the expression of the 1α-hydroxylase enzyme that converts
25(OH)D to the biologically active 1,25-dihydroxyvitamin D. This in turn activates a suite of genes which enhance the production of cathelicidin (Laaksi, 2012; Liu et al., 2006). Our finding that the plasma cathelicidin concentration in the high-level vitamin D status group was significantly higher than in the low-level group and the significant correlation between plasma 25(OH)D and cathelicidin concentrations are in agreement with other studies (Bhan et al., 2011; Dixon et al., 2012; Jeng et al., 2009). Furthermore, vitamin D has been shown to be essential in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes (von Essen et al., 2010).

A novel and potentially important finding of the present study is that vitamin D deficiency was associated with a significant and substantially lower production of pro-inflammatory cytokines by both monocytes and lymphocytes in response to a multi-antigen challenge. The consequence of this could be an impaired immune response to an infectious pathogen, increasing the likelihood of an infection occurring. Our finding is in contrast to other studies that have indicated a reduced pro-inflammatory cytokine response when the biologically active form of vitamin D (1,25(OH)₂D) is added in concentrations of 10-100 nmol/L to stimulated cultures of peripheral blood mononuclear cells (PBMC) (Khoo, Chai, Koenen, Kullberg et al., 2011; Khoo, Chai, Koenen, Sweep et al., 2011). The reason for this discrepancy is unclear. The stimulant we used was a vaccine containing antigens from a virus and both gram-positive and gram-negative bacteria and we used a whole blood culture. The studies by Khoo and colleagues (Khoo, Chai, Koenen, Kullberg et al., 2011; Khoo, Chai, Koenen, Sweep et al., 2011) used lipopolysaccharide (LPS) or Candida albicans to stimulate isolated PBMC. Inhibition of pro-inflammatory cytokine production in these studies was only observed when PBMC were incubated with 1,25(OH)₂D in concentrations that were 50-500-fold above the normal healthy range for plasma 1,25(OH)₂D (50-250 pmol/L), so the
physiological relevance is questionable. Moreover, the multiple antigen challenge used in our study provides valuable information on cytokine production since not all cytokines respond to the same antigen. The capacity of leukocytes to produce cytokines upon adequate challenge (e.g. with mitogen, antigen, endotoxin or pathogen exposure) has potentially far reaching consequences for the entire functional capacity of the immune system. It is highly likely to reflect the capacity of an individual to defend itself against intruding microorganisms and hence is a suitable measure to examine the impact of nutritional interventions (e.g., vitamin D supplementation) designed to boost immune function. We chose to examine whole blood culture rather than isolated PBMC as the former retains the normal cellular, hormonal and cytokine milieu that the leukocytes are normally exposed to in the circulation. This model probably comes closest to the natural environment avoiding artefacts from cell isolation and preparation and allowing natural interactions between immune components and antigens within the normal hormonal milieu. Essentially it is an in vitro method of simulating responses to an infection. Our findings indicate that low vitamin D status is associated with an impaired ability to mount a pro-inflammatory cytokine response to a multi-antigen challenge, whereas the production of anti-inflammatory cytokines (IL-10) was not significantly influenced by vitamin D status. An impaired pro-inflammatory response to antigen challenge could increase the risk of succumbing to infection and increase severity and/or duration of symptoms of infection.

Another interesting finding in the present study is that the salivary SIgA secretion rate in the optimal vitamin D status group was significantly higher than in the other groups. The mucosal immune system, especially SIgA, functions as the first line of defence against pathogen invasion by preventing antigens and microbes adhering to mucosal surfaces and interrupting replication of intracellular pathogens during transcytosis through epithelial cells
(Gleeson, 2007). Previous studies have shown an inverse relationship between salivary SIgA values and URTI prevalence. For example, low salivary SIgA values have been reported to be associated with increased incidence of URTI in athletes (Fahlman & Engels, 2005; Halliday et al., 2011; Neville et al., 2008). The finding in the present study that a significantly lower proportion of subjects in the vitamin D optimal status group experienced URTI episodes during the 4-month study period could be explained partially by the protective effect of their higher salivary SIgA secretion rate. To our knowledge, this is the first study to report an association between salivary SIgA values and plasma vitamin D status. It would be interesting to know if high dose Vitamin D supplementation could elevate SIgA in people with low SIgA secretion.

The inter-individual variation in vitamin D status within our athlete cohort is most likely due to differences in sunlight exposure rather than diet since the D$_3$ form (derived primarily from synthesis in the skin) made up >90% of total plasma 25(OH)D. None of the subjects were taking vitamin supplements but we did not assess dietary vitamin D intake in this study. Vitamin D insufficiency has been reported to be common in athletes (Larson-Meyer & Willis, 2010) especially if exposure to natural sunlight is limited (e.g. when training in the winter months or when training mostly indoors). On the basis of the present data, we found that 38% of athletes had insufficient plasma 25(OH)D values (<50 nmol/L) at the start of the 4-month period and 55% of athletes had plasma 25(OH)D values of less than 50 nmol/L at the end of the study. This proportion is slightly lower than previously reported in a study on UK athletes (Close et al., 2012) that reported 61% had serum 25(OH)D concentrations of less than 50 nmol/L during the winter months. Moreover, we also found that there was a significant drop in plasma 25(OH)D concentration from the start of the 4-month period to the end of the study. This is most likely due to insufficient UV radiation of appropriate wavelength between
November and March in the UK (Loughborough latitude is 53°N) to produce vitamin D in the skin (Webb & Holick, 1988). This probably also explains why there was no difference in plasma 25(OH)D concentration between indoor and outdoor athletes during the winter months in the present study, an observation also reported by other studies (Close et al., 2012; Halliday et al., 2011). Given that the incidence of URTI is generally higher in athletes (Gleeson, 2007) and that low vitamin D status could be a contributing factor, it seems probable that vitamin D supplementation could be desirable for athletes during the winter months. Our results also provide confirmation that the presence of URTI episodes in athletes results in a significant reduction of their training load.

3.5.4 Conclusion

In conclusion, the findings of this study concur with recent reports of illness incidence at major competitive games indicating that female athletes may be more susceptible than their male counterparts to URTI and that lower oral-respiratory mucosal immunity (i.e. lower secretion rates of mucosal AMPs), may account for this in part. Moreover, it has been indicated in the present study that athletes who had experienced prior infection with both CMV and EBV had fewer URTI episodes and fewer symptom days than those with negative serostatus for both CMV and EBV. In addition, our study suggested that low vitamin D status could be an important determinant of URTI risk in endurance athletes. Athletes with low vitamin D concentrations may have a higher risk of URTI and suffer more severe symptoms when URTI is present. This may be due to impaired mucosal and systemic immunity as salivary SIgA secretion, cathelicidin levels and antigen-stimulated pro-inflammatory cytokine production appear to be increased by vitamin D-dependent mechanisms. Vitamin D seems to play important roles in both innate and adaptive immune responses. Therefore, a series of
follow-up studies were also conducted to examine the effect of vitamin D on mucosal and systemic immunity in athletes in this thesis.
Chapter 4:

Influence of vitamin D metabolites on plasma cytokine concentrations in endurance sport athletes and on multi-antigen stimulated cytokine production by whole blood and peripheral blood mononuclear cell cultures
Chapter 4  
Influence of vitamin D metabolites on cytokines

4.1 Abstract

Aim: Our aims were to determine the influence of plasma total 25(OH)D status on the plasma cytokine concentrations in athletes and the \( \textit{in vitro} \) effects of different doses of 1, 25(OH)\(_2\)D\(_3\) on multi-antigen stimulated cytokine production by whole blood and PBMC cultures. Methods: Plasma samples from 43 athletes with high and low levels of 25(OH)D were assayed for the concentrations of cytokines. The whole blood samples and PBMCs from healthy subjects were incubated \( \textit{in vitro} \) with a multi-antigen vaccine and different doses of added 1, 25(OH)\(_2\)D\(_3\). The circulating cytokines and stimulated whole blood and PBMC culture production of cytokines were determined using a biochip assay. Results: The circulating IL-10 and IFN-\( \gamma \) concentrations were significantly higher in the vitamin D sufficient athletes. Furthermore, the production of TNF-\( \alpha \), IL-6, IFN-\( \gamma \), IL-2 and IL-10 by whole blood culture was significantly inhibited by 1, 25(OH)\(_2\)D\(_3\) concentrations of 1000 pmol/L or 10000 pmol/L. Conclusions: We found that the influence of vitamin D on circulating cytokines might be different in athletes compared with non-athletes and cytokines production by whole blood culture were not influenced by 1, 25(OH)\(_2\)D\(_3\) in concentrations within the normal healthy range.
4.2 Introduction

Vitamin D can be obtained either from dietary sources or the epidermal layer of the skin via exposure to sunlight. The endogenously synthesised vitamin D₃ and diet-derived D₂ and D₃ are hydroxylated in the liver to form 25(OH)D. 25(OH)D is the main storage form, which can be stored in muscles and adipose tissue, and is the major circulating metabolite of vitamin D, with a plasma half-life of 2-3 weeks. Therefore, the plasma concentration of 25(OH)D is considered to be the primary indicator of vitamin D status (Baeke et al., 2010). Plasma 25(OH)D values commonly accepted as the reference range are as follows (Heaney et al., 2011; IoM, 2011). In healthy humans, 25(OH)D plasma levels >120 nmol/L are suggested as optimal vitamin D status and levels from 50 to 120 nmol/L are defined as adequate. Plasma levels of 25(OH)D <50 nmol/L are proposed to define inadequate vitamin D concentrations and values <30 nmol/L represent vitamin D deficiency. Subsequently, 25(OH)D can be converted in the kidney to the biologically active form, 1, 25(OH)₂D, by 1-α-hydroxylase, an enzyme which is stimulated by PTH when serum calcium and phosphate concentrations fall below the physiological range. Normal concentrations of circulating 1, 25(OH)₂D are approximately 50-250 pmol/L, about 1000 times lower than its precursor, 25(OH)D; the plasma half-life of 1, 25(OH)₂D is 4-6 hours (Aranow, 2011).

It has only recently been recognised that vitamin D plays an important role in up-regulating immunity (Kamen & Tangpricha, 2010). Several recent studies have found a negative association between vitamin D status and respiratory illness incidence in young and elderly adults and it has been indicated that individuals with low vitamin D status have a higher risk of respiratory illness incidence and suffer more severe symptoms when a respiratory illness is present (Berry et al., 2011; Ginde et al., 2009; He et al., 2013; chapter 3). Moreover, the
presence of the VDR in almost all immune cells, including T lymphocytes, B lymphocytes, neutrophils and antigen presenting cells, such as macrophages and dendritic cells has been demonstrated and these immune cells also express the vitamin D-activating enzyme, 1-\(\alpha\)-hydroxylase, and thus possess the ability to convert 25(OH)D to 1, 25(OH)\(_2\)D. This process is regulated by circulating levels of 25(OH)D or induced by activation of specific TLRs which act as detectors of pathogens (Baeke et al., 2010; Bikle, 2009). Therefore, vitamin D could play vital roles in both innate and adaptive immune responses.

Furthermore, vitamin D may also influence circulating cytokine levels and cytokine production during periods of infection. Within the general population it has been reported that circulating pro-inflammatory cytokine concentrations, such as TNF-\(\alpha\), IFN-\(\gamma\), IL-1\(\beta\) and IL-2, were significantly higher in vitamin D insufficient adults compared with those who were vitamin D sufficient (Barker et al., 2013). However, to our knowledge, there has been no study to compare the difference in circulating cytokine levels between vitamin D insufficient and sufficient athletes. Moreover, the anti-inflammatory effects of exercise training (Gleeson, Bishop, Stensel et al., 2011) might modify the influence of plasma 25(OH)D on circulating cytokine levels in athletes. Thus, it is of interest to determine the influence of plasma 25(OH)D status on circulating cytokine levels in athletes. In addition, several recent studies indicate that 1, 25(OH)\(_2\)D\(_3\) limits the *in vitro* production of TNF-\(\alpha\), IFN-\(\gamma\) and IL-6 by isolated human PBMC stimulated with LPS and *Candida albicans* and seasonal variations in vitamin D levels correlate with alterations in cytokine production by *ex vivo* LPS stimulated PBMC, with higher TNF-\(\alpha\), IFN-\(\gamma\) and IL-6 production in winter (Khoo, Chai, Koenen, Kullberg et al., 2011; Khoo, Chai, Koenen, Sweep et al., 2011). Nevertheless, in our previous vitamin D study (He et al., 2013; chapter 3), we found that vitamin D deficiency was
Influence of vitamin D metabolites on cytokines

associated with substantially lower production of pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β and IL-6, in the whole blood culture by both monocytes and lymphocytes in response to a multi-antigen challenge containing antigens from viruses and both gram-positive and gram-negative bacteria. The reason for this discrepancy is unclear and further research is still needed to understand the influence of vitamin D on multi-antigen stimulated cytokine production.

In the present study, our aims were to determine the influence of plasma 25(OH)D status on the plasma cytokine concentrations of endurance sport athletes and the effects of different doses of 1, 25(OH)₂D₃ on the *in vitro* multi-antigen stimulated cytokine production by whole blood and PBMC cultures.

### 4.3 Methods

**Participants**

A total of 43 healthy individuals from our previous vitamin D study (He et al., 2013; chapter 3) were included in this study. EDTA plasma samples from athletes with high and low vitamin D status (high-level: 25(OH)D >80 nmol/L, n=18; low-level: 25(OH)D <40 nmol/L, n=25) with sufficient volume were selected for assay of the circulating cytokine concentrations. There was no significant difference in the physical characteristics of participants between high-level and low-level vitamin D status groups (Table 4.1).
Table 4.1 Physical characteristics of 43 athletes with high and low vitamin D status

<table>
<thead>
<tr>
<th></th>
<th>high-level vitamin D</th>
<th>low-level vitamin D</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (M:F)</td>
<td>18 (8:10)</td>
<td>25 (19:6)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>20.9 ± 4.1</td>
<td>19.9 ± 2.0</td>
<td>0.294</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>174.0 ± 10.1</td>
<td>177.0 ± 8.5</td>
<td>0.309</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.0 ± 13.5</td>
<td>73.3 ± 9.4</td>
<td>0.516</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>23.3 ± 2.8</td>
<td>23.3 ± 1.8</td>
<td>0.960</td>
</tr>
<tr>
<td>Mean training loads (Mean MET-h/wk)</td>
<td>84.9 ± 46.2</td>
<td>64.0 ± 28.2</td>
<td>0.100</td>
</tr>
</tbody>
</table>

Data are mean ± SD.

M: male; F: female.

P value from independent t test is shown in right hand column.

For the study of 1, 25(OH)$_2$D$_3$ in vitro incubation, 6 healthy male individuals (Age: 28.8 ± 4.1; Height: 176.3 ± 10.0; Weight: 83.9 ± 7.8; BMI: 27.1 ± 2.9) who had not taken any regular medication or antibiotics in the 3 months prior to the study were recruited as subjects from Loughborough University. For the visit to the laboratory, subjects arrived in the morning at 08:30-10:30 following an overnight fast of approximately 12 h. Blood samples were collected as described in Chapter 2. Subjects had to have normal haematology to be included in the study. The whole blood samples and the PBMC isolated from the heparin tubes were used immediately for in vitro incubation with a multi-antigen vaccine and different doses of 1, 25(OH)$_2$D$_3$.

PBMC were isolated by density centrifugation on Ficoll-Hypaque Histopaque 1077 (Sigma Chemicals, Poole, UK). The heparinized whole blood was diluted 1:1 with room temperature
10 mM phosphate buffered saline (Sigma Chemicals, Poole, UK) and layered 20 mL of diluted blood onto 10 mL of room temperature Ficoll-Hypaque. Samples were then centrifuged at 800 g for 30 minutes at 20°C. Cells were collected from interphase and washed twice with RPMI 1640 medium (Sigma Chemicals, Poole, UK) supplemented with 10% Fetal Bovine Serum (Sigma Chemicals, Poole, UK) and then spun at 250 g for 10 minutes at 20°C. Subsequently, cells were resuspended in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum. Cells were counted in an automated cell-counter (Ac.T™5diff haematology analyser, Beckman Coulter, High Wycombe, UK) and the number of cells was adjusted to 2x10^6 cells/mL in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum.

**Plasma 25(OH)D measurements**

Plasma samples were assayed for 25(OH)D concentrations as described in Chapter 2.

**In vitro incubation with 1, 25(OH)2D3 and multi-antigen vaccine**

The whole blood samples and PBMC were incubated *in vitro* with a multi-antigen vaccine and different doses of 1, 25(OH)2D3. The stimulant was a commercially available multi-antigen vaccine (Pediacel Vaccine, Sanofi Pasteur, UK) containing diphtheria, tetanus, acellular pertussis, poliomyelitis and haemophilus influenzae type b antigens. 1, 25(OH)2D3 was purchased from Sigma-Aldrich (Missouri, USA) and dissolved in absolute ethanol to give a stock solution of 10 μM. Aliquots were subsequently diluted with RPMI 1640 medium for use in the *in vitro* incubations.
Stimulated whole blood and PBMC culture production of cytokines (IFN-γ, TNF-α, IL-1β, IL-2, IL-6 and IL-10) were determined as described in Chapter 2. Briefly, for the determination of baseline unstimulated cytokine production, 0.25 mL of heparinized whole blood or PBMC were added to 0.75 mL of RPMI 1640 medium and incubated at 37°C and 5% CO₂ for 24 h. Additionally, 0.25 mL of heparinized whole blood or PBMC were added to 0.70 mL of RPMI 1640 medium containing different doses of 1, 25(OH)₂D₃ (0, 100, 200, 1000 and 10000 pmol/L) with an added 50 µL of Pediacel vaccine cocktail (Sanofi Pasteur msd Limited, Maidenhead, UK) at a dilution of 1:100, before being incubated at 37°C and 5% CO₂ for 24 h. The stimulant dilution of 1:100 used in this study was based on a separate experiment, which established the dose–response curve for the measured cytokines over the dilution range of 1:100–1:20,000. Samples were then centrifuged at 13000 g for 4 min at 4°C, following which the supernatant fluid was harvested and stored at -80°C prior to analysis of cytokine concentrations.

**Cytokine measurements**

Cytokine concentrations were determined as described in Chapter 2. The measured cytokine concentrations for the monocyte-derived cytokines (TNF-α and IL-1β) and lymphocyte-derived cytokines (IL-2 and IFN-γ) were divided by the monocyte and lymphocyte counts, respectively to give cytokine production per 10⁶ cells. Although the production of TNF-α is divided by monocyte counts (the main source), it can be also produced by other cell types, such as neutrophils, NK cells and CD4+ T cells. IL-6 and IL-10 are produced by both monocytes and lymphocytes, so it is not appropriate to normalise their production by cell subset counts.
Chapter 4 Influence of vitamin D metabolites on cytokines

Statistical analysis

The independent t test was used to test for the differences in the physical characteristics of participants between high-level and low-level vitamin D groups. Mann-Whitney U test was used to test for the differences in the circulating cytokine concentrations between high-level and low-level vitamin D groups. The correlation between circulating cytokine and plasma 25(OH)D concentrations was determined using Spearman's rank correlation. Wilcoxon signed rank test performed in the previous studies by Khoo and colleagues (Khoo, Chai, Koenen, Kullberg et al., 2011; Khoo, Chai, Koenen, Sweep et al., 2011) was also used in the present study to examine for the differences in the stimulated whole blood and PBMC culture production of cytokines between different doses of 1, 25(OH)2D3 in vitro incubation. Differences in the multi-antigen stimulated cytokine production without the addition of 1, 25(OH)2D3 by whole blood and PBMC cultures were compared with the Mann-Whitney U test. Data are presented as mean (±SD) or median and IQR and the accepted level of significance was P<0.05.

4.4 Results

Circulating cytokine concentrations and vitamin D status

The plasma 25(OH)D concentrations were 101.3±13.9 and 31.8±3.5 nmol/L in the high-level and low-level vitamin D status groups, respectively. The anti-inflammatory cytokine IL-10 concentration in the high-level vitamin D status group was marginally but significantly higher than in the low-level group (P=0.020), and the pro-inflammatory cytokine IFN-γ concentration in the high-level vitamin D status group was substantially and significantly higher than in the low-level group (P=0.004) (Table 4.2). However, there was no significant difference in the circulating TNF-α, IL-1β, IL-2 and IL-6 concentrations between high-level
and low-level vitamin D status groups (Table 4.2). In addition, there was a positive correlation between the plasma 25(OH)D and IFN-γ concentrations (r=0.374, P=0.014), but plasma 25(OH)D concentrations did not correlate with TNF-α, IL-1β, IL-2, IL-6 or IL-10 levels (Table 4.3).

Table 4.2 Circulating cytokine concentrations between high-level and low-level vitamin D status groups

<table>
<thead>
<tr>
<th></th>
<th>high-level vitamin D</th>
<th>low-level vitamin D</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 (pg/mL)</td>
<td>3.8 (2.6-4.5)</td>
<td>2.5 (0.0-5.4)</td>
<td>0.186</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.3 (1.0-1.9)</td>
<td>0.9 (0.7-1.5)</td>
<td>0.218</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>0.7 (0.6-1.6)</td>
<td>0.6 (0.5-0.8)</td>
<td>0.020</td>
</tr>
<tr>
<td>IFN-γ (pg/mL)</td>
<td>0.9 (0.6-1.4)</td>
<td>0.5 (0.0-0.7)</td>
<td>0.004</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>2.6 (1.8-3.2)</td>
<td>2.4 (2.0-3.3)</td>
<td>0.825</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>4.5 (3.3-5.7)</td>
<td>3.9 (2.8-7.2)</td>
<td>0.912</td>
</tr>
</tbody>
</table>

Data are median and IQR.

P value from Mann-Whitney U test is shown in right hand column.

Table 4.3 The correlation between circulating cytokine and plasma 25(OH)D concentrations

<table>
<thead>
<tr>
<th></th>
<th>IL-2</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman's rank correlation</td>
<td>0.179</td>
<td>0.126</td>
<td>0.257</td>
<td>0.374</td>
<td>-0.157</td>
<td>0.006</td>
</tr>
<tr>
<td>P value</td>
<td>0.250</td>
<td>0.421</td>
<td>0.097</td>
<td>0.014</td>
<td>0.314</td>
<td>0.970</td>
</tr>
</tbody>
</table>
Antigen-stimulated cytokine production by whole blood culture

The antigen-stimulated production of the monocyte-derived cytokine, TNF-α, was both attenuated by 1, 25(OH)2D3, but production of IL-1β was not significantly influenced by 1, 25(OH)2D3. The production of TNF-α was significantly lower in the presence of 1000 pmol/L and 10000 pmol/L of 1, 25(OH)2D3 compared with its production without the addition of 1, 25(OH)2D3 (Figure 4.1). In addition, the antigen-stimulated production of the lymphocyte-derived cytokines, IFN-γ and IL-2, were both attenuated by 1, 25(OH)2D3. The production of IL-2 was significantly lower in the presence of 1000 pmol/L and 10000 pmol/L of 1, 25(OH)2D3 compared with its production without the addition of 1, 25(OH)2D3 and the production of IFN-γ was significantly lower in the presence of 10000 pmol/L of 1, 25(OH)2D3 compared with its production without the addition of 1, 25(OH)2D3 (Figure 4.1). The production of IL-6 was significantly lower in the presence of 10000 pmol/L of 1, 25(OH)2D3 compared with its production without the addition of 1, 25(OH)2D3 (0 pmol/L: 201.8±202.8 pg/mL; 10000 pmol/L: 64.5±53.7 pg/mL; P=0.013), but no differences were observed at the other concentration of added 1, 25(OH)2D3. However, the production of IL-10 was significantly higher in the presence of 10000 pmol/L of 1, 25(OH)2D3 compared with its production without the addition of 1, 25(OH)2D3 (0 pmol/L: 1.5±0.8 pg/mL; 10000 pmol/L: 4.5±2.1 pg/mL; P=0.028), but no differences were observed at the other concentration of added 1, 25(OH)2D3.

Antigen-stimulated cytokine production by PBMC culture

The antigen-stimulated production of the monocyte-derived cytokines of TNF-α and IL-1β were not significantly influenced by 1, 25(OH)2D3. The antigen-stimulated production of the
lymphocyte-derived cytokines, IFN-γ and IL-2, were not significantly influenced by the incubation of different doses of 1, 25(OH)₂D₃ (Figure 4.2). The production of IL-10 was also not significantly influenced by 1, 25(OH)₂D₃. However, the production of IL-6 was significantly lower in the presence of 10000 pmol/L of 1, 25(OH)₂D₃ compared with its production without the addition of 1, 25(OH)₂D₃ (0 pmol/L: 199.2±100.8 pg/mL; 10000 pmol/L: 81.1±34.4 pg/mL; P = 0.038).

Comparison of the whole blood and PBMC culture

The production of the lymphocyte-derived cytokines, IFN-γ and IL-2, and the monocyte-derived cytokine, IL-1β, by multi-antigen stimulated whole blood culture without the addition of 1, 25(OH)₂D₃ were significantly higher than their respective production by the PBMC culture (Table 4.4). However, there was no significant difference in the antigen-stimulated production of TNF-α, IL-6 and IL-10 between the whole blood and PBMC cultures (Table 4.4).
Table 4.4 Comparison of the whole blood and PBMC culture in antigen-stimulated cytokine production

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>whole blood</th>
<th>PBMC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>195.7 (65.9-245.3)</td>
<td>161.5 (156.1-202.1)</td>
<td>0.262</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>1.7 (1.5-1.8)</td>
<td>2.0 (1.6-2.3)</td>
<td>0.297</td>
</tr>
<tr>
<td>IL-2 (pg/10^6 Ly)</td>
<td>9.5 (4.5-16.6)</td>
<td>0.3 (0.2-0.7)</td>
<td>0.016</td>
</tr>
<tr>
<td>IFN-γ (pg/10^6 Ly)</td>
<td>0.6 (0.3-0.9)</td>
<td>0.1 (0.0-0.1)</td>
<td>0.037</td>
</tr>
<tr>
<td>TNF-α (pg/10^6 Mo)</td>
<td>8.4 (3.6-11.9)</td>
<td>7.7 (6.4-11.7)</td>
<td>0.631</td>
</tr>
<tr>
<td>IL-1β (pg/10^6 Mo)</td>
<td>6.4 (4.3-8.8)</td>
<td>1.6 (1.3-2.6)</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Data are median and IQR.  
Ly = lymphocytes; Mo = monocytes  
P value from Mann-Whitney U test is shown in right hand column.
Figure 4.1 The antigen-stimulated production of the lymphocyte-derived cytokines (IL-2 and IFN-γ; left panels) and the monocyte-derived cytokines (TNF-α and IL-1β; right panels) by whole blood culture. *P<0.05 vs 0 pmol/L. Ly = lymphocytes; Mo = monocytes. Data are presented as mean and SD.
Chapter 4  

Influence of vitamin D metabolites on cytokines

Figure 4.2 The antigen-stimulated production of the lymphocyte-derived cytokines (IL-2 and IFN-γ; left panels) and the monocyte-derived cytokines (TNF-α and IL-1β; right panels) by PBMC culture. *P<0.05 vs 0 pmol/L. Ly = lymphocytes; Mo = monocytes. Data are presented as mean and SD.
Chapter 4  
Influence of vitamin D metabolites on cytokines

4.5 Discussion

The aims of this research were to determine the influence of plasma 25(OH)D status on the circulating cytokine levels in endurance sport athletes and the effects of different doses of 1, 25(OH)_{2}D_{3} on the multi-antigen stimulated cytokine production by whole blood and PBMC cultures. The main findings of the present study were that the circulating IL-10 and IFN-γ concentrations were significantly higher in the vitamin D sufficient athletes compared with the vitamin D insufficient athletes and there was a positive correlation between the plasma 25(OH)D and IFN-γ concentrations in endurance athletes. Furthermore, the production of TNF-α, IL-6, IFN-γ, IL-2 and IL-10 by multi-antigen stimulated whole blood culture were significantly influenced by 1, 25(OH)_{2}D_{3} of concentrations of 1000 pmol/L and/or 10000 pmol/L. Nevertheless, a similar effect was only observed for the production of IL-6 by multi-antigen stimulated PBMC culture. In addition, the production of lymphocyte-derived and monocyte-derived cytokines, such as IFN-γ, IL-2 and IL-1β, were significantly different in the multi-antigen stimulated whole blood culture without the addition of 1, 25(OH)_{2}D_{3} compared with its production in the PBMC culture.

On the basis of the present data, we found that the circulating IL-10 and IFN-γ concentrations in the vitamin D sufficient athletes were significantly higher than in the vitamin D insufficient athletes and there was a positive correlation between the plasma 25(OH)D and IFN-γ concentrations in endurance athletes. In contrast, a previous study has reported an elevation of circulating pro-inflammatory cytokines in vitamin D insufficient adults (Barker et al., 2013). Barker et al. (2013) indicated that circulating pro-inflammatory cytokine concentrations, such as TNF-α, IFN-γ, IL-1β and IL-2, were significantly higher in vitamin D insufficient compared to vitamin D sufficient adults but the anti-inflammatory cytokine, IL-
10, was not significantly different between vitamin D insufficient and sufficient adults. Moreover, Barker and colleagues (Barker, Martins, Kjeldsberg, Trawick, & Hill, 2012) found an inverse correlation between the plasma 25(OH)D and IFN-γ concentrations. The reason for this discrepancy is still unclear but may be due to the difference of the cut-off point used for vitamin D insufficiency in the two studies. In healthy humans, 25(OH)D plasma levels >120 nmol/L are suggested as optimal vitamin D status and levels from 50 to 120 nmol/L are defined as adequate. Plasma levels of 25(OH)D <50 nmol/L are proposed to define inadequate vitamin D concentrations and values <30 nmol/L represent vitamin D deficiency (Heaney et al., 2011; IoM, 2011). Therefore, the commonly used cut-off point for vitamin D insufficiency in clinical practice and research reports is the threshold concentration of 25(OH)D of <50 nmol/L (Lai, Lucas, Banks, & Ponsonby, 2012). However, the cut-off point for vitamin D insufficiency used in the Barker et al. (2013) study was <80 nmol/L and the average serum 25(OH)D concentration of vitamin D of insufficient adults was around 60 nmol/L. Thus, the vitamin D insufficient adults in their study were not even close to being vitamin D deficient. In addition, another confounding factor may be due to the anti-inflammatory effects of exercise training. The participants recruited in our previous vitamin D study (He et al., 2013; chapter 3) were the endurance sport athletes engaged in regular sports training (predominantly endurance-based activities such as running, cycling, swimming, triathlon, team games and racquet sports) and their self-reported training loads averaged 10 h/week. The anti-inflammatory effects of exercise training on cytokine responses, such as the release of IL-6 from contracting muscle and the increased levels of circulating IL-10, cortisol and adrenaline, are discussed in the review article by (Gleeson, Bishop, Stensel et al., 2011). It has been demonstrated that the active skeletal muscle significantly increases both cellular and circulating levels of IL-6 during and following exercise of sufficient load and the transient rise in circulating IL-6 during exercise appears to be responsible for a
subsequent rise in circulating levels of anti-inflammatory cytokines, including IL-10, which is a potent promoter of an anti-inflammatory state (Gleeson, Bishop, Stensel et al., 2011). Increases in circulating cortisol and adrenaline during exercise are due to activation of the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system, respectively. Cortisol is known to have potent anti-inflammatory effects and also augmented by the rise in circulating IL-6 from contracting skeletal muscle (Gleeson, Bishop, Stensel et al., 2011). Therefore, the influence of vitamin D on circulating cytokines might be different between athletes and non-athletes. Further research is still needed to understand the influence of vitamin D on circulating cytokines between athletes and non-athletes.

The finding of higher circulating IFN-γ concentrations in the vitamin D sufficient athletes and a positive correlation between the plasma 25(OH)D and IFN-γ concentrations in the present study might be helpful to support the arguments that vitamin D could play a role in reducing both the severity and duration of upper respiratory tract illness symptoms in our previous vitamin D study (He et al., 2013; chapter 3). It has been reported that increasing IFN-γ in the circulation contributes to the conversion of 25(OH)D to its active hormonal form, 1, 25(OH)₂D, in the circulation during inflammatory stress in humans (Barker et al., 2012). In addition, 1, 25(OH)₂D has a vital role in up-regulating the production of antimicrobial proteins and peptides, such as cathelicidin and β-defensin, which are produced by both epithelial cells and macrophages and have a broad range of activities against microorganisms including the direct inactivation of viruses (Liu et al., 2006). 1, 25(OH)₂D also acts to maintain a balance between inflammatory Th1/Th17 cells and immunosuppressive Th2/Treg cells to temper inflammation and tissue damage (Hewison, 2012). Therefore, the vitamin D
sufficient athletes with higher circulating IFN-\(\gamma\) concentrations may have less severe symptoms and shorter duration when the upper respiratory tract illness is present.

According to the findings from the present study, it seems likely that the multi-antigen stimulated cytokine production by whole blood culture was influenced by 1, 25(OH)\(_2\)D\(_3\) only in concentrations that were 1000 pmol/L or more. Although the production of pro-inflammatory cytokines, TNF-\(\alpha\), IL-6, IFN-\(\gamma\) and IL-2 by multi-antigen stimulated whole blood culture were significantly lower in the presence of 1000 pmol/L or 10000 pmol/L of 1, 25(OH)\(_2\)D\(_3\) and the production of anti-inflammatory cytokine, IL-10, was significantly higher in the presence of 10000 pmol/L of 1, 25(OH)\(_2\)D\(_3\), we found that both pro-inflammatory and anti-inflammatory cytokine production by multi-antigen stimulated whole blood culture were not influenced by 1, 25(OH)\(_2\)D\(_3\) in added concentrations of 100 pmol/L and 200 pmol/L. In contrast, the results from our previous study (He et al., 2013; chapter 3) showed that vitamin D status (determined by plasma levels of 25(OH)D) did significantly influence lymphocyte and monocyte pro-inflammatory cytokine production by antigen-stimulated whole blood culture. Individuals with high levels of plasma 25(OH)D would be expected to have higher levels of 1, 25(OH)\(_2\)D so it is perhaps surprising that in the \textit{in vitro} situation variations in 1, 25(OH)\(_2\)D within the normal physiological range appear to have no significant effect on leukocyte cytokine production. In healthy humans, normal concentrations of circulating 1, 25(OH)\(_2\)D are approximately 50-250 pmol/L, about 1000 times lower than its precursor, 25(OH)D (Aranow, 2011). Inhibition of pro-inflammatory cytokine production and elevation of anti-inflammatory cytokine production in the present study were only observed when the whole blood was incubated with 1, 25(OH)\(_2\)D in concentrations that were 10-100-fold above the normal healthy range for plasma 1, 25(OH)\(_2\)D. Therefore, the physiological relevance is
unclear. A limitation of the present study is that the participants’ plasma concentration of 1, 25(OH)$_2$D was not determined. Further research is still needed to understand the influence of the normal healthy range or lower levels of plasma 1, 25(OH)$_2$D on the multi-antigen stimulated cytokine production by whole blood culture.

The data from our study show that there was a significant difference in the production of multi-antigen stimulated lymphocyte-derived and monocyte-derived cytokines, such as IFN-$\gamma$, IL-2, IL-4 and IL-1$\beta$, between the whole blood and PBMC cultures and it seems likely that there was a better dose-dependent response in the cytokine production of multi-antigen stimulated whole blood culture with different doses of 1, 25(OH)$_2$D$_3$ than in the PBMC culture. The whole blood culture retains the normal cellular, hormonal and cytokine milieu that the leukocytes are normally exposed to in the circulation. This model probably comes closest to the natural environment avoiding artefacts from cell isolation and preparation and allowing natural interactions between immune components and antigens within the normal hormonal milieu. Essentially it is an in vitro method of simulating responses to an infection. Moreover, the stimulant we used was a vaccine containing antigens from a virus and both gram-positive and gram-negative bacteria and the multiple antigen challenge used in the present study provides valuable information on cytokine production since not all cytokines respond to the same antigen. The capacity of leukocytes to produce cytokines upon adequate challenge (e.g. with mitogen, antigen, endotoxin or pathogen exposure) has potentially far reaching consequences for the entire functional capacity of the immune system. Therefore, the whole blood culture stimulated with multi-antigen is highly likely to reflect the capacity of an individual to defend itself against intruding microorganisms.
In conclusion, the present study demonstrated that the influence of vitamin D on circulating cytokines might be different in athletes compared with previous studies on non-athletes and the higher circulating IFN-γ concentrations in vitamin D sufficient athletes might be helpful to support the arguments that vitamin D could play a role in reducing both the severity and duration of upper respiratory tract illness symptoms. In addition, we found that both pro-inflammatory and anti-inflammatory cytokines by multi-antigen stimulated whole blood culture were not influenced by 1, 25(OH)₂D₃ in concentrations within the normal healthy range. Furthermore, the whole blood culture stimulated with multi-antigen might be a better \textit{in vitro} method of simulating responses to an infection than cultures using PBMCs.
Chapter 5:
The effect of 14 weeks of vitamin D₃ supplementation on antimicrobial peptides and proteins in athletes
Chapter 5  
14 weeks of vitamin D₃ supplementation

5.1 Abstract

Heavy training is associated with increased respiratory infection risk and antimicrobial proteins are important in defence against oral and respiratory tract infections. We examined the effect of vitamin D₃ supplementation (5000 IU/day) during the winter months on the resting plasma cathelicidin concentration and the salivary secretion rates of SIgA, cathelicidin, lactoferrin and lysozyme in athletes during a winter training period. Blood and saliva were obtained at the start of the study from 39 healthy men who were randomly allocated to vitamin D₃ supplement or placebo. Blood samples were also collected at the end of the study; saliva samples were collected after 7 and 14 weeks. Plasma total 25(OH)D concentration increased by 130% in the vitamin D₃ group and decreased by 43% in the placebo group (both P=0.001). The percentage change of plasma cathelicidin concentration in the vitamin D₃ group was higher than in the placebo group (P=0.025). Only in the vitamin D₃ group, the saliva SIgA and cathelicidin secretion rates increased over time (both P=0.03). A daily 5000 IU vitamin D₃ supplement has a beneficial effect in up-regulating the expression of SIgA and cathelicidin in athletes during a winter training period which could improve resistance to respiratory infections.
5.2 Introduction

Vitamin D can be obtained either from dietary sources or the epidermal layer of the skin via exposure to sunlight. Two forms of vitamin D can be obtained from dietary sources: vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol). While vitamin D₃ is found in food from animal origin, such as cod-liver oil, salmon and egg yolk, vitamin D₂ is present in some plants and fungi. The vitamin D₃ production from ultraviolet-mediated conversion of 7-dehydrocholesterol in the plasma membrane of skin cells provides 80-100% of the body's requirements (Lanteri et al., 2013). The endogenously synthesised vitamin D₃ and diet-derived D₂ and D₃ must first be hydroxylated in the liver into 25(OH)D, the main storage form. In the second hydroxylation, 25(OH)D is converted to the biologically active form, 1, 25(OH)₂D, by 1-α-hydroxylase in the kidney or some cells in non-renal compartments, such as cells of the immune system including T cells, B cells, macrophages and dendritic cells (Aranow, 2011).

It has recently been recognised that vitamin D is a vital mediator of innate immune responses, enhancing the antimicrobial properties of immune cells such as monocytes and macrophages (Bikle, 2009). Vitamin D is a key link between TLR activation and antimicrobial responses in innate immunity. Following activation of the TLR signalling cascade in the presence of microbes, vitamin D has a vital role in up-regulating the production of AMPs, such as cathelicidin and β-defensin (Liu et al., 2006). The biologically active 1, 25(OH)₂D binds to vitamin D receptors and induces expression of vitamin D responsive genes to enhance the production of cathelicidin and β-defensin (Wang et al., 2004). These AMPs have a broad range of activities against microorganisms and may be involved in the direct inactivation of viruses through membrane destabilization (Kamen & Tangpricha, 2010). They are produced
by epithelial cells and macrophages and in the lungs are secreted into the biofilm covering the inner surface of the airways, thereby creating a barrier that is chemically lethal to microbes. Recently, we demonstrated that vitamin D status influences the resting plasma cathelicidin (LL-37) concentration and salivary SIgA secretion rate in endurance athletes during a winter training period (He et al., 2013; chapter 3). The plasma cathelicidin concentration in athletes with high-level vitamin D concentrations was significantly higher than in athletes with low-level vitamin D concentrations and there was a positive correlation between the plasma 25(OH)D and cathelicidin concentrations. In addition, the averaged salivary SIgA secretion rate during the 4-month winter period in the individuals who had relatively high vitamin D concentrations (plasma 25(OH)D >120 nmol/L) was significantly higher than in groups having low vitamin D concentrations (<30 nmol/L).

Vitamin D insufficiency has been reported to be common in athletes in the United Kingdom (UK) especially when training in the winter months (Close et al., 2013; Morton et al., 2012; He et al., 2013; chapter 3). Two studies that assessed the vitamin D status of UK-based professional athletes (latitude 53°N) reported that 62% of athletes (38/61) including professional rugby players, soccer players and jockeys had inadequate serum total 25(OH)D concentrations (<50 nmol/L) in the winter months (Close et al., 2013) and 65% of elite soccer players in the English Premier League (13/20) presented with serum total 25(OH)D concentrations <50 nmol/L in December (Morton et al., 2012). In our previous study on a large student cohort of endurance athletes, we also found that 55% of endurance athletes (100/181) had serum total 25(OH)D concentrations <50 nmol/L at the end of the 4-month winter training period (He et al., 2013; chapter 3). Given that the high prevalence of insufficient vitamin D status in athletes in the UK during winter months, it seems probable
Chapter 5  

14 weeks of vitamin D<sub>3</sub> supplementation

that vitamin D supplementation could be desirable for athletes during this period to increase vitamin D concentrations to up-regulate the expression of AMPs and possibly reduce the risk of respiratory infections.

The purpose of this study was to examine the effect of vitamin D<sub>3</sub> supplementation (5000 IU/day) over the winter months (from December to February, approximately 14 weeks) on the resting plasma cathelicidin concentration and the salivary concentrations and secretion rates of SIgA, cathelicidin, lactoferrin and lysozyme in a population of individuals engaged in regular sport training. It was hypothesized that participants could reach the 25(OH)D level of 120 nmol/L after this duration of vitamin D<sub>3</sub> supplementation and increase their resting salivary AMPs expression during the winter months.

5.3 Methods

Participants

Fifty healthy men aged 20.4 ± 1.9 years who were engaged in regular sports training (such as rugby, volleyball, swimming, triathlon, cycling and racquet sports) from Loughborough University, UK (latitude 53°N) volunteered to participate in the study during November 2013 and their self-reported training loads (determined by a pre-screening questionnaire) averaged 11 ± 4 h/week (mean ± SD). Participants were required to complete a comprehensive health-screening questionnaire prior to starting the study and had not taken any regular medication or antibiotics in the 3 months prior to the study. All participants were fully informed about the rationale for the study and of all experimental procedures to be undertaken. Participants provided written consent to participate in the study, which had earlier received the approval
of Loughborough University ethical advisory committee. The details of inclusion and exclusion criteria were described in Chapter 2. Participants travelling overseas during the study period were also excluded. Participants were not allowed to take any other supplements, other than a daily multivitamin tablet providing no more than the recommended dietary allowances of essential vitamins.

**Study protocol**

Information about the study was given to the participants 1-2 weeks before their first visit to the laboratory. For the first visit to the laboratory, participants arrived in the morning at 09:00-12:00 following a 3-h fast and their body mass and stature were recorded. Participants were required to abstain from any strenuous physical activity for 24 h before coming to the laboratory. Participants sat quietly for 10 min and completed a health-screening questionnaire and inclusion/exclusion criteria questionnaire before signing an informed consent form. Saliva and blood samples were collected as described in Chapter 2.

The participants were randomly allocated to an intervention (vitamin D<sub>3</sub> supplement) group or placebo group for 14 weeks in a double-blind fashion. The vitamin D<sub>3</sub> group received a daily supplement of 5000 IU of vitamin D<sub>3</sub> in capsule form, whereas the placebo group received a visually identical cellulose placebo capsule (Bio-Tech Pharmacal, Arkansas, USA). Participants were given a 7-week supply of vitamin D<sub>3</sub> or placebo capsules and attended the laboratory again after 7 weeks to get another 7-week supply of supplement. Participants were also asked to fill in a standard short form of IPAQ ([http://www.ipaq.ki.se/downloads.htm](http://www.ipaq.ki.se/downloads.htm)) at
weekly intervals, thus providing the quantitative information on training loads in MET-h/week (Craig et al., 2003; Appendix B).

Participants attended the laboratory after 7 and 14 weeks following a 3-h fast and their body mass was recorded again. Participants were also required to abstain from any strenuous physical activity for 24 h before coming to the laboratory. Blood samples were collected again at the end of the study and unstimulated saliva samples were collected after 7 and 14 weeks. A total of 39 participants completed the study and provided sufficient blood for routine haematology and vitamin D status analysis at the start and end of the study and sufficient saliva (1 mL) for analysis of AMPs on all 3 occasions.

**Plasma analysis**

Plasma samples were analysed for total 25(OH)D and cathelicidin concentrations as described in Chapter 2.

**Saliva analysis**

The saliva volume collected and flow rate were estimated as described in Chapter 2. Saliva samples were analysed for SIgA, cathelicidin, lactoferrin and lysozyme as described in Chapter 2. Secretion rates for each of the salivary AMPs were calculated as described in Chapter 2.

**Statistical analysis**
The Shapiro-Wilk test was used to determine if data sets were normally distributed. Anthropometric, self-reported training load (h/week), average IPAQ scores (MET-h/week) and haematological variables were compared between the vitamin D₃ and placebo groups using unpaired t tests for normally distributed data or nonparametric Mann-Whitney U test for non-normally distributed data. Plasma total 25(OH)D and cathelicidin concentrations at the start and end of the study were assessed between the vitamin D₃ and placebo groups using nonparametric Wilcoxon signed ranks test and Mann-Whitney U test. The percentage change of plasma cathelicidin concentration during the study period was also compared between two groups using nonparametric Mann-Whitney U test. The changes in the concentrations and secretion rates of salivary AMPs as well as the salivary flow rates over the 14 weeks of the study in the vitamin D₃ and placebo groups were assessed by two-way ANOVA or nonparametric Friedman tests with post hoc Dunn’s test and Mann-Whitney U test. Data are presented as mean (±SD) for data sets that were normally distributed; for data sets that were not normally distributed, the median and IQR are shown. The accepted level of significance was P<0.05.

5.4 Results

Adherence to the study

Thiry nine participants (vitamin D₃: n=20; placebo: n=19) completed the study and provided sufficient blood for routine haematology and analysis of 25(OH)D and cathelicidin concentrations on 2 occasions and sufficient saliva for analysis of AMPs on all 3 occasions. Reasons for dropout were given as foreign travel (N=3), injury (N=6) or due to undisclosed reasons (N=2).
Baseline characteristics and physical activity levels

There were no significant differences in baseline anthropometrics or haematological variables between the vitamin D₃ and placebo groups (Table 5.1). Analysis of the IPAQ questionnaires indicated that the weekly training loads during the study period were relatively stable within the vitamin D₃ and placebo groups. The training loads averaged over the study period were not significantly different between the vitamin D₃ and placebo group (Table 5.1). Median (IQR) training loads were 69.0 (49.3-80.3) MET-h/week in the vitamin D₃ group which is equivalent to about 11.4 h of moderate-vigorous activity per week and 78.2 (47.6-112.0) MET-h/week in the placebo group which is equivalent to about 12.9 h of moderate-vigorous activity per week.
Table 5.1 Anthropometric, training and haematological variables in the vitamin D₃ and placebo groups at baseline and their training loads determined by IPAQ during the intervention period

<table>
<thead>
<tr>
<th></th>
<th>Vitamin D₃ (n=20)</th>
<th>Placebo (n=19)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>20.1 ± 1.7</td>
<td>21.0 ± 2.3</td>
<td>0.168</td>
</tr>
<tr>
<td>Stature (cm)</td>
<td>181.9 ± 6.9</td>
<td>182.2 ± 8.0</td>
<td>0.907</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>80.8 ± 13.1</td>
<td>80.0 ± 11.2</td>
<td>0.849</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3 ± 3.1</td>
<td>24.1 ± 2.6</td>
<td>0.769</td>
</tr>
<tr>
<td>Previous training load (h/week)</td>
<td>10.1 ± 4.0</td>
<td>11.1 ± 4.8</td>
<td>0.503</td>
</tr>
<tr>
<td>IPAQ (MET-h/week)</td>
<td>69.0 (49.3-80.3)</td>
<td>78.2 (47.6-112.0)</td>
<td>0.374</td>
</tr>
<tr>
<td>RBC count (x10¹²/L)</td>
<td>4.8 ± 0.3</td>
<td>4.9 ± 0.3</td>
<td>0.231</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>44.1 ± 1.9</td>
<td>43.8 ± 2.5</td>
<td>0.687</td>
</tr>
<tr>
<td>Haemoglobin (g/L)</td>
<td>148 ± 7</td>
<td>146 ± 7</td>
<td>0.546</td>
</tr>
<tr>
<td>Leukocyte count (x10⁹/L)</td>
<td>5.5 ± 1.4</td>
<td>5.7 ± 1.2</td>
<td>0.707</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SD or the median and IQR as appropriate. P values are from unpaired t tests apart from IPAQ training load where the Mann-Whitney U test for nonparametric data was used.
Plasma total 25(OH)D and cathelicidin concentrations

The median plasma total 25(OH)D concentration increased from 54.5 to 125.5 nmol/L at week 14 in the vitamin D$_3$ group (P=0.001) and decreased from 57.0 to 32.5 nmol/L in the placebo group at 14 weeks (P=0.001) (Table 5.2). At the end of the study there was a significant difference between groups (P<0.001). Although the median plasma cathelicidin concentration increased in both groups (vitamin D$_3$: 29.5 to 34.0 ng/mL, P=0.003; placebo: 30.9 to 33.6 ng/mL, P=0.048; Wilcoxon signed ranks test) (Table 5.2), the percentage change of cathelicidin concentration in the vitamin D$_3$ group was higher than in the placebo group (vitamin D$_3$: 15.0%; placebo: 5.4%, P=0.025; Mann-Whitney U test) (Figure 5.1).

Figure 5.1 The percentage change of plasma cathelicidin concentration after the 14-week intervention period in the vitamin D$_3$ supplement and placebo groups. Data are median and interquartile range. * Significant difference between groups (P=0.025; Mann-Whitney U test).
Table 5.2 Plasma total 25(OH)D and cathelicidin concentrations in the vitamin D₃ and placebo groups before and after the 14-week intervention period

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total 25(OH)D (nmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>54.5 (43.2-71.0)</td>
<td>125.5 (96.8-149.5) *</td>
<td>0.001</td>
</tr>
<tr>
<td>Placebo</td>
<td>57.0 (38.7-71.0)</td>
<td>32.5 (26.7-49.3)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Cathelicidin (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>29.5 (26.6-32.3)</td>
<td>34.0 (30.0-38.3)</td>
<td>0.003</td>
</tr>
<tr>
<td>Placebo</td>
<td>30.9 (27.7-36.6)</td>
<td>33.6 (28.4-38.1)</td>
<td>0.048</td>
</tr>
</tbody>
</table>

Data are shown as the median and IQR for nonparametric Wilcoxon signed ranks test. Significant difference between groups: * P < 0.001 based on Mann-Whitney U test.


**Salivary variables**

While the concentrations of all salivary AMPs were not significantly altered over time in both vitamin D$_3$ and placebo groups, the salivary flow rates increased over time in the vitamin D$_3$ group (Table 5.3). The resting SIgA and cathelicidin secretion rates increased over time in the vitamin D$_3$ group (but not in the placebo group) (SIgA: 58.8, 87.2, 70.5 µg/min, P=0.026; cathelicidin: 0.076, 0.103, 0.090 ng/min, P=0.030; Friedman tests) (Figure 5.2 a and b). Salivary lysozyme secretion rate increased over time in both groups (time, P=0.002; two-way ANOVA) but lactoferrin secretion rate was unaltered (Figure 5.2 c and d).
Figure 5.2 Changes in salivary secretion rates after 7 and 14 weeks of the intervention period in the vitamin D₃ supplement and placebo groups for (A) SIgA, (B) cathelicidin, (C) lysozyme and (D) lactoferrin. Data are shown as the mean ± SD (lysozyme) or the median and IQR (SIgA, cathelicidin and lactoferrin) as appropriate. The location of significant differences from week 0 within the vitamin D₃ supplemented group only is indicated as follows: * P<0.05; † P=0.05 (Dunn’s post hoc test applied when Friedman test P<0.05). Significant difference from week 0 in both groups is indicated as follows: # P<0.05.
Table 5.3 Salivary flow rate and AMPs concentration in the vitamin D₃ and placebo groups before and after 7 and 14 weeks of the intervention

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 7</th>
<th>Week 14</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saliva flow rate (µL/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>317 (181-420)</td>
<td>462 (254-642) *</td>
<td>358 (274-640) *</td>
<td>0.022</td>
</tr>
<tr>
<td>Placebo</td>
<td>260 (192-496)</td>
<td>331 (242-540)</td>
<td>352 (251-532)</td>
<td>0.143</td>
</tr>
<tr>
<td><strong>SIgA concentration (mg/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>183.8 (142.0-221.8)</td>
<td>170.3 (121.3-235.8)</td>
<td>171.1 (145.6-217.2)</td>
<td>0.861</td>
</tr>
<tr>
<td>Placebo</td>
<td>181.3 (130.4-227.0)</td>
<td>141.4 (120.0-249.9)</td>
<td>171.9 (105.5-245.0)</td>
<td>0.211</td>
</tr>
<tr>
<td><strong>Lactoferrin concentration (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>4715 (2875-5861)</td>
<td>5358 (2995-7596)</td>
<td>4485 (2342-6810)</td>
<td>0.350</td>
</tr>
<tr>
<td>Placebo</td>
<td>4274 (1483-5450)</td>
<td>3772 (1763-5087)</td>
<td>5874 (1235-7550)</td>
<td>0.692</td>
</tr>
<tr>
<td><strong>Lysozyme concentration (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>2472 ± 2515</td>
<td>2986 ± 2907</td>
<td>2397 ± 1644</td>
<td>time*group, 0.460</td>
</tr>
<tr>
<td>Placebo</td>
<td>3367 ± 3135</td>
<td>3205 ± 2500</td>
<td>3018 ± 2389</td>
<td></td>
</tr>
<tr>
<td><strong>Cathelicidin concentration (µg/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>0.22 (0.09-0.53)</td>
<td>0.32 (0.13-0.45)</td>
<td>0.23 (0.09-0.45)</td>
<td>0.463</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.25 (0.05-0.94)</td>
<td>0.16 (0.07-0.37)</td>
<td>0.20 (0.07-0.28)</td>
<td>0.065</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SD for two-way ANOVA or the median and IQR for nonparametric Friedman test with post hoc Dunn’s test. The location of significant differences from week 0 within the group is indicated as follows: * P<0.05 (Dunn’s post hoc test applied when Friedman test P<0.05).
5.5 Discussion

As expected, plasma total 25(OH)D concentration significantly increased in the vitamin D$_3$ group and significantly decreased in the placebo group. The salivary SIgA and cathelicidin secretion rates significantly increased over time in the vitamin D$_3$ group but not in the placebo group. In addition, the percentage change of plasma cathelicidin concentration in the vitamin D$_3$ group was significantly higher than in the placebo group although plasma cathelicidin concentration significantly increased in both groups.

We demonstrated that in athletes following 14 weeks of vitamin D$_3$ supplementation with 5000 IU/day there was a significant increase in the plasma total 25(OH)D concentration from 54.5 to 125.5 nmol/L. This increase in total 25(OH)D is similar to a previous randomised placebo-controlled trial of 5000 IU daily vitamin D$_3$ supplementation, where plasma 25(OH)D levels increased significantly from 29 to 103 nmol/L in the intervention group after 8 weeks of supplementation (Close et al., 2013). During the winter months, 5000 IU of vitamin D$_3$ supplement daily could be a possible way for athletes to elevate 25(OH)D concentrations above a mean of 120 nmol/L as a higher resting salivary SIgA secretion rate was found in the athletes with 25(OH)D >120 nmol/L during the winter period (He et al., 2013; chapter 3). However, the supplementation period and dosage required to increase and maintain serum 25(OH)D concentration to a level necessary for optimal immune function in athletes are still unclear. In addition, it was also observed that plasma total 25(OH)D concentration significantly decreased in the placebo group from December to February probably reflecting seasonal changes and time from last effective ultraviolet exposure. Several other studies that assessed the plasma 25(OH)D concentrations of UK-based athletes have shown that there was no change or a significant decrease in the plasma 25(OH)D levels...
during the winter training period in athletes without vitamin D supplementation (Close et al., 2013; Owens et al., 2014; He et al., 2013; chapter 3). This is most likely due to insufficient ultraviolet radiation of appropriate wavelength (290-315 nm) between November and March in the UK to produce vitamin D in the skin (Webb & Holick, 1988).

In the present study the resting salivary SIgA and cathelicidin secretion rates significantly increased over time in the vitamin D$_3$ group. To our knowledge, this is the first report to show a significant increase in the resting salivary SIgA secretion rates after a period of vitamin D$_3$ supplementation. The reasons for this are still unclear, but could be related to the elevated salivary flow rates after vitamin D$_3$ supplementation. We report that the salivary flow rates significantly increased over time in the vitamin D$_3$ group. Several animal studies have demonstrated that VDRs are present in the parotid, submandibular and sublingual salivary glands which suggest a possible role for vitamin D in regulation of salivary secretion. This is supported by the finding that salivary flow rates were stimulated after treatment with vitamin D$_3$ in vitamin D-deficient rats (Peterfy, Tenenhouse, & Yu, 1988; Stumpf, 2008). Although the influence of vitamin D on salivary flow rate has not yet been demonstrated in humans, the finding that there was a significant positive correlation between plasma total 25(OH)D concentration and salivary flow rate in the endurance athlete cohort (N=225) (unpublished data, r=0.196, $P=0.003$; Pearson correlation test; He et al., 2013; chapter 3) might lend some support to this suggestion. In addition, the resting salivary SIgA and cathelicidin secretion rates in the vitamin D$_3$ group slightly dropped from week 7 to week 14. The reasons for this are unknown. Further studies involving different durations of vitamin D$_3$ supplementation are warranted to determine the effect on the responses of salivary AMPs.
We also found that salivary cathelicidin secretion rates only significantly increased in the participants with vitamin D₃ supplementation. Human cathelicidin is expressed in neutrophils, epithelial cells and salivary glands and can be detected in whole saliva (Davison, Allgrove, & Gleeson, 2009; Murakami, Ohtake, Dorschner, & Gallo, 2002). The elevated salivary cathelicidin secretion rates might be due to the influence of 25(OH)D in the human oral epithelial cells. Human oral epithelial cells which express VDRs and 1-α-hydroxylase have the enzymatic machinery to convert 25(OH)D to 1, 25(OH)₂D and it has been demonstrated that 25(OH)D can induce expression of the vitamin D responsive genes to enhance the production of cathelicidin by binding to VDRs in dose-related manner in human oral keratinocytes (Wang et al., 2013).

Our results indicate that 5000 IU of vitamin D3 supplement daily could have a beneficial effect in up-regulating plasma cathelicidin. We found that the percentage change of cathelicidin concentration in the vitamin D₃ group was significantly higher than in the placebo group, although plasma cathelicidin concentration significantly increased in both groups from November to March. The reason for the increase of plasma cathelicidin concentration in the placebo group is unclear, but could be related to the likely higher respiratory infection incidence over the winter months as cathelicidin production is increased following pathogen exposure and TLR activation (Bucki, Leszczynska, Namiot, & Sokolowski, 2010). Moreover, there was a similar result in another vitamin D₃ supplement study in which 25 healthy participants with plasma 25(OH)D concentration less than 80 nmol/L ingested 50,000 IU vitamin D₃ every other day, for 5 days, and a statistically significant increase of circulating cathelicidin concentration was only found in participants with 25(OH)D levels increasing more than 80 nmol/L after the vitamin D₃ treatment (Bhan et al., 2011). In our study, we also found a significant increase of plasma cathelicidin
concentration in the vitamin D₃ group although there were only 5 participants with 25(OH)D levels increasing more than 80 nmol/L after supplementation. It has been reported that patients with the lowest circulating concentrations of cathelicidin undergoing dialysis are at a greater than two-fold increased risk of death from infectious causes (Gombart et al., 2009). Given the observed associations of circulating cathelicidin concentrations and infection-associated mortality in human studies, a high dose of vitamin D₃ supplementation might be clinically useful in the populations that may be more at risk of infection due to occupational or lifestyle stress (e.g. athletes and military personnel).

Recently, it has been suggested that several salivary AMPs, such as SIgA and cathelicidin, might be associated with the incidence of URTI. Previous studies have shown an inverse relationship between SIgA values and URTI prevalence and low SIgA values have been reported to be associated with increased incidence of URTI in athletes (Fahlman & Engels, 2005; Gleeson et al., 2012; Neville et al., 2008). A one-year follow-up study has reported that the number of URTI episodes was negatively correlated with salivary cathelicidin levels (Usui et al., 2012). Further studies are still needed to establish if the enhancement of salivary SIgA and cathelicidin secretion after vitamin D supplementation could reduce the risk of URTI in athletes.

In conclusion, 5000 IU of vitamin D₃ supplementation daily appears to have a beneficial effect in up-regulating the expression of SIgA and cathelicidin in athletes during a winter training period. Vitamin D supplementation, therefore, could have a positive effect on immune function and lead to decreased incidence of respiratory infections. Further research in a substantially larger athlete cohort is needed to determine this possibility. In addition, it is
still unknown whether participants could reach the 25(OH)D level of 120 nmol/L after a substantially shorter term of high dose vitamin D₃ supplementation to elevate the resting expression of AMPs.
Chapter 6:
The influence of vitamin D status and 4 weeks of vitamin D3 supplementation on salivary antimicrobial peptides and proteins responses to prolonged exercise
6.1 Abstract

The purpose of this study was to determine the influence of vitamin D status and a short term of daily high dose vitamin D₃ supplementation on the salivary concentrations and secretion rates of SIgA, cathelicidin and human neutrophil peptides (HNP) 1-3 in response to prolonged and moderate intensity exercise. Fourteen healthy men cycled for 2 h at 60 % VO₂ max. Unstimulated saliva and blood samples were taken at pre-exercise and the further saliva samples were taken at post-exercise and 1 h post-exercise. After this trial, 7 participants received a daily supplement of three 5000 IU vitamin D₃ capsules for 4 weeks and then cycled for 2 h at 60% VO₂ max again. Unstimulated saliva and blood samples were taken as before. The data showed that there were no significant interaction effects for the concentrations and secretion rates of AMPs in the high-level and low-level vitamin D groups. Although the total 25(OH)D concentrations of participants with vitamin D₃ supplementation increased from 66.3 ± 16.6 to 151.5 ± 27.4 nmol/L, there were no significant interaction effects for the concentrations and secretion rates of AMPs in the pre-intervention and post-intervention trials. We found that there were no significant effects of vitamin D status and a 4-week period of vitamin D₃ supplementation on the salivary AMPs responses to prolonged exercise. However, further studies including more participants with a larger range of vitamin D status or a longer period of vitamin D₃ supplementation are warranted.
6.2 Introduction

Prolonged bouts of strenuous exercise could have a temporary negative impact on both aspects of innate immunity and acquired immunity. Post-exercise immune function depression is most pronounced when the exercise is continuous, prolonged (longer than 1.5 h), of moderate to high intensity (55–75% VO$_2$max), and performed without food intake (Gleeson, 2006). It has been reported that salivary AMPs increased significantly after prolonged exercise, such as cathelicidin and HNP 1-3 (Davison et al., 2009).

It has recently been recognised that vitamin D is a vital mediator of innate immune responses, enhancing the antimicrobial properties of immune cells such as monocytes and macrophages (Bikle, 2009). Vitamin D is a key link between TLR activation and antimicrobial responses in innate immunity. Following activation of the TLR signalling cascade in the presence of microbes, vitamin D has a vital role in up-regulating the production of AMPs, such as cathelicidin (Liu et al., 2006). These AMPs have a broad range of activities against microorganisms and may be involved in the direct inactivation of viruses through membrane destabilization (Kamen & Tangpricha, 2010). They are produced by epithelial cells and macrophages and in the lungs are secreted into the biofilm covering the inner surface of the airways, thereby creating a barrier that is chemically lethal to microbes. A recent study in university athletes reported a higher level of plasma cathelicidin and salivary SIgA secretion in those who had plasma 25(OH)D greater than 120 nmol/L compared with those who had lower vitamin D status (He et al., 2013; Chapter 3) and a follow-up randomised, placebo controlled, double blind vitamin D$_3$ supplementation study (5000 IU/day for 14 weeks) by the same group (He et al., 2015; Chapter 5) reported significant increases in salivary secretion rates of both SIgA and cathelicidin compared with no significant change in the placebo group.
This was due, at least in part to a significant increase in the saliva flow rates over time in the vitamin D group.

However, the relationships between vitamin D status, salivary AMPs, saliva flow rates and prolonged exercise have not yet been explored. Also it is still unknown whether participants could reach the 25(OH)D level of 120 nmol/L after a relatively short term period of daily high dose vitamin D₃ supplementation. Therefore, the purpose of this preliminary study was to determine the influence of vitamin D status and a month of daily high dose vitamin D₃ supplementation on the salivary concentrations and secretion rates of SIgA, cathelicidin and HNP1-3 in response to prolonged and moderate intensity exercise in a population of individuals engaged in regular sport training. We hypothesized that all participants would reach the 25(OH)D level of 120 nmol/L after vitamin D₃ supplementation to elevate their resting salivary AMPs expression and that participants with high level of vitamin D concentrations would exhibit enhanced salivary AMPs expression in response to prolonged exercise.

6.3 Methods

Participants

Fourteen healthy men (age: 22.9 ± 3.4 years, body mass: 79.8 ± 8.7 kg, height: 184.2 ± 7.3 cm, body mass index: 23.6 ± 2.5 kg/m² and VO₂ max: 55.9 ± 13.6 ml/kg/min) who were engaged in regular sports training from Loughborough University, UK (latitude 53 °N) volunteered to participate in the study during May to July 2014 and their self-reported training loads (determined by a pre-screening questionnaire) averaged 12 ± 5 h/week (mean ±
SD). Participants were required to complete a comprehensive health-screening questionnaire prior to starting the study and had not taken any regular medication or antibiotics in the 3 months prior to the study. All participants were fully informed about the rationale for the study and of all experimental procedures to be undertaken. Participants provided written consent to participate in the study, which had earlier received the approval of Loughborough University ethical advisory committee. The details of inclusion and exclusion criteria were described in Chapter 2.

**Study protocol**

Approximately 1 week before the main trials, participants completed a continuous incremental test on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) to volitional exhaustion to determine their maximal oxygen uptake (VO$_2$ max). After a 3-min warm-up cycling at 70 W, the test began at 95 W with increments of 35 W every 3 min. Verbal encouragement was provided to ensure maximal effort. Expired-gas samples were collected in Douglas bags (Harvard Apparatus, Edenbridge, UK) during the third minute of each work-rate increment, and heart rate was measured continuously using short-range radio telemetry (Polar, Kempele, Finland). An oxygen/carbon dioxide analyzer (Servomex 1400, Crowbridge, UK) was used along with a dry gas meter (Harvard Apparatus, Edenbridge, UK) to determine V$_E$, VO$_2$, and VCO$_2$. From the VO$_2$–work-rate relationship, the work rate equivalent to 60% VO$_2$ max was interpolated. After a 15 minute recovery, participants cycled for 20 min at a steady state work rate equivalent to 60 % VO$_2$ max with expired gas samples collected after 10 and 20 min in order to familiarize the subjects with the exercise protocol for subsequent trials and to ensure that the calculated work rate elicited the desired. After this trial the participants were familiarized with the saliva-collection procedure.
For the main trials, all the participants arrived at 12:00 following a 3-h fast and their body mass and stature were recorded. Participants were required to abstain from any strenuous physical activity for 24 h before coming to the laboratory. Saliva and blood samples were collected as described in Chapter 2. Participants then cycled for 120 min at 60% VO₂ max on a stationary cycle ergometer in a laboratory maintained at 21 ± 1 °C and drank water ad libitum during exercise. Expired gas was collected after 15, 45, 75, and 105 min of exercise to determine VO₂. Heart rate was measured using short-range telemetry (Polar, Kempele, Finland) at 30-min intervals. Immediately after completing the exercise task, unstimulated saliva samples were taken. Another saliva samples were taken at 60 minutes after exercise. After centrifugation for 2 min at 5000 g to remove cells and insoluble matter, the saliva samples were stored frozen at -20 °C prior to analysis. The EDTA blood was centrifuged for 10 min at 1500 g and 4 °C and the plasma stored at -20ºC prior to analysis.

After the main trials, participants were randomly allocated to a vitamin D₃ supplement group (n=7) or a placebo group (n=7); those in the supplemented group received a daily supplement of three 5000 IU vitamin D₃ capsules (one at breakfast, one at lunch and one at dinner); those in the placebo group received 3 placebo (cellulose) capsules. After the 4-week supplementation period, the participants returned to the laboratory at 12:00 following a 3-h fast and their body mass and stature were recorded again. They were also required to abstain from any strenuous physical activity for 24 h before coming to the laboratory. A resting unstimulated saliva sample and venous blood sample (10 ml) were taken as before. During the second main trial, the participants in the vitamin D₃ supplement group performed the same exercise, 120 min cycling at 60% VO₂ max and further saliva samples were taken as...
before. Two of the participants in the placebo group dropped out and the remaining 5 participants had a resting blood sample taken after 4 weeks as the control group for plasma 25(OH)D concentration.

**Plasma analysis**

Plasma samples were analysed in duplicate for total 25(OH)D concentrations. The best measure of vitamin D status is considered to be the sum of the 25-hydroxy metabolites of D₂ and D₃ (25(OH)D₃ and 25(OH)D₂). The resting EDTA plasma samples were assayed in duplicate for 25(OH)D concentration using a commercially available immunoassay kit (25(OH)-Vitamin D Xpress ELISA kit K2107, Immunodiagnostik AG, Bensheim, Germany) according to the manufacturer’s instructions which has been shown by the manufacturer to have excellent agreement ($R^2=0.920$) with the ‘gold-standard’ high pressure liquid chromatography-tandem mass spectrometer method. It has also shown a good agreement ($R^2=0.832$) from our own samples that we analysed with both methods prior to the study. The intra-assay CV was ±5.1% across a working range of 16–250 nmol/L. Plasma samples were also analysed for cathelicidin concentrations as described in Chapter 2.

**Saliva analysis**

The saliva volume collected and flow rate were estimated as described in Chapter 2. Saliva samples were analysed for SIgA, cathelicidin and HNP1-3 as described in Chapter 2. Secretion rates for each of the salivary AMPs were calculated as described in Chapter 2.
**Statistical analysis**

The Shapiro-Wilk test was used to determine if data sets were normally distributed. Anthropometric, haematological variables, self-reported training loads, VO\textsubscript{2} max, physiological variables and total 25(OH)D concentrations were compared between the high-level and low-level vitamin D groups using unpaired t tests. The changes in the concentrations and secretion rates of salivary AMPs as well as the salivary flow rates over the exercise trial in the high-level and low-level vitamin D groups were assessed by two-way mixed ANOVA. To compare the pre-intervention and post-intervention trials, the paired t test and two-way repeated measures ANOVA were used. Data are presented as mean (±SD) and the accepted level of significance was P<0.05.

### 6.4 Results

**The plasma total 25(OH)D concentrations**

The total 25(OH)D concentration at baseline from 14 participants was 73.2 ± 26.8 nmol/L. The total 25(OH)D concentration from 5 participants classified in the high-level (>80 nmol/L) group was 102.7 ± 17.8 nmol/L and the total 25(OH)D concentration from 5 participants classified in the low-level (<60 nmol/L) was 49.9 ± 13.5 nmol/L. The total 25(OH)D concentrations from 4 participants excluded in these two group were 66.9, 66.6, 66.5 and 61.7 nmol/L.

In addition, the total 25(OH)D concentrations of 7 participants with vitamin D\textsubscript{3} supplementation increased from 66.3 ± 16.6 to 151.5 ± 27.4 nmol/L (P<0.001) and the total
25(OH)D concentrations from 5 participants without vitamin D₃ supplementation were unaltered (63.5 ± 23.0 to 66.1 ± 21.2 nmol/L, P=0.531).

Physiological and salivary variables in the high-level and low-level vitamin D groups

There were no significant differences in baseline anthropometrics, haematological variables, training loads and VO₂max between the high-level and low-level vitamin D groups (Table 6.1). The mean relative exercise intensity (% VO₂ max), power output and water intake over the 2 h exercise are also illustrated in Table 6.1 and there were no significant differences in any of the physiological variables during the trial between the two groups.

Table 6.1 Physiological variables in the high-level and low-level vitamin D groups

<table>
<thead>
<tr>
<th></th>
<th>High-level</th>
<th>Low-level</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>22.4 ± 2.1</td>
<td>25.0 ± 4.5</td>
<td>0.272</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>180.2 ± 8.3</td>
<td>187.2 ± 5.0</td>
<td>0.140</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>77.9 ± 4.8</td>
<td>80.0 ± 11.2</td>
<td>0.579</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.2 ± 2.9</td>
<td>23.0 ± 2.4</td>
<td>0.521</td>
</tr>
<tr>
<td>RBC count (x10¹²/L)</td>
<td>4.2 ± 0.5</td>
<td>4.3 ± 0.6</td>
<td>0.688</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>38.7 ± 4.1</td>
<td>39.6 ± 5.7</td>
<td>0.781</td>
</tr>
<tr>
<td>Haemoglobin (g/L)</td>
<td>142 ± 6</td>
<td>145 ± 13</td>
<td>0.694</td>
</tr>
<tr>
<td>Leukocyte count (x10⁹/L)</td>
<td>6.5 ± 2.5</td>
<td>5.1 ± 1.1</td>
<td>0.296</td>
</tr>
<tr>
<td>Training load (h/week)</td>
<td>11.4 ± 2.6</td>
<td>8.6 ± 2.2</td>
<td>0.103</td>
</tr>
<tr>
<td>VO₂ max (ml/kg/min)</td>
<td>55.0 ± 8.0</td>
<td>55.3 ± 13.1</td>
<td>0.966</td>
</tr>
<tr>
<td>% VO₂ max</td>
<td>57.2 ± 0.7</td>
<td>59.1 ± 3.4</td>
<td>0.250</td>
</tr>
<tr>
<td>Power output (W)</td>
<td>182 ± 44</td>
<td>184 ± 37</td>
<td>0.940</td>
</tr>
<tr>
<td>Water intake (ml)</td>
<td>1116 ± 635</td>
<td>1192 ± 744</td>
<td>0.866</td>
</tr>
</tbody>
</table>
There were no significant interaction effects for the concentrations and secretion rates of SIgA, cathelicidin and HNP1-3 although there were significant overall effects of time for the concentrations of SIgA, cathelicidin and HNP1-3 and the secretion rates of cathelicidin, all of which were increased from pre- to post-exercise (Table 6.2). In addition, there were no significant overall effects of group for the concentrations and secretion rates of SIgA, cathelicidin and HNP1-3 between the high-level and low-level vitamin D groups (Table 6.2). Furthermore, there were no any significant effects for salivary flow rates (Table 6.2).
Table 6.2 Salivary variables during exercise in the high-level and low-level vitamin D groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-Ex</th>
<th>Post-Ex</th>
<th>1 h Post-Ex</th>
<th>Main Effects P values (group; time; group x time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva flow rate (mL/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-level</td>
<td>0.504 ± 0.178</td>
<td>0.345 ± 0.143</td>
<td>0.406 ± 0.137</td>
<td>0.492; 0.131; 0.223</td>
</tr>
<tr>
<td>Low-level</td>
<td>0.331 ± 0.246</td>
<td>0.307 ± 0.199</td>
<td>0.373 ± 0.240</td>
<td></td>
</tr>
<tr>
<td>SIgA concentration (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-level</td>
<td>159.3 ± 55.4</td>
<td>251.2 ± 60.7</td>
<td>264.8 ± 84.4</td>
<td>0.343; 0.026; 0.952</td>
</tr>
<tr>
<td>Low-level</td>
<td>243.8 ± 123.9</td>
<td>316.5 ± 180.9</td>
<td>331.5 ± 195.7</td>
<td></td>
</tr>
<tr>
<td>SIgA secretion rate (µg/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-level</td>
<td>73.9 ± 15.1</td>
<td>82.1 ± 23.2</td>
<td>110.2 ± 59.8</td>
<td>0.475; 0.079; 0.941</td>
</tr>
<tr>
<td>Low-level</td>
<td>65.5 ± 31.6</td>
<td>70.0 ± 34.9</td>
<td>92.4 ± 39.4</td>
<td></td>
</tr>
<tr>
<td>Cathelicidin concentration (µg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-level</td>
<td>4.0 ± 3.0</td>
<td>15.5 ± 3.1</td>
<td>14.0 ± 6.4</td>
<td>0.360; 0.001; 0.094</td>
</tr>
<tr>
<td>Low-level</td>
<td>7.0 ± 4.7</td>
<td>11.6 ± 5.5</td>
<td>9.1 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Cathelicidin secretion rate (ng/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-level</td>
<td>1.9 ± 1.4</td>
<td>5.1 ± 1.3</td>
<td>5.7 ± 3.0</td>
<td>0.128; 0.010; 0.076</td>
</tr>
<tr>
<td>Low-level</td>
<td>2.1 ± 1.8</td>
<td>3.1 ± 2.5</td>
<td>2.6 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>HNP 1-3 concentration (µg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-level</td>
<td>310 ± 435</td>
<td>1272 ± 680</td>
<td>984 ± 897</td>
<td>0.381; 0.043; 0.140</td>
</tr>
<tr>
<td>Low-level</td>
<td>1234 ± 1097</td>
<td>1422 ± 904</td>
<td>1165 ± 660</td>
<td></td>
</tr>
<tr>
<td>HNP 1-3 secretion rate (ng/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-level</td>
<td>138.5 ± 186.7</td>
<td>411.1 ± 242.8</td>
<td>367.9 ± 357.9</td>
<td>0.931; 0.064; 0.300</td>
</tr>
<tr>
<td>Low-level</td>
<td>282.2 ± 212.4</td>
<td>355.1 ± 293.4</td>
<td>317.0 ± 180.7</td>
<td></td>
</tr>
</tbody>
</table>
Physiological and salivary variables in the pre-intervention and post-intervention trials

Seven participants (age: 24.3 ± 3.9 years, body mass: 78.3 ± 7.3 kg, height: 186.7 ± 4.3 cm, body mass index: 22.5 ± 1.9 kg/m² and VO₂ max: 57.0 ± 12.2 ml/kg/min) received a 4-week period of 15000 IU daily vitamin D₃ supplementation and then performed the same exercise trial.

There were no significant differences in baseline haematological variables between pre-intervention and post-intervention trials. The mean exercise intensity (% VO₂ max), power output and water intake over the 2 h exercise were illustrated in Table 6.3 and there were no significant differences in any physiological variables during 2 h exercise between pre-intervention and post-intervention trials.

Table 6.3 Physiological variables in the pre-intervention and post-intervention trials

<table>
<thead>
<tr>
<th>Variable</th>
<th>PRE-Intervention</th>
<th>POST-Intervention</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC count (x10¹²/L)</td>
<td>4.3 ± 0.6</td>
<td>4.6 ± 0.2</td>
<td>0.168</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>39.4 ± 5.4</td>
<td>42.4 ± 1.7</td>
<td>0.165</td>
</tr>
<tr>
<td>Haemoglobin (g/L)</td>
<td>140 ± 7</td>
<td>136 ± 7</td>
<td>0.057</td>
</tr>
<tr>
<td>Leukocyte count (x10⁹/L)</td>
<td>4.8 ± 1.0</td>
<td>4.3 ± 0.6</td>
<td>0.071</td>
</tr>
<tr>
<td>% VO₂ max</td>
<td>59.1 ± 3.1</td>
<td>58.9 ± 3.2</td>
<td>0.889</td>
</tr>
<tr>
<td>Power output (W)</td>
<td>197 ± 36</td>
<td>197 ± 36</td>
<td>1.000</td>
</tr>
<tr>
<td>Water intake (ml)</td>
<td>1226 ± 734</td>
<td>1236 ± 414</td>
<td>0.960</td>
</tr>
</tbody>
</table>
There were no significant interaction effects for the concentrations and secretion rates of SIgA, cathelicidin and HNP1-3 although there were significant overall effects of time for the concentrations of SIgA, cathelicidin and HNP1-3 which all increased from pre- to post-exercise (Table 6.4). In addition, there were no significant overall effects of trial for the concentrations and secretion rates of SIgA, cathelicidin and HNP1-3 between pre-intervention and post-intervention trials (Table 6.4). Furthermore, there were no any significant effects for salivary flow rates (Table 6.4).
### Table 6.4 Salivary variables during exercise in the pre-intervention and post-intervention trials

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-Ex</th>
<th>Post-Ex</th>
<th>1 h Post-Ex</th>
<th>Main Effects P values (trial; time; trial x time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva flow rate (mL/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE-Intervention</td>
<td>0.374 ± 0.191</td>
<td>0.314 ± 0.141</td>
<td>0.377 ± 0.178</td>
<td>0.186; 0.211; 0.255</td>
</tr>
<tr>
<td>POST-Intervention</td>
<td>0.435 ± 0.204</td>
<td>0.360 ± 0.145</td>
<td>0.508 ± 0.201</td>
<td></td>
</tr>
<tr>
<td>SIgA concentration (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE-Intervention</td>
<td>248.3 ± 117.7</td>
<td>339.2 ± 192.5</td>
<td>306.5 ± 133.0</td>
<td>0.184; 0.041; 0.427</td>
</tr>
<tr>
<td>POST-Intervention</td>
<td>230.3 ± 71.7</td>
<td>307.4 ± 177.0</td>
<td>213.9 ± 110.8</td>
<td></td>
</tr>
<tr>
<td>SIgA secretion rate (μg/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE-Intervention</td>
<td>75.1 ± 19.0</td>
<td>89.6 ± 40.8</td>
<td>101.2 ± 34.0</td>
<td>0.459; 0.564; 0.793</td>
</tr>
<tr>
<td>POST-Intervention</td>
<td>93.4 ± 39.0</td>
<td>103.1 ± 56.6</td>
<td>100.8 ± 57.0</td>
<td></td>
</tr>
<tr>
<td>Cathelicidin concentration (μg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE-Intervention</td>
<td>7.4 ± 5.3</td>
<td>14.9 ± 7.3</td>
<td>8.6 ± 3.1</td>
<td>0.161; 0.044; 0.447</td>
</tr>
<tr>
<td>POST-Intervention</td>
<td>7.8 ± 6.8</td>
<td>10.6 ± 3.8</td>
<td>6.7 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>Cathelicidin secretion rate (ng/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE-Intervention</td>
<td>2.3 ± 1.7</td>
<td>4.2 ± 2.2</td>
<td>2.9 ± 0.9</td>
<td>0.545; 0.226; 0.924</td>
</tr>
<tr>
<td>POST-Intervention</td>
<td>2.7 ± 2.1</td>
<td>4.1 ± 2.9</td>
<td>3.3 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>HNP 1-3 concentration (μg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE-Intervention</td>
<td>1085.9 ± 1070.5</td>
<td>1481.9 ± 911.8</td>
<td>874.0 ± 568.1</td>
<td>0.259; 0.023; 0.771</td>
</tr>
<tr>
<td>POST-Intervention</td>
<td>855.3 ± 773.7</td>
<td>1202.3 ± 1045.2</td>
<td>811.4 ± 688.5</td>
<td></td>
</tr>
<tr>
<td>HNP 1-3 secretion rate (ng/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE-Intervention</td>
<td>272.1 ± 220.6</td>
<td>399.7 ± 248.6</td>
<td>275.6 ± 166.1</td>
<td>0.630; 0.229; 0.665</td>
</tr>
<tr>
<td>POST-Intervention</td>
<td>250.7 ± 216.3</td>
<td>415.7 ± 340.3</td>
<td>376.0 ± 352.3</td>
<td></td>
</tr>
</tbody>
</table>
6.5 Discussion

This was a preliminary study to investigate the influence of vitamin D status and a short-term of vitamin D₃ supplementation on salivary AMP responses to prolonged exercise. The main findings of the present study were as follows: (1) there were no significant interaction effects for the salivary flow rates as well as the concentrations and secretion rates of SIgA, cathelicidin and HNP1-3 between the high-level and low-level vitamin D groups; (2) the plasma total 25(OH)D concentrations significantly increased after a 4-week period of vitamin D₃ supplementation; (3) there were no significant interaction effects for salivary flow rates or the concentrations and secretion rates of SIgA, cathelicidin and HNP1-3 between pre-intervention and post-intervention trials.

To our knowledge, this is the first study to examine the influence of vitamin D status on salivary AMP responses to prolonged exercise. We found that there were no significant interaction effects on the any salivary variable responses to prolonged exercise between the high-level and low-level vitamin D groups. It has been previously reported that vitamin D status influences the salivary SIgA secretion rates in endurance athletes during a winter training period (He et al., 2013; chapter 3). The median SIgA secretion rate during the 4-month winter period in the individuals who had relatively high vitamin D concentrations (plasma 25(OH)D >120 nmol/L) was significantly higher than in groups having lower vitamin D status. However, in the present study, there was no significant effect of vitamin D status on the SIgA secretion rates at rest or in response to prolonged exercise. This is likely because the difference of plasma total 25(OH)D concentrations classified in the high-level group in the present study from the previous study. But it is worthy of note that the participants with higher vitamin D status tended to have higher SIgA, cathelicidin and HNP1-
3 secretion rates at baseline and after exercise than those had lower vitamin D status in the present study and a significant limitation of the present study was the relatively small number of participants. Further research including more participants with a larger range of vitamin D status might be warranted to understand the effect of vitamin D status on the salivary AMP responses to prolonged exercise. In addition, we reported that the concentrations of SIgA, cathelicidin and HNP1-3 significantly increased after exercise in both the high-level and low-level vitamin D groups, which is in accordance with previous studies involving prolonged exercise (Allgrove et al., 2009; Davison et al., 2009).

On the basis of the present data, we found that the plasma total 25(OH)D concentrations significantly increased from 66.3 ± 16.6 to 151.5 ± 27.4 nmol/L after a 4-week period of 15000 IU daily vitamin D₃ supplementation. It was also observed that the total 25(OH)D concentrations from participants without vitamin D₃ supplementation were unaltered from May to July. It seems likely that the sunlight from May to July in the UK is only enough to maintain vitamin D status instead of increasing the total 25(OH)D concentrations. A randomised, placebo controlled, double blind vitamin D₃ supplementation study (5000 IU/day for 14 weeks) reported significant increases in salivary secretion rates of both SIgA and cathelicidin compared with no significant change in the placebo group (He et al., 2015; Chapter 5). Moreover, it has been shown that the resting salivary flow rates were increased after 7 and 14 weeks of 5000 IU daily vitamin D₃ supplementation (He et al., 2015; Chapter 5). However, in the present study there were no significant interaction effects for salivary flow rates and the expression of AMPs between pre-intervention and post-intervention trials. It might be due to the shorter period of vitamin D₃ supplementation in the present study. Nevertheless, the salivary flow rates and the secretion rates of SIgA and cathelicidin tended to increase at baseline after 4 weeks of vitamin D₃ supplementation. Therefore, the further
study of vitamin D₃ supplementation for a longer period is needed to clarify the effects of vitamin D₃ supplementation on the salivary AMP responses to prolonged exercise.

We acknowledge that there were limitations in this pilot study design regarding the supplemental dose of vitamin D₃ used and possible adverse responses. We used the dose of 15,000 IU/day which is 50% higher than the European Food Safety Authority’s recently revised tolerable upper intake level of 10,000 IU/day. Also we did not measure any markers of vitamin D toxicity. However, no participants in the current trial reported any of the symptoms of vitamin D toxicity which are attributable to hypercalcaemia, including nausea, dehydration and lethargy. Furthermore, both the IoM and the Endocrine Society concluded that the upper limit of 25(OH)D concentration of 250 nmol/L was safe and would not cause vitamin D intoxication (IoM, 2011; Holick et al., 2011).

In summary, the results of current study showed that there were no significant effects of vitamin D status and a 4-week period of vitamin D₃ supplementation on the salivary AMP responses to prolonged exercise. However, further studies including more participants with a larger range of vitamin D status or a longer period of vitamin D₃ supplementation are warranted.
Chapter 7:

General discussion
7.1 Overview of the experimental studies in this thesis

Female athletes may be more susceptible than their male counterparts to URTI and that lower mucosal immunity may account for this in part. Moreover, athletes with positive serostatus for both CMV and EBV may have fewer URTI episodes than those with negative serostatus. In addition, athletes with low vitamin D concentrations may have a higher risk of URTI. This may be due to impaired mucosal and systemic immunity.

Vitamin D seems to play important roles in both innate and adaptive immune responses. Therefore, a series of follow-up studies were also conducted to examine the effect of vitamin D on mucosal and systemic immunity in athletes in this thesis.

The influence of vitamin D on circulating cytokines might be different in athletes compared with previous studies on non-athletes. In addition, we found that both pro-inflammatory and anti-inflammatory cytokines by multi-antigen stimulated whole blood culture were not influenced by 1, 25(OH)2D3 in concentrations within the normal healthy range.

5000 IU of vitamin D3 supplementation daily appears to have a beneficial effect in up-regulating the expression of SIGA and cathelicidin in athletes during a winter training period.

There were no significant effects of a short term of vitamin D3 supplementation or between high and low levels of vitamin D on the salivary AMP responses to prolonged exercise.

Figure 7.1 Overview of the experimental studies
7.2 The main findings of the thesis

The main findings in Chapter 3 were:

✧ The female athletes had more URTI days and their URTI episodes lasted several days longer than the male athletes.

✧ Saliva flow rate was ~17% lower in female athletes and the secretion rates of lactoferrin and lysozyme were significantly lower in females.

✧ In both sexes, saliva AMP secretion rates increased over time which could be due to the significant fall in the training loads of the athletes that was observed in the middle part of the study period.

✧ There was no difference in the proportion of subjects who presented with symptoms of infection between CMV/EBV positive and negative groups.

✧ Athletes with prior infection of both CMV and EBV had fewer URTI episodes and symptom days and higher numbers of circulating lymphocytes than athletes who were seronegative for both CMV and EBV.

✧ A higher proportion (67%) of athletes who had vitamin D concentrations (<30 nmol/L) experienced one or more URTI episodes during the 4-month study period.

✧ The plasma cathelicidin concentration was positively associated with vitamin D concentrations.

✧ The averaged salivary SIgA secretion rate during the 4-month winter period in athletes who had vitamin D concentrations (>120 nmol/L) was significantly higher than in groups having vitamin D concentrations (<30 nmol/L).

✧ Pro-inflammatory cytokine production in response to multi-antigen stimulation was substantially lower in athletes who had vitamin D concentrations (<30 nmol/L).
The main findings in Chapter 4 were:

✧ The circulating IL-10 and IFN-γ concentrations were significantly higher in the vitamin D sufficient athletes (>50 nmol/L) compared with the vitamin D insufficient athletes (<50 nmol/L).

✧ The production of TNF-α, IL-6, IFN-γ, IL-2 and IL-10 by multi-antigen stimulated whole blood culture were significantly influenced by 1, 25(OH)₂D₃ of concentrations of 1000 pmol/L and/or 10000 pmol/L.

✧ The production of lymphocyte-derived and monocyte-derived cytokines, such as IFN-γ, IL-2, IL-4 and IL-1β, were significantly different in the multi-antigen stimulated whole blood culture without the addition of 1, 25(OH)₂D₃ compared with its production in the PBMC culture.

The main findings in Chapter 5 were:

✧ The salivary SIgA and cathelicidin secretion rates significantly increased over time in the vitamin D₃ group but not in the placebo group.

✧ The salivary flow rates increased over time in the vitamin D₃ group but not in the placebo group.

✧ The change of plasma cathelicidin concentration in the vitamin D₃ group was significantly higher than in the placebo group.

The main findings in Chapter 6 were:

✧ There were no significant interaction effects for the salivary flow rates and AMPs responses to prolonged exercise between the high-level and low-level vitamin D participants.
The plasma total 25(OH)D concentrations significantly increased after a short term of high dose vitamin D₃ supplementation.

There were no significant interaction effects for the salivary flow rates and AMPs responses to prolonged exercise between pre-intervention and post-intervention trials.

7.3 The limitations of the thesis

The phase of the menstrual cycle (when blood and saliva samples were taken) was not determined in this research but we did establish that 40% of the females were taking oral contraceptives.

We did not attempt to distinguish between symptoms of an infectious/illness nature vs inflammation throughout the winter months of the study design. We used the validated Jackson score questionnaire which is a conservative instrument requiring a substantial symptom score criterion threshold to define a URTI episode.

The participants’ plasma concentration of 1, 25(OH)₂D was not determined in the study that we added different doses of 1, 25(OH)₂D in the multi-antigen stimulated whole blood culture and the lower doses of 1, 25(OH)₂D within the normal physiological range could be used in the whole blood culture.

There was a limitation in the pilot study design regarding supplemental dose. We used the dose of 15,000 IU/day which is 50% higher than the European Food Safety Authority’s recently revised tolerable upper intake level of 10,000 IU/day. However, no participants in the current trial had a plasma 25(OH)D concentration higher than 250 nmol/L. Both the IoM and the Endocrine Society concluded that an upper limit of
Chapter 7

General discussion

25(OH)D concentration of 250 nmol/L was safe and would not cause vitamin D intoxication (IoM, 2011; Holick et al., 2011).

7.4 Future directions

Following on from the studies presented in this thesis, a number of key questions still remain to be answered:

✧ Is it possible that the same training load could have a greater depressive effect on humoral and systemic immunity (e.g. lower secretion rates of mucosal AMPs and fewer numbers of circulating B cells and NK cells) for women than for men?

✧ Further research is needed to understand the influence of previous CMV or EBV infection (or both) on the immune system and clarify whether it has an important influence on the risk of URTI.

✧ It is unknown that the influence of prior infection with CMV and EBV alone or in combination on the white blood cell subsets and salivary antimicrobial proteins response to prolonged exercise.

✧ Is there an optimal vitamin D level for athletes to improve immune function and prevent URTI during the winter months?

✧ Further research is needed to understand the effect of the normal healthy range or lower levels of plasma 1, 25(OH)₂D on multi-antigen stimulated cytokine production by whole blood culture.

✧ Different durations of vitamin D₃ supplementation are warranted to determine the effects on the responses of salivary AMPs.
It is still unknown if the enhancement of salivary SIgA and cathelicidin secretion after vitamin D$_3$ supplementation could reduce the risk of URTI in athletes.

Further research including a large cohort of participants with various ranges of vitamin D status might be warranted to understand the effect of vitamin D status on the salivary AMP responses to prolonged exercise.

7.5 Conclusions

A series of studies in this thesis have demonstrated the influence of various factors (sex differences, CMV/EBV serostatus and vitamin D status) on susceptibility to URTI among athletes. Female athletes may be more susceptible than their male counterparts to URTI during winter training periods. Moreover, athletes who had experienced prior infection with both CMV and EBV may have fewer URTI episodes than those with negative serostatus for both CMV and EBV. In addition, athletes with low vitamin D status may have a higher risk of URTI and suffer more severe symptoms when URTI is present. Furthermore, it was suggested in this thesis that vitamin D$_3$ supplementation could have a positive effect on immune function and lead to decreased incidence of respiratory infections.
References


References


154


References


Appendices
Appendix A: Jackson Score Common Cold Symptom Questionnaire

Name ..............................................Subject Number……. Date…………………..

Do you think that you are suffering from a common cold or flu today?
Fill in the circle if your answer is YES    o

If yes please complete all the questions below-
Are any of the following symptoms of the common cold or flu present today? Please indicate your response by filling in one circle for each of the following symptoms:

<table>
<thead>
<tr>
<th>SYMPTOM</th>
<th>DEGREE OF DISCOMFORT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None at all</td>
</tr>
<tr>
<td>Sneezing</td>
<td>o</td>
</tr>
<tr>
<td>Headache</td>
<td>o</td>
</tr>
<tr>
<td>Malaise (feeling of being generally unwell, run down or out of sorts)</td>
<td>o</td>
</tr>
<tr>
<td>Nasal discharge (runny nose)</td>
<td>o</td>
</tr>
<tr>
<td>Nasal obstruction (blocked nose)</td>
<td>o</td>
</tr>
<tr>
<td>Sore throat</td>
<td>o</td>
</tr>
<tr>
<td>Cough</td>
<td>o</td>
</tr>
<tr>
<td>Fever</td>
<td>o</td>
</tr>
<tr>
<td>Hoarseness</td>
<td>o</td>
</tr>
<tr>
<td>Earaches</td>
<td>o</td>
</tr>
<tr>
<td>Chilliness (question in Jackson original)</td>
<td>o</td>
</tr>
<tr>
<td>Joint aches and pains</td>
<td>o</td>
</tr>
</tbody>
</table>

Have you taken your supplement today? Fill in the circle if your answer is YES    o
Appendix B: International Physical Activity Questionnaire

Name ............................................... Subject Number…… Date……………………

The following questions relate to the amount of physical activity you have undertaken in the past week.

For the previous 7 days, please consider the amount, type and intensity of physical activity/training that you have undertaken. If this is more or less than normal, this should be reflected in your answers. This type of activity can take any form and can include normal everyday activities such as walking to the shops.

Vigorous physical activity refers to activities that take hard physical effort to complete and make you breathe/work much harder than normal.

Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

If in training, try to classify each whole session as either vigorous or moderate and record appropriately in the boxes provided.

Be as honest as possible.

1. In the last seven days, on how many days did you partake in vigorous physical activities like heavy lifting, aerobics, hard training, cycling, swimming or running?

   ■ ■ ■ Days  If none, answer 00 and skip to question 3

2. On days when you did vigorous physical activity how much time did you usually spend doing it?

   ■ ■ Hours ■ ■ Minutes

3. In the last 7 days, on how many days did you partake in moderate physical activities like lifting light loads, moderate training, cycling, swimming or running?

   ■ ■ ■ Days  If none, answer 00 and skip to question 5
4. On days when you did moderate physical activity how much time did you usually spend doing it?

   Hours   Minutes

5. In the last 7 days, on how many days did you walk for at least 10 minutes at a time?

   Days   If none, answer 00 and skip to question 7

6. On days when you walked for at least 10 minutes at a time, in total, how much time did you spend walking?

   Hours   Minutes

7. How much time did you spend sitting on a usual day? This may include time spent studying, visiting friends, reading or watching television.

   Hours   Minutes