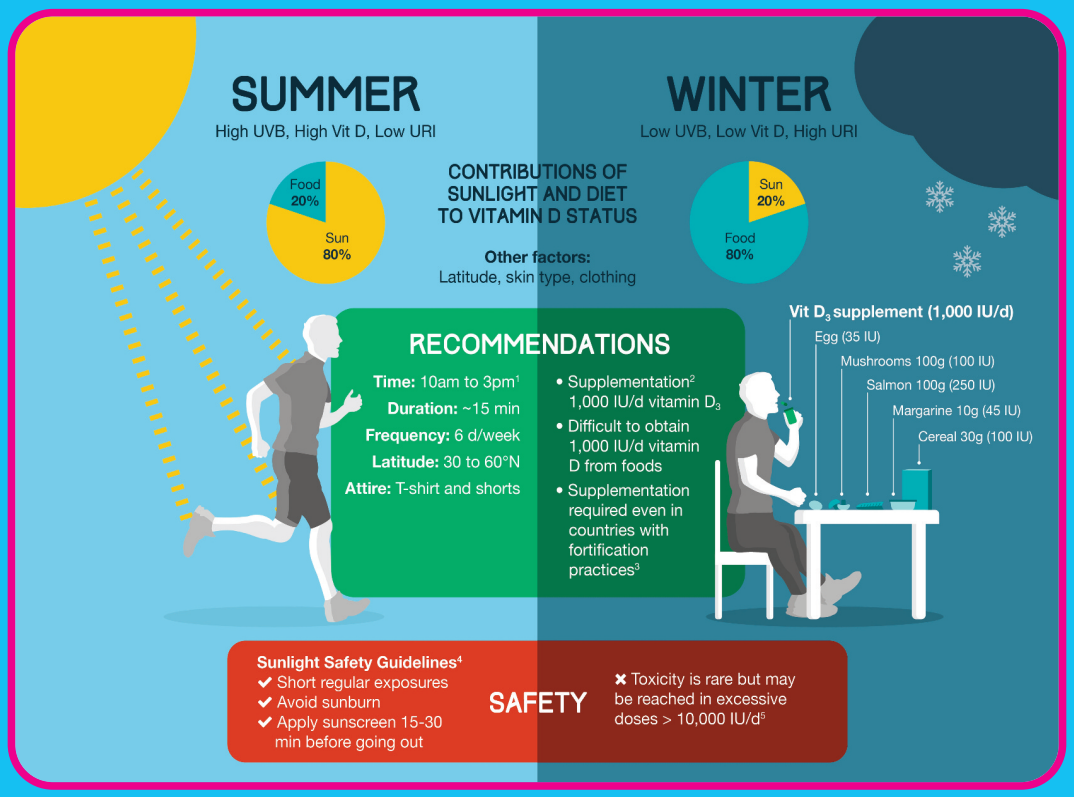
EXERCISE IMMUNOLOGY REVIEW



VOLUME 22 • 2016

The International Society of
Exercise and Immunology

DGSP
Deutsche Gesellschaft für
Sportmedizin und Prävention -
Deutscher Sportärztebund

EXERCISE IMMUNOLOGY REVIEW

An official Publication of
ISEI and DGSP

EXERCISE IMMUNOLOGY REVIEW

VOLUME 22 • 2016

CONTENTS

Editorial Statement

Exercise Immunology Review, an official publication of the International Society of Exercise Immunology and of the German Society of Sports Medicine and Prevention, is committed to developing and. enriching knowledge in all aspects of immunology that relate to sport, exercise, and regular physical ativity. In recognition of the broad range of disciplines that contribute to the understanding of immune function, the journal has adopted an interdisciplinary focus. This allows dissemination of research findings from such disciplines as exercise science, medicine, immunology, physiology, behavioral science, endocrinology, pharmacology, and psychology.

Exercise Immunology Review publishes review articles that explore: (a) fundamental aspects of immune function and regulation during exercise; (b) interactions of exercise and immunology in the optimization of health and protection against acute infections: (c) deterioration of immune function resulting from competitive stress and overtraining; (d) prevention or modulation of the effects of aging or disease (including HIV infection; cancer; autoimmune, metabolic or transplantation associated disorders) through exercise. (e) instrumental use of exercise or related stress models for basic or applied research in any field of physiology, pathophysiology or medicine with relations to immune function.

Exercise Immunology Review (ISSN 1077-5552) is published and printed by TOM-Systemdruck GmbH, Hansanring 125.

Postmaster:
Justus-Liebig-Universität Gießen, Abteilung für Sportmedizin
Exercise Immunology Review, Kugelberg 62, 35394 Gießen.

Editor: Dr. Karsten Krüger
Past Editor: Prof. Dr. Hinnak Northoff

Send editorial correspondence to:
Justus-Liebig- Universität Gießen
Abteilung für Sportmedizin
Dr. Karsten Krüger
Kugelberg 62
35394 Gießen
karsten.krueger@sport.uni-giessen.de

 Exercise Immunology Review is indexed in Sport Database, Sport Discus, Sport Search, SciSearch, EMBASE/ Excerpta Medica, Focus on: Sports Science & Medicine, Index Medicus, MEDLINE, Physical Education Index, Research Alert, International Bibliography of Periodical Literature, International Bibliography of Book Reviews, and CINAHL database.

To subscribe or renew subscription go to http://www.isei.dk/ or e-mail: karsten.krueger@sport.uni-giessen.de

From the Editors

This year's issue contains seven scientific articles, three which are concerned with exercise immunology itself, while the other four are more clinically oriented. The submissions made it difficult again to focus on special topics. Instead, the issue is a multifaceted compilation of articles on topics that are sufficiently novel and interesting for our readership.

EIR22 starts with a review article by Simpson and colleagues who describe the effects of infection history on the immune responses to exercise. They show that host infection history and the ability to regulate dormant pathogens are likely to play a key role in our understanding of how the immune system responds to both acute and chronic exercise. The second article by Heaney et al. defines reference ranges and responses of salivary immunoglobulin free light chains to exercise. The next article by He et al. represents a comprehensive overview about the role of vitamin D in exercise immunology.

The chronic disease section starts with an article by Perandini et al. who describe the effects of acute exercise on leukocyte inflammatory gene expression in patients with systemic lupus erythematosus. The second article by LaVoy et al. addresses the topic of exercise effects on cancer-related fatigue in cancer survivors. Perry et al. included original data in a review article about the relation between muscle inflammation and atrophy related signaling pathways in type II diabetes. Finally, James Markworth and David Cameron-Smith discuss the roles of lipid mediators in mediating immunological and adaptive skeletal muscle responses to muscle injury and exercise.

This year's issue of EIR appears with the support of the new Associate Editors Rickie Simpson and Neil Walsh, who closely supported Jonathan Peake and me. Unfortunately, Mike Gleeson left the team. Thank you, Mike, for your strong and close support of EIR, specifically for supporting me during the early times of my editorial work.

For EIR23 and the future, we want most contributions to be topical review articles. In the case of original research articles, we encourage the authors to embed their new data into review articles. Please note that the submission deadline for EIR23 is 31st July 2016. We hope you enjoy reading the new issue. Similar to EIR21, this issue will only be published online.

Thank you, Rickie Simpson, Neil Walsh and Jonathan Peake, for the close and friendly teamwork.

Thank you (all ISEI members), and all members of the Editorial Board for the confidence you have placed in us.

Thank you all for your ongoing support of EIR.
A special thanks to all the authors of EIR22.

On behalf of the Editors,
Karsten Krüger

Human cytomegalovirus infection and the immune response to exercise

Richard J. Simpson[1], Austin B. Bigley[1], Guillaume Spielmann[1,2], Emily C.P. LaVoy[1], Hawley Kunz[1] and Catherine M. Bollard[3]

[1] Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, 3855 Holman Street, Houston, Texas, 77204, USA.
[2] School of Kinesiology, Louisiana State University, 112 Long Fieldhouse, Baton Rouge, Louisiana 70803, USA.
[3] Program for Cell Enhancement and Technologies for Immunotherapy, Children's National Health System and The George Washington University, Washington D.C., USA

ABSTRACT

Human cytomegalovirus (HCMV) is a ubiquitous β-herpes virus that has co-evolved with its host since the very beginning of human life. The vast majority of adults worldwide carry the virus in a latent state, which has striking effects on the composition and function of both T-cells and NK-cells. While there is evidence to suggest that prior exposure to HCMV can have beneficial effects in the immune competent host, poor control of the virus may contribute to T-cell exhaustion and the early onset of immunosenescence. The interaction between HCMV and exercise has garnered a lot of recent research attention. This stemmed from observations that people with HCMV redeploy greater numbers of CD8+ T-cells in response to a single exercise bout, while NK-cell mobilization is, conversely, impaired. Moreover, athletes with latent HCMV infection may be better protected against symptoms of upper respiratory illness (URI), and it has been suggested that the host's ability to control HCMV (i.e. keeping HCMV in a latent state) may connect apparent bidirectional effects of exercise volume on host immunity and infection risk. This work has set a new paradigm that immune responses to both acute and chronic exercise might be governed by the infection history of the host. In this review, we summarize current knowledge on the effects of HCMV infection on T-cells and NK-cells and synthesize the literature on HCMV and the immune response to both single exercise bouts and prolonged periods of exercise training. We also discuss potential clinical and practical applications of this work including the use of HCMV reactivation as a biomarker of immune depression in athletes, its relevance in immunosenescence and the associated immune risk profile, and the potential for exercise to augment vaccine responses and the manufacture of immune cells for adoptive transfer immunotherapy. Although research in this area is still in its infancy, we conclude that host infection history and the ability to regulate dormant pathogens is likely to play a key role in our understanding of how the immune system responds to both acute and chronic exercise across the entire exercise volume continuum.

Keywords: Immunosenescence, T-cell, NK-cell, acute stress response; vaccination; adoptive transfer immunotherapy; athletes

Frequently Used Abbreviations
AML: acute myeloid leukemia
β_2-AR: β_2-adrenergic receptor
CCR: chemokine receptor
CD: cluster of differentiation
CM: central memory
EBV: Epstein-Barr virus
EM: effector memory
EMRA: CD45RA+ effector memory
HCMV: human cytomegalovirus
HLA: human leukocyte antigen
HNP: human neutrophil protein
HSCT: hematopoietic stem cell transplantation
HSV: herpes simplex virus
IE-1: immediate early antigen 1
Ig: Immunoglubulin
KIR: killer inhibitor receptor
KLRG1: killer-lectin like receptor G1
MHC: major histocompatibility complex
NKCA: natural Killer cell cytotoxic Activity
NKG: natural killer group
PD: programed death
pp65: phosphoprotein 65
TCR: T-cell receptor
URI: upper respiratory illness

Corresponding Author
Dr Richard J. Simpson BSc PhD FACSM
Associate Professor in Exercise Physiology/Immunology
Laboratory of Integrated Physiology
Department of Health and Human Performance
University of Houston, 3855 Holman Street, Houston, Texas, USA 77204
Phone: (713) 743-9270, Fax: (713) 743-9860
Email: rjsimpson@uh.edu

INTRODUCTION

Human cytomegalovirus (HCMV), also known as human herpes-virus 5 (HHV-5), is a ubiquitous β-herpes virus that infects 40-70% of the adult population in the United States (10) and 45-100% of adults worldwide depending on age, ethnicity and geographical location (30). The virus is mostly transmitted through direct contact with bodily fluids such as saliva, urine or breast milk. It can also be contracted through sexual intercourse, organ transplantation and blood transfusions. Primary infection usually occurs asymptomatically before the virus establishes latency and persists for the lifetime of the host. Compared to other herpesviruses, HCMV has a very large and divergent genome which allows it to evade immune detection (152) and appears to persist in monocytes and CD34+ myeloid progenitor cells (129). While often dismissed as an innocuous infection in otherwise healthy people, HCMV is a major cause of morbidity and mortality in immunocompromised patients and is one of the leading causes of death after solid organ and hematopoietic stem cell transplantation (HSCT) (55, 129). The virus is also capable of periodic reactivation causing large-scale expansions of cytotoxic T-cells and NK-cells that seem to linger long after the infection has been curtailed. Consequently, and regardless of age, people with a latent HCMV infection have substantially increased numbers and proportions of CD8+ (and to some extent CD4+) T-cells with a highly differentiated phenotype; and more recently, it has been shown that latent HCMV infection markedly increases the proportion of NK-cells expressing the activating receptor, NKG2C (82). HCMV has also been implicated in *immunosenescence,* the term used to describe the progressive demise of the immune system that is associated with aging, and has been linked with weakened vaccine responses in both young and older subjects (85, 140).

The effects of HCMV on the phenotype and functional properties of T-cells and NK-cells has featured heavily in mainstream immunology literature over the last two decades. This is largely due to the striking ability of HCMV to alter the composition of T-cell and NK-cell subsets in peripheral blood, its propensity for reactivation due to physical and psychological stress, and its implied role as a 'driver' of immunosenescence (64). However, the interaction between HCMV and exercise has only recently been investigated (123). This interest stemmed from two sources: firstly, the observation that people with HCMV redeploy greater numbers of CD8+ T-cells in response to a single exercise bout, due mostly to the ability of HCMV to expand 'exercise-responsive' subsets of memory T-cells (139); and secondly, because regular exercise is associated with better immune responses in the elderly, it has been suggested that exercise interventions aimed at improving immunity may act on HCMV-dependent pathways of immunosenescence (127, 128). Thus, the impact of HCMV on the immune system has set a new paradigm that the immune response to both acute and chronic exercise may largely depend on the infection history of the host. In this review, we summarize current knowledge on the effects of HCMV infection on T-cells and NK-cells and synthesize the literature on HCMV and the immune response to both acute and chronic exercise. Finally, we discuss potential clinical and practical ramifications for this work in the context of immunotherapy, biomarker profiling of athletes, vaccination and *immunosenescence.*

HCMV and T-cells

Primary HCMV infection is characterized by an intense viral replication and a profound T-cell response that may last for several months (5), with both CD8+ cytotoxic and CD4+ helper T-cells playing central roles in the resolution of acute primary infection and the maintenance of long-term memory during viral persistence. HCMV-specific cytotoxicity is predominantly performed by CD8+ T-cells, although HCMV-specific CD4+ T-cells also have the ability to lyse infected target cells as well as maintain the upkeep of the CD8+ T-cell population (7, 135). It is clear that HCMV has a colossal impact on the memory T-cell pool, with ~10% of all memory CD4+ and CD8+ T-cells in blood being specific to HCMV proteins in individuals with even a latent infection (135). The proportion of the peripheral T-cell compartment devoted to the control of HCMV is highly variable, reported to range from <1% to over 40% in HCMV seropositive individuals (135, 145). The high variability is likely due to a number of factors including the size of the viral inoculum, the length of time the infection has been carried, and the immunocompetence of the host while infected (8).

Adequate HCMV control requires large numbers of differentiated T-cells with an effector memory (EM; $CD45RA^{neg}/CD62L^{neg}/CCR7^{neg}$) or effector memory RA^{pos} (EMRA; $CD45RA^{pos}/CD62L^{neg}/CCR7^{neg}$) phenotype, with the proportions of these cell subsets within both CD8+ and CD4+ blood T-cells remaining elevated long after primary infection. Increased proportions of EM and EMRA subsets among CD8+ T-cells have long been considered a hallmark of aging, although it is not uncommon for children or young adults infected with HCMV to have EM and EMRA proportions that are comparable or even greater than non-infected adults 25-50 years their senior (130, 131). While cells with this phenotype were long considered to be 'terminally-differentiated' or 'senescent', this does not apply, at least to a portion of HCMV-specific T-cells that are still able to secrete cytokines, proliferate in response to HCMV peptide stimulation, kill HCMV-infected target cells, and control viral dissemination *in vitro* (62, 143, 159). Moreover, even after massive clonal expansion, many EMRA cells can revert from a CD45RA to a CD45RO phenotype and re-express CCR7 (143), which challenges previous models of T-cell memory that surmise a unidirectional linear differentiation pathway (6, 115) and fail to accurately reflect the inherent plasticity of memory T-cell responses (129).

While many EMRA cells are specific for antigenic epitopes in the pp65 and IE-1 HCMV proteins, T-cells specific to other HCMV antigens including US3, pp71, UL28 and IE-2 are also mostly comprised of EMRA cells, indicating that HCMV-specific T-cells have a similar composition of T-cell subsets regardless of their antigen specificity (62). Moreover, it appears that HCMV-specific T-cell responses are highly varied among individuals, with some people mounting a more diverse response and others a more restricted and focused response to fewer HCMV proteins and antigenic epitopes (62). While the antigenic diversity of HCMV-specific T-cells

appears unrelated to age, older individuals tend to have a greater number of cells responding to HCMV proteins, and both the magnitude and diversity of these responses are stable over time (at least up to 2-years) (62). Nevertheless, it is still likely that HCMV, particularly if improperly controlled as a result of chronic immune depression or dysregulation, can drive T-cells to clonal and functional exhaustion (44). Telomere length is considered a robust measure of T-cell clonal capacity, with shorter telomeres indicative of excess T-cell proliferation and the impending end of the clonal lifespan (replicative senescence). While HCMV does not appear to impact T-cell telomere length in the young, average telomere lengths among isolated CD8+ T-cells were found to be shorter in HCMV-infected older individuals compared to their age-matched non-infected counterparts (142), indicating that HCMV may drive replicative senescence in some individuals. Riddell et al. measured telomere length in HCMV (NLV)-specific CD8+ T-cells contrasted by age and surface phenotype (CD45RA/CD27 combinations) and found that the NLV-specific cells from older subjects had shorter telomeres than the young across all surface phenotypes (111). Interestingly, they also reported that telomere length was shortest in the CD45RA+/CD27+ (normally considered to be a 'naïve' or 'low differentiated' phenotype) CD8+ subset, indicating that these cells had undergone excessive rounds of cell division *in vivo* and are certainly not naïve cells (111). Truly 'exhausted' T-cells that fail to undergo further proliferation or secrete cytokines in response to stimulation are likely to be phenotypically identical to those fully functional viral-specific cells displaying surface markers of high-differentiation (i.e. KLRG1, CD57). Thus, a simple identification of T-cell subset distribution through surface markers is insufficient to assess immunocompetence, but is still likely to provide a representative footprint of the host's infection history. At least in healthy individuals, aging (>65 yrs) does not appear to be associated with exhausted HCMV-specific CD8+ T-cells (121), and increased proportions of truly 'exhausted' or 'senescent' HCMV-specific T-cells might only manifest in the very old following a lifetime of poor HCMV control (96), after primary infection (5), or perhaps following a period of intense HCMV reactivation. Although apparently exhausted HCMV-specific T-cells have been found to express PD-1, blocking the receptor can restore their pro-inflammatory cytokine profile and antigen-specific proliferative responses suggesting that PD-1 associated exhaustion is reversible (5, 40). Nevertheless, in contrast to HIV-specific CD8+ T-cells, PD-1 expression is very low, or even absent, on HCMV-specific CD8+ T-cells (103, 138), indicating that HCMV does not induce clonal exhaustion in most immunocompetent people.

Recently, more attention has been paid to the impact of HCMV on the frequency and function of γδ T cells, which predominantly reside in the gut mucosa. Unlike their αβ T-cell counterparts, γδ T cells are present in the blood in relatively small numbers and, despite expressing the pan T-cell marker CD3, are mostly negative for both CD4 and CD8 (105). The long-term persistence of the $V\delta2^{neg}$ cell population in peripheral blood has become a hallmark feature of HCMV infection (105) and, although these are not HCMV-specific cells by strict definition, they are still capable of killing HCMV-infected fibroblasts through upregulation of endothelial protein C receptor (EPCR) and ICAM-1 on the stressed target cells (158). Aging is usually associated with a marked reduction in the frequency of γδ T cells in peripheral blood regardless of whether or not the host is carrying a latent HCMV infection (104).

HCMV and NK-cells

NK-cells express a range of inhibitory and activating surface receptors that tightly regulate their cytotoxic functions. These include killer-cell immunoglobulin-like receptors (KIR) that, despite having much less polymorphism than the TCR, are able to deliver inhibitory and/or activating signals to the NK-cell via human leukocyte antigens (HLA) expressed on healthy host cells and transformed/target cells (70). NK-cells play a crucial role in curtailing HCMV and other viral infections in immunocompetent individuals through expression of a series of activating receptors (e.g. NKG2D, DNAM-1, and NKp46) that allow them to recognize and eliminate HCMV and other virus-infected cells (33, 83). This immune selection pressure has resulted in HCMV acquiring many immune evasive strategies to avoid detection by host NK-cells (69, 81). For instance, HCMV can control the expression of several genes that code for ligands of NK-cell inhibitory and activating receptors in a manner that avoids detection and elimination (13, 83, 153). The virus has also been shown to induce expression of HLA homologues, which ligate with NK-cell inhibitory receptors to prevent destruction of HCMV-infected cells (12, 108). NK-cells have also acquired strategies of their own to override the immune evasive properties of HCMV. A striking example of this is the HLA-E-dependent expansion of $NKG2C^{pos}/NKG2A^{neg}$ NK-cells (112). Both receptors ligate with the (non-classic) class 1b MHC molecule HLA-E (141) that is upregulated in HCMV-infected cells (137), tumor cell lines of major lymphoid and nonlymphoid lineages (80), and primary acute myeloid leukemia and multiple myeloma cells (94, 155). Signaling through the inhibitory receptor NKG2A is dominant, thus only $NKG2C^{pos}/NKG2A^{neg}$ NK-cells are able to effectively lyse HLA-E-expressing target cells (71) . These NKG2Cpos/NKG2Aneg NK-cells are often referred to as "memory NK-cells", because their frequency remains elevated after resolution of HCMV viremia (15, 82) and NK-cells expressing Ly49H (the mouse equivalent of NKG2C) have been shown to mount apparent recall responses to HCMV (134). Whether or not these are truly 'memory' responses is subject to debate as these mechanisms are not nearly as precise as TCR/MHC/peptide interactions (136). However, it is clear that HCMV leaves a stable 'imprint' on the NK-cell KIR repertoire that allows the host to maintain long lasting HCMV control in a manner that goes well beyond typical features of innate immunity (129, 136). The clonal-like proliferation of $NKG2C^{pos}/NKG2A^{neg}$ NK-cells in those with HCMV results in high expression of the putative terminal differentiation marker CD57 and chronic skewing of the KIR repertoire, as only licensed (i.e. cells expressing inhibitory KIR for self-HLA molecules) NK-cells proliferate (15, 32, 46). The preferential expansion of $NKG2C^{pos}$ NK-cells observed with HCMV infection is unique amongst the Herpesvirus family and has not been reported in response to any other viruses (22, 27, 102).

$NKG2C^{pos}$ NK-cells are not merely an artifact of HCMV infection as they also serve a protective function. For exam-

ple, a higher percentage of NKG2C^{pos} NK-cells is associated with a lower risk of acute HCMV infection in patients undergoing solid organ transplantation or HSCT (54, 63). Further, NKG2C^{pos} NK-cells taken from HCMV-infected donors show enhanced expansion and function in response to HCMV reactivation in HSCT recipients when compared to NKG2C^{pos} NK-cells derived from HCMV^{neg} donors (45, 54). Thus, it seems clear that NKG2C^{pos} NK-cells play a critical role in the suppression of acute HCMV infection and achievement of long-term viral control. The functional benefits of NKG2C^{pos} NK-cells, however, go beyond their ability to contain HCMV. For example, NKG2C^{pos} NK-cells with high cytotoxicity have been observed to expand in response to active Hantavirus, Chikungunya, HIV, and Hepatitis B infections, but only in individuals previously infected with HCMV (22, 27, 102). Thus, it is suggested that HCMV infection "primes" NKG2C+ NK-cells to respond to other active viral infections, some of which are also associated with upregulation of HLA-E in infected cells (22). Beyond viral immunity, we have shown in healthy subjects that latent HCMV infection is associated with increased NK-cell cytotoxic activity (NKCA) against multiple myeloma, leukemia, and lymphoma cell lines expressing HLA-E (18), and HCMV reactivation during allogeneic HSCT has been associated with strikingly lower occurrences of relapse in acute myeloid leukemia (AML) patients (9% in patients with HCMV reactivation vs. 42% in those without) (11, 43). This is due, in part, to the HCMV-induced expansion of NKG2C^{pos}/NKG2A^{neg} NK-cells (46) which are able to effectively lyse HLA-E^{pos} targets, including AML blasts (94) and several other 'liquid' cancers (80). However, it has also been reported that HCMV associated NKG2C^{pos} NK-cell expansions may be involved in the development of *de novo* head/neck and colorectal cancers in liver transplant patients (1), indicating that HCMV-induced expansion of highly cytotoxic NK-cell subsets could be a double edged sword that needs to be tightly controlled.

HCMV and Immunosenescence

For well over a decade, HCMV infection was purported to 'drive' immunosenescence (98, 99). This was largely due to the finding that HCMV serostatus predicted mortality over 2, 4 and 6 years in Swedish octogenarian and nonagenarian subjects (100). The impact of HCMV was an accumulation of the so-called senescent T-cells, an excess numbers of HCMV-specific T-cell clones, an inverted CD4:CD8 T-cell ratio, and a lower proportion of naïve cells. These parameters formed the 'immune risk profile', which predicted mortality and morbidity in several cohorts of very old subjects (157). Excess HCMV-specific T-cell clones were considered to signify restricted T-cell diversity and a polarization of the memory T-cell response to a single virus, whereas low numbers and proportions of naïve cells were thought to compromise immune responses to novel infectious agents and vaccine antigens. In this regard, HCMV was believed to take up a large portion of the 'immunological space' required by the host to mount efficient memory responses to novel infectious and vaccine antigens (28). However, it has since been established that HCMV actually has very little impact on the naïve T-cell compartment, but rather increases the pool of memory T-cells with a differentiated phenotype without affecting naïve T-cell numbers (87, 144, 146, 154). Thus, at least in younger, healthy individuals, there is little evidence that the so-called immunological space is fixed, particularly if thymic output and homeostatic proliferation remains functional and continues to maintain the diversity of the peripheral T-cell pool.

In contrast to the Swedish octogenarian and nonagenarian studies, a recent study completed in an elderly Dutch population found that lower frequencies of naïve and higher frequencies of late differentiated cells among the total peripheral CD8+ T-cell pool was actually associated with *increased* survival at 7-years follow up (39). Moreover, while weaker immune responses to vaccines (i.e. influenza vaccine) have been attributed to HCMV serostatus (85, 140), this has not been consistently reported (36, 149). Indeed, it was reported recently that HCMV infection *enhances* influenza vaccine responses in young adults (48). These equivocal reports, coupled with the finding that HCMV-specific T-cells with an apparent 'senescent' phenotype remain highly functional in most people, casts a great deal of dubiety on the HCMV and immunosenescence paradigm. In this regard, HCMV may actually be a 'passenger' rather than a 'driver' of immunosenescence and, at least in some cases, carrying HCMV might even be beneficial (116). Notwithstanding, the ability to control HCMV decreases with age (133), which is likely to cause large-scale T-cell clonal expansions that lead to immune 'exhaustion', inefficient vaccine responses and an immune senescent profile. It will be important for future studies to determine the impact of HCMV control, and not just serostatus, on immunosenescence. Measuring IgG antibody titers in serum is considered by some to be a crude measure that merely indicates prior exposure, and provides little information on viral load or host HCMV control over time (79). Indeed, a recent study of community dwelling elderly reported no change in HCMV antibody titers over a 12-year period (79). The presence of HCMV DNA in blood monocytes has been used as a marker of poor HCMV control, with approximately 56% of elderly individuals having HCMV DNA positive monocytes (78, 79). Moreover, a positive relationship exists between HCMV DNA+ monocytes, the numbers of HCMVpp65-specific CD8+ T-cells (78, 79), and serum neopterin (a marker of monocyte/macrophage activation) levels (77), independently of serum IgG antibody titers. Poor HCMV control might also explain the apparently weaker vaccine responses that have been reported in those with a positive IgG titer, but this has yet to be determined. A major criticism of the HCMV immunosenescence paradigm is the centric focus on the T-cell compartment, and there is a clear need to explore the relationship between HCMV and immune aging as it pertains to other lymphocytes affected by the virus, including NK-cells, B-cells and γδ T cells (8). This will be important to determine whether or not persistent HCMV infection can be considered beneficial or detrimental to immunity over the natural course of aging.

Blood Lymphocytes and Acute Exercise

Blood lymphocyte numbers increase dramatically upon engaging in a single bout of acute dynamic exercise. This exercise-induced lymphocytosis is almost instantaneous, with the mobilized lymphocytes consisting mostly of NK-cells, followed by CD8+ T-cells, CD4+ T-cells, B-cells, and lastly γδ T cells. However, when compared to lymphocyte numbers

in resting blood, the relative change in the absolute number of γδ T cells is greater than those of CD8+ T-cells but still less than NK-cells (3). Upon cessation of exercise, there is a rapid lymphocytopenia that occurs within 30-60-minutes and may persist for up to 6-24h later depending on the intensity and duration of the bout. For a more detailed overview on the lymphocyte response to acute exercise and the underpinning mechanisms, we direct the reader to the most recent ISEI position statements (150, 151). Although this response is very well characterized, the influence of infection history on the redeployment of lymphocytes and other leukocyte subtypes to single exercise bouts has only recently been investigated.

Acute Exercise Preferentially Redeploys 'Mature' subsets of T-cells and NK-cells

Within the CD8+ T-cell compartment, acute dynamic exercise has consistently been shown to evoke a preferential redeployment of antigen-specific T-cells with a differentiated effector memory phenotype (29, 124-126, 139). Cells with an EMRA phenotype are redeployed in relatively greater numbers than EM cells, followed by CM and lastly naïve cells (29). Even when other phenotypic identifiers of T-cell differentiation are used (i.e. CD27/CD28 or KLRG1/CD28 combinations), a preferential mobilization of the most differentiated, or 'late' (CD27-/CD28-; KLRG1+/CD28-) cells is evident followed by the 'intermediate' (CD27-/CD28+; KLRG1+/CD28+) cells and lastly the cells with an 'early' (CD27+/CD28+; KLRG1-/CD28+) differentiated phenotype (124, 139).

Our early interpretations of this work intimated that acute exercise preferentially redeployed 'senescent' CD8+ T-cells (124-126). This was based on prior observations that CD8+ T-cells expressing KLRG1 and/or CD57 lacked proliferative capabilities and that HCMV-specific CD8+ T-cells predominantly bore this phenotype (59, 96, 147). However, just as blocking PD-1 can restore cytokine secretion, blocking KLRG1 can restore T-cell proliferation indicating that KLRG1 is an inhibitor of T-cell clonal expansion but not a marker of replicative senescence per se (57). So although some cells bearing this phenotype may in fact be senescent, the term itself is a misnomer due to diversity of cell types, both functional and dysfunctional, that may express these surface markers. There are also other indicators that exercise mobilizes highly functional cells despite large proportions of the mobilized cells having a so-called senescent phenotype. We found that the average telomere length among isolated CD8+ T-cells was actually longer among the post-exercise cells (124), and that CD8+ T-cells in blood immediately post-exercise were still capable of secreting a wide array of cytokines following mitogen stimulation, even if bearing a $CD27^{neg}$ differentiated phenotype (75). Thus, the contribution of truly senescent or exhausted cells to the preferential mobilization of highly differentiated CD8+ T-cells with exercise and the physiological significance of such a response remains to be determined.

We recently reported that the redeployment of NK-cells with exercise is non-uniform, and like CD8+ T-cells, there is preferential mobilization of the most differentiated NK-cell subsets (17). NK-cell differentiation is defined by acquisition of inhibitory KIR expression followed by loss of the inhibitory receptor NKG2A (14) and gain of the "terminal differentiation" marker CD57 (23). As NK-cells differentiate, they lose the ability to proliferate and express IFN-γ in response to pro-inflammatory cytokines, while their capacity to kill a wide range of target cells increases (14, 23). In response to 30-minutes of steady state cycling exercise, NK-cell subsets were redeployed in a stepwise manner in accordance with differentiation status [highly-differentiated ($KIR^{pos}/NKG2A^{neg}/CD57^{pos}$) > medium-differentiated ($KIR^{pos}/NKG2A^{pos}/CD57^{neg}$) > low-differentiated (KIRneg-/NKG2Apos/CD57neg)] and the effect was consistent across multiple exercise intensities ranging from -5% to +15% of the individual blood lactate threshold (17). However, $NKG2C^{pos}$ NK-cells, despite having potent NKCA against specific target cells expressing non-classical HLA-E, are redeployed with exercise in relatively few numbers regardless of differentiation status. This causes the proportion of $NKG2C^{pos}$ NK-cells in blood to increase during exercise recovery due to a preferential egress of NK-cells lacking this receptor (17). In other words, as NK-cells are redeployed from the blood to the tissues during exercise recovery, most of the $NKG2C^{pos}$ cells are 'left behind'. While confirming previous reports that exercise did not affect NKCA against the K562 leukemic target cell line on a per NK-cell basis (88, 92, 95) we showed that NKCA per cell was markedly elevated against the HLA-E transfected 221.AEH lymphoma cell line and multiple myeloma target cells expressing classical HLA-C (U266 and RPMI-8226) during the recovery phase of exercise (+1h after exercise cessation) (17, 18). This 1h post-exercise increase in NKCA per cell was positively associated with the proportions of NK-cells expressing the activating receptor NKG2C, and lacking inhibitory KIR for classical HLA molecules (CD158b) (17). Although it is often suggested that exercise evokes a redeployment of the most cytotoxic lymphocyte subtypes, it is somewhat of a conundrum why $NKG2C^{pos}$ NK-cells, given their potent cytotoxic effector functions against target cells expressing non-classical HLA-E, are not preferentially redeployed with exercise as well. This appears to be due to their lower expression of the β2-adrenergic receptor (β_2-AR) and insensitivity to synthetic catecholamine stimulation compared to NK-cells lacking NKG2C (18). Taken together, these findings indicate that the effects of acute exercise on NK-cell function are strongly influenced by proportional shifts in NK-cell subsets and target cell expression of classical and non-classical HLA receptors.

HCMV Infection and Acute Exercise

HCMV Infection and the T-cell Response to Acute Exercise

The preferential mobilization of EM and EMRA cells with exercise led to the hypothesis that HCMV carriers, as a result of having greater numbers and proportions of these cell types in resting blood, would display an amplified mobilization of highly differentiated T-cells with exercise. Turner et al. were the first to show that 60-minutes of treadmill running exercise evoked a mobilization and egress of total CD8+ and EMRA CD8+ T-cells that was ~2 and ~6 times greater in those with a latent HCMV infection compared to non-infected participants (139). This amplified effect of HCMV on the redeployment of CD8+ T-cells to exercise has also been reported by our group, with the high-differentiated CD8+ T-cell subsets (KLRG1+/CD28-) accounting for the vast majority of the

HCMV effect (72, 130). Although individuals with HCMV carry other herpesvirus infections, Epstein-Barr virus (EBV) and herpes simplex virus-1 (HSV-1) serostatus does not appear to confound or alter the magnitude of the HCMV effect (16, 72). Interestingly, while aging is associated with an impaired ability to redeploy T-cells in response to a single bout of exercise (31, 84), we found that this only applies to older individuals who do not carry HCMV. On the other hand, older HCMV infected individuals mobilized CD8+ T-cells similarly to young HCMV-infected participants and almost twice as much as the non-infected young, again with the highly-differentiated subsets accounting for the effect (130). However, the redeployment (ingress and egress) of naïve/early-differentiated cells with acute exercise appears to be impaired with aging regardless of HCMV serostatus (130). HCMV infection also increases the mobilization of highly-differentiated CD4+ T-cells with exercise, although the magnitude of this response is not sufficient to amplify the redeployment of total CD4+ T-cells (72, 130, 139). It is important to note that although HCMV is associated with an amplified redeployment of differentiated T-cells, it is likely that the effects are mostly due to the unique ability of the virus to alter the composition of the peripheral T-cell pool in favour of the more exercise-responsive subtypes (72, 76). Because HSV-1 and EBV do not expand the EM and EMRA T-cell subsets in blood (37), this probably explains why these viruses are not associated with an amplified exercise redeployment of total CD8+ T-cells independently of HCMV (16, 72). It is likely, however, that other persistent viral infections that are able to expand the proportions of highly-differentiated T-cell subsets, such as Hepatitis C (6), will also contribute to an augmented redeployment of CD8+ T-cells in response to acute stress and exercise regardless of whether or not a HCMV co-infection is present. Indeed, well-treated HIV-infected men show a greater redeployment of highly differentiated CD8+ T-cells in response to acute maximal exercise compared to healthy controls (41), although it is not known if this effect is due to a HCMV co-infection as those with HIV tend to harbor HCMV also (68).

We showed that a single bout of exercise increases the number of HCMV-specific T-cells in peripheral blood 2-5-fold (130). This, in conjunction with a previous observation that the number of HCMV-specific T-cells increased after an acute psychological stress task (9), indicates that HCMV-specific T-cells are redeployed with exercise under the influence of catecholamines and β-adrenergic receptors. Phenotypic analysis of CD8+ T-cells specific to an NLV epitope of the HCMVpp65 antigen using an HLA-A2-restricted MHC class I Pentamer revealed that ~25% of resting HCMV-specific cells had a high differentiated (KLRG1+/CD28-) phenotype, increasing to ~49% immediately post exercise (130). We also found that the number of cells responding to HCMVpp65 and HCMV IE-I peptides increased dramatically post-exercise, and that the mobilized cells had broad HCMV antigen epitope specificity. In a more recent study, we stimulated a fixed number of PBMCs before and after 30-minutes of steady state cycling exercise (at +15% of the individual blood lactate threshold) with synthetic 15mer peptides specific for 4 antigens derived from HCMV (pp65 and IE-1) and EBV (LMP-2 and BMLF-1) in the presence of growth cytokines (IL-4, IL-7, IL-15) for 8-days (Spielmann et al. Unpublished). While the expansion protocol elicited, on average, a 2.1 to 13.5-fold increase in the number of HCMV and EBV specific cells (enumerated using an IFN-γ ELISPOT assay) generated from resting PBMCs, the number of viral-specific cells present in the post exercise cultures were strikingly greater; on average, up to ~4.7 and ~70.4 times greater, for HCMV and EBV-specific T-cells, respectively (Spielmann et al. Unpublished). The number of viral-specific cells generated after 8-days of cell culture relative to the input number of T-cells and viral-specific T-cells was still greater after exercise, indicating that the exercise effect is not merely due to greater numbers of viral-specific cells among the cells stimulated with viral peptides at day 0. Moreover, the viral-specific T-cell lines expanded after exercise maintained their ability to kill autologous peptide-pulsed target cells in an MHC dependent manner, and while there was a greater proportion of EM and EMRA cells among the post-exercise T-cells at day 0, the composition of T-cell subsets did not differ between the before and after exercise cell cultures at day 8 (Spielmann et al. Unpublished). Taken together, these findings indicate that latent HCMV infection not only amplifies CD8+ T-cell redeployment in response to exercise, but that many of the mobilized cells are specific to HCMV antigens and, despite the majority of the mobilized cells having a late-differentiated phenotype, they appear to be highly functional with broad epitope diversity and the capacity for massive clonal expansion in response to peptide stimulation *in vitro* (130).

Latent HCMV infection also amplifies the redeployment of γδ T cells to a single bout of exercise in healthy young adults and, like CD8+ T-cells, the interaction effect between HCMV and exercise is independent of co-infections such as EBV, HSV-1 and parvovirus-B19 (104). Aging, however, was associated with an impaired redeployment of γδ T cells regardless of HCMV serostatus, indicating that latent HCMV infection helps maintain robust exercise-induced redeployment of CD8+ αβ T-cells but not γδ T cells with aging (104). This is likely due to the differential effects of age and HCMV infection on the γδ T cell compartment (113). Although we did not look at the relative exercise response of γδ T cell subsets (104), we expect HCMV to augment the mobilization of the $V\delta2^{neg}$ subset, given that they are overexpressed in those with HCMV and predominantly consist of the exercise-responsive EMRA phenotype (2, 4, 105, 113). It is not known, however, if γδ T cell function is affected by exercise and future studies should determine if exercise alters their proliferative responses to phosphoantigens (i.e. zoledronic acid), which are typically used to expand γδ T cells *in vitro* for immunotherapeutic purposes (61).

HCMV Infection and the NK-cell Response to Acute Exercise

Turner et al. reported that CD8+ T-cell redeployment was amplified in those with HCMV, yet total lymphocyte redeployment was unaffected (139). This provided indirect evidence that the redeployment of other lymphocyte subtypes to exercise might actually be impaired in people with HCMV. We tested this hypothesis by examining the effects of latent HCMV infection on the redeployment of NK-cells and their subtypes, and found that HCMV serostatus was associated with a strikingly impaired redeployment of NK-cells (16)

(Bigley et al., 2012). The relative blunting effect of HCMV infection on the exercise-induced redeployment of NK-cells was actually larger in magnitude than its augmenting effect on CD8+ T-cell redeployment (illustrated in Figure 1). Although we are the only group so far to report that HCMV infection inhibits NK-cell redeployment in response to acute exercise, we have found this consistently in three separate subject cohorts and shown that the effect is independent of both age and baseline blood NK-cell numbers (16, 18, 21). Moreover, HCMV only impairs the NK-cell response to exercise at intensities above the blood lactate threshold (18), the point at which blood lactate and catecholamine concentrations increase above pre-exercise levels (106). As HCMV did not affect the lactate or catecholamine response to exercise, this indicated that HCMV might be associated with decreased NK-cell catecholamine sensitivity and/or β_2-AR activity (18). To test this hypothesis, we compared NK-cell β_2-AR expression and cyclic AMP production in response to *in vitro* isoproterenol (non-preferential synthetic β-agonist) stimulation between HCMV seronegative and seropositive subjects (18). Those with HCMV had a lower expression of the β^2-AR and an impaired cyclic AMP response compared to non-infected subjects. Moreover, cyclic AMP production was inversely correlated with the proportion of NKG2C^{pos}/CD57^{pos} cells within the isolated NK-cells (18). This, in conjunction with the observation that NKG2C^{pos}/CD57^{pos} NK-cells are not preferentially mobilized with exercise, indicates that it is the accumulation of catecholamine insensitive NKG2C^{pos}/CD57^{pos} NK-cells (a cell population that is practically absent in those without HCMV) that links HCMV infection with impaired NK-cell redeployment to exercise.

HCMV also affects NKCA both at rest and in response to exercise. The increased proportions of NKG2C^{pos} NK-cells in

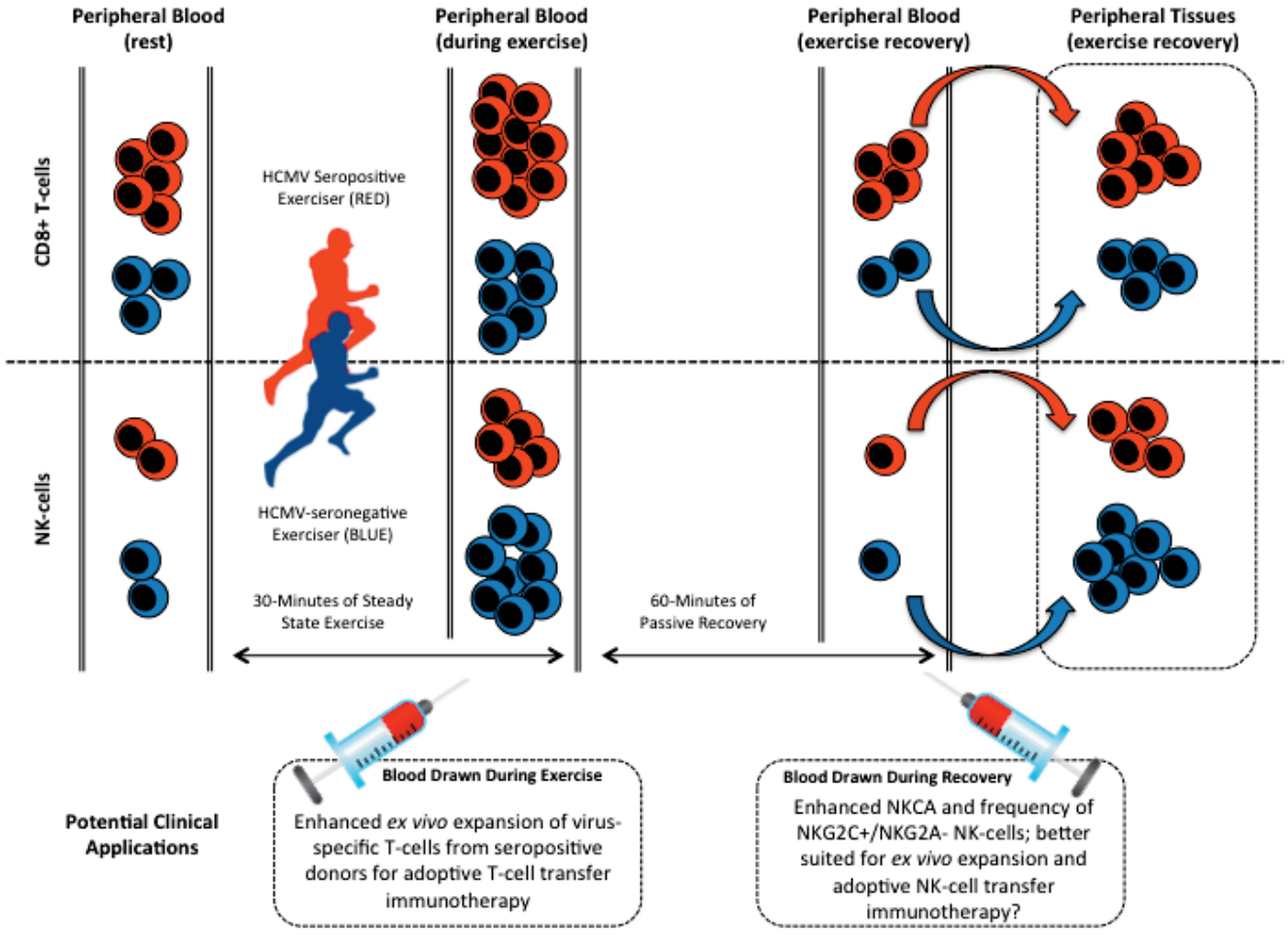


Figure 1. Divergent effects of HCMV infection on the exercise-induced redeployment of CD8+ T-cells and NK-cells and their potential clinical applications. HCMV seropositive participants mobilize 1.5-2.5 x more CD8+ T-cells than HCMV seronegative participants in response to fixed intensity steady state exercise, although the relative change in CD8+ T-cell number is similar between HCMV seropositive and seronegative participants (~2-fold increase in total CD8+ T-cells). HCMV increases the number and proportion of the catecholamine-sensitive EM and EMRA CD8+ subtypes, making the CD8+ T-cell population as a whole more responsive to exercise. The mobilized cells contain 'primed' virus-specific T-cells that may be obtained from the blood during exercise before they would normally egress the blood and infiltrate the peripheral tissues. These cells can then be used to augment the ex vivo expansion of multi-virus-specific T-cells for use in the allogeneic adoptive transfer immunotherapy setting. In contrast, HCMV seronegative participants mobilize 2-4 x more NK-cells than HCMV seropositive participants despite having similar resting NK-cell numbers. This appears to be due to a greater proportion of catecholamine-insensitive NKG2C+ NK-cells in people with HCMV. Although NK-cell cytotoxic activity (NKCA) does not change at the individual cell level during exercise, individual NKCA in blood is enhanced during exercise recovery. This is due to an increased proportion of NKG2C+/NKG2A- NK-cells in blood that are able to effectively recognize and kill HLA-E expressing tumor target cells. NK-cells with these phenotypic and functional properties might be better suited for ex vivo expansion and allogeneic adoptive NK-cell transfer.

those with HCMV enhances resting NKCA against a wide-range of tumor target cells *in vitro*, with the magnitude of the HCMV effect being positively associated with HLA-E expression on the target cells (18). However, when compared to baseline, only those without HCMV demonstrated increased NKCA per cell against the U266, RPMI-8226, and 221.AEH target cell lines 1h post-exercise. Thus, due to marked changes in the composition of NK-cell subsets, both acute exercise and HCMV are able to enhance NK-cell function, although the effects are not synergistic (18).

Divergent effects of HCMV on the Exercise-Induced Redeployment of T-cells and NK-cells

We have shown that latent HCMV infection has dichotomous effects on the redeployment of NK-cells and T-cells in response to a single exercise bout (Figure 1). While HCMV-specific CD8+ T-cells appear to be highly stress-responsive, the so-called HCMV-specific (NKG2C^{pos}) NK-cells respond poorly to catecholamines and are redeployed in comparatively fewer numbers with exercise. From a historical perspective, physical exertion was performed only when hunting, working or evading predators and is therefore considered to be an evolutionary conserved mechanism to 'prime' the immune system during situations when physical injury and infection are more likely to occur (38). As such, the current dogma is that exercise mobilizes lymphocytes and other leukocytes that have high tissue migration and effector functions as part of the 'flight or fight' response to facilitate immunosurveillance, promote wound healing and regenerate damaged tissue (24). It is perplexing, therefore, why NKG2C^{pos} NK-cells, given their importance in controlling viral infections such as HCMV and their ability to recognize and destroy malignant cells expressing HLA-E (e.g. multiple myeloma, AML) (94, 155), are 'left behind' in the bloodstream while other NK-cell subsets are redeployed to the tissues during exercise recovery. Interestingly, it is not just the exercise responses of NK-cells and T-cells that are divergent in those infected with HCMV, it is the immune response to HCMV itself.

Despite the well-established benefits of NKG2C^{pos} NK-cells to HCMV containment and overall immunity, the proportion of NKG2C^{pos} NK-cells is highly variable amongst HCMV-seropositive individuals (53). One likely explanation for this variation is that HCMV-specific NK-cell and T-cell responses are reciprocally related in subjects with good viral control (20), most likely due to the rheostat-like capacity of NK-cells to limit viral-specific T-cell responses (148). Thus, people with HCMV can contain the virus through an NK-cell or T-cell-mediated response, but not both. Considering the link between HCMV-driven T-cell responses and immunosenescence (116, 156), and the broad functionality of NKG2C^{pos} NK-cells (46), it is likely that an NK-cell-mediated response to HCMV would be preferable in most cases. However, due to the poor exercise responsiveness of NKG2C^{pos} NK-cells, it is precisely these subjects who drive the impaired NK-cell response to exercise in those with HCMV.

Does HCMV Infection Affect Other Immune Responses to Acute Exercise?

Although HCMV has profound effect on the composition and function of the blood lymphocyte compartment, neutrophils and monocytes are also highly exercise responsive and their redeployment with exercise might be affected by HCMV as well, particularly monocytes which are believed to harbor the virus during latency. However, we did not find HCMV serostatus to affect neutrophil or monocyte numbers following a 75-km cycling time trial (76). Moreover, neutrophil and monocyte phagocytosis and oxidative burst activity, as well as plasma levels of the cytokines IL-6, IL-8, IL-10 and TNF-α, and the lipid peroxidation marker F_2-isoprostanes, increased after the exercise bout but were not affected by HCMV serostatus (76). The concentration and/or secretion of salivary antimicrobial proteins (AMPs) such as salivary IgA, LL-37, HNP 1-3, lactoferrin, α-amylase and lysozyme are known to increase after a single bout of exercise (67). However, in a retrospective analysis of this cohort (67), in which ~53% of the participants were found to be HCMV seropositive, previous exposure to the virus had no impact on either the resting or the exercise-induced change in the concentration or secretion of these salivary AMPs (Kunz et al. unpublished). Thus, although the number of studies investigating the effects of infection history on other immune responses to exercise is small, it has so far only been shown that HCMV infection impacts the redeployment and function of T-cells and NK-cells in response to a single bout of exercise.

HCMV Infection and Chronic Exercise

Few studies have examined the impact of HCMV on immune responses to long-term exercise training. Participation in moderate-intensity regular exercise has been associated with a less-differentiated T-cell profile (132), while, conversely, habitual high volume exercise training has been associated with a more-differentiated T-cell profile and reduced thymic output (26, 107), which are hallmark features of immunosenescence. Thus, at least from the available cross-sectional data, it appears that moderate intensity exercise has anti-immunosenescence effects whereas high-volume exercise, such as the type practiced by highly competitive athletes, has mostly pro-immunosenescence effects. Although current empirical data is lacking, we postulate that viral control will be strongly linked to this bidirectional relationship between exercise volume and immunosenescence (Figure 2). Here we discuss the potential effects of high and low/moderate volume exercise training on the host's ability to control HCMV and other herpesviruses and how these might be linked to the apparent bidirectional effects of exercise volume on host infection risk.

High Volume Exercise Training and HCMV Control

Infection history is likely to have important implications for the immune system of athletes and other occupational personnel exposed to prolonged periods of high physical training loads. Indeed, Brown et al. reported that HCMV infection was more prevalent among female soccer players compared to age-matched controls (25, 26) despite training status being associated with fewer differentiated T-cells (26), while Moro-Garcia et al. (91) reported that older athletes with HCMV presented with lower numbers of CD4+ T-cells. Although measures of viral serology are becoming more common in exercise training investigations, studies that have examined the effects of high volume exercise training on latent viral reactivation in athletes are scarce, and none, to our knowledge, have focused

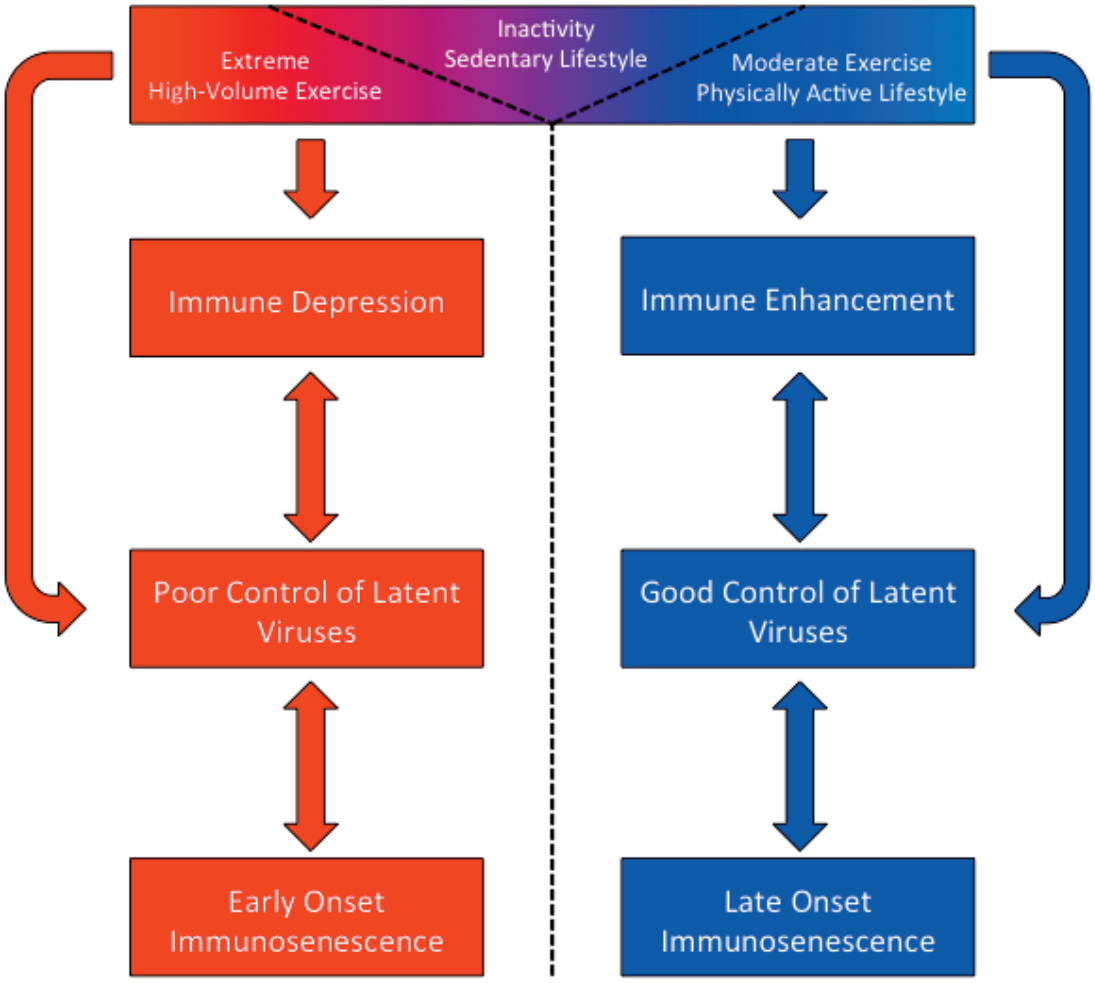


Figure 2. Theoretical framework depicting the bidirectional effects of exercise on immune function and latent viral control. Regular moderate intensity exercise and physically active lifestyles are associated with enhanced immunity that results in better control of latent viruses. Conversely, excessive high volume exercise (such as the type practiced by high performance athletes) is associated with immune impairment that may result in poor control of latent viral infections. The bidirectional effects of exercise and physical activity on immune function and viral control is likely to have important health implications across the lifespan. Individuals with strong immune function and good viral control will likely spend a greater proportion of their lives in good health (increased helathspan) with immunosenescence occurring at an older chronological age compared to those with weak immunity and poor viral control.

specifically on HCMV. In contrast, latent viral reactivation has been studied extensively in astronauts and it has been shown that crewmembers who shed viral DNA [HCMV, (EBV) and varicella-zoster virus (VZV)] have a skewed Type I plasma cytokine profile compared to their non-shedding counterparts (86). Moreover, viral DNA in astronauts has been found in samples collected even prior to spaceflight indicating that it is a stress-induced phenomenon (35). Indeed, viral reactivation has been linked with acute and chronic stress (50, 117) and these may be exacerbated by frequent training and competition (150).

Symptoms of upper respiratory illness (URI) continue to be a problem for high performance athletes. Gleeson et al. (51) reported that EBV serostatus was associated with increased self-reported symptoms of URTI in a group of elite swimmers following a 30-day period of intensive training. Further, EBV DNA was detectable in saliva (indicative of viral shedding) prior to the manifestation of URI symptoms, indicating that EBV reactivation and URI incidence during periods of intensive training could be causally related (51). However, in a follow up study, prophylactic administration of the anti-viral drug Valtrex was able to prevent EBV reactivation but failed to prevent symptoms of URI (34). In contrast, a recent study found that prior exposure to both HCMV and EBV protected endurance athletes from URI during periods of increased training volume (56). In a study of 246 athletes over a 4-month winter training period, those athletes with HCMV/EBV (25% of the cohort) reported fewer URI symptom days (median: 2 vs. 4 days) compared to non-infected athletes. Seropositivity to EBV alone provided no protective effect on URI symptoms, indicating that either prior exposure to HCMV alone, or a synergistic effect between HCMV and EBV, offers protection against future illness (56). As most HCMV-infected people are co-infected with EBV (133), it is difficult to determine the effects of HCMV independently of EBV. These apparent protective effects of prior HCMV exposure against infectious symptoms in athletes may not be surprising, as greater numbers of circulating EM and EMRA CD8+ T-cells and an amplified redeployment of these cell types in response to a single exercise bout may actually enhance immunosurveillance. In addition, those with a

HCMV positive IgG antibody titer also display increased resting NKCA against a wide range of NK-cell target cells (Bigley et al., Unpublished). It is not known if any of the athletes in the He et al. (56) study displayed signs of poor viral control, as HCMV IgG antibody titers and viral shedding were not reported. Moreover, the discrepancy in findings between Gleeson et al. (51) and He et al. (56) might be due to the failure of the latter study to differentiate between latent and active forms of infection. It is possible, therefore, that prior HCMV exposure boosts immunosurveillance and reduces infection risk in the immunocompetent athlete, but if excessive training leads to immune dysregulation and HCMV or EBV is allowed to reactivate, the viral replication might overburden the immune system and increase the risk for opportunistic infections. Although Brown et al. (25) recently reported that 2-weeks of increased training volume did not alter the composition of T-cell subsets in elite level soccer players regardless of HCMV serostatus, it is not known if the HCMV positive athletes experienced a reactivation of the virus as only serostatus was determined. Thus, it is important that future studies determine the link between viral control and immune competence during periods of heavy training and competition.

Low/Moderate Volume Exercise Training and HCMV Control

As persistent HCMV infection has been implicated in the etiology of immunosenescence, it is intuitive to speculate that appropriate lifestyle interventions aimed at boosting immunity and keeping the virus in check would be advantageous to the host. While many studies have investigated the effects of long-term, moderate-intensity exercise training on various aspects of immunity, particularly in the elderly and the obese (128), it is somewhat surprising that there are currently no studies focused on viral seroprevalence or viral control. Notwithstanding, signature features of both latent and chronic HCMV infection tend to be less marked in habitual moderate-intensity exercisers compared to their sedentary counterparts (127, 128). The beneficial effects of habitual moderate intensity exercise include enhanced vaccine responses, lower numbers of differentiated T-cells, lower circulating levels of pro-inflammatory cytokines, increased mitogen-induced T-cell proliferation, and longer blood leukocyte telomere lengths (128). Hence, it is possible that HCMV regulation connects the beneficial effects of habitual moderate-intensity exercise with improvements in immune function. Future studies should test this hypothesis through randomized clinical trials utilizing longitudinal exercise training interventions.

Habitual low volume exercise may also have indirect effects on viral control through its ability to modulate human stress levels (93), as HCMV reactivation has been shown to occur during periods of acute (order of days to weeks) and chronic (order of months to years) stress (35). In a large occupational cohort that included over 300 HCMV seropositive participants, Rector et al. (109) reported that higher HCMV IgG serum antibody titers were associated with questionnaire-based measures of elevated anxiety, depression, vital exhaustion, and lower scores of mental health. These associations withstood adjustment for a large number of potential confounding factors such as age and socioeconomic status. Because exercise has been shown to ameliorate symptoms of psychological stress and improve health and overall wellbeing (93), it is likely that frequent participation in structured exercise training or physical activity will mediate these relationships between measures of psychological stress and poor control of HCMV.

HCMV Infection and Exercise: Potential Clinical and Practical Applications

Latent Viral Reactivation: A biomarker of immune depression in athletes?

While there are several anecdotal reports of athletes contracting infectious mononucleosis, developing shingles and/or having frequent cold sores (110), no study to our knowledge has shown that these manifestations of primary or reactivated herpesvirus infections are more frequent in athletes compared to their non-athlete counterparts. However, latent viruses can often reactivate without conspicuous symptoms of infection, particularly during or shortly after periods of high physical and/or psychological stress. Other models of both acute and chronic stress have shown evidence of latent viral reactivation without accompanying infectious symptoms, such as academic stress, military training/deployment, bereavement and caregiving (35). So not only does the shedding of viral DNA have the potential to directly cause disease, but the subclinical reactivation of a latent virus may serve as a reliable indicator of immune depression in athletes and provide an early indication that they might be susceptible to opportunistic infections. HCMV reactivation can be detected through shedding of viral DNA in urine (86, 133), the presence of HCMV DNA in blood monocytes (78), and/or pp65 antigenemia in peripheral blood leukocytes (66); with changes in HCMV IgG antibody titers being used by some researchers as an indicator of viral load (20, 36, 109). Thus, the shedding of viral DNA could potentially be used to predict overtraining/underperformance and allow coaches and team physicians to make decisions on the training, nutritional and recuperation regimens of an athlete with an active herpesvirus infection. While the molecular biology techniques required to confirm active viral infections may not be practical in large groups, high performance athletes should at least be screened for HCMV serology and other persistent infections so that those previously exposed can be monitored periodically for viral load using more sensitive measures (i.e. viral antigenemia; shedding of viral DNA) as they prepare for major competitions.

Immunosenescence and the Immune Risk Profile

Whether or not the plasticity of the immune system can be modulated by exercise during the natural course of aging is of great interest to both exercise immunologists and immunogerontologists. It has been suggested that regular exercise may help prevent and/or rejuvenate ‘older looking’ immune systems (127, 128). Although HCMV infection features heavily in immunosenescence and the associated IRP, the contemporary view is that HCMV might be a 'passenger' rather than a 'driver' of immunosenescence, and the reactivation of HCMV and the consequential effects it has on immunity are secondary to immunosenescence itself (116). Thus, it is possible that exercise may have greater involvement in preventing immunosenescence that leads to HCMV reactivation and its effects on the peripheral T-cell pool in particular. If regular exercise is found to improve HCMV control, especial-

ly in the elderly, this could be one mechanism by which exercise helps curtail immunosenescence.

It is equally important to determine if exercise can exert rejuvenating effects downstream of immunosenescence. One possible mechanism would be for regular exercise to alter the composition of the peripheral T-cell pool in the direction of a less senescent profile. We previously hypothesized that regular moderate intensity exercise performed over a very protracted period of time (likely in the order of months to years) might 'make space' for new and fully functional T-cells by causing older, exhausted memory T-cell subsets to undergo apoptosis (122). These new cells would likely come from increased thymic output, sites of extrathymic T-cell maturation or homeostatic clonal expansion of existing T-cells (122). The 'exercise makes space' hypothesis may also have an indirect component in that regular moderate exercise may exert better viral control, thus resulting in less frequent shedding of HCMV. As the frequency of viral shedding decreases, the antigenic stimulus and the need to maintain large numbers of HCMV-specific T-cells also decreases and the HCMV-specific T-cells are therefore selectively 'deleted' over time. So, in this instance, exercise is not directly causing the deletion of excess viral T-cell clones, but the accompanying improvement in HCMV control eliminates the need for their existence in large numbers. There is evidence for this 'use them or lose them' idea with other viral infections such as adenovirus, where large numbers of adenovirus-specific cells are more likely to be found in children (due to recent infection) than adults and persistence of memory cells is relatively short-term (order of a few years) (118). However, it should be noted that HCMV-specific T-cell clonotypes may persist for up to 4-years even in healthy people with low antigenic load (58), so any alteration in the frequency of HCMV-specific T-cells with exercise as a result of better viral control may only be seen after a protracted period of time.

It is important to note that the 'exercise makes space' hypothesis is merely a theoretical framework that is a long way off from having the empirical support required to be an accepted mechanism by which exercise can rejuvenate the aged immune system. The main challenge to the credibility of this framework is that it was integrated with the dogmatic view of that time that there is a fixed 'immunological space' that restricts total T-cell numbers (28). This limitation in 'space' was thought to reflect homeostatic control of peripheral T-cell numbers as opposed to a shortage of actual physical space, but this idea has recently been challenged (87, 144, 154). It is also unknown if T-cell apoptosis is required to trigger the production of new T-cells, although a recent study by Mooren and Kruger (90) showed that adoptive transfer of apoptotic lymphocytes in rodents triggered the release of hematopoietic progenitor cells into the circulation. Whether or not apoptotic T-cells can trigger thymic output or homeostatic T-cell proliferation in a similar manner remains to be determined.

Regardless of what the underpinning mechanism might be, there are no longitudinal randomized control trials to date that show exercise training can rejuvenate older looking immune systems (128). However, as exercise training has been shown to improve vaccine outcomes in previously sedentary community dwelling elderly (65, 160), it is likely that direct improvements in immune function are involved. Future clinical trials involving exercise training and outcome measures of immune function should focus on cohorts with an apparent 'senescent' or 'immune risk profile' at baseline, as exercise is likely to exert larger beneficial effects in these subject groups. For instance, individuals with several hallmark features of immunosenescence (i.e. inverted CD4:CD8 T-cell ratio, low naïve T-cell numbers, increased PD-1^{pos} HCMV-specific T-cells) and/or those with poor HCMV control (high HCMV IgG antibody titers, HCMV DNA^{pos} monocytes) might benefit more from an exercise training intervention compared to those who, despite being chronologically older, display few signs of immunosenescence and impaired HCMV control. Moreover, a limitation of the current HCMV and immunosenescence literature is that it is polarized almost entirely to the T-cell compartment. Recent evidence has indicated that certain individuals control HCMV predominantly through NK-cells, which may shoulder the burden of HCMV control to preserve T-cell function and prevent excess T-cell clonal expansion and functional exhaustion (20). It is therefore important that future studies determine the impact of exercise and other lifestyle interventions on HCMV control and the interaction between T-cell and NK-cell responses to the virus during the natural course of aging.

Exercise as a Vaccine Adjuvant: is HCMV Infection a Mediator?

Improvements in vaccine response after exercise training have provided the strongest evidence to date that the plasticity of the immune system can be positively affected by exercise in non-diseased people. Woods et al. (160) reported that community-dwelling elderly randomized to a 10-month cardiovascular exercise-training program had increased seroprotection rates following immunization with the trivalent influenza vaccine compared to controls who performed flexibility/stretching exercise up to 24 weeks after inoculation. Kohut et al. (65) also reported that older adults immunized with a trivalent influenza vaccine before and after a 10-month aerobic exercise training intervention had a greater mean fold increase in antibody titre to H1N1 and H3N2 strains of influenza A virus compared to non-exercised controls. Several studies have shown that single bouts of exercise performed immediately prior to vaccination are also effective, but these are not always consistent (97). These studies have involved whole body dynamic exercise and localized resistance exercise designed to evoke a local inflammatory response at the site of vaccination. Exercise and vaccine studies typically focus on the influenza, tetanus toxoid, diphtheria, pneumococcal and meningococcal vaccines, and the subjects range from young, healthy adults to community dwelling elderly (97). In response to acute exercise, the majority of studies report that exercise enhances the response against those vaccine strains that elicited the poorest response in the control group, suggesting that immune responses to those vaccine antigens with low immunogenicity are most likely to be enhanced by acute exercise (97).

While the mechanisms underpinning these exercise-induced improvements in vaccine responses are likely to be different between single exercise bouts and chronic exercise training, it

is possible that individuals with poor HCMV control (i.e. weaker immunity) will benefit most from the adjuvant effects of exercise. Although weaker immune responses to vaccines (i.e. influenza vaccine) have been attributed to HCMV serostatus (85, 140) this has not been consistently reported (36), and these equivocal findings might be due to the failure of these studies to account for HCMV control using sensitive methods such as viral shedding or the presence of HCMV DNA^{pos} monocytes (78, 86). We postulate that the inverse associations between HCMV serostatus and vaccine efficacy will be more marked in those with poor HCMV control, and that chronic exercise training will, in turn, enhance vaccine efficacy by improving HCMV control and lowering the overall burden placed on the immune system. Moreover, because vaccines already elicit robust immune responses in the vast majority of healthy people, the adjuvant effects of the acute exercise response might not be apparent in those with good HCMV control. At the very least, acute exercise might help those with HCMV mount vaccine responses that are comparable to non-HCMV infected people at rest. In a study of young healthy adults, acute eccentrically biased resistance exercise was found to enhance immune responses to the seasonal influenza vaccine in men(42), with the exercise bout affecting those with and without HCMV equally (140). This occurred despite latent HCMV infection being associated with weaker vaccine responses (140). Future research should determine the impact of both acute and chronic exercise on vaccine efficacy in the context of HCMV infection across a wide age range, considering both prior exposure and the ability of the host to keep the virus in a latent state. This will be particularly important to study in the elderly who are known to have impaired vaccine responses and are more likely to have poor control of HCMV due to immunosenescence.

Acute Exercise and Adoptive Transfer Immunotherapy

Another clinical procedure that may benefit from the immune-enhancing effects of acute exercise is adoptive transfer immunotherapy - the passive infusion of *ex vivo* expanded donor-derived or autologous immune cells to a cancer patient recipient. HSCT is used to treat many hematologic malignancies, but is associated with significant morbidity and mortality especially due to viral infections (i.e. HCMV, EBV and adenovirus), relapse, and graft-versus-host disease (GvHD) (49, 55). Viral infections and relapse can be controlled by adoptive transfer of antigen-specific T-cells that have been expanded *ex vivo* from an MHC compatible donor. Current viral-specific T-cell manufacturing processes involve stimulating PBMCs with overlapping viral antigen peptides *in vitro* to expand the population of memory T-cells with anti-viral activity (49). Clinically sufficient numbers can be obtained in 8-21 days before they are delivered to the patient. Although adoptive T-cell transfer is often successful in curtailing viral infections after HSCT, the inadequate restoration of immunity in some cases may be due to the failure to generate sufficient numbers of functional antigen-specific T-cells that are able to recognize and destroy target cells in vivo and persist in the host after transfusion. Using viral antigen peptides to expand memory T-cells from peripheral blood, we have shown that a single bout of exercise is capable of augmenting the manufacture of highly functional cytotoxic T-cell lines specific to multiple virus antigens (Spielmann et al. Unpublished). Thus, exercise might serve as a simple and economical method to augment the rapid generation of multi-virus specific T-cells from healthy donors for subsequent adoptive transfer to immunocompromised patients after HSCT. Exercise therefore has the potential to amplify the total number of viral specific cells generated from a fixed volume of blood ensuring a faster delivery of a product that is enriched with broad virus specific activity to the patient. Moreover, exercising donors during blood collections may also reduce the need for apheresis, cost and the overall burden placed on the donor. As the mechanisms for viral-specific T-cell mobilization with exercise are likely to involve interactions between catecholamines and β-AR, it is also possible that the exercise effects might be reproducible in resting donors administered a synthetic β-AR agonist (3). Although this remains to be determined, eliminating the need for exercise would be preferable for some donors and may increase the applicability of the technique to the autologous adoptive transfer immunotherapy setting also. For instance, cancer patients required to donate their own cells for reinfusion (i.e. autologous adoptive T-cell transfer) might be too ill to perform a single exercise bout but may be able to tolerate the administration of a β-AR agonist for the purposes of mobilizing their antigen-specific T-cells to the peripheral blood prior to *ex vivo* expansion.

Although cytotoxic CD8+ and CD4+ T-cells are often preferred for adoptive transfer immunotherapy because of their antigen specificity and ability to proliferate and persist in the host after infusion, allogeneic adoptive transfer of NK-cells has also shown promise as a means of controlling or reversing the spread of multiple human malignancies including multiple myeloma, AML, and non-small cell lung cancer (60, 89, 120, 148). This immunotherapeutic procedure has shown a consistently high safety profile and has increased survival in poor prognosis cancer patients (89, 114) and is preferred by some over T-cell transfer because NK-cells do not cause GvHD (114). However, multiple issues remain that undermine the efficacy of long-term cancer treatment using adoptive transfer of NK-cells. Existing pre-transfer expansion protocols are able to generate large numbers of NK-cells (47, 119), but alloreactivity of donor NK-cells is highly variable (101, 114, 120) and expression of NKG2A is far greater than NKG2C (119), which limits the capacity of NK-cells to kill tumor cells expressing classical HLA molecules and HLA-E (101, 120). As such, the rapid expansion of alloreactive, HLA-E-targeting NK-cells needs to be improved. Our work shows that exercise has great potential as an adjuvant for NK-cell immunotherapy as it primes NK-cells to kill HLA-expressing tumor cells that are typically resistant to NK-cells (17, 18). Interestingly, however, the phenotypic and functional properties of NK-cells in the blood during the recovery phase of exercise appear better suited for the allogeneic adoptive transfer setting compared to NK-cells in blood at rest or immediately after exercise (17) (Figure 1). Indeed, 1h after completing a 30-minute cycling protocol, there are increased proportions of $NKG2C^{pos}/NKG2A^{neg}$ NK-cells and their ability to kill target cells expressing both classic and non-classic HLA molecules is markedly elevated, particularly in HCMV seronegative donors (18). Thus, it might be better to expand NK-cells during exercise recovery as opposed to during or immediately after exercise, especially from HCMV seronegative donors,

who tend to have lower numbers of NKG2C^{pos}/NKG2A^{neg} NK-cells and lower NKCA against HLA-E-target cells at rest (18). We have also shown that a single bout of exercise can augment the manufacture of monocyte-derived dendritic cells and T-cells recognizing tumor antigens (73, 74) from healthy people, indicating that exercise has great potential as a simple and economical adjuvant to boost the manufacture of various cell types for use in the allogeneic adoptive transfer immunotherapy setting (19).

SUMMARY

It has been suggested that HCMV has likely co-evolved with its host since the very beginning of human life (136). Carrying the infection was long considered to exert mostly negative effects on immunity that may accelerate the biological aging of the human immune system and the onset of immunosenescence (64). This viewpoint has changed somewhat in that prior exposure to HCMV, provided that the host is immune competent and can adequately keep the virus in check, might actually strengthen immunity (116). Evidence for this comes from studies that have found enhanced immune responses to the influenza vaccine in young people with latent HCMV infection (48), and a putative virus-versus-tumor effect that has been documented both *in vitro* and *in vivo* (20, 43). Specifically the increase in NKG2C expression on NK-cells from people with even a latent HCMV infection enhances NKCA against certain cancer (i.e. AML) and other viruses (i.e. Hantavirus) that are characterized by the upregulation of HLA-E on malignant or transformed cells (20, 22); whereas clinical HCMV reactivation and the subsequent accumulation of NKG2C+ NK-cells have been linked to a markedly decreased risk of relapse in AML patients (43, 52). Despite the longstanding association between humans and HCMV, studies investigating the effects of exercise in the context of HCMV infection only began in earnest within the last 5-years. It has become apparent that HCMV infection has a profound influence on the redistribution of CD8+ T-cells, γδ T-cells and NK-cells in response to a single exercise bout, and that these effects are independent of co-infections with other herpesviruses such as HSV-1 and EBV (16, 72). As such, studies that are concerned with harnessing the acute stress response to improve clinical outcomes (i.e. vaccination, adoptive transfer immunotherapy, surgical outcomes) should consider the role that host infection history plays in this response.

We postulate that the bidirectional effects of exercise volume on host immunity will be directly linked to the ability of the host to control HCMV and other persistent infections (Figure 2). It is vitally important for future research to determine if regular moderate-intensity exercise training and/or physical activity can improve control of HCMV (and other latent viruses) and preserve host immunity. Moreover, whether or not improved viral control can contribute to immune rejuvenation through exercise and lifestyle interventions is a key question that still remains unanswered (127). The interactions between habitual exercise/physical activity and psychological stress on HCMV reactivation and immune function should also be thoroughly explored as this will add to our understanding of the role that lifestyle and the social environment might play in the etiology of immunosenescence. In contrast to habitual moderate-intensity exercise training and physical activity, high volume prolonged exercise training may compromise immunity and impair host HCMV control, which could lead to detrimental pro-senescence effects for the high performance athlete in both the short and long term. Although research in this area is still in its infancy, we conclude that host infection history and the ability to regulate dormant pathogens is likely to play a key role in our understanding of how the immune system responds to both acute and chronic exercise across the entire exercise volume continuum.

ACKNOWLEDGMENTS

This work was supported by NASA grants NNJ10ZSA003N and NNJ14ZSA001N-FLAGSHIP to R.J. Simpson and NIH grant P01 CA148600-01A1 to C.M. Bollard.

REFERENCES

1. Achour A, Baychelier F, Besson C, Arnoux A, Marty M, Hannoun L, Samuel D, Debre P, Vieillard V, and Group KGS. Expansion of CMV-mediated NKG2C+ NK cells associates with the development of specific de novo malignancies in liver-transplanted patients. J Immunol 192: 503-511, 2014.
2. Alejenef A, Pachnio A, Halawi M, Christmas SE, Moss PA, and Khan N. Cytomegalovirus drives Vdelta2neg gammadelta T cell inflation in many healthy virus carriers with increasing age. Clin Exp Immunol 176: 418-428, 2014.
3. Anane LH, Edwards KM, Burns VE, Drayson MT, Riddell NE, van Zanten JJ, Wallace GR, Mills PJ, and Bosch JA. Mobilization of gammadelta T lymphocytes in response to psychological stress, exercise, and beta-agonist infusion. Brain Behav Immun 23: 823-829, 2009.
4. Anane LH, Edwards KM, Burns VE, Zanten JJ, Drayson MT, and Bosch JA. Phenotypic characterization of gammadelta T cells mobilized in response to acute psychological stress. Brain Behav Immun 24: 608-614, 2010.
5. Antoine P, Olislagers V, Huygens A, Lecomte S, Liesnard C, Donner C, and Marchant A. Functional exhaustion of CD4+ T lymphocytes during primary cytomegalovirus infection. J Immunol 189: 2665-2672, 2012.
6. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, and Rowland-Jones SL. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 8: 379-385, 2002.
7. Appay V, Zaunders JJ, Papagno L, Sutton J, Jaramillo A, Waters A, Easterbrook P, Grey P, Smith D, McMichael AJ, Cooper DA, Rowland-Jones SL, and Kelleher AD. Characterization of CD4(+) CTLs ex vivo. J Immunol 168: 5954-5958, 2002.
8. Arens R, Remmerswaal EB, Bosch JA, and van Lier RA. 5(th) International Workshop on CMV and Immunosenescence - A shadow of cytomegalovirus infection on immunological memory. Eur J Immunol 45: 954-957, 2015.

9. Atanackovic D, Schnee B, Schuch G, Faltz C, Schulze J, Weber CS, Schafhausen P, Bartels K, Bokemeyer C, Brunner-Weinzierl MC, and Deter HC. Acute psychological stress alerts the adaptive immune response: stress-induced mobilization of effector T cells. J Neuroimmunol 176: 141-152, 2006.
10. Bate SL, Dollard SC, and Cannon MJ. Cytomegalovirus seroprevalence in the United States: the national health and nutrition examination surveys, 1988-2004. Clin Infect Dis 50: 1439-1447, 2010.
11. Beck JC, Wagner JE, DeFor TE, Brunstein CG, Schleiss MR, Young JA, Weisdorf DH, Cooley S, Miller JS, and Verneris MR. Impact of cytomegalovirus (CMV) reactivation after umbilical cord blood transplantation. Biol Blood Marrow Transplant 16: 215-222, 2010.
12. Beck S, and Barrell BG. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. Nature 331: 269-272, 1988.
13. Bennett NJ, Ashiru O, Morgan FJ, Pang Y, Okecha G, Eagle RA, Trowsdale J, Sissons JG, and Wills MR. Intracellular sequestration of the NKG2D ligand ULBP3 by human cytomegalovirus. J Immunol 185: 1093-1102, 2010.
14. Beziat V, Descours B, Parizot C, Debre P, and Vieillard V. NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. PLoS One 5: e11966, 2010.
15. Beziat V, Liu LL, Malmberg JA, Ivarsson MA, Sohlberg E, Bjorklund AT, Retiere C, Sverremark-Ekstrom E, Traherne J, Ljungman P, Schaffer M, Price DA, Trowsdale J, Michaelsson J, Ljunggren HG, and Malmberg KJ. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. Blood 121: 2678-2688, 2013.
16. Bigley AB, Lowder TW, Spielmann G, Rector JL, Pircher H, Woods JA, and Simpson RJ. NK-cells have an impaired response to acute exercise and a lower expression of the inhibitory receptors KLRG1 and CD158a in humans with latent cytomegalovirus infection. Brain Behav Immun 26: 177-186, 2012.
17. Bigley AB, Rezvani K, Chew C, Sekine T, Pistillo M, Crucian B, Bollard CM, and Simpson RJ. Acute exercise preferentially redeploys NK-cells with a highly-differentiated phenotype and augments cytotoxicity against lymphoma and multiple myeloma target cells. Brain Behav Immun 39: 160-171, 2014.
18. Bigley AB, Rezvani K, Pistillo M, Reed J, Agha N, Kunz H, O'Connor DP, Sekine T, Bollard CM, and Simpson RJ. Acute exercise preferentially redeploys NK-cells with a highly-differentiated phenotype and augments cytotoxicity against lymphoma and multiple myeloma target cells. Part II: Impact of latent cytomegalovirus infection and catecholamine sensitivity. Brain Behav Immun 2015.
19. Bigley AB, and Simpson RJ. NK cells and exercise: implications for cancer immunotherapy and survivorship. Discovery medicine 19: 433-445, 2015.
20. Bigley AB, Spielmann G, Agha N, O'Connor DP, and Simpson RJ. Dichotomous effects of latent CMV infection on the phenotype and functional properties of CD8+ T-cells and NK-cells. Cell Immunol 2015.
21. Bigley AB, Spielmann G, Agha N, and Simpson RJ. The Effects of Age and Latent Cytomegalovirus Infection on NK-Cell Phenotype and Exercise Responsiveness in Man. Oxidative medicine and cellular longevity 2015: 979645, 2015.
22. Bjorkstrom NK, Lindgren T, Stoltz M, Fauriat C, Braun M, Evander M, Michaelsson J, Malmberg KJ, Klingstrom J, Ahlm C, and Ljunggren HG. Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. J Exp Med 208: 13-21, 2011.
23. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, Bjorklund AT, Flodstrom-Tullberg M, Michaelsson J, Rottenberg ME, Guzman CA, Ljunggren HG, and Malmberg KJ. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood 116: 3853-3864, 2010.
24. Bosch JA, Berntson GG, Cacioppo JT, and Marucha PT. Differential mobilization of functionally distinct natural killer subsets during acute psychologic stress. Psychosom Med 67: 366-375, 2005.
25. Brown FF, Bigley AB, Ross JC, LaVoy EC, Simpson RJ, and Galloway SD. T-lymphocyte populations following a period of high volume training in female soccer players. Physiol Behav 152: 175-181, 2015.
26. Brown FF, Bigley AB, Sherry C, Neal CM, Witard OC, Simpson RJ, and Galloway SD. Training status and sex influence on senescent T-lymphocyte redistribution in response to acute maximal exercise. Brain Behav Immun 39: 152-159, 2014.
27. Brunetta E, Hudspeth KL, and Mavilio D. Pathologic natural killer cell subset redistribution in HIV-1 infection: new insights in pathophysiology and clinical outcomes. J Leukoc Biol 88: 1119-1130, 2010.
28. Brunner S, Herndler-Brandstetter D, Weinberger B, and Grubeck-Loebenstein B. Persistent viral infections and immune aging. Ageing Res Rev 10: 362-369, 2010.
29. Campbell JP, Riddell NE, Burns VE, Turner M, van Zanten JJ, Drayson MT, and Bosch JA. Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. Brain Behav Immun 23: 767-775, 2009.
30. Cannon MJ, Schmid DS, and Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol 20: 202-213, 2010.
31. Ceddia MA, Price EA, Kohlmeier CK, Evans JK, Lu Q, McAuley E, and Woods JA. Differential leukocytosis and lymphocyte mitogenic response to acute maximal exercise in the young and old. Med Sci Sports Exerc 31: 829-836, 1999.
32. Charoudeh HN, Terszowski G, Czaja K, Gonzalez A, Schmitter K, and Stern M. Modulation of the natural killer cell KIR repertoire by cytomegalovirus infection. Eur J Immunol 43: 480-487, 2013.
33. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, and Chalupny NJ. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 14: 123-133, 2001.
34. Cox AJ, Gleeson M, Pyne DB, Saunders PU, Clancy RL, and Fricker PA. Valtrex therapy for Epstein-Barr virus reactivation and upper respiratory symptoms in elite runners. Med Sci Sports Exerc 36: 1104-1110, 2004.
35. Crucian B, Simpson RJ, Mehta S, Stowe R, Chouker A, Hwang SA, Actor JK, Salam AP, Pierson D, and Sams C. Terrestrial stress analogs for spaceflight associated immune system dysregulation. Brain Behav Immun 39: 23-32, 2014.

36. den Elzen WP, Vossen AC, Cools HJ, Westendorp RG, Kroes AC, and Gussekloo J. Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities. Vaccine 29: 4869-4874, 2011.
37. Derhovanessian E, Maier AB, Hahnel K, Beck R, de Craen AJ, Slagboom EP, Westendorp RG, and Pawelec G. Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans. J Gen Virol 92: 2746-2756, 2011.
38. Dhabhar FS. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. Neuroimmunomodulation 16: 300-317, 2009.
39. Di Benedetto S, Derhovanessian E, Steinhagen-Thiessen E, Goldeck D, Muller L, and Pawelec G. Impact of age, sex and CMV-infection on peripheral T cell phenotypes: results from the Berlin BASE-II Study. Biogerontology 2015.
40. Dirks J, Egli A, Sester U, Sester M, and Hirsch HH. Blockade of programmed death receptor-1 signaling restores expression of mostly proinflammatory cytokines in anergic cytomegalovirus-specific T cells. Transplant infectious disease : an official journal of the Transplantation Society 15: 79-89, 2013.
41. Dirksen C, Hansen BR, Kolte L, Haugaard SB, and Andersen O. T-lymphocyte subset dynamics in well-treated HIV-infected men during a bout of exhausting exercise. Infectious diseases 47: 919-923, 2015.
42. Edwards KM, Campbell JP, Ring C, Drayson MT, Bosch JA, Downes C, Long JE, Lumb JA, Merry A, Paine NJ, and Burns VE. Exercise intensity does not influence the efficacy of eccentric exercise as a behavioural adjuvant to vaccination. Brain Behav Immun 24: 623-630, 2010.
43. Elmaagacli AH, Steckel NK, Koldehoff M, Hegerfeldt Y, Trenschel R, Ditschkowski M, Christoph S, Gromke T, Kordelas L, Ottinger HD, Ross RS, Horn PA, Schnittger S, and Beelen DW. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. Blood 118: 1402-1412, 2011.
44. Faist B, Fleischer B, and Jacobsen M. Cytomegalovirus infection- and age-dependent changes in human CD8+ T-cell cytokine expression patterns. Clinical and vaccine immunology : CVI 17: 986-992, 2010.
45. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, Anasetti C, Weisdorf D, and Miller JS. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. Journal of immunology 189: 5082-5088, 2012.
46. Foley B, Cooley S, Verneris MR, Pitt M, Curtsinger J, Luo X, Lopez-Verges S, Lanier LL, Weisdorf D, and Miller JS. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. Blood 119: 2665-2674, 2012.
47. Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, Eldridge P, Leung WH, and Campana D. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer research 69: 4010-4017, 2009.
48. Furman D, Jojic V, Sharma S, Shen-Orr SS, Angel CJ, Onengut-Gumuscu S, Kidd BA, Maecker HT, Concannon P, Dekker CL, Thomas PG, and Davis MM. Cytomegalovirus infection enhances the immune response to influenza. Science translational medicine 7: 281ra243, 2015.
49. Gerdemann U, Keirnan JM, Katari UL, Yanagisawa R, Christin AS, Huye LE, Perna SK, Ennamuri S, Gottschalk S, Brenner MK, Heslop HE, Rooney CM, and Leen AM. Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. Mol Ther 20: 1622-1632, 2012.
50. Glaser R, Friedman SB, Smyth J, Ader R, Bijur P, Brunell P, Cohen N, Krilov LR, Lifrak ST, Stone A, and Toffler P. The differential impact of training stress and final examination stress on herpesvirus latency at the United States Military Academy at West Point. Brain Behav Immun 13: 240-251, 1999.
51. Gleeson M, Pyne DB, Austin JP, Lynn Francis J, Clancy RL, McDonald WA, and Fricker PA. Epstein-Barr virus reactivation and upper-respiratory illness in elite swimmers. Med Sci Sports Exerc 34: 411-417, 2002.
52. Green ML, Leisenring WM, Xie H, Walter RB, Mielcarek M, Sandmaier BM, Riddell SR, and Boeckh M. CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. Blood 122: 1316-1324, 2013.
53. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, and Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood 104: 3664-3671, 2004.
54. Hadaya K, de Rham C, Bandelier C, Bandelier C, Ferrari-Lacraz S, Jendly S, Berney T, Buhler L, Kaiser L, Seebach JD, Tiercy JM, Martin PY, and Villard J. Natural killer cell receptor repertoire and their ligands, and the risk of CMV infection after kidney transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 8: 2674-2683, 2008.
55. Hanley PJ, and Bollard CM. Controlling cytomegalovirus: helping the immune system take the lead. Viruses 6: 2242-2258, 2014.
56. He CS, Handzlik M, Muhamad A, and Gleeson M. Influence of CMV/EBV serostatus on respiratory infection incidence during 4 months of winter training in a student cohort of endurance athletes. Eur J Appl Physiol 113: 2613-2619, 2013.
57. Henson SM, Franzese O, Macaulay R, Libri V, Azevedo RI, Kiani-Alikhan S, Plunkett FJ, Masters JE, Jackson S, Griffiths SJ, Pircher HP, Soares MV, and Akbar AN. KLRG1 signaling induces defective Akt (ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. Blood 113: 6619-6628, 2009.
58. Iancu EM, Corthesy P, Baumgaertner P, Devevre E, Voelter V, Romero P, Speiser DE, and Rufer N. Clonotype selection and composition of human CD8 T cells specific for persistent herpes viruses varies with differentiation but is stable over time. J Immunol 183: 319-331, 2009.
59. Ibegbu CC, Xu YX, Harris W, Maggio D, Miller JD, and Kourtis AP. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. J Immunol 174: 6088-6094, 2005.
60. Iliopoulou EG, Kountourakis P, Karamouzis MV, Doufexis D, Ardavanis A, Baxevanis CN, Rigatos G, Papamichail M, and Perez SA. A phase I trial of adoptive transfer of allogeneic natural killer cells in patients with advanced non-small cell lung cancer. Cancer immunology, immunotherapy : CII 59: 1781-1789, 2010.

61. Izumi T, Kondo M, Takahashi T, Fujieda N, Kondo A, Tamura N, Murakawa T, Nakajima J, Matsushita H, and Kakimi K. Ex vivo characterization of gammadelta T-cell repertoire in patients after adoptive transfer of Vgamma9Vdelta2 T cells expressing the interleukin-2 receptor beta-chain and the common gamma-chain. Cytotherapy 15: 481-491, 2013.
62. Jackson SE, Mason GM, Okecha G, Sissons JG, and Wills MR. Diverse specificities, phenotypes, and antiviral activities of cytomegalovirus-specific CD8+ T cells. J Virol 88: 10894-10908, 2014.
63. Kheav VD, Busson M, Scieux C, Peffault de Latour R, Maki G, Haas P, Mazeron MC, Carmagnat M, Masson E, Xhaard A, Robin M, Ribaud P, Dulphy N, Loiseau P, Charron D, Socie G, Toubert A, and Moins-Teisserenc H. Favorable impact of natural killer cell reconstitution on chronic graft-versus-host disease and cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation. Haematologica 99: 1860-1867, 2014.
64. Koch S, Larbi A, Ozcelik D, Solana R, Gouttefangeas C, Attig S, Wikby A, Strindhall J, Franceschi C, and Pawelec G. Cytomegalovirus infection: a driving force in human T cell immunosenescence. Ann N Y Acad Sci 1114: 23-35, 2007.
65. Kohut ML, Arntson BA, Lee W, Rozeboom K, Yoon KJ, Cunnick JE, and McElhaney J. Moderate exercise improves antibody response to influenza immunization in older adults. Vaccine 22: 2298-2306, 2004.
66. Kulkarni A, Westmoreland D, and Fox JD. Molecular-based strategies for assessment of CMV infection and disease in immunosuppressed transplant recipients. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 7: 179-186, 2001.
67. Kunz H, Bishop NC, Spielmann G, Pistillo M, Reed J, Ograjsek T, Park Y, Mehta SK, Pierson DL, and Simpson RJ. Fitness level impacts salivary antimicrobial protein responses to a single bout of cycling exercise. Eur J Appl Physiol 115: 1015-1027, 2015.
68. Lang DJ, Kovacs AA, Zaia JA, Doelkin G, Niland JC, Aledort L, Azen SP, Fletcher MA, Gauderman J, Gjerset GJ, and et al. Seroepidemiologic studies of cytomegalovirus and Epstein-Barr virus infections in relation to human immunodeficiency virus type 1 infection in selected recipient populations. Transfusion Safety Study Group. Journal of acquired immune deficiency syndromes 2: 540-549, 1989.
69. Lanier LL. Evolutionary struggles between NK cells and viruses. Nat Rev Immunol 8: 259-268, 2008.
70. Lanier LL. NK cell recognition. Annu Rev Immunol 23: 225-274, 2005.
71. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nature immunology 9: 495-502, 2008.
72. Lavoy EC, Bigley AB, Spielmann G, Rector JL, Morrison MR, O'Connor DP, and Simpson RJ. CMV Amplifies T-cell Redeployment to Acute Exercise Independently of HSV-1 Serostatus. Med Sci Sports Exerc 2013.
73. LaVoy EC, Bollard CM, Hanley PJ, Blaney JW, O'Connor DP, Bosch JA, and Simpson RJ. A single bout of dynamic exercise enhances the expansion of MAGE-A4 and PRAME-specific cytotoxic T-cells from healthy adults. Exerc Immunol Rev 21: 144-153, 2015.
74. LaVoy EC, Bollard CM, Hanley PJ, O'Connor DP, Lowder TW, Bosch JA, and Simpson RJ. A single bout of dynamic exercise by healthy adults enhances the generation of monocyte-derived-dendritic cells. Cell Immunol 295: 52-59, 2015.
75. Lavoy EC, Bosch JA, Lowder TW, and Simpson RJ. Acute aerobic exercise in humans increases cytokine expression in CD27(-) but not CD27(+) CD8(+) T-cells. Brain Behav Immun 27: 54-62, 2013.
76. LaVoy EC, Nieman DC, Henson DA, Shanely RA, Knab AM, Cialdella-Kam L, and Simpson RJ. Latent cytomegalovirus infection and innate immune function following a 75 km cycling time trial. Eur J Appl Physiol 113: 2629-2635, 2013.
77. Leng SX, Li H, Xue QL, Tian J, Yang X, Ferrucci L, Fedarko N, Fried LP, and Semba RD. Association of detectable cytomegalovirus (CMV) DNA in monocytes rather than positive CMV IgG serology with elevated neopterin levels in community-dwelling older adults. Exp Gerontol 46: 679-684, 2011.
78. Leng SX, Qu T, Semba RD, Li H, Yao X, Nilles T, Yang X, Manwani B, Walston JD, Ferrucci L, Fried LP, Margolick JB, and Bream JH. Relationship between cytomegalovirus (CMV) IgG serology, detectable CMV DNA in peripheral monocytes, and CMV pp65(495-503)-specific CD8+ T cells in older adults. Age (Dordr) 33: 607-614, 2011.
79. Li H, Weng P, Najarro K, Xue QL, Semba RD, Margolick JB, and Leng SX. Chronic CMV infection in older women: longitudinal comparisons of CMV DNA in peripheral monocytes, anti-CMV IgG titers, serum IL-6 levels, and CMV pp65 (NLV)-specific CD8(+) T-cell frequencies with twelve year follow-up. Exp Gerontol 54: 84-89, 2014.
80. Lo Monaco E, Tremante E, Cerboni C, Melucci E, Sibilio L, Zingoni A, Nicotra MR, Natali PG, and Giacomini P. Human leukocyte antigen E contributes to protect tumor cells from lysis by natural killer cells. Neoplasia 13: 822-830, 2011.
81. Lopez-Botet M, Angulo A, and Guma M. Natural killer cell receptors for major histocompatibility complex class I and related molecules in cytomegalovirus infection. Tissue Antigens 63: 195-203, 2004.
82. Lopez-Verges S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, Houchins JP, Miller S, Kang SM, Norris PJ, Nixon DF, and Lanier LL. Expansion of a unique CD57(+)NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. Proc Natl Acad Sci U S A 108: 14725-14732, 2011.
83. Magri G, Muntasell A, Romo N, Saez-Borderias A, Pende D, Geraghty DE, Hengel H, Angulo A, Moretta A, and Lopez-Botet M. NKp46 and DNAM-1 NK-cell receptors drive the response to human cytomegalovirus-infected myeloid dendritic cells overcoming viral immune evasion strategies. Blood 117: 848-856, 2011.
84. Mazzeo RS, Rajkumar C, Rolland J, Blaher B, Jennings G, and Esler M. Immune response to a single bout of exercise in young and elderly subjects. Mech Ageing Dev 100: 121-132, 1998.
85. McElhaney JE, Zhou X, Talbot HK, Soethout E, Bleackley RC, Granville DJ, and Pawelec G. The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines. Vaccine 30: 2060-2067, 2012.
86. Mehta SK, Crucian BE, Stowe RP, Simpson RJ, Ott CM, Sams CF, and Pierson DL. Reactivation of latent viruses is associated with increased plasma cytokines in astronauts. Cytokine 61: 205-209, 2013.

87. Mekker A, Tchang VS, Haeberli L, Oxenius A, Trkola A, and Karrer U. Immune senescence: relative contributions of age and cytomegalovirus infection. PLoS pathogens 8: e1002850, 2012.
88. Miles MP, Mackinnon LT, Grove DS, Williams NI, Bush JA, Marx JO, Kraemer WJ, and Mastro AM. The relationship of natural killer cell counts, perforin mRNA and CD2 expression to post-exercise natural killer cell activity in humans. Acta Physiol Scand 174: 317-325, 2002.
89. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, McKenna D, Le C, Defor TE, Burns LJ, Orchard PJ, Blazar BR, Wagner JE, Slungaard A, Weisdorf DJ, Okazaki IJ, and McGlave PB. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood 105: 3051-3057, 2005.
90. Mooren FC, and Kruger K. Apoptotic lymphocytes induce progenitor cell mobilization after exercise. Journal of applied physiology 119: 135-139, 2015.
91. Moro-Garcia MA, Fernandez-Garcia B, Echeverria A, Rodriguez-Alonso M, Suarez-Garcia FM, Solano-Jaurrieta JJ, Lopez-Larrea C, and Alonso-Arias R. Frequent participation in high volume exercise throughout life is associated with a more differentiated adaptive immune response. Brain Behav Immun 39: 61-74, 2014.
92. Moyna NM, Acker GR, Weber KM, Fulton JR, Robertson RJ, Goss FL, and Rabin BS. Exercise-induced alterations in natural killer cell number and function. European journal of applied physiology and occupational physiology 74: 227-233, 1996.
93. Muller L, and Pawelec G. Aging and immunity - Impact of behavioral intervention. Brain Behav Immun 2013.
94. Nguyen S, Dhedin N, Vernant JP, Kuentz M, Al Jijakli A, Rouas-Freiss N, Carosella ED, Boudifa A, Debre P, and Vieillard V. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. Blood 105: 4135-4142, 2005.
95. Nieman DC, Ahle JC, Henson DA, Warren BJ, Suttles J, Davis JM, Buckley KS, Simandle S, Butterworth DE, Fagoaga OR, and et al. Indomethacin does not alter natural killer cell response to 2.5 h of running. Journal of applied physiology 79: 748-755, 1995.
96. Ouyang Q, Wagner WM, Voehringer D, Wikby A, Klatt T, Walter S, Muller CA, Pircher H, and Pawelec G. Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). Exp Gerontol 38: 911-920, 2003.
97. Pascoe AR, Fiatarone Singh MA, and Edwards KM. The effects of exercise on vaccination responses: a review of chronic and acute exercise interventions in humans. Brain Behav Immun 39: 33-41, 2014.
98. Pawelec G, Akbar A, Beverley P, Caruso C, Derhovanessian E, Fulop T, Griffiths P, Grubeck-Loebenstein B, Hamprecht K, Jahn G, Kern F, Koch SD, Larbi A, Maier AB, Macallan D, Moss P, Samson S, Strindhall J, Trannoy E, and Wills M. Immunosenescence and Cytomegalovirus: where do we stand after a decade? Immun Ageing 7: 13, 2010.
99. Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, and Wikby A. Human immunosenescence: is it infectious? Immunol Rev 205: 257-268, 2005.
100. Pawelec G, and Derhovanessian E. Role of CMV in immune senescence. Virus Res 157: 175-179, 2011.
101. Pende D, Castriconi R, Romagnani P, Spaggiari GM, Marcenaro S, Dondero A, Lazzeri E, Lasagni L, Martini S, Rivera P, Capobianco A, Moretta L, Moretta A, and Bottino C. Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction. Blood 107: 2030-2036, 2006.
102. Petitdemange C, Becquart P, Wauquier N, Beziat V, Debre P, Leroy EM, and Vieillard V. Unconventional repertoire profile is imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. PLoS Pathog 7: e1002268, 2011.
103. Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, Precopio ML, Schacker T, Roederer M, Douek DC, and Koup RA. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. J Exp Med 203: 2281-2292, 2006.
104. Pistillo M, Bigley AB, Spielmann G, LaVoy EC, Morrison MR, Kunz H, and Simpson RJ. The effects of age and viral serology on gammadelta T-cell numbers and exercise responsiveness in humans. Cell Immunol 284: 91-97, 2013.
105. Pitard V, Roumanes D, Lafarge X, Couzi L, Garrigue I, Lafon ME, Merville P, Moreau JF, and Dechanet-Merville J. Long-term expansion of effector/memory Vdelta2-gammadelta T cells is a specific blood signature of CMV infection. Blood 112: 1317-1324, 2008.
106. Podolin DA, Munger PA, and Mazzeo RS. Plasma catecholamine and lactate response during graded exercise with varied glycogen conditions. J Appl Physiol 71: 1427-1433, 1991.
107. Prieto-Hinojosa A, Knight A, Compton C, Gleeson M, and Travers PJ. Reduced thymic output in elite athletes. Brain Behav Immun 2014.
108. Prod'homme V, Griffin C, Aicheler RJ, Wang EC, McSharry BP, Rickards CR, Stanton RJ, Borysiewicz LK, Lopez-Botet M, Wilkinson GW, and Tomasec P. The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1+ but activates LIR-1- NK cells. J Immunol 178: 4473-4481, 2007.
109. Rector JL, Dowd JB, Loerbroks A, Burns VE, Moss PA, Jarczok MN, Stalder T, Hoffman K, Fischer JE, and Bosch JA. Consistent associations between measures of psychological stress and CMV antibody levels in a large occupational sample. Brain Behav Immun 38: 133-141, 2014.
110. Reid VL, Gleeson M, Williams N, and Clancy RL. Clinical investigation of athletes with persistent fatigue and/or recurrent infections. Br J Sports Med 38: 42-45, 2004.
111. Riddell NE, Griffiths SJ, Rivino L, King DC, Teo GH, Henson SM, Cantisan S, Solana R, Kemeny DM, MacAry PA, Larbi A, and Akbar AN. Multifunctional cytomegalovirus (CMV)-specific CD8(+) T cells are not restricted by telomere-related senescence in young or old adults. Immunology 144: 549-560, 2015.
112. Rolle A, Pollmann J, Ewen EM, Le VT, Halenius A, Hengel H, and Cerwenka A. IL-12-producing monocytes and HLA-E control HCMV-driven NKG2C+ NK cell expansion. The Journal of clinical investigation 124: 5305-5316, 2014.

113. Roux A, Mourin G, Larsen M, Fastenackels S, Urrutia A, Gorochov G, Autran B, Donner C, Sidi D, Sibony-Prat J, Marchant A, Stern M, Sauce D, and Appay V. Differential impact of age and cytomegalovirus infection on the gammadelta T cell compartment. J Immunol 191: 1300-1306, 2013.
114. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF, and Velardi A. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 295: 2097-2100, 2002.
115. Sallusto F, Lenig D, Forster R, Lipp M, and Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401: 708-712, 1999.
116. Sansoni P, Vescovini R, Fagnoni FF, Akbar A, Arens R, Chiu YL, Cicin-Sain L, Dechanet-Merville J, Derhovanessian E, Ferrando-Martinez S, Franceschi C, Frasca D, Fulop T, Furman D, Gkrania-Klotsas E, Goodrum F, Grubeck-Loebenstein B, Hurme M, Kern F, Lilleri D, Lopez-Botet M, Maier AB, Marandu T, Marchant A, Mathei C, Moss P, Muntasell A, Remmerswaal EB, Riddell NE, Rothe K, Sauce D, Shin EC, Simanek AM, Smithey MJ, Soderberg-Naucler C, Solana R, Thomas PG, van Lier R, Pawelec G, and Nikolich-Zugich J. New advances in CMV and immunosenescence. Exp Gerontol 55: 54-62, 2014.
117. Sarid O, Anson O, Yaari A, and Margalith M. Human cytomegalovirus salivary antibodies as related to stress. Clin Lab 48: 297-305, 2002.
118. Sester M, Sester U, Alarcon Salvador S, Heine G, Lipfert S, Girndt M, Gartner B, and Kohler H. Age-related decrease in adenovirus-specific T cell responses. J Infect Dis 185: 1379-1387, 2002.
119. Shah N, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, Decker WK, Li S, Robinson SN, Sekine T, Parmar S, Gribben J, Wang M, Rezvani K, Yvon E, Najjar A, Burks J, Kaur I, Champlin RE, Bollard CM, and Shpall EJ. Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity. PloS one 8: e76781, 2013.
120. Shi J, Tricot G, Szmania S, Rosen N, Garg TK, Malaviarachchi PA, Moreno A, Dupont B, Hsu KC, Baxter-Lowe LA, Cottler-Fox M, Shaughnessy JD, Jr., Barlogie B, and van Rhee F. Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. British journal of haematology 143: 641-653, 2008.
121. Shin MS, Lee JS, Lee N, Lee WW, Kim SH, and Kang I. Maintenance of CMV-specific CD8+ T cell responses and the relationship of IL-27 to IFN-gamma levels with aging. Cytokine 61: 485-490, 2013.
122. Simpson RJ. Aging, persistent viral infections, and immunosenescence: can exercise "make space"? Exerc Sport Sci Rev 39: 23-33, 2011.
123. Simpson RJ, and Bosch JA. Special issue on exercise immunology: current perspectives on aging, health and extreme performance. Brain Behav Immun 39: 1-7, 2014.
124. Simpson RJ, Cosgrove C, Chee MM, McFarlin BK, Bartlett DB, Spielmann G, O'Connor DP, Pircher H, and Shiels PG. Senescent phenotypes and telomere lengths of peripheral blood T-cells mobilized by acute exercise in humans. Exercise Immunology Review 16: 36-51, 2010.
125. Simpson RJ, Cosgrove C, Ingram LA, Florida-James GD, Whyte GP, Pircher H, and Guy K. Senescent T-lymphocytes are mobilised into the peripheral blood compartment in young and older humans after exhaustive exercise. Brain Behav Immun 22: 544-551, 2008.
126. Simpson RJ, Florida-James GD, Cosgrove C, Whyte GP, Macrae S, Pircher H, and Guy K. High-intensity exercise elicits the mobilization of senescent T lymphocytes into the peripheral blood compartment in human subjects. J Appl Physiol 103: 396-401, 2007.
127. Simpson RJ, and Guy K. Coupling aging immunity with a sedentary lifestyle: has the damage already been done?--a mini-review. Gerontology 56: 449-458, 2010.
128. Simpson RJ, Lowder TW, Spielmann G, Bigley AB, Lavoy EC, and Kunz H. Exercise and the aging immune system. Ageing Res Rev 11: 404-420, 2012.
129. Sissons JG, and Wills MR. How understanding immunology contributes to managing CMV disease in immunosuppressed patients: now and in future. Medical microbiology and immunology 204: 307-316, 2015.
130. Spielmann G, Bollard CM, Bigley AB, Hanley PJ, Blaney JW, LaVoy EC, Pircher H, and Simpson RJ. The effects of age and latent cytomegalovirus infection on the redeployment of CD8+ T cell subsets in response to acute exercise in humans. Brain Behav Immun 39: 142-151, 2014.
131. Spielmann G, Johnston CA, O'Connor DP, Foreyt JP, and Simpson RJ. Excess body mass is associated with T cell differentiation indicative of immune ageing in children. Clin Exp Immunol 176: 246-254, 2014.
132. Spielmann G, McFarlin BK, O'Connor DP, Smith PJ, Pircher H, and Simpson RJ. Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. Brain Behav Immun 25: 1521-1529, 2011.
133. Stowe RP, Kozlova EV, Yetman DL, Walling DM, Goodwin JS, and Glaser R. Chronic herpesvirus reactivation occurs in aging. Exp Gerontol 42: 563-570, 2007.
134. Sun JC, Lopez-Verges S, Kim CC, DeRisi JL, and Lanier LL. NK cells and immune "memory". Journal of immunology 186: 1891-1897, 2011.
135. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, Sleath PR, Grabstein KH, Hosken NA, Kern F, Nelson JA, and Picker LJ. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med 202: 673-685, 2005.
136. Terrazzini N, and Kern F. Cell-mediated immunity to human CMV infection: a brief overview. F1000prime reports 6: 28, 2014.
137. Tomasec P, Braud VM, Rickards C, Powell MB, McSharry BP, Gadola S, Cerundolo V, Borysiewicz LK, McMichael AJ, and Wilkinson GW. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science 287: 1031, 2000.
138. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, Routy JP, Haddad EK, and Sekaly RP. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. Nat Med 12: 1198-1202, 2006.
139. Turner JE, Aldred S, Witard OC, Drayson MT, Moss PM, and Bosch JA. Latent cytomegalovirus infection amplifies CD8 T-lymphocyte mobilisation and egress in response to exercise. Brain Behav Immun 24: 1362-1370, 2010.

140. Turner JE, Campbell JP, Edwards KM, Howarth LJ, Pawelec G, Aldred S, Moss P, Drayson MT, Burns VE, and Bosch JA. Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults. Age (Dordr) 36: 287-297, 2014.
141. Vales-Gomez M, Reyburn HT, Erskine RA, Lopez-Botet M, and Strominger JL. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. EMBO J 18: 4250-4260, 1999.
142. van de Berg PJ, Griffiths SJ, Yong SL, Macaulay R, Bemelman FJ, Jackson S, Henson SM, ten Berge IJ, Akbar AN, and van Lier RA. Cytomegalovirus infection reduces telomere length of the circulating T cell pool. J Immunol 184: 3417-3423, 2010.
143. van Leeuwen EM, Gamadia LE, Baars PA, Remmerswaal EB, ten Berge IJ, and van Lier RA. Proliferation requirements of cytomegalovirus-specific, effector-type human CD8+ T cells. J Immunol 169: 5838-5843, 2002.
144. van Leeuwen EM, Koning JJ, Remmerswaal EB, van Baarle D, van Lier RA, and ten Berge IJ. Differential usage of cellular niches by cytomegalovirus versus EBV- and influenza virus-specific CD8+ T cells. J Immunol 177: 4998-5005, 2006.
145. Vescovini R, Biasini C, Fagnoni FF, Telera AR, Zanlari L, Pedrazzoni M, Bucci L, Monti D, Medici MC, Chezzi C, Franceschi C, and Sansoni P. Massive load of functional effector CD4+ and CD8+ T cells against cytomegalovirus in very old subjects. J Immunol 179: 4283-4291, 2007.
146. Vescovini R, Fagnoni FF, Telera AR, Bucci L, Pedrazzoni M, Magalini F, Stella A, Pasin F, Medici MC, Calderaro A, Volpi R, Monti D, Franceschi C, Nikolich-Zugich J, and Sansoni P. Naive and memory CD8 T cell pool homeostasis in advanced aging: impact of age and of antigen-specific responses to cytomegalovirus. Age (Dordr) 36: 625-640, 2014.
147. Voehringer D, Koschella M, and Pircher H. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1). Blood 100: 3698-3702, 2002.
148. Waggoner SN, Cornberg M, Selin LK, and Welsh RM. Natural killer cells act as rheostats modulating antiviral T cells. Nature 481: 394-398, 2012.
149. Wald A, Selke S, Magaret A, and Boeckh M. Impact of human cytomegalovirus (CMV) infection on immune response to pandemic 2009 H1N1 influenza vaccine in healthy adults. J Med Virol 85: 1557-1560, 2013.
150. Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, Shephard RJ, Oliver SJ, Bermon S, and Kajeniene A. Position statement. Part two: Maintaining immune health. Exerc Immunol Rev 17: 64-103, 2011.
151. Walsh NP, Gleeson M, Shephard RJ, Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, and Simon P. Position statement. Part one: Immune function and exercise. Exerc Immunol Rev 17: 6-63, 2011.
152. Wang A, Ren L, Abenes G, and Hai R. Genome sequence divergences and functional variations in human cytomegalovirus strains. FEMS immunology and medical microbiology 55: 23-33, 2009.
153. Welte SA, Sinzger C, Lutz SZ, Singh-Jasuja H, Sampaio KL, Eknigk U, Rammensee HG, and Steinle A. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Immunol 33: 194-203, 2003.
154. Wertheimer AM, Bennett MS, Park B, Uhrlaub JL, Martinez C, Pulko V, Currier NL, Nikolich-Zugich D, Kaye J, and Nikolich-Zugich J. Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T cell subsets in humans. J Immunol 192: 2143-2155, 2014.
155. Wieten L, Sarkar S, Gelder Mv, Noort W, Xu Y, Rouschop K, Bloois Lv, Germeraad W, Groen R, Tilanus M, Martens A, and Bos G. HLA-E: an important regulator of Natural Killer cell immunity against Multiple Myeloma. . In Dutch Hematology Congress, Papendal, Arnhem, Netherlands 2014.
156. Wikby A, Johansson B, Olsson J, Lofgren S, Nilsson BO, and Ferguson F. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. Experimental gerontology 37: 445-453, 2002.
157. Wikby A, Mansson IA, Johansson B, Strindhall J, and Nilsson SE. The immune risk profile is associated with age and gender: findings from three Swedish population studies of individuals 20-100 years of age. Biogerontology 9: 299-308, 2008.
158. Willcox CR, Pitard V, Netzer S, Couzi L, Salim M, Silberzahn T, Moreau JF, Hayday AC, Willcox BE, and Dechanet-Merville J. Cytomegalovirus and tumor stress surveillance by binding of a human gammadelta T cell antigen receptor to endothelial protein C receptor. Nature immunology 13: 872-879, 2012.
159. Wills MR, Okecha G, Weekes MP, Gandhi MK, Sissons PJ, and Carmichael AJ. Identification of naive or antigen-experienced human CD8(+) T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T cell response. J Immunol 168: 5455-5464, 2002.
160. Woods JA, Keylock KT, Lowder T, Vieira VJ, Zelkovich W, Dumich S, Colantuano K, Lyons K, Leifheit K, Cook M, Chapman-Novakofski K, and McAuley E. Cardiovascular exercise training extends influenza vaccine seroprotection in sedentary older adults: the immune function intervention trial. J Am Geriatr Soc 57: 2183-2191, 2009.

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

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

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

ABSTRACT

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

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

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

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

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

INTRODUCTION

Introduction to free light chains and their production in healthy individuals

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

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

Free light chains as a biomarker in clinical populations

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

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

Free light chains as a biomarker in the general population

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

Biological role of free light chains

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

Free light chains and exercise

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

Potential of free light chains as a salivary biomarker

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

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

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

METHODS

Participants

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

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

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

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

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

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

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

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

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

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

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

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

Saliva sample collection and analyses

Saliva samples were collected using the same technique across cohorts. Participants were instructed to refrain from eating, drinking (accept water) or brushing their teeth for a minimum of 1 h before arriving to sample collection; no drinking was permitted 10 min before samples were collected. Unstimulated whole saliva samples were collected by passive dribble into pre-weighed tubes for a timed period of 2–4 min. Saliva volume was calculated by re-weighing the tube post-collection assuming a density of 1g/mL. Saliva flow rates (mL/min) were determined by dividing the volume of saliva by the collection time. Samples were centrifuged to separate cells and insoluble matter and the supernatant was removed and stored at –20°C until assay. Salivary kappa and lambda FLCs were quantified using highly sensitive sandwich ELISAs developed by the Clinical Immunology Service at the University of Birmingham. These assays use monoclonal antibodies (mAbs) that have been characterised and validated previously (24).The microtitre wells were coated with mAbs that specifically target either human kappa or lambda FLC and thus do not bind light chain in whole immunoglobulin (Abingdon Health, York, UK). After a blocking period of 1 h to prevent non-specific binding, standards, controls and saliva samples were added to the plate in duplicate. After 1 h incubation, plates were washed to remove any unbound sample. Kappa or lambda detection antibody labelled with horseradish peroxidase was then added to the plate and left to incubate for 1 h. Detection antibodies were mAbs specific for kappa or lambda light chains either free or bound, that recognise an epitope distinct from the one used to capture the FLCs from saliva (Abingdon Health, York, UK). Plates were washed again and substrate solution was added; after 10 min incubation, the reaction was stopped and the optical density was measured at 450 nm. The intra-assay CVs were 9.9% for kappa and 6.7% for lambda and inter-assay CVs were 15.3% for kappa and 16.4% for lambda. The limits of detection were calculated by serially diluting normal serum 1 in 2 in assay buffer and selecting the lowest concentration determined by the assays above the blank (well containing only assay buffer). Limits were 0.004 mg/L and 0.001 mg/L for kappa and lambda FLCs, respectively. The calibration ranges used in the assay were 0.00028–2.82 mg/L for kappa and 0.0003–2.96 mg/L for lambda; saliva samples were diluted 1 in 15 with 0.01M phosphate buffered saline.

Serum sample collection and analyses

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

Free light chain parameters

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

Statistical analyses

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

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

RESULTS

Ranges of free light chains in healthy young and older adults

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

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

Free light chain parameters controlling for potential confounding variables

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

FLC responses to acute exercise in young and older adult populations

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

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

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

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

Saliva

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

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

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

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

Serum

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

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

Saliva

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

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

Serum

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

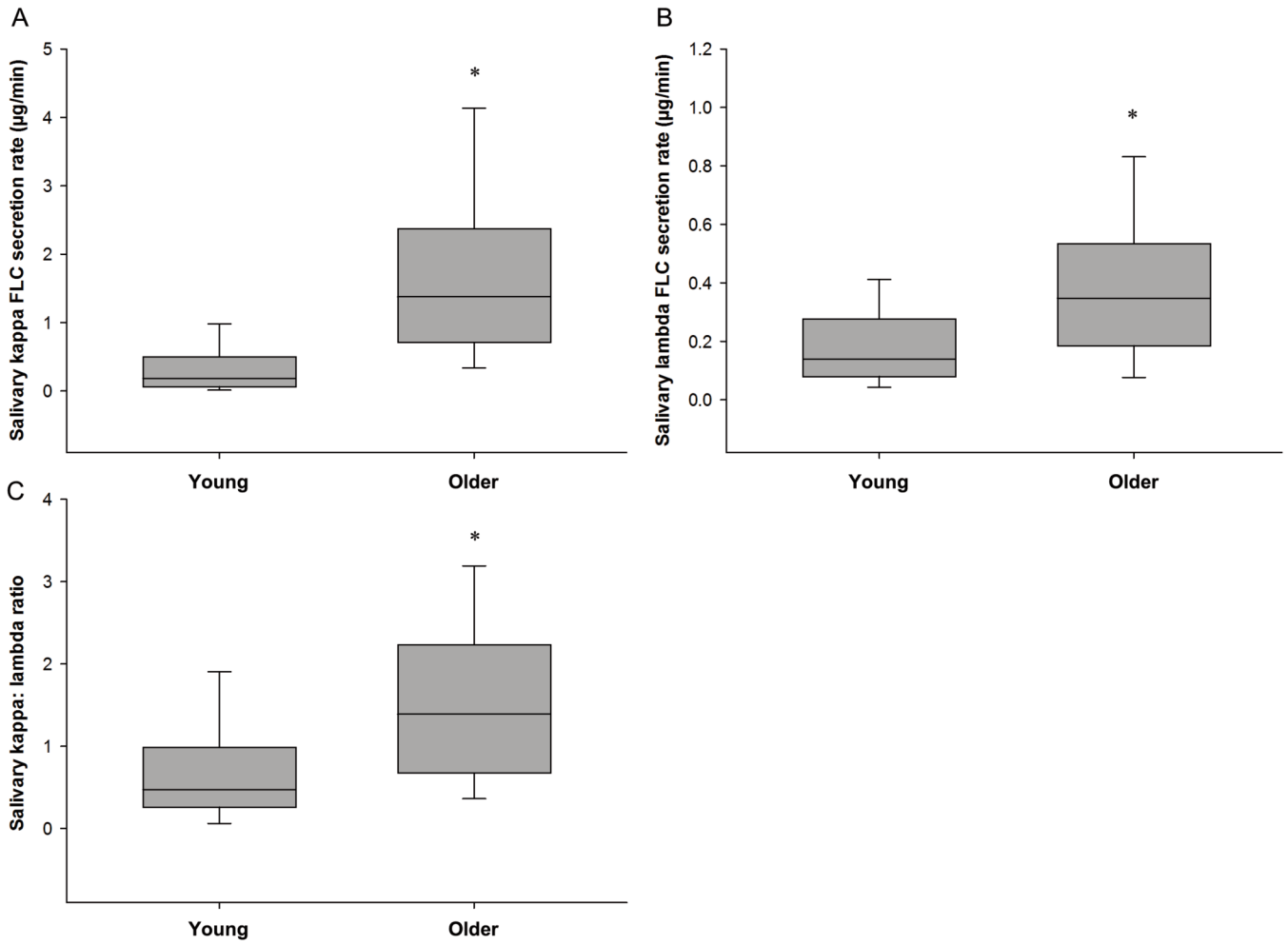


Figure 1. Salivary kappa (A) and lambda (B) free light chain (FLC) secretion rates and the kappa:lambda ratio controlling for flow rate (C) in young and older adults. Boxes represent the 25–75th percentile, with the line indicating the median, and whiskers show the 10–90th percentile. * Significantly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

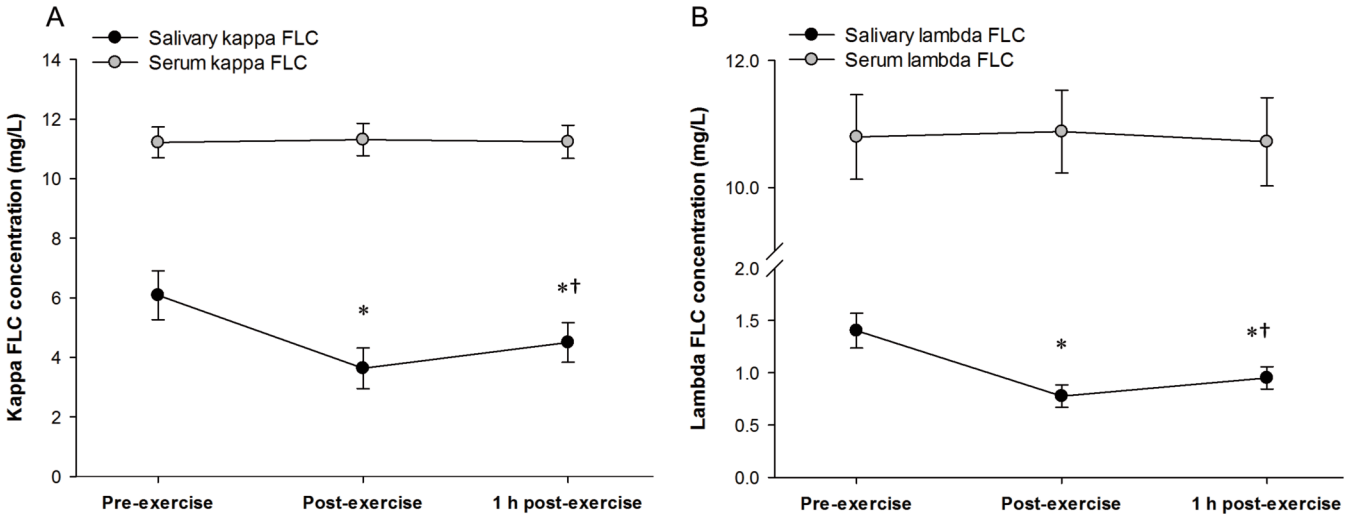


Figure 2. Kappa (A) and lambda (B) free light chain (FLC) concentrations in serum and saliva in response to submaximal exercise in older adults * Kappa (A) and lambda (B) FLC concentrations were significantly reduced post-exercise and 1 h post-exercise compared with pre-exercise in saliva, $p < 0.001$;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

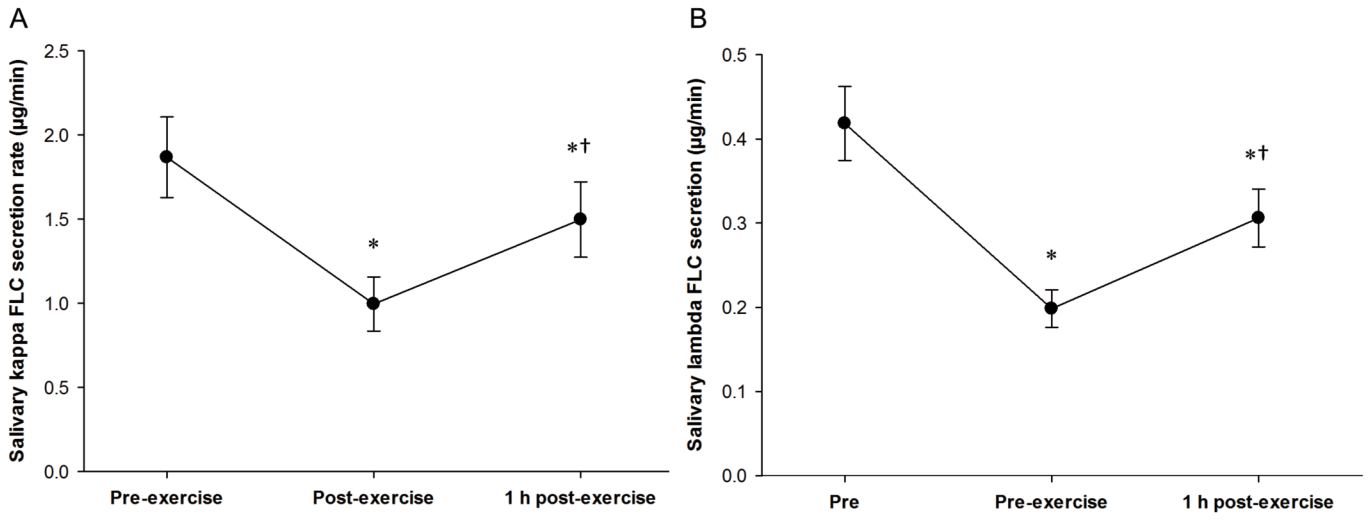


Figure 3. Salivary kappa (A) and lambda (B) free light chain (FLC) secretion rates in response to submaximal exercise in older adults. * Kappa (A) and lambda (B) FLC secretion rates were significantly reduced post-exercise ($p < 0.001$) and 1 h post-exercise ($p < 0.01$) compared with pre-exercise in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

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

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

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

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

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

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

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

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

CONCLUSIONS

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

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

Study Funding: PepsiCo Inc, NY, USA funded the study involving highly trained cyclists included as part of this manuscript; The Clinical Immunology Service, University of Birmingham carried out serum sample analyses; Abingdon Health, UK, provided ELISA kits for saliva sample analysis.

Acknowledgements: The authors would like to thank Dr Gareth Wallis and Dr Victoria Burnsfor providing saliva samples used as in initial assay validation and Dr James Turner for his help with data collection.

REFERENCES

1. Suki WN, and Massry SG, eds. Suki and Massry's Therapy of Renal Diseases and Related Disorders. Kluer Academic Publishers, 1998.
2. Katzmann JA, Clark RJ, Abraham RS, Bryant S, Lymp JF, Bradwell AR, et al. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains.Clin Chem 48: 1437-1444, 2002.
3. Nakano T, Matsui M, Inoue I, Awata T, Katayama S, and Murakoshi T. Free immunoglobulin light chain: its biology and implications in diseases.Clin Chim Acta 412: 843-849, 2011.
4. Waldmann TA, Strober W, and Mogielnicki RP. The renal handling of low molecular weight proteins. II. Disorders of serum protein catabolism in patients with tubular proteinuria, the nephrotic syndrome, or uremia.J Clin Invest 51: 2162-2174, 1972.
5. Brekke OH, and Sandlie I. Therapeutic antibodies for human diseases at the dawn of the twenty-first century.Nat Rev Drug Discov 2: 52-62, 2003.
6. Davids MS, Murali MR, and Kuter DJ. Serum free light chain analysis.Am J Hematol 85: 787-790, 2010.
7. Rajkumar SV, Dimopoulos MA, Palumbo A, Blade J, Merlini G, Mateos M-V, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma.The Lancet Oncology 15: e538-e548, 2014.
8. Dispenzieri A, Kyle R, Merlini G, Miguel JS, Ludwig H, Hajek R, et al. International Myeloma Working Group guidelines for serum-free light chain analysis in multiple myeloma and related disorders.Leukemia 23: 215-224, 2009.
9. Pratt G. The evolving use of serum free light chain assays in haematology.Br J Haematol 141: 413-422, 2008.
10. Drayson MT. Using single protein biomarkers to predict health and disease in diverse patient populations: a new role for assessment of immunoglobulin free light chains.Mayo Clin Proc 87: 505-507, 2012.
11. Dispenzieri A, Zhang L, Katzmann JA, Snyder M, Blood E, Degoey R, et al. Appraisal of immunoglobulin free light chain as a marker of response.Blood 111: 4908-4915, 2008.
12. Brebner JA, and Stockley RA. Polyclonal free light chains: a biomarker of inflammatory disease or treatment target?F1000 Med Rep 5: 1, 2013.
13. van der Heijden M, Kraneveld A, and Redegeld F. Free immunoglobulin light chains as target in the treatment of chronic inflammatory diseases.Eur J Pharmacol 533: 319-326, 2006.
14. Hutchison CA, Harding S, Hewins P, Mead GP, Townsend J, Bradwell AR, et al. Quantitative assessment of serum and urinary polyclonal free light chains in patients with chronic kidney disease.Clin J Am Soc Nephrol 3: 1684-1690, 2008.
15. Dispenzieri A, Katzmann JA, Kyle RA, Larson DR, Therneau TM, Colby CL, et al. Use of nonclonal serum immunoglobulin free light chains to predict overall survival in the general population.Mayo Clin Proc 87: 517-523, 2012.
16. Cohen G, Rudnicki M, and Horl WH. Uremic toxins modulate the spontaneous apoptotic cell death and essential functions of neutrophils.Kidney Int Suppl 78: S48-52, 2001.
17. Braber S, Thio M, Blokhuis BR, Henricks PA, Koelink PJ, Groot Kormelink T, et al. An association between neutrophils and immunoglobulin free light chains in the pathogenesis of chronic obstructive pulmonary disease.Am J Respir Crit Care Med 185: 817-824, 2012.
18. Hutchinson AT, Jones DR, and Raison RL. The ability to interact with cell membranes suggests possible biological roles for free light chain.Immunol Lett 142: 75-77, 2012
19. Redegeld FA, Thio M, and Groot Kormelink T. Polyclonal immunoglobulin free light chain and chronic inflammation.Mayo Clin Proc 87: 1032-1033, 2012.
20. Matsumori A, Shimada M, Jie X, Higuchi H, Groot Kormelink T, and Redegeld FA. Effects of free immunoglobulin light chains on viral myocarditis.Circ Res 106: 1533-1540, 2010.
21. Campbell JP, Eijsvogels TMH, Wang Y, Hopman MTE, Drayson MT, and Jacobs JF. Changes to serum free light chain levels in healthy adults immediately after marathon running.Clin Chem Lab Med, In press.
22. Jacobs JF, Eijsvogels TM, van der Geest KS, Koenen HJ, Hutchison CA, Boots AM, et al. The impact of exercise on the variation of serum free light chains.Clin Chem Lab Med 52: e239-242, 2014.
23. Tanaka H, Monahan KD, and Seals DR. Age-predicted maximal heart rate revisited.J Am Coll Cardiol 37: 153-156, 2001.
24. Campbell JP, Cobbold M, Wang Y, Goodall M, Bonney SL, Chamba A, et al. Development of a highly-sensitive multi-plex assay using monoclonal antibodies for the simultaneous measurement of kappa and lambda immunoglobulin free light chains in serum and urine.J Immunol Methods 391: 1-13, 2013.
25. Siegel D, Bilotti E, and van Hoeven K. Serum Free Light Chain Analysis for Diagnosis, Monitoring, and Prognosis of Monoclonal Gammopathies.Lab Med 40: 363-366, 2009.
26. Oliver SJ, Laing SJ, Wilson S, Bilzon JL, Walters R, and Walsh NP. Salivary immunoglobulin A response at rest and after exercise following a 48 h period of fluid and/or energy restriction.Br J Nutr 97: 1109-1116, 2007.
27. Saunders RH, and Handelman SL. Effects of hyposalivatory medications on saliva flow rates and dental caries in adults aged 65 and older.Spec Care Dentist 12: 116-121, 1992.
28. Gupta A, Epstein JB, and Sroussi H. Hyposalivation in elderly patients.J Can Dent Assoc 72: 841-846, 2006.
29. Bradwell AR. Immunoassays for free light chain measurement Serum free light chain analysis (plus Heavylite) 6th Edition. The Binding Site Group Ltd, Birmingham, UK, 2010.
30. Hofman LF. Human saliva as a diagnostic specimen.J Nutr 131: 1621S-1625S, 2001.
31. Brandtzaeg P. Do salivary antibodies reliably reflect both mucosal and systemic immunity?Ann N Y Acad Sci: 288-311, 2007.

32. Grant MM, Creese AJ, Barr G, Ling MR, Scott AE, Matthews JB, et al. Proteomic analysis of a noninvasive human model of acute inflammation and its resolution: the twenty-one day gingivitis model.J Proteome Res 9: 4732-4744, 2010.
33. Vining RF, McGinley RA, and Symons RG. Hormones in saliva: mode of entry and consequent implications for clinical interpretation.Clin Chem 29: 1752-1756, 1983.
34. Miletic ID, Schiffman SS, Miletic VD, and Sattely-Miller EA. Salivary IgA secretion rate in young and elderly persons.Physiol Behav 60: 243-248, 1996.
35. Nagler RM. Salivary glands and the aging process: mechanistic aspects, health-status and medicinal-efficacy monitoring.Biogerontology 5: 223-233, 2004.
36. Ekström J, Khosravani N, Castagnola M, and Messana I. Saliva and the Control of Its Secretion. In: Ekberg O, ed. Dysphagia. Medical Radiology: Springer Berlin Heidelberg; 2012:19-47.
37. NHS Information Centre. Oral health and function – a report from the Adult Dental Health Survey 2009. Available at: http://www.dhsspsni.gov.uk/theme1_oralhealthandfunction.pdf.
38. NHS Information Centre. Disease and related disorders – a report from the Adult Dental Health Survey 2009. Available at: http://www.hscic.gov.uk/catalogue/PUB01086/adul-dent-heal-surv-summ-them-the2-2009-rep4.pdf.
39. Eke PI, Dye BA, Wei L, Thornton-Evans GO, and Genco RJ. Prevalence of periodontitis in adults in the United States: 2009 and 2010.J Dent Res 91: 914-920, 2012.
40. Makela M, Soderling E, Paunio K, Talonpoika J, and Hyyppa T. Protein composition of crevicular fluid before and after treatment.Scand J Dent Res 99: 413-423, 1991.
41. Baylis D, Bartlett DB, Patel HP, and Roberts HC. Understanding how we age: insights into inflammaging.Longev Healthspan 2: 2046-2395, 2013.
42. Franceschi C, and Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases.J Gerontol A Biol Sci Med Sci 69, 2014.
43. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation.Nat Rev Immunol 15: 30-44, 2015.
44. Evans P, Der G, Ford G, Hucklebridge F, Hunt K, and Lambert S. Social class, sex, and age differences in mucosal immunity in a large community sample.Brain Behav Immun 14: 41-48, 2000.
45. Bishop N. Acute exercise and aquired immune function In: Gleeson M, ed. Immune Function in Sport and Exercise Churchill Livingstone Elsevier, 2006:107-109.
46. Chicharro JL, Lucia A, Perez M, Vaquero AF, and Urena R. Saliva composition and exercise.Sports Med 26: 17-27, 1998.
47. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position statement. Part one: Immune function and exercise.Exerc Immunol Rev 17: 6-63, 2011.

Is there an optimal vitamin D status for immunity in athletes and military personnel?

Cheng-Shiun He[1], Xin Hui Aw Yong[2], Neil P. Walsh[2] and Michael Gleeson[1]

[1] School of Sport, Exercise and Health Sciences, Loughborough University, United Kingdom.
[2] School of Sport, Health and Exercise Sciences, Bangor University, United Kingdom.

ABSTRACT

Vitamin D is mainly obtained through sunlight ultraviolet-B (UVB) exposure of the skin, with a small amount typically coming from the diet. It is now clear that vitamin D has important roles beyond its well-known effects on calcium and bone homeostasis. Immune cells express the vitamin D receptor, including antigen presenting cells, T cells and B cells, and these cells are all capable of synthesizing the biologically active vitamin D metabolite, 1, 25 dihydroxy vitamin D. There has been growing interest in the benefits of supplementing vitamin D as studies report vitamin D insufficiency (circulating 25(OH)D < 50 nmol/L) in more than half of all athletes and military personnel tested during the winter, when skin sunlight UVB is negligible. The overwhelming evidence supports avoiding vitamin D deficiency (25(OH)D < 30 nmol/L) to maintain immunity and prevent upper respiratory illness (URI) in athletes and military personnel. Recent evidence supports an optimal circulating 25(OH)D of 75 nmol/L to prevent URI and enhance innate immunity and mucosal immunity and bring about anti-inflammatory actions through the induction of regulatory T cells and the inhibition of pro-inflammatory cytokine production. We provide practical recommendations for how vitamin D sufficiency can be achieved in most individuals by safe sunlight exposure in the summer and daily 1, 000 IU vitamin D_3 supplementation in the winter. Studies are required in athletes and military personnel to determine the impact of these recommendations on immunity and URI; and, to demonstrate the purported benefit of achieving 25(OH)D > 75 nmol/L.

Keywords: Exercise; Immune; Infection; Cholecalciferol; Ergocalciferol

1. INTRODUCTION

Stress-induced immune dysregulation is widely acknowledged to have negative implications for health (48). Those working in the field of exercise immunology have shown us that individuals who undertake heavy physical exertion, particularly when combined with periods of psychological stress, nutritional inadequacy and sleep disruption (e.g. athletes and military personnel), risk compromising host defence and increasing their susceptibility to respiratory viral infections such as the common cold and possibly to other infectious microorganisms (58, 136, 137). In 1981, the British general practitioner and celebrated epidemiologist, R. Edgar Hope-Simpson was the first to hypothesise that respiratory viral infections (e.g. epidemic influenza) have a 'seasonal stimulus' intimately associated with solar radiation. He observed an increased incidence of respiratory viral infections during the winter that appeared to be more strongly related to the amount of solar radiation than the presence of anti-viral antibodies. The nature of this 'seasonal stimulus' remained undiscovered until the important immuno-modulatory effects of the sunlight-dependent secosteroid vitamin D were fully recognised (Figure 1) (24); indeed, vitamin D levels in the human body are known to fall to a nadir during the peak influenza season and peak when influenza is scarce (95).

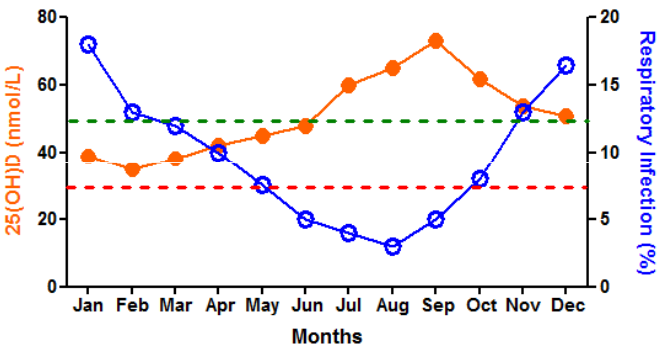


Figure 1. The association between circulating 25(OH)D (closed circles) and respiratory infection (open circles) in British adults. Adapted from Berry et al. (16). Upper dotted line indicates vitamin D sufficiency threshold (50 nmol/L) and lower dotted line indicates vitamin D deficiency threshold (30 nmol/L) as suggested by the Institute of Medicine (66).

Vitamin D refers to a group of fat-soluble secosteroids responsible for enhancing intestinal absorption of calcium, iron, magnesium, phosphate and zinc (63). In humans, vitamin D can be obtained either from sunlight exposure at the skin or in

Corresponding Author:
Prof. Neil P. Walsh FACSM, School of Sport, Health and Exercise Sciences, Bangor University, Bangor, LL57 2PZ, UK.
Email: n.walsh@bangor.ac.uk, Telephone: + 44 1248 383480

the foods we eat and by consuming dietary supplements. Vitamin D production as a result of sunlight ultraviolet (UV) B radiation penetrating the skin typically provides 80-100% of the body's vitamin D requirements. In humans, the most important compounds in the vitamin D group are vitamin D_3 (also known as cholecalciferol) and vitamin D_2 (ergocalciferol). Both cholecalciferol and ergocalciferol can be ingested from the daily diet and from supplements. Unlike other fat- and water-soluble vitamins, the body can also synthesise vitamin D (specifically cholecalciferol) in the skin, from cholesterol, when exposure from sunlight UVB is adequate. Evidence indicates the synthesis of vitamin D from sunlight UVB exposure is regulated by a negative feedback loop that prevents toxicity, but because of uncertainty about the cancer risk from overexposure to sunlight, currently no recommendations are issued by national bodies regarding the amount of sunlight exposure required to meet vitamin D requirements. Accordingly, the recommended daily dietary intake of vitamin D for adults (5 µg or 200 IU in the European Union and 15 µg or 600 IU in the USA) assumes that no synthesis occurs and all of a person's vitamin D is from food intake, although that will rarely occur in practice. As vitamin D can be synthesised in adequate amounts by humans and most other mammals exposed to sunlight, it is not strictly a vitamin (i.e. an organic compound and a vital nutrient that an organism requires in limited amounts), and following its hydroxylation in the body to 1, 25 dihydroxy vitamin D (1, 25$(OH)_2$D) it may be considered a hormone as its synthesis and biological activity occur in different locations. Its discovery in the 1930s can be attributed to key contributions by the chemist Adolf Windaus that included the elucidation of the chemical structures of vitamin D (144).

Inadequate nutrition in terms of dietary energy, macro- or micronutrients is a potential cause of depressed immune function in those engaging in heavy training regimens (137). While most individuals undergoing heavy training who consume a varied diet sufficient to meet their energy needs should meet their micronutrient requirements, one exception can be the failure to achieve adequate vitamin D status during the winter months due to limited vitamin D synthesis from reduced sunlight exposure (106). Therefore, dietary sources of vitamin D and oral vitamin D supplementation are of particular importance during the winter as will be discussed in this review.

The focus of this review is on the effects of vitamin D on immune function and susceptibility to infection and its potential importance for health maintenance in athletes and military personnel. After covering the structure, sources, metabolism and measurement of vitamin D we will present evidence showing that vitamin D deficiency (defined by the Institute of Medicine (IoM) as a circulating 25(OH)D concentration < 30 nmol/L and used hereafter) occurs commonly in athletes and military personnel. The influence of vitamin D status on innate and adaptive immunity, wound repair and respiratory infection with specific reference to those undergoing heavy training schedules will follow. Then we will discuss whether a circulating 25(OH)D level ≥ 75 nmol/L represents an optimal vitamin D status for immune function and host defence with some simple practical guidance on safe summer sunlight exposure and safe oral vitamin D supplementation during the autumn and winter. The reader is referred to other recent reviews for a consideration of the influence of vitamin D on bone health and risk of fractures, cancer prevention, hypertension and mortality (15, 20) and the emerging role of vitamin D in optimising muscle function and athletic performance (6, 80, 99, 105, 106, 123, 130).

1.1 Vitamin D structure and sources

All forms of vitamin D belong to a family of lipids called secosteroids which are very similar in structure to steroids except that two of the B-ring carbon atoms of the typical four steroid rings are not joined, whereas in steroids they are (Figure 2A). The biologically active metabolite, 1, 25$(OH)_2$D, acts very much like a steroid, binding to nuclear receptors and modulating gene expression and subsequently the synthesis of specific proteins.

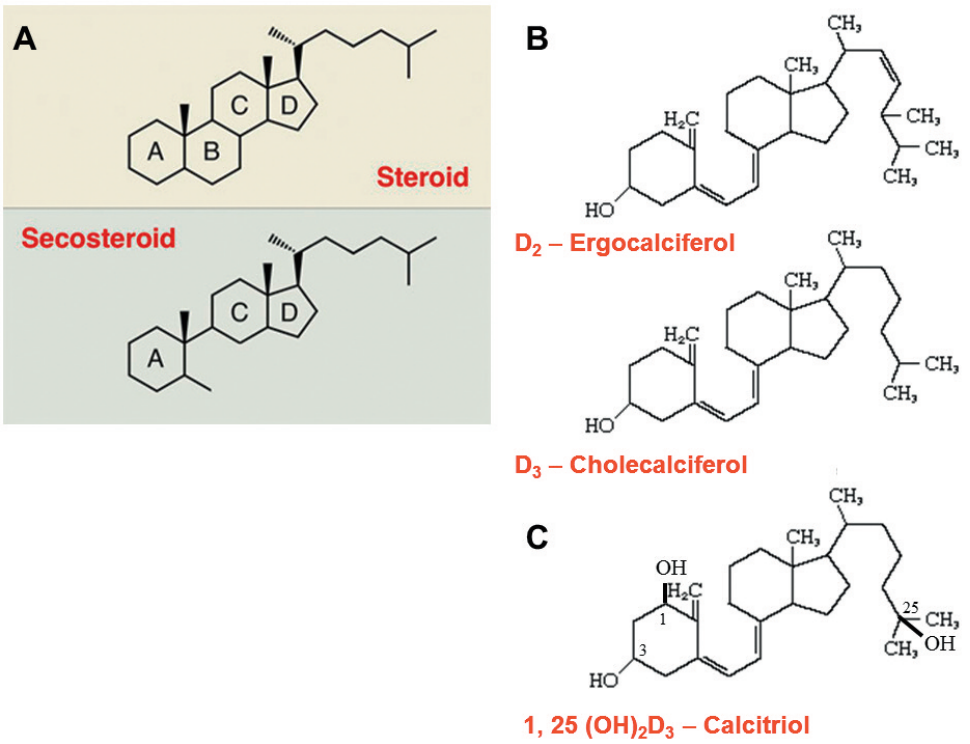


Figure 2. General structure of a secosteroid compared with that of a traditional steroid (A) and the structure of the vitamin D secosteroids (B): Ergocalciferol (D_2) is produced by UV irradiation of ergosterol, a membrane sterol which is produced by some kinds of plankton, invertebrates, yeasts and fungi. Cholecalciferol (D_3) is produced by ultraviolet B irradiation of 7-dehydrocholesterol in the skin which supplies 80-100% of the body's vitamin D requirements. Also shown (C) is the biologically active form of vitamin D, 1, 25-dihydroxy-vitamin D (1, 25$(OH)_2$D), known as calcitriol or calciferol.

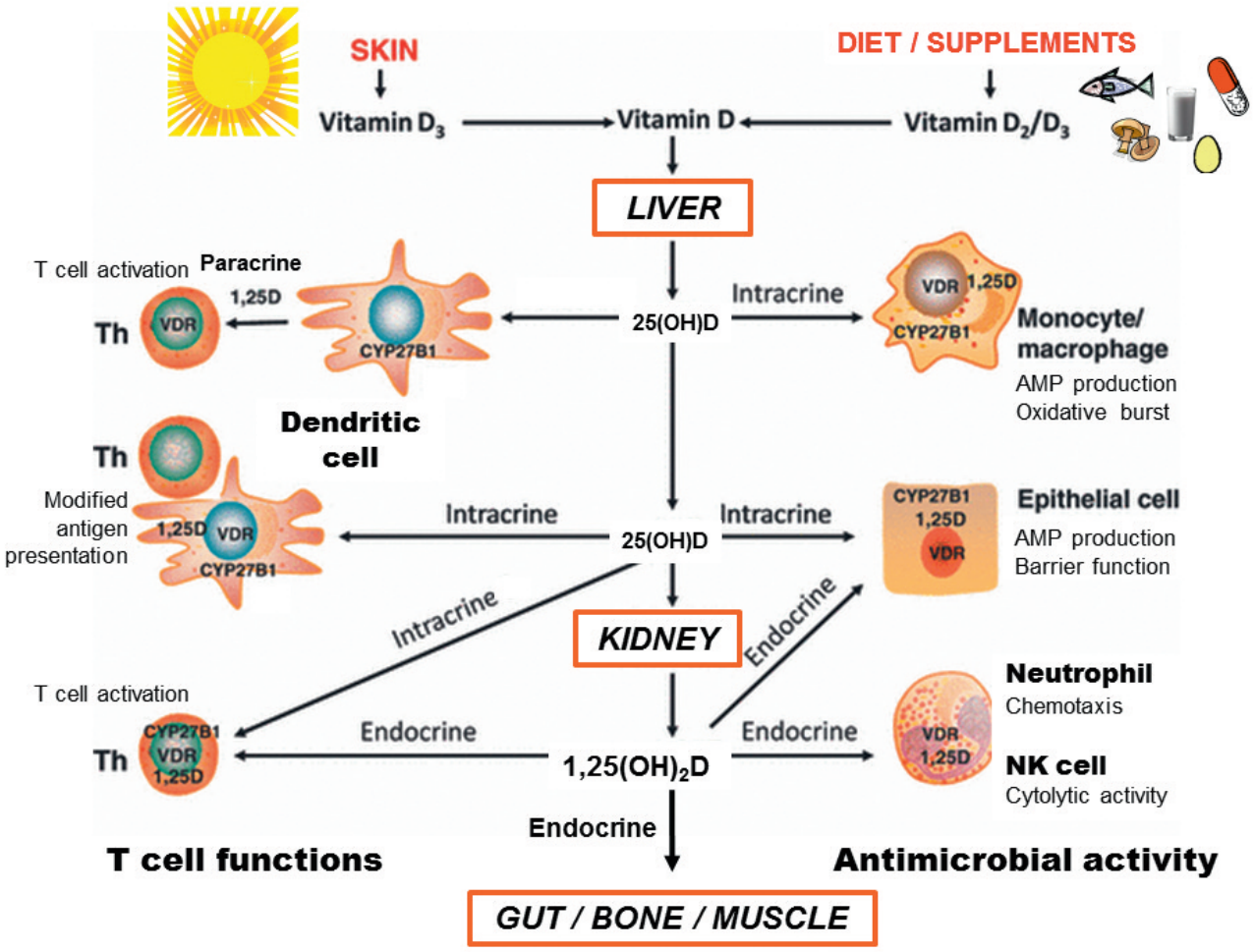


Figure 3. Mechanisms for innate and adaptive immune responses to vitamin D. Ergocalciferol (vitamin D_2) from the diet and cholecalciferol (vitamin D_3) from the diet or produced from the action of UVB on the skin are metabolised in the liver to form 25-hydroxyvitamin D (25(OH)D), the main circulating form of vitamin D. Target cells such as monocytes, macrophages and dendritic cells expressing the mitochondrial vitamin D-activating enzyme 1-α hydroxylase (CYP27B1) and the cytoplasmic vitamin D receptor (VDR) can then utilise 25(OH)D for intracrine responses via localised conversion to 1, 25-dihydroxy-vitamin D (1, 25$(OH)_2$D; calcitriol, shown in the Figure as 1,25D for intracellular locations). In monocytes and macrophages this promotes antibacterial responses to infection. In dendritic cells, intracrine synthesis of 1, 25$(OH)_2$D inhibits dendritic cell maturation, thereby modulating helper T-helper (Th) cell function. Th cell responses to 25(OH)D may also be mediated in a paracrine fashion, via the actions of dendritic cell-generated 1, 25$(OH)_2$D. Intracrine immune effects of 25(OH)D also occur in epithelial cells expressing the VDR and the 1-α hydroxylase (CYP27B1). However, other leukocytes such as neutrophils and natural killer (NK) cells do not appear to express CYP27B1 and are therefore likely to be directly affected by circulating levels of 1, 25$(OH)_2$D synthesised by the kidneys or locally produced in and secreted from tissue macrophages and dendritic cells. VDR-expressing Th cells are also potential targets for systemic 1, 25$(OH)_2$D, although intracrine mechanisms have also been proposed. In a similar fashion, epithelial cells can respond in an intracrine fashion to 25(OH)D, but may also respond to systemic 1, 25$(OH)_2$D to promote antibacterial responses.

Two forms of vitamin D can be obtained from dietary sources (Figure 2B): vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). While vitamin D_3 is found in food from animal origin, such as oily fish, egg yolk, liver and milk, vitamin D_2 is present in some plants and mushrooms (derived from UVB exposure of fungi and yeast ergosterols). Some foods including cereals, margarine and dairy products may be fortified, usually with vitamin D_3. The fractional absorption of both forms of vitamin D from lipid micelles (with the aid of bile salts) in the gut is about 50%. After uptake by intestinal mucosal cells they are incorporated into chylomicrons and enter the circulation via the lymphatic system.

Under optimal conditions of skin sunlight exposure, vitamin D_3 production from UVB-mediated conversion of 7-dehydrocholesterol in the plasma membrane of skin cells provides 80-100% of the body's vitamin D requirements (78). This process is rapid and the production of vitamin D_3 in the skin after only a few minutes of appropriate sunlight easily exceeds dietary sources. The UVB radiation (wavelength of 290-320 nm) promotes photolytic cleavage of 7-dehydrocholesterol into previtamin D in the epidermis, which is subsequently converted into vitamin D_3 by a spontaneous thermal isomerisation. Newly synthesised vitamin D_3 (and its metabolites) are bound to vitamin D-binding protein (VDBP) for systemic transport. Vitamin D_2 is more rapidly metabolised than vitamin D_3, is less well bound to VDBP and therefore has a shorter half-life.

1.2 Metabolism of vitamin D

Vitamin D needs to be hydroxylated twice to achieve the biologically active form, 1, 25$(OH)_2$D (Figure 2C). The endogenously synthesised vitamin D_3 and diet-derived D_2 and D_3

must first be hydroxylated in the liver into 25(OH)D (calcidiol or calcifediol) at the carbon 25-position by the enzyme, 25-hydroxylase. The main storage form of vitamin D, 25(OH)D is found in muscles and adipose tissue, and 25(OH)D is the major circulating metabolite of vitamin D, with a half-life of 2-3 weeks. Therefore, the total plasma concentration of 25(OH)D is considered to be the primary indicator of vitamin D status (11).

In the second hydroxylation, 25(OH)D is converted in the kidney to the biologically active form, 1, 25$(OH)_2$D (calcitriol or calciferol), by 1-α-hydroxylase, an enzyme which is stimulated by parathyroid hormone (PTH) when serum calcium and phosphate concentrations fall below their normal physiological range of 2.1–2.6 mmol/L and 1.0–1.5 mmol/L, respectively. 1, 25$(OH)_2$D, is released into the circulation from the kidney which is considered as a vital endocrine source of hormone (Figure 3). 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; the plasma half-life of 1, 25$(OH)_2$D is 4-6 hours. Some cells other than kidney cells also express 1-α-hydroxylase and have the enzymatic machinery to convert 25(OH)D to 1, 25$(OH)_2$D in non-renal compartments including cells of the immune system as illustrated in Figure 3 (8). Importantly, 1, 25$(OH)_2$D limits its own activity in a negative feedback loop by inducing 24-hydroxylase, which converts 1, 25$(OH)_2$D into the biologically inactive metabolite, 1, 24, 25$(OH)_3$D. In addition, 1, 25$(OH)_2$D also inhibits the expression of renal 1-α-hydroxylase. This negative feedback loop reduces the likelihood of hypercalcaemia by preventing excessive vitamin D signalling, thus maintaining bone health.

1.3 Mode of action of 1, 25$(OH)_2$D

1, 25$(OH)_2$D exerts its functions by acting as a modulator of over 900 genes (73). Circulating 1, 25$(OH)_2$D passes through the plasma membrane of target cells and binds to the vitamin D receptor (VDR) in the cytoplasm. The VDR is a nuclear receptor and ligand-activated transcription factor. It 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$(OH)_2$D 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$(OH)_2$D-VDR-RXR heterodimer translocates to the nucleus where it binds to VDRE located in the regulatory regions of 1, 25$(OH)_2$D target genes and then induces expression of the vitamin D responsive genes (8).

1.4 Vitamin D measurement

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 in whole blood. 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 (2, 7, 143). For example, storage of serum samples for up to 3 years at -20°C does not affect serum 25(OH)D concentrations (2) and 25(OH)D concentrations in serum samples that have been thawed and refrozen up to four times are still reliable (7).

Plasma or serum 25(OH)D concentration can be measured by competitive protein binding assay, immunoassay, high pressure liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (40). 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 (29, 38). 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 (40, 135). 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 an 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 (D_2 and D_3) (40, 135). 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$(OH)D_3$ (135, 146). 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. Nonetheless, the use of LC-MS/MS is not without limitations. Significant inter-assay variability of 16.4% has been reported for 25(OH)D measurement using in-house standards and can only be avoided if laboratories use common standards (26) as well as adopt the similar preparation and calibration methods (39).

1.5 Classical biological role of vitamin D for bone health

The classic function of vitamin D is to maintain the health of bones and teeth by influencing calcium homeostasis. Vitamin D influences bone health by upregulating the expression of genes for several calcium transport proteins that enhance calcium absorption from the diet in the small intestine and increase calcium reabsorption in the renal tubules (in association with elevated PTH). Vitamin D also stimulates bone cell differentiation to promote calcium homeostasis and bone health (63). In the general population, individuals who maintain higher vitamin D status have higher bone mineral density in the hip and lumbar spine (63). In physically active populations, sufficient vitamin D is important for the prevention of stress fractures. For example, in Finnish military recruits stress fracture risk was 3.6 times higher in those with relatively low vitamin D status (25(OH)D concentration < 75 nmol/L) compared to those with higher status (118). A randomised, placebo-controlled, double-blind trial of vitamin D_3 supplementation (daily 800 IU with 2 g calcium) found a 20% reduction in stress fracture incidence in female US naval recruits compared with those taking a placebo (79).

1.6 Is there a consensus of opinion on vitamin D status classifications for immune health?

The simple answer to this question is ‘no’. The IoM has recommended a circulating 25(OH)D level above 50 nmol/L to

Table 1. Classification of vitamin D status suggested by the Institute of Medicine[1].

Vitamin D status	Circulating 25(OH)D concentration (nmol/L)
Deficient	< 30
Inadequate	30 - 50
Sufficient	> 50

[1]Institute of Medicine (66).

achieve 'good bone health' in virtually all of the population but there are no such classifications for vitamin D status in relation to immunity and resistance to common infections (Table 1) (117). In fact, right now there is still no definitive consensus of opinion on the thresholds for vitamin D status and bone health. For example, the circulating 25(OH)D level below which represents deficiency for bone health has been proposed as 30 nmol/L by the IoM (117) but 50 nmol/L by the Endocrine Society and a number of world-leading researchers in the field (20, 64). Furthermore, based on the studies relating 25(OH)D with circulating PTH levels, as well as other evidence for reducing risk of fracture, improving muscle strength and preventing chronic diseases, the Endocrine Society recommends that vitamin D sufficiency should be defined as circulating 25(OH)D > 75 nmol/L (64). Also, a recent and comprehensive review that summarises the various studies that have attempted to evaluate threshold levels for circulating 25(OH)D levels in relation to bone mineral

Table 2. Vitamin D status in athletes and military personnel.

Season	Location (latitude)	Population	N	Age (years)	Circulating 25(OH)D concentration (nmol/L)			Ref
					% < 30 nmol/L[1] Deficient	% < 50 nmol/L[1] Insufficient	% < 75 nmol/L[2] Suboptimal	
Winter								
	Finland (60 - 70 ºN)	Finnish military recruits	196	18 - 28	19% < 25	78% < 40	-	Laaksi et al.(77)
	Liverpool, UK (53 °N)	Elite soccer players	20	24	-	65%	-	Morton et al. (100)
	Liverpool, UK (53 °N)	UK club athletes	30	20 - 24	20%	57%	-	Close et al. (34)
	Liverpool, UK (53 °N)	Professional UK athletes	61	18 - 27	35%	64%	-	Close et al.(33)
	Loughborough, UK (53 ºN)	Recreational to elite athletes	225	21	8%	38%	-	He et al. (56)
	Barcelona (41 ºN)	Professional basketball players	21	25	10% < 25	57%	-	Garcia & Guisado(42)
Autumn								
	Washington, USA (47 °N)	Collegiate athletes	39	18 - 33	-	3%	26% < 80	Storlie et al. (127)
	Australia (35 °S)	Australian female gymnasts	18	10 - 17	-	33%	83%	Lovell (87)
Summer								
	Finland (60 - 70 ºN)	Finnish military recruits	756	18 - 29	-	4% < 40	-	Laaksi et al.(76)
	California, USA (34 °N)	Collegiate athletes	223	-	-	3%	34% < 80	Villacis et al.(133)
	Doha, Qatar (25 ºN)	Middle-eastern sportsmen	93	13 - 45	59% < 25	91%	100%	Hamilton et al.(51)
	Doha, Qatar (25 ºN)	Professional Qatar based footballers	342	16 - 33	12% < 25	56%	84%	Hamilton et al.(52)
All seasons								
	Carolina, USA (35 °N)	Young active military personnel	312	-	-	-	52%	Wentz et al.(142)
Not reported								
	East Germany (53 ºN)	Competitive gymnasts	85	8 - 27	37% < 25	-	-	Bannert et al.(12)
	Pittsburgh, USA (40 °N)	National football league players	80	22 - 37	-	26%	69%	Maroon et al.(92)
	Jerusalem, Israel (32 °N)	Athletes and dancers	98	10 - 30	-	-	73%	Constantini et al.(35)
	Texas, USA (31 °N)	Overweight and obese soldiers	314	31	-	21%	72% < 72	Funderburk et al.(41)

Age is presented as mean or range. Hyphen '-' indicates not reported.
[1]Values are based on current recommendations for bone health, where circulating 25(OH)D < 30 nmol/L is defined as deficient and < 50 nmol/L is defined as insufficient (66). Note: not all authors have used the IoM classification as reflected in the table.
[2]Evidence suggests that those with circulating 25(OH)D < 75 nmol/L have a higher adjusted odds of acute respiratory infections compared with individuals with 25(OH)D levels ≥ 75 nmol/L (97).

density, lower limb function, dental health, cancer prevention, risk of falls, fractures, incident hypertension and mortality concludes that for all endpoints, circulating levels of 25(OH)D < 50 nmol/L are associated with adverse effects or no benefit, while the most advantageous circulating levels for 25(OH)D appeared to be close to 75 nmol/L (20). Further research is clearly required to investigate whether a circulating 25(OH)D > 75 nmol/L is necessary to optimise immune function, as will be discussed in more detail in section 5.3. For the purposes of this review, vitamin D deficiency is denoted as circulating 25(OH)D < 30 nmol/L in line with the current IoM recommendations (117).

2. IS VITAMIN D DEFICIENCY A PROBLEM FOR ATHLETES AND MILITARY PERSONNEL?

The answer to this question appears to be 'yes' although to date we know of no evidence indicating that athletes are at greater risk of vitamin D deficiency than non-athletes. A summary of the current evidence on vitamin D status in athletes and military personnel is provided in Table 2. As logic dictates, vitamin D deficiency is more prevalent in the winter when skin sunlight UVB exposure and endogenous synthesis of vitamin D is low and in those who cover their skin whilst training outdoors in the summer (51) or who train predominantly indoors (12). In the winter months more than half of the athletes and military personnel studied could be considered to have insufficient vitamin D status (circulating 25(OH)D < 50 nmol/L) and as many as 35% could be considered vitamin D deficient. Important considerations when interpreting the data on the incidence of vitamin D deficiency in athletes and military personnel (Table 2) include: sunlight avoidance behaviour (fear of sunburn and skin cancer); season; latitude; skin type; clothing and sunscreen use, all of which will be discussed in section 7.

3. EMERGING BIOLOGICAL ACTIONS OF VITAMIN D

Many tissues other than kidney, including brain, lung, muscle, skin, adipose tissue and cells of the immune system possess both the 1-α-hydroxylase and VDR and are able to produce the biologically active 1, 25$(OH)_2$D from circulating 25(OH)D (11). It is important to note that extra-renal 1-α-hydroxylase differs from renal 1-α-hydroxylase in that it is not regulated by circulating PTH, calcium and phosphate concentrations (145). In recent years it has been established that vitamin D is not only important for calcium homeostasis and bone health but also for the optimal function of skeletal muscle and immune function.

3.1 Vitamin D and skeletal muscle function

Vitamin D can modulate skeletal muscle function by both genomic and nongenomic events. 1, 25$(OH)_2$D induces muscle gene transcription and protein synthesis to influence muscle cell proliferation and differentiation, calcium uptake and phosphate transport across the sarcolemma (50). The nongenomic responses include modulation of calcium uptake across the sarcolemma and the activation of mitogen-activated protein kinase signalling pathways in muscle fibres (50). Vitamin D also up-regulates expression of insulin-like growth factor-1 (IGF-1) (5), which has a well-recognised role in muscle remodelling, hypertrophy and strength gains (74). IGF-1, which is mostly produced by the liver and bound by insulin-like growth factor binding protein 3 (IGFBP-3) in the serum, is a key component in muscle regeneration and could induce proliferation, differentiation and hypertrophy of skeletal muscle (5, 122). IGFBP-3 expression could be regulated by vitamin D as there are vitamin D response elements in the promoter region of the human IGFBP-3 gene which might lead to higher circulating amounts of IGFBP-3 and so delay the normally rapid clearance of IGF-1 in the bloodstream (50, 83). The obvious implication of these findings is that vitamin D status and vitamin D supplementation might affect muscle strength, endurance and athletic performance. This has received considerable attention over the past decade and the results of these studies have been the main focus of numerous recent reviews about vitamin D and the athlete (6, 80, 99, 105, 106, 123, 130). The general consensus at present is vitamin D deficiency could negatively impact athletic performance due to the influence of vitamin D on muscle function. However, there is insufficient evidence from a limited number of cross sectional vitamin D status studies and longitudinal, randomised, placebo-controlled vitamin D_3 supplementation studies in athletes to conclude that vitamin D is a direct performance enhancer (46).

4. VITAMIN D AND IMMUNE FUNCTION

Vitamin D is known to have important effects on both innate and adaptive immune function with implications for host defence. These issues are the main focus of the remainder of this review.

The discovery of VDR in almost all immune cells, including T lymphocytes, B lymphocytes, neutrophils and antigen presenting cells, such as monocytes, macrophages and dendritic cells prompted the idea that vitamin D could have a vital role in the regulation of immune responses (11). These immune cells also express the mitochondrial vitamin D-activating enzyme, 1-α-hydroxylase (CYP27B1) and thus possess the ability to convert 25(OH)D to 1, 25$(OH)_2$D. This conversion is regulated by circulating levels of 25(OH)D and can also be induced by activation of specific toll-like receptors (TLRs) (18) which act as pathogen detectors. Thus, 1, 25$(OH)_2$D could play important roles in both innate and adaptive immune responses (Figure 3). Four potential mechanisms by which vitamin D can influence immune function have been proposed: 1) direct endocrine actions on immune cells mediated by circulating 1, 25$(OH)_2$D formed in the kidney; 2) direct intracellular actions of 1, 25$(OH)_2$D following intracrine conversion of 25(OH)D to 1, 25$(OH)_2$D within immune cells; 3) paracrine actions of 1, 25$(OH)_2$D produced in and secreted from antigen presenting cells on local lymphocytes and neutrophils and 4) indirect effects on antigen presentation to T cells mediated by influence of circulating 1, 25$(OH)_2$D on antigen presenting cells (60, 112). The proposed actions of 1, 25$(OH)_2$D on the human immune system are summarised in

Table 3. The proposed effects of 1, 25 dihydroxy vitamin D on the immune system.

Target site	Actions of 1, 25 $(OH)_2D$
Antigen presenting cells	Upregulation of the production of antimicrobial proteins and peptides (AMPs) (e.g. cathelicidin, β-defensins)
	Increased generation of reactive oxygen species and the expression of inducible nitric oxide synthase
	Increased macrophage phagocytosis
	Upregulation of CD14 expression
	Downregulation of CD40 (required for B cell activation)
	Downregulation of CD80/86 (required for T cell activation)
	Downregulation of MHCII expression
	Elevation of IL-10 production
	Inhibition of production of pro-inflammatory cytokines
Saliva	Increased saliva flow and AMP secretion
Epithelial cells	Upregulation of genes for gap junction, adherens junction and tight junction proteins to strengthen barrier function
Natural Killer cells	Downregulation of production of IFN-γ
	Upregulation of expression of NK cytotoxicity receptors NKp30 and NKp44
	Augmentation of IL-2 activated cytolysis
T cells	Increased vitamin D receptor expression
	Suppression of T helper (Th) type 1 and induction of Th2
	Inhibition of production of pro-inflammatory cytokines IL-2 and IFN-γ by Th1 cells
	Elevation of IL-4 production by Th2 cells
	Suppression the development of Th17 cells and inhibition of the production of cytokines by Th17 cells
	Induction of Treg cells
	Increased IL-10 production by Treg cells
	Upregulation of phospholipase C-gamma 1 expression leading to increased antigen-specific T cell activation and proliferation
B cells	Increased vitamin D receptor expression
	Suppression of B cell proliferation and immunoglobulin production
	Inhibition of the differentiation of B cell precursors into plasma cells

Table 3. Although the actions of vitamin D do not alter numbers of circulating leukocytes, neutrophils, monocytes or lymphocytes, the proportions of lymphocyte subsets, particularly within the T cell compartment, can be modified as can the functions of various immune cells associated with both innate and acquired immunity.

4.1 Vitamin D, innate immunity and mucosal immunity

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 through the induction of antimicrobial proteins (AMPs) and stimulation of autophagy and autophagosome activity (18, 31). 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 (85, 138). The AMPs have a broad range of activities against microorganisms, particularly bacteria, and may also be involved in the direct inactivation of viruses through membrane destabilisation (68). 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. Both macrophages 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, $25(OH)_2D$, can induce expression of the vitamin D responsive genes to enhance the production of cathelicidin and β-defensin by binding to VDREs as described previously in section 1.3. The stimulation of TLRs by interaction with pathogen associated molecular patterns in macrophages or by wounding the epidermis in keratinocytes results in increased expression of both the VDR and the 1-α-hydroxylase enzyme, which up-regulates the production of 1, $25(OH)_2D$ to stimulate the expression of cathelicidin and β-defensins in the presence of adequate 25(OH)D as illustrated in Figure 4 (31, 85). 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 NF-κB transcription factor function (84).

In addition to its effects on AMPs, 1, $25(OH)_2D$ strengthens epithelial barrier functions by up-regulating genes for the proteins required in tight junctions (e.g. occludin), gap juctions (e.g. connexin 43) and adherens junctions (e.g. E-cadherin) in epithelial cells, fibroblasts and keratinocytes (32, 47, 107). Furthermore, 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 (124) as well as augmenting IL-1β secretion and up-regulating the expression of CD14, the lipopolysaccharide (LPS) receptor.

Recent studies on natural killer (NK) cell function indicate that 1, $25(OH)_2D$ upregulates the expression of NK cell surface cytotoxicity receptors NKp30, NKp44 and NKG2D, downregulates the expression of the killer inhibitory receptor CD158 and enhances NK cell cytolytic activity (3). Vitamin D appears to have rather limited effects on neutrophil function. Although neutrophils are recognised as an important source of

cathelicidin and do express VDRs, they seem to have no 1-α hydroxylase activity that would enable them to convert 25(OH)D into the biologically active 1, $25(OH)_2D$ necessary to initiate cathelicidin gene expression (59). However, neutrophils can be influenced directly by circulating 1, $25(OH)_2D$ and, as in monocytes, expression of CD14 on the cell surface is augmented by 1, $25(OH)_2D$ (129). Previous exposure of neutrophils to pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) or granulocyte macrophage colony-stimulating factor or with the VDBP leads to alterations in complement activation peptide C5a-mediated neutrophil functions, including enhanced chemotaxis (19). A recent study on VDBP knockout mice reported that neutrophil recruitment to the lung in both C5a- and CXCL1-induced alveolitis was 50% lower than in the wild type controls (131) and that the reduced neutrophil response in VDBP knockout mice could be restored to wild-type levels by administering exogenous VDBP suggesting that VDBP may have a more significant role in neutrophil recruitment than previously recognised. These various effects of vitamin D can be suggested as improving innate immunity and could conceivably contribute to a reduced susceptibility to infections.

4.1.1 Vitamin D, innate immunity and mucosal immunity in athletes

A recent study in university athletes reported a higher level of plasma cathelicidin and salivary secretory immunoglobulin A (SIgA) secretion in those who had plasma 25(OH)D greater than 120 nmol/L compared with those who had lower vitamin D status (56) and a follow-up randomised, placebo controlled, double blind vitamin D_3 supplementation study (5, 000 IU/day for 14 weeks) by the same group (55) reported significant increases in salivary secretion rates of both SIgA and cathelicidin compared with no significant changes in the placebo group. This was due, at least in part to a significant increase in saliva flow rates 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 points to a possible role for vitamin D in the 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 (108, 128). The mechanism for how vitamin D affects salivary flow rates requires elucidation. But it was suggested that vitamin D might stimulate salivary secretion through the regulation of calcium as the rapid efflux of calcium plays a role in the stimulation of fluid secretion (108).

In summary, the discovery of increased VDR and 1α-hydroxylase (CYP27B1) expression in macrophages following a pathogen challenge, and the subsequent enhancement of AMP production, oxidative burst and autophagosome activity has underlined the importance of intracrine vitamin D as a key enhancer of innate immune function. It is now clear that both macrophages and dendritic cells are able to respond to 25(OH)D, the major circulating vitamin D metabolite, thereby providing a link between the function of these cells and the variations in vitamin D status among humans. Although the evidence is limited, recent studies in athletes show beneficial effects of high circulating vitamin D (> 120 nmol/L) on innate immunity and mucosal immunity.

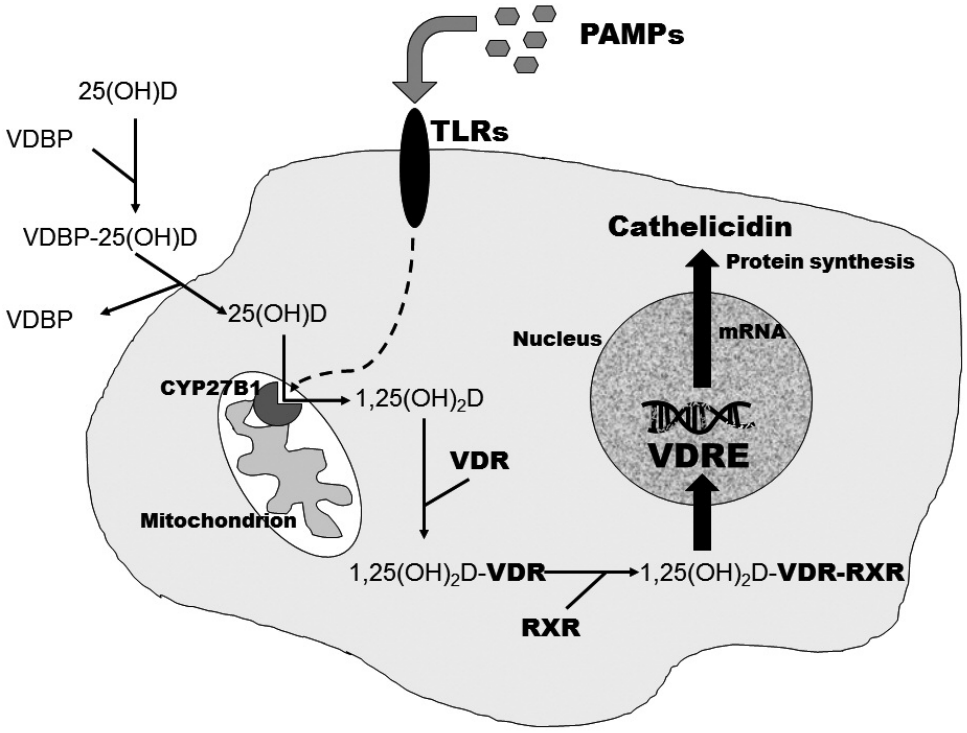


Figure 4. Cathelicidin induction via activation of TLRs and vitamin D. 25-hydroxy vitamin D (25(OH)D) is transported in the circulation bound to the vitamin D binding protein (VDBP). Pathogen associated molecular patterns (PAMPs) on invading microorganisms trigger toll-like receptors (TLR 1/2 and TLR4) and subsequent downstream signalling (dashed arrow) induces the mitochondrial 1-α hydroxylase (CYP27B1), increasing the intracellular conversion of 25(OH)D to 1, $25(OH)_2D$ which after binding to the vitamin D receptor (VDR) along with the retinoid X receptor (RXR) in the cytoplasm translocates to the nucleus where it binds to cognate vitamin D response elements (VDRE) located in the regulatory regions of 1, $25(OH)_2D$ target genes and then induces transcription of the vitamin D responsive genes leading to increased amounts of antimicrobial proteins (e.g. cathelicidin, β-defensin) being produced.

4.2 Vitamin D, adaptive immunity and inflammation

In contrast with the innate immune responses, many of the reported actions of vitamin D on adaptive immunity are indicative of anti-inflammatory and suppressive mechanisms, which could be beneficial for those with autoimmune disorders. The effect of 1, 25$(OH)_2$D on antigen presenting cells is to induce IL-10 and suppress IL-12 production, inhibit dendritic cell activation by down-regulating expression of costimulatory molecules CD40 and CD80/86 while up-regulating the production of AMPs and autophagosome activity (1). Furthermore, 1, 25$(OH)_2$D can 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 (81). Studies using human T cell cultures have shown that 1, 25$(OH)_2$D inhibits T cell proliferation and production of IL-2 and interferon gamma (IFN-γ) (102, 116, 132). In contrast, 1, 25$(OH)_2$D enhances cytokine production by Th2 cells (e.g. IL-4) that are associated with humoral immunity (21). Thus, vitamin D could help limit the inflammation and tissue damage associated with excessive Th1 cellular immunity by shifting the balance to a Th2 cell phenotype. 1, 25$(OH)_2$D also has an influence on the activity of Th17 cells, which are linked to inflammatory tissue damage. It appears that 1, 25$(OH)_2$D can suppress the development of Th17 cells and inhibit the production of cytokines by Th17 cells (27). In addition, it has been shown that treatment of naive CD4 T cells with 1, 25$(OH)_2$D potently induces the development of regulatory T cells (Treg) which are capable of producing cytokines that block Th1 development (49). Vitamin D also increases synthesis of the primary anti-inflammatory cytokine IL-10 by Treg cells and dendritic cells (45, 120). Overall, vitamin D is suggested to maintain a balance between inflammatory Th1/Th17 cells and immunosuppressive Th2/Treg cells to temper inflammation and tissue damage (59). It has also been demonstrated that 1, 25$(OH)_2$D 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 (30).

The actions of vitamin D on adaptive immunity appear to be mostly suppressive or inhibitory, so why does this not impair immune responses to pathogens and increase susceptibility to infection? The answer to this paradox may be found in the recent studies indicating that vitamin D is essential in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes (73, 134) 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 signalling 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 up-regulation 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 signalling via the alternative mitogen-activated protein kinase p38 pathway. Thus, initial TCR signalling via p38 leads to successive induction of VDR and PLC-γ1, which are required for subsequent classical TCR signalling 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. The aforementioned suppressive actions of vitamin D on adaptive immunity may therefore be a reaction to prevent the development of an exaggerated immune response and excessive inflammation following T cell activation. This is, of course, important as the ideal immune response is rapid, proportionate, and effective but finite; an inflammatory response which is disproportionate or lasts too long risks injury to the host. The recognition that in adaptive immunity vitamin D is needed for its effective activation when challenged by pathogens is more in keeping with its role in promoting innate immunity and the reduction in respiratory infection incidence with improved vitamin D status which has been reported in several large scale studies in both the general population (44) and athletes (56) which are discussed in more detail in section 5 of this review.

It is also important to recognise that the primary influence of 1, 25$(OH)_2$D may vary with the tissue site. Systemic levels of 1, 25$(OH)_2$D may aid in maintaining tonic immunosuppression and thus prevent trivial antigenic stimuli from initiating an immune response. Upon initiation of an immune response to a significant antigenic challenge 1, 25$(OH)_2$D may, in concert with other suppressor mechanisms, limit the extent of the host response by inhibition of IL-2 and IFN-γ production. At local sites of chronic inflammation concentrations of 1, 25$(OH)_2$D may be elevated and may act in an autocrine or paracrine fashion to alter the immune response, for example, by increasing IL-1β production and antigen presentation by tissue macrophages. The activation of T cells is associated with increased expression of VDRs, thus potentially limiting T cell proliferation in the presence of the 1, 25$(OH)_2$D. Thus, the end result of the opposing effects of 1, 25$(OH)_2$D on immune cells and their secretory products may vary with the specific cells involved, their state of maturation and activation, and the local concentrations of 1, 25$(OH)_2$D.

The identification of hundreds of primary 1, 25$(OH)_2$D target genes in immune cells has provided new insight into the role of vitamin D in the adaptive immune system, such as the modulation of antigen-presentation and T cell proliferation and phenotype, with the over-arching effects being to suppress inflammation and promote immune tolerance, while also being able to activate the acquired immune response in the presence of pathogen challenge. Thus variations in 25(OH)D levels have the potential to influence both innate and adaptive immune responses.

4.3 Vitamin D and cytokine responses

The studies that have reported modulation of pro- and anti-inflammatory cytokine production by vitamin D have generally administered 1, 25$(OH)_2$D *in vivo* in animals (25, 147) or *in vitro* in human peripheral blood mononuclear cell cultures (70, 101, 115, 116) and observed increases in anti-inflammatory cytokines such as transforming growth factor-β, IL-4 and IL-10 and reductions in pro-inflammatory cytokines including IL-2, IL-6, IFN-γ and TNF-α. However, these studies have used supraphysiological (nanomolar) concentrations of 1, 25$(OH)_2D_3$ to determine mitogen- or bacteria-stimulated

cytokine production in human peripheral blood mononuclear cell cultures (the normal human plasma 1, 25$(OH)_2D_3$ concentration is 50-250 pmol/L). Furthermore, this experimental approach is not a true reflection of differences in vitamin D status, where marked differences in circulating 25(OH)D concentration may exist and might have more influence on immune cell functions than changes in levels of circulating 1, 25$(OH)_2$D. Using *in vitro* antigen-stimulated human whole blood culture, inhibition of IL-2, IL-6, IFN-γ and TNF-α production was only observed at 1, 25$(OH)_2D_3$ concentrations of 1, 000 or 10, 000 pmol/L and not within the more realistic range of 0 to 200 pmol/L (54). This suggests that antigen-stimulated cytokine production is unchanged within the normal reference range of 1, 25$(OH)_2D_3$ concentrations.

4.3.1 Vitamin D and cytokine responses in athletes

A recent study in athletes indicated that athletes deficient in 25(OH)D (circulating 25(OH)D < 30 nmol/L) had substantially lower *in vitro* antigen-stimulated production of the pro-inflammatory cytokines (IL-6, IFN-γ and TNF-α) by whole blood culture than athletes with high vitamin D status (circulating 25(OH)D > 90 nmol/L) (56). This is similar to a report of decreased macrophage IL-6, IL-1β and TNF-α production following *in vitro* LPS stimulation of peritoneal macrophages in vitamin D deficient mice (69). In that study the authors also reported that TNF-α and IL-6 concentrations in serum were ~50% lower following *in vivo* administration of LPS in vitamin-D deficient mice indicating that vitamin D deficiency does result in a defect of cytokine production. A higher pro-inflammatory cytokine production in response to an antigen challenge with better vitamin D status could be seen as being beneficial to host defence against pathogenic microorganisms. Indeed, athletes with high vitamin D status had fewer upper respiratory illness (URI) episodes during a 4-month winter period than those with vitamin D deficiency (56).

Further studies are warranted to understand the mechanisms by which vitamin D affects adaptive immunity and the implications for both infectious and autoimmune diseases. In particular, studies in athletes and military personnel are required to examine the influence of seasonal changes in vitamin D status and vitamin D supplementation (see section 6) on *in vivo* immune measures with known clinical endpoints such as the antibody response to vaccination (136).

It is also worth noting that some cytokines have an influence on vitamin D metabolism, For example IFN-γ is a Th1 pro-inflammatory cytokine that influences vitamin D metabolism in human monocytes (37, 126) and macrophages (72) by increasing 1α-hydroxylase activity which mediates the conversion of 25(OH)D to 1, 25$(OH)_2$D. In contrast to IFN-γ, IL-4 is a Th2 anti-inflammatory cytokine that initiates the catabolism of 25(OH)D to the biologically inactive 24, 25$(OH)_2$D (37). Furthermore, recent genome-wide analyses (31) have highlighted how cytokine signalling pathways can influence the intracrine vitamin D system and either enhance or abrogate responses to 25(OH)D.

4.4 Vitamin D, wound repair and rehabilitation from injury

The emerging evidence for an influence of vitamin D status during musculoskeletal rehabilitation following injury or surgery is of potential importance to athletes. One study reported that vitamin D status influenced strength and recovery in young, recreationally active individuals following anterior cruciate ligament repair (14). In this study, those with circulating 25(OH)D concentration below 75 nmol/L recovered more slowly and had significantly attenuated increases in peak isometric force compared to those with concentrations above 75 nmol/L. Another study by the same group reported that following an intense single limb exercise bout a faster recovery of muscle strength occurred with higher pre-exercise levels of circulating 25(OH)D (13). Studies in athlete populations are currently lacking but a few studies of patients in rehabilitation units support the idea that vitamin D may be important for rehabilitation (13, 71, 121). A study in a general rehabilitation unit found that vitamin D deficiency delayed rehabilitation and increased length of stay by 19% (71). Another randomised trial in female stroke patients found that supplementation with 1, 000 IU vitamin D/day improved muscle strength and increased the relative number and size of type II muscle fibres (121).

5. VITAMIN D STATUS AND RESPIRATORY INFECTION

5.1 Vitamin D status and respiratory infection in the general population

Several cross-sectional and cohort studies have reported a negative association between vitamin D status and respiratory infection incidence. In the National Health, Nutrition and Examination Survey involving 18, 883 participants above 12 years, those with circulating 25(OH)D < 25 nmol/L were 1.4 times more likely to report recent URI compared to those with 25(OH)D ≥ 75 nmol/L, even after adjusting for demographics and clinical data (season, body mass index, smoking history, asthma and chronic obstructive pulmonary disease) (44). The proportion of participants who had a self-reported URI was also significantly different between vitamin D groups (24% in those with circulating 25(OH)D levels < 25 nmol/L vs. 20% with levels of 25-75 nmol/L *vs.* 17% with levels of ≥ 75 nmol/L) (44). In a cohort study over 3.5 months in 198 healthy adults, there was a significant inverse association between circulating 25(OH)D concentration and risk of acute viral respiratory tract infection (45% in those with 25(OH)D < 95 nmol/L *vs.* 17% in those with circulating 25(OH)D ≥ 95 nmol/L). Circulating 25(OH)D > 95 nmol/L was also associated with a significant two-fold reduction in the risk of developing acute respiratory tract infections (119). The main strength of the study was that infection was confirmed by determination of pathogens in swabs collected from participants who exhibited symptoms of respiratory tract infection. Furthermore, in a nationwide study involving 6, 789 middle-aged British adults, 12% of those with circulating 25(OH)D < 25 nmol/L had a respiratory infection in the month prior to blood sampling compared to 6% in those with 25(OH)D > 100 nmol/L. Circulating 25(OH)D was inversely associated with risk of acute respiratory infection even after taking into account lifestyle and socio-economic factors. Each 10 nmol/L increase in circulating 25(OH)D significantly reduced the risk of self-reported acute respiratory infection by 7% (16). Hence, these population-wide studies indicate an inverse rela-

tionship between circulating 25(OH)D and the incidence of URI.

5.2 Vitamin D status and respiratory infection in military personnel and athletes

In 756 young Finnish conscripts who were starting military training during the summer time (July), 4% had low circulating 25(OH)D concentrations (stated by the authors as < 40 nmol/L). Although only a minority showed such low levels of circulating 25(OH)D, this group had significantly more duty days lost due to respiratory infection during the following 6 months of training to January (median: 4 *vs.* 2 days) than those with circulating 25(OH)D > 40 nmol/L. Those with low 25(OH)D were also 1.6 times more likely to miss duty due to respiratory infection (76). However, the study only measured circulating 25(OH)D at the start of military training and thus failed to account for any changes in 25(OH)D during the training period that might have influenced respiratory infection incidence.

Studies in athletic populations have yielded similar findings. Vitamin D status was assessed in a group of elite athletes who reported to a physician with URI symptoms. Athletes who had positive virology/bacteriology results (infectious group; mean ± SD circulating 25(OH)D 79 ± 164 nmol/L) or had mild to moderate leukocytosis (suggestive group; circulating 25(OH)D 77 ± 95 nmol/L) had significantly lower circulating 25(OH)D levels than those who had negative virology/bacteriology results and normal differential leukocyte counts (unknown group; 168 ± 251 nmol/L) (36). Irrespective of the high SDs reported, which suggest large between-participant variability in circulating vitamin D levels, the vitamin D level in the infectious and suggestive groups appear relatively high (means > 75 nmol/L): it's unclear if this finding can be explained by the assay used to determine circulating 25(OH)D as the assay method is not mentioned. In another group of endurance athletes, a significantly greater proportion of those with circulating 25(OH)D < 30 nmol/L presented with URI symptoms than those with 25(OH)D > 120 nmol/L (56). Furthermore, the total number of URI symptom days and the median symptom-severity score in athletes with circulating 25(OH)D < 30 nmol/L was significantly higher than those with 25(OH)D > 120 nmol/L (56).

In summary, though causality cannot be established from cross-sectional comparisons, studies in military personnel and athletes agree with the large general population studies (that used powerful logistical regression techniques to identify contributing factors to URI) by showing an inverse relationship between circulating 25(OH)D and the incidence of URI.

5.3 Is there an optimal vitamin D status to prevent respiratory infections?

As mentioned previously, circulating 25(OH)D is recommended to be > 50 nmol/L for optimum bone health as this represents the level that reduces circulating PTH to a minimum and allows maximum calcium absorption (66). Nonetheless, Chapuy et al. (28) reported an inverse relationship between circulating levels of 25(OH)D and PTH up to 75 nmol/L, at which point the decrease in PTH in response to increasing 25(OH)D levelled out. As such, many experts now agree that a circulating 25(OH)D concentration of at least 75 nmol/L is desirable (4, 20, 64, 109). In accordance with this recommendation, one large scale study involving 14, 108 participants over 16 years of age (NHANES, 2001–2006) supports the proposed circulating 25(OH)D cut-off level of 75 nmol/L for the prevention of respiratory infection as there was a near linear inverse relationship between circulating 25(OH)D levels and the cumulative frequency of acute respiratory infection up to 25(OH)D levels ~75 nmol/L (97). Interestingly, in another study, a partition analysis determined that a circulating 25(OH)D cut-off level of 95 nmol/L best discriminated between groups that did or did not develop viral infections and it has been reported that adults with 25(OH)D status < 95 nmol/L had a significant two-fold increase in the risk of developing acute respiratory infection during winter months compared with those whose 25(OH)D status was > 95 nmol/L (119). Therefore, the optimal circulating 25(OH)D level required to prevent URI in athletes and military personnel has yet to be determined, but based on the limited evidence available, is likely to be 75 nmol/L or possibly higher (e.g. 95 nmol/L). Continued research using randomised-controlled trials of vitamin D supplementation (see the next section) is required to substantiate the purported 75 nmol/L cut-off for circulating 25(OH)D to prevent URI in athletes and military personnel.

6. THE EFFECTS OF ORAL VITAMIN D SUPPLEMENTATION AND UVB IRRADIATION ON VITAMIN D STATUS, IMMUNITY AND RESPIRATORY INFECTION

As described in section 5, a consistent observation in the extant literature is that vitamin D insufficiency is associated with increased URI incidence and symptom duration. Therefore, adopting strategies to avoid vitamin D insufficiency e.g. taking a daily oral vitamin D supplement during the winter and, where possible, practising safe summer sunlight exposure is important to optimise vitamin D status and defence against URI. The information covered in this section considered alongside the sections that follow on factors affecting vitamin D status (section 7) and toxicity (section 8) will form the backdrop for the closing section on simple recommendations to optimise vitamin D status and immune health for athletes and military personnel (section 10).

6.1 The effects of oral vitamin D supplementation on vitamin D status, immunity and respiratory infection

Although vitamin D_2 and D_3 are available as oral supplements, vitamin D_3 supplementation is more commonly used as it has a greater efficacy in raising circulating 25(OH)D compared to vitamin D_2 (65). Current evidence (Table 4) indicates that oral vitamin D supplementation enhances innate responses to mycobacterial infection (specifically, *Mycobacterium bovis* in the BCG-lux assay) (93) and increases circulating levels of the AMP cathelicidin (17, 55). A shift towards an anti-inflammatory cytokine profile (91, 125) and an increase in circulating regulatory T cells (111) has also been demonstrated with oral vitamin D supplementation. Nevertheless, there are weaknesses with some of these studies that limit the interpretation in terms of the

Table 4. Summary of evidence regarding the effects of oral vitamin D supplementation on immune function.

Study design	Population[1]	Season or month	Supplementation	Change in circulating 25(OH)D concentration[2] (pre to post, nmol/L)	Immune outcome	Ref
UT	25 healthy adults, 39 years	-	50,000 IU Vit D_2 every other day for 5 days	Vit D group: ↑ 62 from pre < 80	Group with largest increase in plasma 25(OH)D (80 - 160 nmol/L) showed increase in plasma cathelicidin	Bhan et al.(17)
RCT DB	39 athletes, 20 years	Winter	5,000 IU Vit D_3 or placebo daily for 14 weeks	Vit D group: ↑ 55 to 126 Placebo group: ↓ 57 to 33	Vit D group: Plasma cathelicidin ↑ 15% Placebo group: Plasma cathelicidin ↓ 9%	Heet al.(55)
RCT DB	131 healthy adults, 25 - 45 years	Winter and Spring	Single dose of 100,000 IU Vit D_2 or placebo at baseline	Vit D group: ↑ 35 to 67 Placebo group: -	Vit D group: Ability of whole blood to restrict BCG-lux luminescence ↑ 20% compared to placebo No difference in whole blood antigen-stimulated IFN-γ secretion	Martineau et al.(93)
RCT DB	39 MS patients	-	1,000 IU Vit D_3 and 800 mg calcium or placebo daily for 6 months	Vit D group: ↑ 42 to 70 Placebo group: -	Vit D group: TGF-ß1 levels ↑ 28% Placebo group: No effect	Mahon et al.(91)
UT	46 healthy adults, 31 years	Feb to Jun	140,000 IU of Vit D_3 at baseline and week 4	Vit D group: ↑ 60 to 145	Vit D group: % Tregs↑ 17%	Prietlet al.(111)
UT	15 MS patients	Oct to Dec	20,000 IU Vit D_3 daily for 3 months	Vit D group: ↑ 50 to 380 (median)	Vit D group: Proportion of IL-10^+ $CD4^+$ T cells ↑ 92% Ratio between IFN-γ^+ and IL-4^+ $CD4^+$ T cells ↓ 19%	Smolders et al.(125)

[1]Mean, median or range is provided for age as reported.
[2]Mean values are reported unless stated otherwise.
Hyphen '-' indicates not reported. RCT = Randomised controlled trial. DB = Double-blinded. UT = Uncontrolled trial. BCG = Bacille Calmette Guérin, a vaccine against tuberculosis, prepared from a strain of *Mycobacterium bovis*. IFN-γ = Interferon gamma. MS = Multiple sclerosis.

Table 5. Summary of evidence regarding the effects of oral vitamin D supplementation on self-reported URI.

Study design	Population[1]	Season or month	Supplementation	Change in circulating 25(OH)D[2] (pre to post, nmol/L)	URI outcome	Sig.	Ref
RCT DB	162 healthy adults, 18 - 80 years	Winter	2,000 IU Vit D_3 or placebo daily for 3 months	Vit D group: ↑ 64 to 89 Placebo group: ↓ 63 to 61	Episodes per group Vit D group: 48 Placebo group: 50 Symptom duration (days) Vit D group: 5 Placebo group: 5	NS NS	Li-Ng et al.(82)
RCT DB	164 young Finnish conscripts, 18 - 28 years	Autumn and Winter	400 IU Vit D_3 or placebo daily for 6 months	Vit D group: ↓ 79 to 72 Placebo group: ↓ 74 to 51	Symptom incidence (%, Vit D *vs*. Placebo) Cough 65 *vs*. 57 Runny nose 74 *vs*. 75 Sore throat 48 *vs*. 45 Fever 31 *vs*. 38 Absent from duty due to URI (days) Vit D group: 2 Placebo group: 3	NS NS NS NS NS	Laaksi et al.(75)
RCT DB	322 healthy adults in New Zealand, 47 years	Feb to Nov	Initial dose of 200,000 IU Vit D_3, 200,000 IU a month later, subsequently 100,000 IU monthly or placebo for 18 months	Vit D group: ↑ 72 to > 120 Placebo group: ↓ 70 to < 50	Episodes per person Vit D group: 4 Placebo group: 4 Missed work due to URI (days) Vit D group: 1 Placebo group: 1 Symptom duration (days) Vit D group: 12 Placebo group: 12	NS NS NS	Murdoch et al.(103)

[1]Average age or age range provided where reported.
[2]Mean values are reported unless stated otherwise.
RCT = Randomised controlled trial. DB = Double-blinded. NS = Non-significant.

influence of vitamin D supplementation on immunity; including, the lack of experimental control (111, 125) and co-supplementation with calcium (91). In addition, a few of these studies were conducted using Multiple Sclerosis patients (91, 125), an autoimmune disease characterised by an inflammatory profile, and there is a large discrepancy amongst studies with regards the oral vitamin D dosing regimens (Table 4). As such, more randomised-controlled trials are needed in young, healthy athletic populations to confirm these findings.

Currently, there is little evidence to support vitamin D supplementation to reduce URI incidence and duration (Table 5). Three randomised-controlled trials showed no difference between oral vitamin D supplementation and placebo for URI incidence and duration (75, 82, 103). This is despite the fact that vitamin D supplementation increased circulating 25(OH)D compared to the placebo. The lack of an observed effect of oral vitamin D supplementation on URI in these studies may be due to participants having relatively high baseline vitamin D levels at the outset (baseline circulating 25(OH)D levels range from 64 to 79 nmol/L). Indeed, it has been suggested that boosting the 25(OH)D level in those with vitamin D deficiency (circulating 25(OH)D level < 30 nmol/L) activates various innate and adaptive immune responses that are critical in the control of some respiratory viral infections; however, boosting from a higher starting level of 25(OH)D probably provides no additional benefit (22). Randomised-control trials of oral vitamin D supplementation are sorely needed in athletes and military personnel around the winter-time nadir in circulating 25(OH)D level when evidence indicates vitamin D deficiency in up to 35% of individuals (Table 2). Moreover, for convenience, rather than confirming the presence of pathogens in oral/nasopharyngeal swabs, studies have tended to rely on self-report of URI using common-cold symptom questionnaires that have been criticised (36). Notwithstanding this limitation, symptoms of URI without a detectable pathogen appear to be common in those under heavy training stress (36) and likely have a negative impact upon training and performance (113).

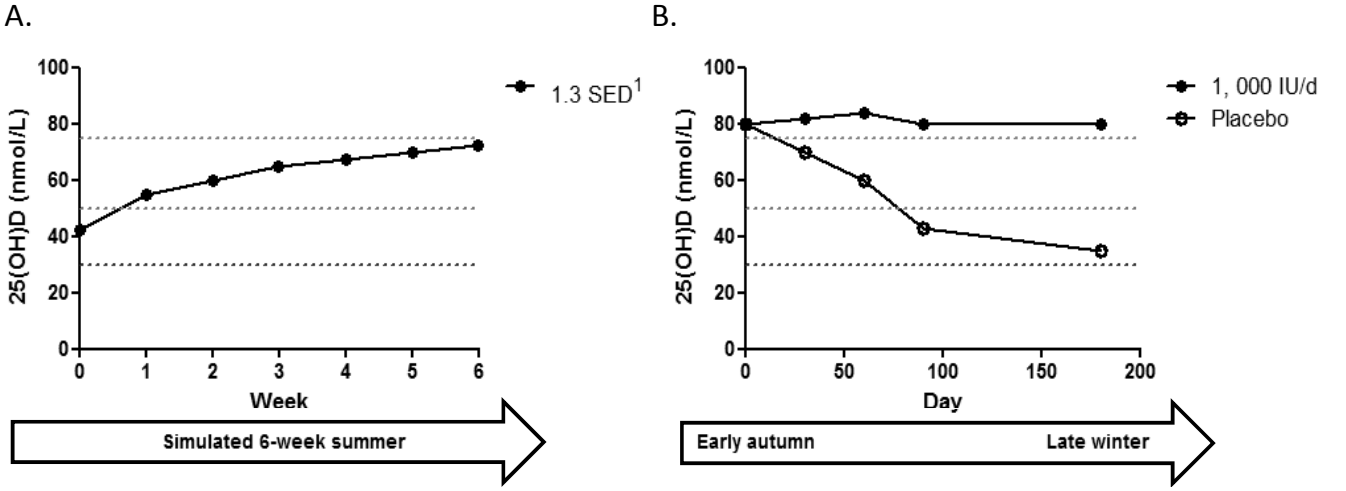


Figure 5. (A) Impact of simulated summer sunlight exposures on circulating 25(OH)D, adapted from Rhodes et al. (114). White adult Caucasians from Greater Manchester, UK (53.5 °N) received simulated summer sunlight exposures, specifically 1.3 standard erythemal dose (SED), three times weekly for 6 weeks, while wearing T-shirt and shorts. **(B)** Influence of daily 1, 000 IU oral vitamin D_3 supplementation (close circle) or placebo (open circle) for 6 months from early autumn to late winter in adults living in New Zealand (46 °S), adapted from Logan et al. (86). Dotted horizontal lines indicate Institute of Medicine thresholds for deficiency (30 nmol/L) and sufficiency (50 nmol/L) and the proposed optimal threshold (75 nmol/L) for circulating 25(OH)D to prevent URI (97). [1]To avoid the confounding influence of skin exposure to ambient UVB the study commenced in wintertime (53.5 °N) when sunlight is negligible and circulating 25(OH)D is at its nadir.

6.2 The effects of UVB irradiation on vitamin D status and immunity

As introduced in section 1.1, for a range of skin colours and latitudes between 30 and 60 °N, the majority of vitamin D can be obtained through short-lasting skin exposures to natural UVB irradiation from summer sunlight (~15 min each day). Importantly, prolonged exposures give diminishing returns in terms of vitamin D formation (61) and raise the risk of erythema (i.e. sunburn) for fairer skin types (140) and skin cancer (10). For example, a single minimum erythemal dose (MED: the minimum amount of sunlight that burns the skin) typically provides an oral equivalent vitamin D dose of 10, 000 to 25, 000 IU (140). As such, experts recommend short, frequent exposures to a standard erythemal dose (SED: equates to ~ ¼ to ½ MED for the white UK population) in shorts and t-shirt that does not burn the skin and provides the oral equivalent vitamin D dose of ~1, 000 IU (Figure 5). To ensure safety and efficacy, exposing a large surface area over a shorter duration as opposed to a small surface area for prolonged periods is recommended to increase circulating levels of vitamin D. For example, full body exposure for 2 min is preferable to over-exposing a (bald) head and neck for 20 min (139).

It remains unknown if UVB exposure of the skin (either from sunlight or a sun cabinet) has additional benefits on immune function, health and performance independent of the synthesis of vitamin D. For example, UV radiation generates nitric oxide locally at the skin which has been associated with benefits to cardiovascular health via a decrease in systemic blood pressure (67). In addition to its effects on vasodilatation, nitric oxide may also influence neurotransmission, immune defence, regulation of cell death (apoptosis) and cell motility (67). The potential for mood enhancement (possibly mediated via increased β-endorphins) and stress reduction with skin sunlight or artificial UVB exposure to influence immune function, health and performance should not be overlooked. Indeed, it's possible, but remains unknown, that UVB exposure of the skin improves immune function and exercise performance to a greater extent than the equivalent oral vitamin D supplementation due to enhanced levels of nitric oxide, mood or some as yet unknown mechanism.

A method to replicate safe summer sunlight exposure using sub-erythemal solar simulated radiation in a laboratory-based irradiation cabinet has been developed by Rhodes and colleagues (Figure 5A) (114). During the winter months, when

vitamin D status was low, this method restored adequate circulating levels of vitamin D (25(OH)D level > 50 nmol/L) in the majority of volunteers. Importantly, in line with policy recommendations in the UK, this method simulates summer sunlight exposure (at latitudes between 30 and 60 °N for most skin types), on several occasions each week, for ~15 min wearing t-shirt and shorts without producing sunburn (140). Whether this method to restore adequate circulating vitamin D in the winter improves immunity and host defence remains largely unknown and requires investigation. Promising work shows a threefold increase in circulating Tregs ($CD4+CD25^{hi}FoxP3^{+}$) that correlated positively with the change in circulating 25(OH)D in patients with immune-mediated skin disease (e.g. psoriasis) after 4 weeks of phototherapy treatment (96).

7. FACTORS AFFECTING VITAMIN D STATUS IN ATHLETES AND MILITARY PERSONNEL

Vitamin D can be increased through natural food sources or the exposure of skin to UVB radiation. The vitamin D production in the skin from sufficient UVB exposure provides 80-100% of body requirements (78). In particular, several factors can affect the production of vitamin D via UVB exposure, these include season and latitude as well as age, skin colour, clothing and sunscreen use.

7.1 Season and latitude

The solar zenith angle (SZA) is the angle between the local vertical and the position of the sun in the sky. During the summer and at low latitudes the SZA is small. Conversely, the SZA is large during the winter and at high latitudes. At a large SZA, UVB radiation (290 – 320 nm) travels a longer path through the atmosphere and there is greater attenuation of the radiation compared to a small SZA (139). Consequently, the amount of UVB radiation reaching the Earth's surface is reduced and scattered over a larger area (139). This explains why vitamin D deficiency is more prevalent in countries at high latitudes and particularly during the winter months (43). Furthermore, it has been estimated that the contribution of sunlight to vitamin D status is only 20% during the winter (88) and 80% (89) during the spring and summer thus suggesting the importance of the increase in the contribution of diet during winter in order to prevent vitamin D deficiency.

7.2 Age

As one ages, cutaneous vitamin D production declines. This is due to the decrease in the amount of 7-dehydrocholesterol available in the epidermal layer of the skin, where the majority of vitamin D is formed following exposure to UVB radiation (90). Despite the decline in vitamin D_3 production with age, the elderly can still achieve adequate amounts of vitamin D in the summer through regular skin exposure to sunlight (141) (section 6.2). Nonetheless, older athletes living in the northern latitude may wear more clothing and train mostly indoors. As regular skin sunlight exposure is limited, this group of elderly people is at a greater risk of vitamin D deficiency. Therefore, a combination of regular sun exposure where possible and increased vitamin D intake from the daily diet and oral supplementation are important considerations to ensure that older athletes have adequate vitamin D.

7.3 Skin colour

The amount of melanin pigment in the skin can interfere with vitamin D synthesis by absorbing UVB radiation and blocking the wavelength of sunlight required to synthesise vitamin D (89), thus preventing the cutaneous production of pre-vitamin D_3. Melanin content is higher in dark-skinned individuals compared to fair-skinned individuals. Hence, for a given dose of UVB, a dark-skinned individual will produce less pre-vitamin D_3 than a fair-skinned individual (30). An analysis of the vitamin D status in 63 elite UK track and field athletes reported 7% of dark-skinned athletes with 25(OH)D < 50 nmol/L compared to only 1% of fair-skinned athletes during the summer (110). When individuals were given UVB doses adjusted for their skin colour, it was discovered that there was a tendency for dark-skinned individuals to show a smaller increase in 25(OH)D (9). Nonetheless, it should be noted that, dark-skinned individuals can produce equivalent amounts of vitamin D_3 as their fair-skinned counterparts when exposed to adequate amounts of UVB radiation (30).

7.4 Clothing

Clothing can act as a physical barrier preventing UVB radiation from reaching the skin. As the majority of vitamin D is synthesised in the skin, any area covered by clothing will reduce the exposed skin surface area to sunlight. Broadly speaking, in terms of vitamin D synthesis, there is an inverse relationship between the surface area of the skin exposed to sunlight and the duration of exposure. To illustrate this relationship, a fully clothed person with the head and neck exposed for 20 min would synthesise an equivalent amount of vitamin D to exposing the whole body for 2 min (139). In the summer, athletes who train and compete for prolonged periods in short-sleeved tops and shorts may not receive adequate sun protection. In contrast, military recruits who train in long-sleeved uniform and wear helmets (and those who train indoors or who cover their skin for religious reasons) are at risk of a lack of sun exposure and vitamin D deficiency. In these groups at risk of vitamin D deficiency, alternative methods to increase vitamin D levels e.g. solar-simulated radiation or oral supplementation warrant investigation (see recommendations in section 10).

7.5 Sunscreen use

The use of sunscreen interferes with vitamin D_3 formation by absorbing and reflecting UVB radiation, thus preventing UVB radiation from reaching the target skin cells. Topical application of a sunscreen of sun protection factor 8 was found to limit vitamin D_3 production in protected compared to unprotected participants (94). Although sunscreen use can be beneficial in preventing sunburn and skin cancer, it should be used appropriately. For example, in the summer, athletes exposed to UVB radiation for prolonged periods are at increased risk of sunburn and should be encouraged to apply a broad-spectrum water-resistant sunscreen of at least SPF 30-50 every 2 to 4 hours (53). On the other hand, the elderly have a reduced ability to synthesise vitamin D cutaneously and are at greater risk of vitamin D deficiency. It has been recommended that they expose their hands, face, arms and legs to summer sun-

Table 6. Dietary sources of vitamin D[1].

Sources	Vitamin D content
Natural foods	
Cod liver oil	~ 400 - 1000 IU/teaspoon vitamin D_3
Salmon (fresh, wild)	~ 600 - 1000 IU/100 g vitamin D_3
Salmon (fresh, farmed)	~ 100 - 250 IU/100 g vitamin D_3
Salmon (canned)	~ 300 - 600 IU/100 g vitamin D_3
Sardines (canned)	~ 300 IU/100 g vitamin D_3
Mackerel (canned)	~ 250 IU/100 g vitamin D_3
Tuna (canned)	~ 230 IU/100 g vitamin D_3
Herring in oil	~ 800 IU/100 g vitamin D_3
Pickled herring	~ 480 IU/100 g vitamin D_3
Shiitake mushrooms (fresh)	~ 100 IU/100 g vitamin D_2
Shiitake mushrooms (dried)	~ 1600 IU/100 g vitamin D_2
Egg yolk	~ 20 - 50 IU/yolk vitamin D_3
Cheese	~ 7 - 28 IU/100 g vitamin D_3
Cow's milk	~ 0.4 - 1.2 IU/100 ml vitamin D_3
Fortified foods[2]	
Fortified milk	~ 100 IU/237 ml vitamin D_3
Fortified orange juice	~ 100 IU/237 ml vitamin D_3
Fortified yoghurts	~ 100 IU/237 ml vitamin D_3
Fortified butter	~ 50 IU/100 g vitamin D_3
Fortified margarine	~ 430 IU/100 g vitamin D_3
Fortified cheeses	~ 100 IU/85 g vitamin D_3
Fortified breakfast cereals	~ 100 IU/30 g vitamin D_3

[1]Adapted from Holick et al. (63) and Pludowski et al. (109).
[2]Countries with fortification policies include Australia, Finland, UK and the USA (77, 89, 98, 104).
IU denotes international unit. 1 IU is equivalent to 0.025 μg. To convert μg to IU multiply by 40.

light two to three times a week for only ~¼ of the duration that will take for them to reach mid sunburn and apply SPF ≥ 15 on all exposed skin for any further time spent outdoors (61, 62). This will allow the elderly to obtain the beneficial effects of sunlight for vitamin D nutrition whilst avoiding the detrimental effects of overexposure.

7.6 Natural food sources

As mentioned earlier, vitamin D can be obtained from the diet (Table 6) by consuming foods such as oily fish (e.g. tuna, mackerel, salmon), shiitake mushrooms and egg yolks (63). Interestingly, an analysis of the vitamin D_3 content in a variety of oily fish showed that farmed salmon contains only 25% of the vitamin D_3 found in wild caught Alaskan salmon (30), suggesting that wild-type fish are a better source of vitamin D_3 than farmed varieties. In countries such as America and Canada, some foods such as milk, breakfast cereals and margarine are also fortified with vitamin D in order to increase vitamin D intake (23) (Table 6).

8. VITAMIN D TOXICITY

Excessive intake of vitamin D can result in vitamin D intoxication, which is characterised by hypercalcaemia (total serum calcium corrected for albumin > 2.6 mmol/L), renal stones and renal calcification, with kidney failure and death (57). Except for infrequent cases of accidental or intentional poisoning, this is extremely rare. Both the intoxication literature and several controlled dosing studies show no cases of confirmed intoxication at circulating 25(OH)D levels below 500 nmol/L. Correspondingly, the oral intakes needed to produce such levels are in excess of 20, 000 IU/day in otherwise healthy adults and 10, 000 IU/day (which is substantially more than is apparently needed for any recognised efficacy endpoint) is considered as the tolerable upper intake level (57). Incidentally, it is worth noting that whole-body skin sun exposure, such as might be achieved in a few minutes on a summer day, produces an endogenous vitamin D production of 10, 000 to 20, 000 IU, depending upon skin type (9). Thus, frequent summer sun exposures produce inputs of the same magnitude as the proposed upper intake level (can be characterised as a "physiological") and there has never been a case of vitamin D intoxication reported as a result of sun exposure.

The toxicity of intakes of high oral doses of vitamin D has been established on the basis of relatively short-term studies and there has to be some concern about the longer-term implications for health of high vitamin D intakes over a lifetime. The IoM indicates that sparse data are available for upper circulating 25(OH)D levels in humans, and values above 125-150 nmol/L should raise concerns about potential adverse effects because of several large scale studies indicating an increased multivariable-adjusted risk of all-cause mortality not only for circulating 25(OH)D levels below 30 nmol/L, but also for levels above 125 nmol/L (66). The all-cause mortality data emerging from the examination of national survey data as well as observational studies suggest adverse effects at circulating 25(OH)D levels much lower than those associated with the toxicity demonstrated by short-term acute hypervitaminosis D. In general, these studies, as expected, indicated that low circulating 25(OH)D levels akin to < 30 nmol/L are associated with an increased risk of mortality. Furthermore, as circulating 25(OH)D levels increase up to a point mortality is lowered. However, some, but not all, of the studies have observed a troubling U-shaped relationship with a statistically significant trend between increasing circulating 25(OH)D levels and lower odds ratios for all-cause mortality. For these reasons, a circulating 25(OH)D of above 125-150 nmol/L is not recommended, corresponding to intakes of not more than 5, 000 IU/day in the absence of adequate sun exposure.

9. CONCLUSIONS

A multitude of studies have suggested that vitamin D deficiency (circulating 25(OH)D level < 30 nmol/L) not only has negative consequences on bone health but also increases the risk for many acute and chronic illnesses, including respiratory infections. Recent work in athletes shows beneficial effects of optimising vitamin D status on innate immunity and mucosal immunity and vitamin D exerts anti-inflammatory actions through the induction of regulatory T cells and the inhibition of pro-inflammatory cytokine production. Although the incidence of vitamin D insufficiency (circulating 25(OH)D level < 50 nmol/L) appears to be similar in athletic and non-athletic populations, studies show that more than half of all athletes and military personnel are vitamin D insufficient in the winter months and as many as 35% are vitamin D deficient. To date,

studies point to the benefits of avoiding vitamin D deficiency to maintain immunity and reduce the burden of URI in athletes and military personnel. In answer to the question posed in the title of this review, there is broad agreement that a circulating 25(OH)D level of 75 nmol/L represents an optimal vitamin D status for the prevention of URI (97). Fruitful future lines of enquiry include verification of this proposed optimal vitamin D status to maintain immunity and resistance against URI in athletes and military personnel. In addition, investigations should explore whether UVB exposure of the skin (either from sunlight or an irradiation cabinet) has additional benefits on immune function, health and exercise performance independent of the synthesis of vitamin D.

10. PRACTICAL RECOMMENDATIONS (FIGURE 6)

In Figure 6 we attempt to provide some simple practical recommendations for athletes and military personnel on how vitamin D sufficiency can be achieved in the summer and maintained during the winter. Mindful of key factors such as latitude and skin type, as little as 15 min of exposure to summer sunlight between 10am and 3pm wearing t-shirts and shorts on most days can achieve vitamin D sufficiency in most individuals and levels deemed optimal in some (Figure 5A and 6) (114, 140). Dietary sources of vitamin D and vitamin D supplements become important considerations during the winter months when skin sunlight as a source of vitamin D is absent or drastically reduced (Figure 6). Studies have shown that consuming a 1, 000 IU/day vitamin D_3 supplement during the winter can achieve vitamin D sufficiency in most individuals (86, 89) and maintain end-of-summer 25(OH)D levels throughout the autumn and winter (Figure 5B) (86). Finally, the recommendation to take a 1, 000 IU/day vitamin D_3 supplement in the autumn-winter may also be suitable for those who cannot achieve the safe summer sunlight guidance (Figure 6). For example, individuals training indoors in the summer or those required to wear clothing (for protective or religious reasons) that restricts skin sunlight exposure in the summer may benefit from 1, 000 IU/day vitamin D_3 supplementation year-round as there is evidence that these individuals can suffer vitamin D deficiency, even during the summer months (Table 2) (51, 52). Further research endeavours are required to determine whether following these recommendations for vitamin D benefit athletes and military personnel by maintaining immunity and increasing resistance against URI.

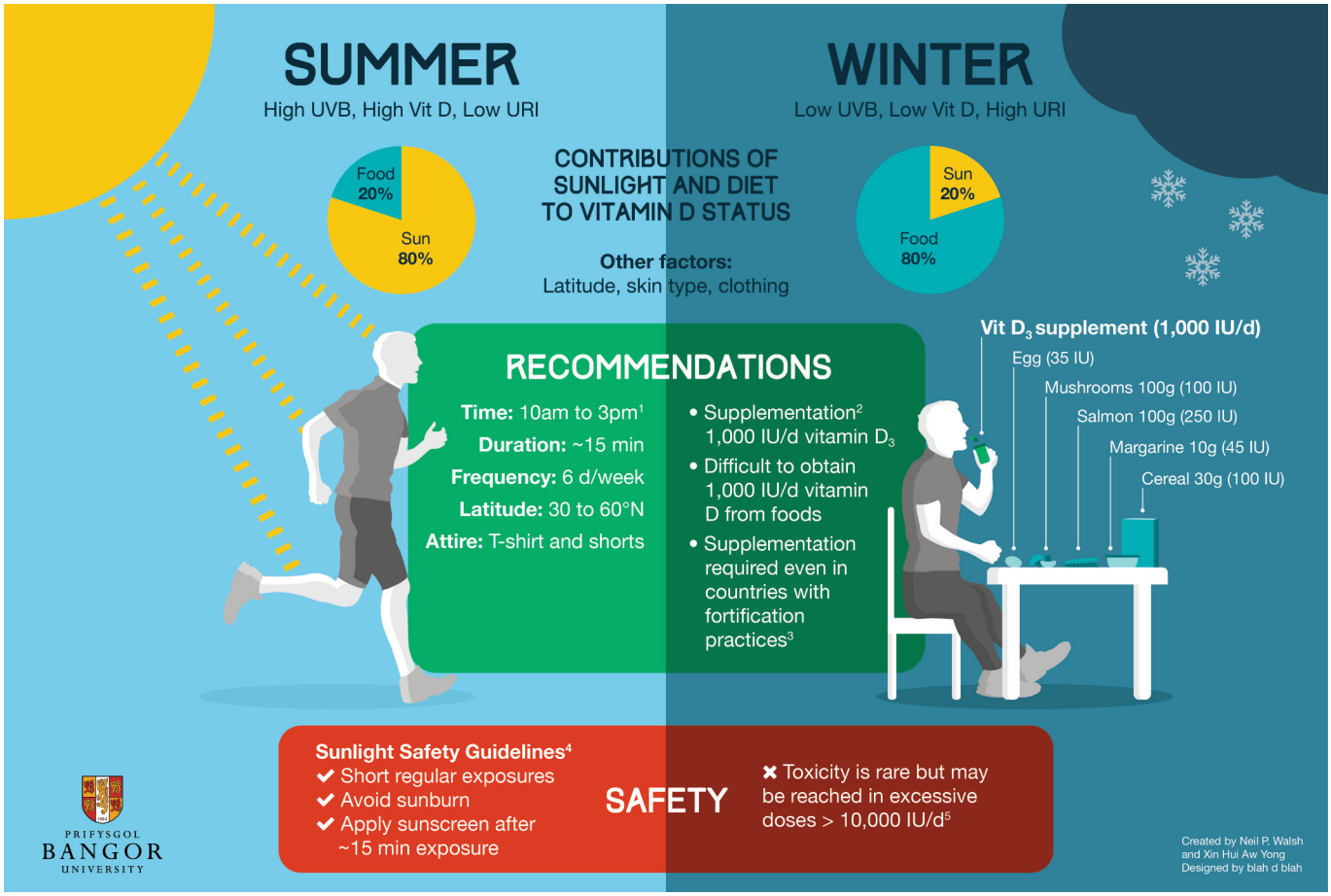


Figure 6. Practical recommendations for athletes and military personnel on how vitamin D sufficiency can be achieved in the summer and maintained during the winter. [1]Recommended summer sunlight exposure (114, 140). [2]Recommended oral vitamin D_3 supplementation (86, 89). [3]Countries with fortification policies include Australia, Finland, UK and the USA (77, 89, 98, 104) but average dietary vitamin D intake remains below 1, 000 IU/day. [4]Sunlight safety recommendations (139, 140). [5]Safety recommendations for oral vitamin D supplementation (57). UVB = ultraviolet-B. URI = upper respiratory illness.

REFERENCES

1. Adorini L and Penna G. Dendritic cell tolerogenicity: a key mechanism in immunomodulation by vitamin D receptor agonists. Hum Immunol 70: 345-352, 2009.
2. Agborsangaya CB, Surcel HM, Toriola AT, Pukkala E, Parkkila S, Tuohimaa P, Lukanova A and Lehtinen M. Serum 25-hydroxyvitamin D at pregnancy and risk of breast cancer in a prospective study. Eur J Cancer 46: 467-470, 2010.
3. Al-Jaderi Z and Maghazachi AA. Effects of vitamin D_3, calcipotriol and FTY720 on the expression of surface molecules and cytolytic activities of human natural killer cells and dendritic cells. Toxins 5: 1932-1947, 2013.
4. Alshahrani F and Aljohani N. Vitamin D: deficiency, sufficiency and toxicity. Nutrients 5: 3605-3616, 2013.
5. Ameri P, Giusti A, Boschetti M, Murialdo G, Minuto F and Ferone D. Interactions between vitamin D and IGF-1: from physiology to clinical practice. Clin Endocrinol 79: 457-463, 2013.
6. Angeline ME, Gee AO, Shindle M, Warren RF and Rodeo SA. The effects of vitamin D deficiency in athletes. Am J Sports Med 41: 461-464, 2013.
7. Antoniucci DM, Black DM and Sellmeyer DE. Serum 25-hydroxyvitamin D is unaffected by multiple freeze-thaw cycles. Clin Chem 51: 258-261, 2005.
8. Aranow C. Vitamin D and the immune system. J Invest Med 59: 881-886, 2011.
9. Armas LA, Dowell S, Akhter M, Duthuluru S, Huerter C, Hollis BW, Lund R and Heaney RP. Ultraviolet-B radiation increases serum 25-hydroxyvitamin D levels: the effect of UVB dose and skin color. J Am Acad Dermatol 57: 588-593, 2007.
10. Armstrong BK and Kricker A. The epidemiology of UV induced skin cancer. J Photochem Photobiol B 63: 8-18, 2001.
11. Baeke F, Takiishi T, Korf H, Gysemans C and Mathieu C. Vitamin D: modulator of the immune system. Curr Opin Pharmacol 10: 482-496, 2010.
12. Bannert N, Starke I, Mohnike K and Frohner G. Parameters of mineral metabolism in children and adolescents in athletic training. Kinderarztl Prax 59: 153-156, 1991.
13. Barker T, Henriksen VT, Martins TB, Hill HR, Kjeldsberg CR, Schneider ED, Dixon BM and Weaver LK. Higher serum 25-hydroxyvitamin D concentrations associate with a faster recovery of skeletal muscle strength after muscular injury. Nutrients 5: 1253-1275, 2013.
14. Barker T, Martins TB, Hill HR, Kjeldsberg CR, Trawick RH, Weaver LK and Traber MG. Low vitamin D impairs strength recovery after anterior cruciate ligament surgery. J Evid Based Complementary Altern Med 16: 201-209, 2011.
15. Bendik I, Friedel A, Roos FF, Weber P and Eggersdorfer M. Vitamin D: a critical and essential micronutrient for human health. Front Physiol 5: 248, 2014.
16. Berry DJ, Hesketh K, Power C and Hyppönen E. Vitamin D status has a linear association with seasonal infections and lung function in British adults. Br J Nutr 106: 1433-1440, 2011.
17. Bhan I, Camargo CA, Wenger J, Ricciardi C, Ye J, Borregaard N and Thadhani R. Circulating levels of 25-hydroxyvitamin D and human cathelicidin in healthy adults. J Allergy Clin Immunol 127: 1302-1304, 2011.
18. Bikle D. Nonclassic actions of vitamin D. J Clin Endocrinol Metab 94: 26-34, 2009.
19. Binder R, Kress A, Kan G, Herrmann K and Kirschfink M. Neutrophil priming by cytokines and vitamin D binding protein (Gc-globulin): impact on C5a-mediated chemotaxis, degranulation and respiratory burst. Mol Immunol 36: 885-892, 1999.
20. Bischoff-Ferrari HA. Optimal serum 25-hydroxyvitamin D levels for multiple health outcomes. Adv Exp Med Biol 810: 500-525, 2014.
21. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF and O'Garra A. 1, 25-Dihydroxyvitamin D3 has a direct effect on naive CD4+ T cells to enhance the development of Th2 cells. J Immunol 167: 4974-4980, 2001.
22. Bryson KJ, Nash AA and Norval M. Does vitamin D protect against respiratory viral infections? Epidemiol Infect 142: 1789-1801, 2014.
23. Calvo MS, Whiting SJ and Barton CN. Vitamin D fortification in the United States and Canada: current status and data needs. Am J Clin Nutr 80: 1710S-1716S, 2004.
24. Cannell JJ, Vieth R, Umhau JC, Holick MF, Grant WB, Madronich S, Garland CF and Giovannucci E. Epidemic influenza and vitamin D. Epidemiol Infect 134: 1129-1140, 2006.
25. Cantorna MT, Woodward WD, Hayes CE and DeLuca HF. 1, 25-Dihydroxyvitamin D3 is a positive regulator for the two anti-encephalitogenic cytokines TGF-ß and IL-4. J Immunol 160: 5314-5319, 1998.
26. Carter GD and Jones JC. Use of a common standard improves the performance of liquid chromatography-tandem mass spectrometry methods for serum 25-hydroxyvitamin-D. Ann Clin Biochem 46: 79-81, 2009.
27. Chang SH, Chung Y and Dong C. Vitamin D suppresses Th17 cytokine production by inducing C/EBP homologous protein (CHOP) expression. J Biol Chem 285: 38751-38755, 2010.
28. Chapuy MC, Preziosi P, Maamer M, Arnaud S, Galan P, Hercberg S and Meunier PJ. Prevalence of vitamin D insufficiency in an adult normal population. Osteoporosis Int 7: 439-443, 1997.
29. Chen J, Olivares-Navarrete R, Wang Y, Herman TR, Boyan BD and Schwartz Z. Protein-disulfide isomerase-associated 3 (Pdia3) mediates the membrane response to 1, 25-dihydroxyvitamin D3 in osteoblasts. J Biol Chem 285: 37041-37050, 2010.
30. Chen TC, Chimeh F, Lu Z, Mathieu J, Person KS, Zhang A, Kohn N, Martinello S, Berkowitz R and Holick MF. Factors that influence the cutaneous synthesis and dietary sources of vitamin D. Arch Biochem Biophys 460: 213-217, 2007.
31. Chun RF, Liu PT, Modlin RL, Adams JS and Hewison M. Impact of vitamin D on immune function: lessons learned from genome-wide analysis. Front Physiol 5: 151, 2014.
32. Clairmont A, Tessmann D, Stock A, Nicolai S, Stahi W and Sies H. Induction of gap junctional intercellular communication by vitamin D in human skin fibroblasts is dependent on the nuclear vitamin D receptor. Carcinogenesis 17: 1389-1391, 1996.
33. Close GL, Russell J, Cobley JN, Owens DJ, Wilson G, Gregson W, Fraser WD and Morton JP. Assessment of vitamin D concentration in non-supplemented professional athletes and healthy adults during the winter months in the UK: implications for skeletal muscle function. J Sports Sci 31: 344-353, 2013.

34. Close GL, Leckey J, Patterson M, Bradley W, Owens DJ, Fraser WD and Morton JP. The effects of vitamin D3 supplementation on serum total 25 [OH] D concentration and physical performance: a randomised dose-response study. Br J Sports Med 47: 692-696, 2013.
35. Constantini NW, Arieli R, Chodick G and Dubnov-Raz G. High prevalence of vitamin D insufficiency in athletes and dancers. Clin J Sport Med 20: 368-371, 2010.
36. Cox AJ, Gleeson M, Pyne DB, Callister R, Hopkins WG and Fricker PA. Clinical and laboratory evaluation of upper respiratory symptoms in elite athletes. Clin J Sport Med 18: 438-445, 2008.
37. Edfeldt K, Liu PT, Chun R, Fabri M, Schenk M, Wheelwright M, Keegan C, Krutzik SR, Adams JS, Hewison M and Modlin RL. T-cell cytokines differentially control human monocyte antimicrobial responses by regulating vitamin D metabolism. Proc Natl Acad Sci U S A 107: 22593-22598, 2010.
38. Farrell CJ, Martin S, McWhinney B, Straub I, Williams P and Herrmann M. State-of-the-art vitamin D assays: a comparison of automated immunoassays with liquid chromatography-tandem mass spectrometry methods. Clin Chem 58: 531-542, 2012.
39. Fraser WD. Standardization of vitamin D assays: art or science? Ann Clin Biochem 46: 3-4, 2009.
40. Fraser WD and Milan AM. Vitamin D assays: past and present debates, difficulties, and developments. Calcif Tissue Int 92: 118-127, 2013.
41. Funderburk LK, Daigle K and Arsenault JE. Vitamin D status among overweight and obese soldiers. Mil Med 180: 237-240, 2015.
42. Garcia RB and Guisado FR. Low levels of vitamin D in professional basketball players after wintertime: relationship with dietary intake of vitamin D and calcium. Nutr Hosp 26: 945-951, 2011.
43. Genuis SJ, Schwalfenberg GK, Hiltz MN and Vaselenak SA. Vitamin D status of clinical practice populations at higher latitudes: analysis and applications. Int J Environ Res Public Health 6: 151-173, 2009.
44. Ginde AA, Mansbach JM and Camargo CA, Jr. Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. Arch Intern Med 169: 384-390, 2009.
45. Ginde AA, Mansbach JM and Camargo CA, Jr. Vitamin D, respiratory infections, and asthma. Curr Allergy Asthma Rep 9: 81-87, 2009.
46. Girgis CM, Clifton-Bligh RJ, Turner N, Lau SL and Gunton JE. Effects of vitamin D in skeletal muscle: falls, strength, athletic performance and insulin sensitivity. Clin Endocrinol 80: 169-181, 2014.
47. Gniadecki R, Gajkowska B and Hansen M. 1, 25-dihydroxyvitamin D3 stimulates the assembly of adherens junctions in keratinocytes: involvement of protein kinase C. Endocrinology 138: 2241-2248, 1997.
48. Godbout JP and Glaser R. Stress-induced immune dysregulation: implications for wound healing, infectious disease and cancer. J Neuroimmune Pharm 1: 421-427, 2006.
49. Gorman S, Kuritzky LA, Judge MA, Dixon KM, McGlade JP, Mason RS, Finlay-Jones JJ and Hart PH. Topically applied 1, 25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+ CD25+ cells in the draining lymph nodes. J Immunol 179: 6273-6283, 2007.
50. Hamilton B. Vitamin D and human skeletal muscle. Scand J Med Sci Sports 20: 182-190, 2010.
51. Hamilton B, Grantham J, Racinais S and Chalabi H. Vitamin D deficiency is endemic in middle eastern sportsmen. Public Health Nutr 13: 1528-1534, 2010.
52. Hamilton B, Whiteley R, Farooq A and Chalabi H. Vitamin D concentration in 342 professional football players and association with lower limb isokinetic function. J Sci Med Sport 17: 139-143, 2014.
53. Harrison SC and Bergfeld WF. Ultraviolet light and skin cancer in athletes. Sports Health 1: 335-340, 2009.
54. He CS, Fraser WD and Gleeson M. Influence of vitamin d metabolites on plasma cytokine concentrations in endurance sport athletes and on multiantigen stimulated cytokine production by whole blood and peripheral blood mononuclear cell cultures. ISRN Nutr: 820524, 2014.
55. He CS, Gleeson M, Tanqueray E, Rudland-Thomas J, Brown K and Renwick S. The effect of chronic vitamin D3 supplementation on antimicrobial peptides and proteins in athletes. J Sports Sci 32, S6-S7, 2014.
56. He CS, Handzlik M, Fraser WD, Muhamad A, Preston H, Richardson A and Gleeson M. Influence of vitamin D status on respiratory infection incidence and immune function during 4 months of winter training in endurance sport athletes. Exerc Immunol Rev 19: 86-101, 2013.
57. Heaney RP. Vitamin D: criteria for safety and efficacy. Nutr Rev 66: S178-S181, 2008.
58. Hellard P, Avalos M, Guimaraes F, Toussaint JF and Pyne DB. Training-related risk of common illnesses in elite swimmers over a 4-yr period. Med Sci Sports Exerc 47: 698-707, 2015.
59. Hewison M. Vitamin D and immune function: an overview. P Nutr Soc 71: 50-61, 2012.
60. Hewison M. Vitamin D and immune function: autocrine, paracrine or endocrine? Scand J Clin Lab Invest Suppl 72: 92-102, 2012.
61. Holick MF. Environmental factors that influence the cutaneous production of vitamin D. Am J Clin Nutr 61: 638S-645S, 1995.
62. Holick MF. Vitamin D: A millenium perspective. J Cell Biochem 88: 296-307, 2003.
63. Holick MF. Vitamin D deficiency. New Engl J Med 357: 266-281, 2007.
64. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH and Weaver CM. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 96: 1911-1930, 2011.
65. Houghton LA and Vieth R. The case against ergocalciferol (vitamin D2) as a vitamin supplement. Am J Clin Nutr 84: 694-697, 2006.
66. Institute of Medicine (IOM). Dietary reference intakes for calcium and vitamin D. Washington DC: National Academies Press, 2011.
67. Juzeniene A and Moan J. Beneficial effects of UV radiation other than via vitamin D production. Dermatoendocrinol 4: 109-117, 2012.
68. Kamen DL and Tangpricha V. Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. J Mol Med 88: 441-450, 2010.

69. Kankova M, Luini W, Pedrazzoni M, Riganti F, Sironi M, Bottazzi B, Mantovani A and Vecchi A. Impairment of cytokine production in mice fed a vitamin D3-deficient diet. Immunology 73: 466-471, 1991.
70. Khoo AL, Chai LYA, Koenen HJPM, Kullberg BJ, Joosten I, van der Ven AJ and Netea MG. 1, 25-dihydroxyvitamin D3 modulates cytokine production induced by Candida albicans: impact of seasonal variation of immune responses. J Infect Dis 203: 122-130, 2011.
71. Kiebzak GM, Moore NL, Margolis S, Hollis B and Kevorkian CG. Vitamin D status of patients admitted to a hospital rehabilitation unit: relationship to function and progress. Am J Phys Med Rehabil 86: 435-445, 2007.
72. Koeffler HP, Reichel H, Bishop JE and Norman AW. Gamma-interferon stimulates production of 1, 25-dihydroxyvitamin D 3 by normal human macrophages. Biochem Bioph Res Commun 127: 596-603, 1985.
73. Kongsbak M, Levring TB, Geisler C and Von Essen MR. The vitamin D receptor and T cell function. Front Immunol 4: 148, 2013.
74. Kraemer WJ and Ratamess NA. Hormonal responses and adaptations to resistance exercise and training. Sports Med 35: 339-361, 2005.
75. Laaksi I, Ruohola JP, Mattila V, Auvinen A, Ylikomi T and Pihlajamäki H. Vitamin D supplementation for the prevention of acute respiratory tract infection: a randomized, double-blinded trial among young Finnish men. J Infect Dis 202: 809-814, 2010.
76. Laaksi I, Ruohola JP, Tuohimaa P, Auvinen A, Haataja R, Pihlajamäki H and Ylikomi T. An association of serum vitamin D concentrations < 40 nmol/L with acute respiratory tract infection in young Finnish men. Am J Clin Nutr 86: 714-717, 2007.
77. Laaksi IT, Ruohola JS, Ylikomi TJ, Auvinen A, Haataja RI, Pihlajamäki HK and Tuohimaa PJ. Vitamin D fortification as public health policy: significant improvement in vitamin D status in young Finnish men. Eur J Clin Nutr 60: 1035-1038, 2006.
78. Lanteri P, Lombardi G, Colombini A and Banfi G. Vitamin D in exercise: physiologic and analytical concerns. Clin Chim Acta 415: 45-53, 2013.
79. Lappe J, Cullen D, Haynatzki G, Recker R, Ahlf R and Thompson K. Calcium and vitamin D supplementation decreases incidence of stress fractures in female navy recruits. J Bone Miner Res 23: 741-749, 2008.
80. Larson-Meyer DE and Willis KS. Vitamin D and athletes. Curr Sports Med Rep 9: 220-226, 2010.
81. Lemire JM. Immunomodulatory actions of 1, 25-dihydroxyvitamin D 3. J Steroid Biochem Mol Biol 53: 599-602, 1995.
82. Li-Ng M, Aloia JF, Pollack S, Cunha BA, Mikhail M, Yeh J and Berbari N. A randomized controlled trial of vitamin D3 supplementation for the prevention of symptomatic upper respiratory tract infections. Epidemiol Infect 137: 1396-1404, 2009.
83. Liao L, Chen X, Wang S, Parlow AF and Xu J. Steroid receptor coactivator 3 maintains circulating insulin-like growth factor I (IGF-I) by controlling IGF-binding protein 3 expression. Mol Cell Biol 28: 2460-2469, 2008.
84. Liu PT, Schenk M, Walker VP, Dempsey PW, Kanchanapoomi M, Wheelwright M, Vazirnia A, Zhang X, Steinmeyer A and Zugel U. Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. PLoS One 4: e5810, 2009.
85. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schauber J, Wu K, Meinken C, Kamen DL, Wagner M, Bals R, Steinmeyer A, Zügel U, Gallo RL, Eisenberg D, Hewison M, Adams JS, Bloom BR and Modlin RL. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 311: 1770-1773, 2006.
86. Logan VF, Gray AR, Peddie MC, Harper MJ and Houghton LA. Long-term vitamin D3 supplementation is more effective than vitamin D2 in maintaining serum 25-hydroxyvitamin D status over the winter months. Br J Nutr 109: 1082-1088, 2013.
87. Lovell G. Vitamin D status of females in an elite gymnastics program. Clin J Sport Med 18: 159-161, 2008.
88. Macdonald HM, Mavroeidi A, Fraser WD, Darling AL, Black AJ, Aucott L, O'Neill F, Hart K, Berry JL and Lanham-New SA. Sunlight and dietary contributions to the seasonal vitamin D status of cohorts of healthy postmenopausal women living at northerly latitudes: a major cause for concern? Osteoporosis Int 22: 2461-2472, 2011.
89. Macdonald HM. Contributions of sunlight and diet to vitamin D status. Calcif Tissue Int 92: 163-176, 2013.
90. MacLaughlin J and Holick MF. Aging decreases the capacity of human skin to produce vitamin D3. J Clin Invest 76: 1536-1538, 1985.
91. Mahon BD, Gordon SA, Cruz J, Cosman F and Cantorna MT. Cytokine profile in patients with multiple sclerosis following vitamin D supplementation. J Neuroimmunol 134: 128-132, 2003.
92. Maroon JC, Mathyssek CM, Bost JW, Amos A, Winkelman R, Yates AP, Duca MA and Norwig JA. Vitamin D profile in national football league players. Am J Sports Med 43: 1241-1245, 2015.
93. Martineau AR, Wilkinson RJ, Wilkinson KA, Newton SM, Kampmann B, Hall BM, Packe GE, Davidson RN, Eldridge SM, Maunsell ZJ, Rainbow SJ, Berry JL and Griffiths CJ. A single dose of vitamin D enhances immunity to mycobacteria. Am J Resp Crit Care Med 176: 208-213, 2007.
94. Matsuoka LY, Ide L, Wortsman J, MacLaughlin J and Holick MF. Sunscreens suppress cutaneous vitamin D3 synthesis. J Clin Endocrinol Metab 64: 1165-1168, 1987.
95. Maxwell JD. Seasonal variation in Vitamin D. P Nutr Soc 53: 533-543, 1994.
96. Milliken SV, Wassall H, Lewis BJ, Logie J, Barker RN, Macdonald H, Vickers MA and Ormerod AD. Effects of ultraviolet light on human serum 25-hydroxyvitamin D and systemic immune function. J Allergy Clin Immunol 129: 1554-1561, 2012.
97. Monlezun DJ, Bittner EA, Christopher KB, Camargo CA and Quraishi SA. Vitamin D status and acute respiratory infection: Cross sectional results from the United States National Health and Nutrition Examination Survey, 2001-2006. Nutrients 7: 1933-1944, 2015.
98. Moore C, Murphy MM, Keast DR and Holick MF. Vitamin D intake in the United States. J Am Diet Assoc 104: 980-983, 2004.
99. Moran DS, McClung JP, Kohen T and Lieberman HR. Vitamin D and physical performance. Sports Med 43: 601-611, 2013.
100. Morton JP, Iqbal Z, Drust B, Burgess D, Close GL and Brukner PD. Seasonal variation in vitamin D status in professional soccer players of the English Premier League. Appl Physiol Nutr Metab 37: 798-802, 2012.

101. Muller K, Haahr PM, Diamant M, Rieneck K, Kharazmi A and Bendtzen K. 1, 25-dihydroxyvitamin D 3 inhibits cytokine production by human blood monocytes at the post-transcriptional level. Cytokine 4: 506-512, 1992.
102. Muller K, Ødum N and Bendtzen K. 1, 25-Dihydroxyvitamin D 3 selectively reduces interleukin-2 levels and proliferation of human T cell lines in vitro. Immunol Lett 35: 177-182, 1993.
103. Murdoch DR, Slow S, Chambers ST, Jennings LC, Stewart AW, Priest PC, Florkowski CM, Livesey JH, Camargo CA and Scragg R. Effect of vitamin D3 supplementation on upper respiratory tract infections in healthy adults: the VIDARIS randomized controlled trial. JAMA 308: 1333-1339, 2012.
104. Nowson CA and Margerison C. Vitamin D intake and vitamin D status of Australians. Med J Aust 177: 149-152, 2002.
105. Ogan D and Pritchett K. Vitamin D and the athlete: risks, recommendations, and benefits. Nutrients 5: 1856-1868, 2013.
106. Owens DJ, Fraser WD and Close GL. Vitamin D and the athlete: Emerging insights. Eur J Sport Sci 15: 73-84, 2015.
107. Palmer HG, Gonzalez-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, Quintanilla M, Cano A, de Herreros AG, Lafarga M and Munoz A. Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of ß-catenin signaling. J Cell Biol 154: 369-388, 2001.
108. Peterfy C, Tenenhouse A and Yu E. Vitamin D and parotid gland function in the rat. J Physiol 398: 1, 1988.
109. Pludowski P, Karczmarewicz E, Bayer M, Carter G, Chlebna-Sokól D, Czech-Kowalska J, Debski R, Decsi T, Dobrzanska A, Franek E, Gluszko P, Grant W, Holick M, Yankovskaya L, Konstantynowicz J, Ksiazyk J, Ksiezopolska-Orlowska K, Lewinski A, Litwin M, Lohner S, Lorenc R, Lukaszkiewicz J, Marcinowska-Suchowierska E, Milewicz A, Misiorowski W, Nowicki M, Povoroznyuk V, Rozentryt P, Rudenka E, Shoenfeld Y, Socha P, Solnica B, Szalecki M, Talalaj M, Varbiro S and Zmijewski M. Practical guidelines for the supplementation of vitamin D and the treatment of deficits in Central Europe-recommended vitamin D intakes in the general population and groups at risk of vitamin D deficiency. Endokrynol Pol 64: 319-327, 2013.
110. Pollock N, Dijkstra P, Chakraverty R and Hamilton B. Low 25 (OH) vitamin D concentrations in international UK track and field athletes. S Afr J Sports Med 24: 55-59, 2012.
111. Prietl B, Pilz S, Wolf M, Tomaschitz A, Obermayer-Pietsch B, Graninger W and Pieber TR. Vitamin D supplementation and regulatory T cells in apparently healthy subjects: vitamin D treatment for autoimmune diseases? Isr Med Assoc J 12: 136-139, 2010.
112. Prietl B, Treiber G, Pieber TR and Amrein K. Vitamin D and immune function. Nutrients 5: 2502-2521, 2013.
113. Pyne DB, Hopkins WG, Batterham AM, Gleeson M and Fricker PA. Characterising the individual performance responses to mild illness in international swimmers. Br J Sports Med 39: 752-756, 2005.
114. Rhodes LE, Webb AR, Fraser HI, Kift R, Durkin MT, Allan D, O'Brien SJ, Vail A and Berry JL. Recommended summer sunlight exposure levels can produce sufficient (≥ 20 ng ml-1) but not the proposed optimal (≥ 32 ng ml-1) 25 (OH) D levels at UK latitudes. J Invest Dermatol 130: 1411-1418, 2010.
115. Rigby WF, Denome S and Fanger MW. Regulation of lymphokine production and human T lymphocyte activation by 1, 25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA. J Clin Invest 79: 1659-1664, 1987.
116. Rigby WF, Stacy T and Fanger MW. Inhibition of T lymphocyte mitogenesis by 1, 25-dihydroxyvitamin D3 (calcitriol). J Clin Invest 74: 1451-1455, 1984.
117. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL and Jones G. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab 96: 53-58, 2011.
118. Ruohola JP, Laaksi I, Ylikomi T, Haataja R, Mattila VM, Sahi T, Tuohimaa P and Pihlajamäki H. Association between serum 25 (OH) D concentrations and bone stress fractures in Finnish young men. J Bone Miner Res 21: 1483-1488, 2006.
119. Sabetta JR, DePetrillo P, Cipriani RJ, Smardin J, Burns LA and Landry ML. Serum 25-hydroxyvitamin d and the incidence of acute viral respiratory tract infections in healthy adults. PLoS One 5: e11088, 2010.
120. Sandhu MS and Casale TB. The role of vitamin D in asthma. Ann Allerg Asthma Immunol 105: 191-199, 2010.
121. Sato Y, Iwamoto J, Kanoko T and Satoh K. Low-dose vitamin D prevents muscular atrophy and reduces falls and hip fractures in women after stroke: a randomized controlled trial. Cerebrovasc Dis 20: 187-192, 2005.
122. Schertzer JD, Gehrig SM, Ryall JG and Lynch GS. Modulation of insulin-like growth factor (IGF)-I and IGF-binding protein interactions enhances skeletal muscle regeneration and ameliorates the dystrophic pathology in mdx mice. Am J Pathol 171: 1180-1188, 2007.
123. Shuler FD, Wingate MK, Moore GH and Giangarra C. Sports health benefits of vitamin D. Sports Health 4: 496-501, 2012.
124. Sly LM, Lopez M, Nauseef WM and Reiner NE. 1, 25-Dihydroxyvitamin D3-induced monocyte antimycobacterial activity is regulated by phosphatidylinositol 3-kinase and mediated by the NADPH-dependent phagocyte oxidase. J Biol Chem 276: 35482-35493, 2001.
125. Smolders J, Peelen E, Thewissen Ml, Cohen Tervaert JW, Menheere P, Hupperts R and Damoiseaux J. Safety and T cell modulating effects of high dose vitamin D3 supplementation in multiple sclerosis. PLoS One 5: e15235, 2010.
126. Stoffels K, Overbergh L, Giulietti A, Verlinden L, Bouillon R and Mathieu C. Immune regulation of 25-hydroxyvitamin-D3-1alpha-hydroxylase in human monocytes. J Bone Miner Res 21: 37-47, 2006.
127. Storlie DM, Pritchett K, Pritchett R and Cashman L. 12-Week vitamin D supplementation trial does not significantly influence seasonal 25 (OH) D status in male collegiate athletes. Int J Health Nutr 2: 8-13, 2011.
128. Stumpf WE. Vitamin D and the digestive system. Eur J Drug Metab Pharmacokinet 33: 85-100, 2008.
129. Takahashi K, Nakayama Y, Horiuchi H, Ohta T, Komoriya K, Ohmori H and Kamimura T. Human neutrophils express messenger RNA of vitamin D receptor and respond to 1alpha,25-dihydroxyvitamin D3. Immunopharmacol Immunotoxicol 24: 335-347, 2002.
130. Todd JJ, Pourshahidi LK, McSorley EM, Madigan SM and Magee PJ. Vitamin D: Recent advances and implications for athletes. Sports Med 45: 213-229, 2015.

131. Trujillo G, Habiel DM, Ge L, Ramadass M, Cooke NE and Kew RR. Neutrophil recruitment to the lung in both C5a-and CXCL1-induced alveolitis is impaired in vitamin D-binding protein-deficient mice. J Immunol 191: 848-856, 2013.
132. Tsoukas C, Watry D, Escobar S, Provvedini D, Dinarello C, Hustmyer F and Manolagas S. Inhibition of interleukin-1 production by 1, 25-dihydroxyvitamin D3. J Clin Endocrinol Metab 69: 127-133, 1989.
133. Villacis D, Yi A, Jahn R, Kephart CJ, Charlton T, Gamradt SC, Romano R, Tibone JE and Hatch GFR. Prevalence of abnormal vitamin D levels among Division I NCAA athletes. Sports Health 6: 340-347, 2014.
134. Von Essen MR, Kongsbak M, Schjerling P, Olgaard K, Ødum N and Geisler C. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. Nat Immunol 11: 344-349, 2010.
135. Wallace AM, Gibson S, De La Hunty A, Lamberg-Allardt C and Ashwell M. Measurement of 25-hydroxyvitamin D in the clinical laboratory: current procedures, performance characteristics and limitations. Steroids 75: 477-488, 2010.
136. Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, Shephard RJ, Oliver SJ, Bermon S and Kajeniene A. Position statement part two: maintaining immune health. Exerc Immunol Rev 17: 64-103, 2011.
137. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A and Simon P. Position statement part one: immune function and exercise. Exerc Immunol Rev 17: 6-63, 2011.
138. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, Tavera-Mendoza L, Lin R, Hanrahan JH, Mader S and White JH. Cutting edge: 1, 25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J Immunol 173: 2909-2912, 2004.
139. Webb AR. Who, what, where and when-influences on cutaneous vitamin D synthesis. Prog Biophys Mol Biol 92: 17-25, 2006.
140. Webb AR, Kift R, Berry JL and Rhodes LE. The vitamin D debate: translating controlled experiments into reality for human sun exposure times. Photochem Photobiol 87: 741-745, 2011.
141. Webb AR, Pilbeam C, Hanafin N and Holick MF. An evaluation of the relative contributions of exposure to sunlight and of diet to the circulating concentrations of 25-hydroxyvitamin D in an elderly nursing home population in Boston. Am J Clin Nutr 51: 1075-1081, 1990.
142. Wentz L, Berry-Cabán C, Eldred J and Wu Q. Vitamin D correlation with testosterone concentration in US army special operations personnel. The FASEB J 29: 733-735, 2015.
143. Wielders JP and Wijnberg FA. Preanalytical stability of 25(OH)-vitamin D3 in human blood or serum at room temperature: solid as a rock. Clin Chem 55: 1584-1585, 2009.
144. Wolf G. The discovery of vitamin D: the contribution of Adolf Windaus. J Nutr 134: 1299-1302, 2004.
145. Wu S, Ren S, Nguyen L, Adams JS and Hewison M. Splice variants of the CYP27b1 gene and the regulation of 1, 25-dihydroxyvitamin D3 production. Endocrinology 148: 3410-3418, 2007.
146. Zerwekh JE. Blood biomarkers of vitamin D status. Am J Clin Nutr 87: 1087S-1091S, 2008.
147. Zhu Y, Mahon BD, Froicu M and Cantorna MT. Calcium and 1, 25-dihydroxyvitamin D3 target the TNF-alpha pathway to suppress experimental inflammatory bowel disease. Eur J Immunol 35: 217-224, 2005.

Effects of acute aerobic exercise on leukocyte inflammatory gene expression in systemic lupus erythematosus

Perandini LA (PhD)[1], Sales-de-Oliveira D (BSc)[1], Almeida, DC (PhD)[2], Azevedo H (MSc)[3], Moreira-Filho CA (PhD)[3], Cenedeze MA (MSc)[2], Benatti FB (PhD)[1,5], Lima FR (MD, PhD)[1], Borba E (MD, PhD)[1], Bonfa E (MD, PhD)[1], Sá-Pinto AL (MD, PhD)[1], Roschel H (PhD)[1,4], Camara NO (MD, PhD)[4], Gualano B (PhD)[1,5].

[1] Rheumatology Division, School of Medicine, University of Sao Paulo, Sao Paulo, Brazil.
[2] Laboratory of Clinical and Experimental Immunology, Department of Medicine, Division of Nephrology, Federal University of Sao Paulo, Sao Paulo, Brazil.
[3] Laboratory of Pediatric Genomics, Department of Pediatrics, School of Medicine, University of Sao Paulo, Sao Paulo, Brazil.
[4] Laboratory of Transplantation Immunobiology, Department of Immunology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil.
[5] School of Physical Education and Sport, University of Sao Paulo, Sao Paulo, Brazil.

ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease with a persistent systemic inflammation. Exercise-induced inflammatory response in SLE remains to be fully elucidated. The aim of this study was to assess the effects of acute exercise on leukocyte gene expression in active (SLE_{ACTIVE}) and inactive SLE ($SLE_{INACTIVE}$) patients and healthy controls (HC). ***Methods****: All subjects (n = 4 per group) performed a 30-min single bout of acute aerobic exercise (~70% of VO_2 peak) on a treadmill, and blood samples were collected for RNA extraction from circulating leukocyte at baseline, at the end of exercise, and after three hours of recovery. The expression of a panel of immune-related genes was evaluated by a quantitative PCR array assay. Moreover, network-based analyses were performed to interpret transcriptional changes occurring after the exercise challenge.* ***Results:*** *In all groups, a single bout of acute exercise led to the down-regulation of the gene expression of innate and adaptive immunity at the end of exercise (e.g., TLR3, IFNG, GATA3, FOXP3, STAT4) with a subsequent up-regulation occurring upon recovery. Exercise regulated the expression of inflammatory genes in the blood leukocytes of the SLE patients and HC, although the SLE groups exhibited fewer modulated genes and less densely connected networks (number of nodes: 29, 40 and 58; number of edges: 29, 60 and 195; network density: 0.07, 0.08 and 0.12, for SLE_{ACTIVE}, $SLE_{INACTIVE}$ and HC, respectively).* ***Conclusion:*** *The leukocytes from the SLE patients, irrespective of disease activity, showed a down-regulated inflammatory gene expression immediately after acute aerobic exercise, followed by an up-regulation at recovery. Furthermore, less organized gene networks were observed in the SLE patients, suggesting that they may be deficient in triggering a normal exercise-induced immune transcriptional response.*

Key words: physical activity, inflammation, autoimmunity, gene array, exercise immunology, network analysis, hubs.

Correspondence:
Rheumatology Division - School of Medicine
University of Sao Paulo - Dr. Arnaldo Avenue, 455 –3rd floor - room 3190; Sao Paulo, SP - Brazil; ZIP code: 01246-903
Phone: + 55 11 30617492; FAX: +55 11 30617490

Abbreviations:
SLE, systemic lupus erythematosus
$SLE_{INACTIVE}$, inactive systemic lupus erythematosus patients
SLE_{ACTIVE}, active systemic lupus erythematosus patients
BMI, body mass index
SLEDAI, systemic lupus erythematosus disease activity index
HC, healthy controls
VAT, ventilatory anaerobic threshold
RCP, respiratory compensation point
VE, ventilation
VCO_2, carbon dioxide output
Baseline, prior to exercise
End-ex, end of exercise
Recovery, three hours of recovery
HR, heart rate
VO_2, oxygen uptake
DE, differentially expressed

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease characterized by an immune-mediated inflammation of multiple organs (65). Although mechanisms underlying SLE pathogenesis have not yet been fully elucidated, patients with active (SLE_{ACTIVE}) and inactive ($SLE_{INACTIVE}$) disease exhibit up-regulation of the expression of genes involved in both innate and adaptive immunity when compared with their healthy counterparts (18, 23). This molecular dysfunction may result in auto-antibody production, immune complex deposition, and complement system activation (11), ultimately leading to chronic inflammation (11, 33). The consequent exacerbated inflammatory response has been implicated in some SLE-related comorbidities, such as atherosclerosis and endothelial dysfunction (26), which have been thought to be the main causes of mortality in SLE patients (59).

Physical exercise has emerged as a potential tool for counteracting SLE comorbidities (45), endothelial dysfunction (19), aerobic deconditioning (12), cardiac dysautonomia (29), and inflammation (46). In fact, exercise has been shown to exert anti-inflammatory effects in a number of chronic diseases, including inflammatory rheumatic diseases. It has been specu-

lated that such a role of exercise may be due to an anti-inflammatory environment induced by successive acute exercise bouts. In response to a single bout of exercise, skeletal muscle releases interleukin-6 (IL-6) in an exponential fashion according to the duration and intensity of exercise (41, 42). Because IL-6 has classically been considered a pro-inflammatory cytokine primarily secreted by stimulated immune cells (*e.g.*, monocytes and macrophages) (2), its increase in response to a single bout of exercise was initially regarded as a pro-inflammatory response. However, the increase in IL-6 is not preceded by an increase in tumor necrosis factor alpha (TNF-α) and, most importantly, is followed by increased levels of anti-inflammatory cytokines, namely IL-1 receptor antagonist (IL-1ra) and IL-10 (41, 63). Therefore, rather than pro-inflammatory, the acute exercise-induced increase in IL-6 may actually lead to an anti-inflammatory environment (9). Studies with recombinant IL-6 (rhIL-6) infusion further support the anti-inflammatory consequences of the exercise-induced increase in IL-6. When healthy subjects are exposed to a low-dose of *E. Coli* endotoxin in a "low-grade inflammation model", the infusion of rhIL-6 (mimicking the exercise-induced IL-6 response) blunts the otherwise expected increase in TNF-α (62). This anti-inflammatory role of IL-6 has been associated with the transitory increase of this cytokine secreted by the muscle in response to a single bout of exercise (43), while persistent high serum levels of IL-6 have been associated with a persistent inflammatory response and chronic disabilities (20).

Even though consecutive bouts of exercise may potentially exert anti-inflammatory effects, there is limited evidence showing that regular exercise training may attenuate inflammation in chronic diseases. In this regard, Adamopoulos et al. (1) observed a reduction in resting TNF-α, IL-6, soluble TNF receptors 1 and 2 (sTNFR1 and sTNFR2, respectively) in patients with congestive heart failure after 12 weeks of aerobic training [5 sessions per week, 30 min per session, 80% of maximal heart rate (HR_{max})]. Conversely, Niebauer et al. (37) failed to observe any changes in cytokines in patients with the same disease after a similar training program. In patients with coronary heart disease, 12 weeks of aerobic training (3 sessions per week, 45 min per session, 70-80% HR_{max}) resulted in reductions in IL-1, IL-6, interferon-gamma (IFN-γ) and C-reactive protein, and increases in IL-10 (22). Likewise, patients with fibromyalgia showed a reduction in C-reactive protein serum levels and pro-inflammatory cytokines production (IL-1β and TNF-α) in *ex vivo* stimulated monocytes, after 8 months of aquatic aerobic exercises (2 sessions per week, 60 min per session, 40-75% HR_{max}) (39, 40). In contrast, patients with chronic obstructive pulmonary disease did not experience any change in IL-6, TNF-α, sTNFR1 and sTNFR2 after an 8-week aerobic training (5 sessions per week, 60 min per session, 90% peak power) (51). Studies involving patients with autoimmune diseases are scarce. The three studies assessing the effects of aerobic exercise (2 to 5 sessions per week, 30 to 60 minutes per session, 3 to 8 weeks at either 60% VO_{2peak} or 75% HR_{max}) in patients with multiple sclerosis did not show any changes in pro- or anti-inflammatory cytokines (6, 13, 58).

The effects of exercise training upon inflammation in autoimmune inflammatory rheumatic diseases are also controversial. Baslund et al. (7) showed that 8 weeks of an interval aerobic training (4 to 5 sessions per week, 30 min per session, 80% VO_{2max}) did not elicit any changes in a number of immune parameters, namely natural-killer cell activity, monocyte count, lymphocyte proliferative responses or circulating levels of IL-6, IL-1α and IL-1β in patients with rheumatoid arthritis with low disease activity. Likewise, Rall et al. (56) did not observe changes in peripheral blood mononuclear cells production of TNF-α, IL-6, IL-2, IL-1β or lymphocyte proliferation after 12 weeks of a progressive resistance exercise program (2 sessions per week, 3 sets of 8 repetitions, 80% of 1RM) in patients with rheumatoid arthritis. Bearne et al. (8) examined the acute and chronic effects a 5-week lower-body strength exercise program (2 sessions per week, 4 sets of 6 isometric contractions at maximal voluntary contraction) on the cytokine response in patients with rheumatoid arthritis. Before the exercise program, a single bout of strength exercise did not elicit any significant changes on IL-1, IL-6 and TNF-α levels, which supports the safety of exercise in these patients. Similarly, after the training program, no significant changes were observed in resting levels of these cytokines. However, IL-6 level was decreased after an acute single exercise session at the end of the intervention. TNF-α response was also acutely decreased, although this did not reach statistical significance. Although the training program did not change baseline cytokine levels in these patients, it is conceivable to speculate that the chronic exercise may have affected, in a positive way, the inflammatory response to a single bout of exercise. Interestingly, Nader et al. (34) reported decreased expression of pro-inflammatory and pro-fibrotic gene networks (using microarray technique) after 7 weeks of resistance training [3 sessions per week, 45 min per session (3 sets of 10RM in 5 muscle groups)] in patients with dermatomyositis and polymyositis, indicating an important local anti-inflammatory effect of exercise in these patients, which might be associated with improvements in clinical symptoms.

Recently, our group investigated the effects a 12-week aerobic training program [2 sessions per week, 30 to 50 minutes per session, from ventilatory anaerobic threshold (VAT) to 10% below respiratory compensation point (RCP)] in patients with SLE in remission (47). Prior to and after the regular training program, cytokines and sTNFRs were assessed at rest and in response to single bouts of acute moderate and intense exercises. Exercise training led to a decrease in resting sTNFR2 and a trend towards reduction in IL-10. In response to a bout of moderate exercise, area under the curve of IL-10 was reduced and area under the curve of IL-10, IL-6, TNF-α, and sTNFR1 approached comparable values to those of the healthy control group after the exercise training program in SLE group. In response to a bout of intense exercise, area under the curve of IL-10 was also reduced in SLE; additionally, area under the curve of sTNFR2 tended to decrease after training. Altogether, these findings indicate that an exercise training program did not exacerbate inflammation in women with SLE in remission at rest or in response to single bouts of acute exercise, irrespective of exercise intensity. Moreover, exercise training attenuated the exacerbated inflammatory milieu, suggesting that this intervention may promote a homeostatic immunomodulatory effect in SLE.

Mechanisms underlying exercise-induced changes in cytokine pattern are not yet fully elucidated in healthy subjects and patients with an inflammatory disease; however, it has been postulated that modulation in gene expression of leukocyte cells could explain alterations in inflammatory mediators in response to exercise (60). This concept has been supported by gene array studies (10, 15, 16, 53, 54). A growing number of studies have demonstrated that a single bout of exercise can modulate gene expression in circulating leukocytes in healthy subjects (60). Notably, these studies have revealed that exercise can simultaneously stimulate anti- and pro-inflammatory genes as well as growth and repair genes, suggesting a coordinated counterbalanced response to the stress imposed by exercise (53).

Connolly et al. (16) showed that a 30-minute cycling exercise (80% VO_{2peak}) led to an up-regulation of genes related to stress and inflammation in peripheral blood mononuclear cells in healthy subjects. Importantly, altered expression levels of pro-inflammatory genes returned to baseline 60 minutes into recovery, whereas *IL-1RA* gene, which is thought to be an anti-inflammatory mediator, increased during this stage. This response was paralleled by an increase in IL-1ra and IL-6 circulating levels. Heat shock proteins *(HSP)* genes, which have been shown to inhibit NF-κB (27), were also upregulated during the exercise. Based on these findings, the authors suggested that changes in *IL-1RA* and *HSP* gene expression might be related to the anti-inflammatory effect of exercise. Büttner et al. (10) also showed a significant up-regulation of leukocytes genes involved in energy metabolism, extracellular matrix, heat-shock response and inflammation in response to a higher- and lower-intensity exercise (80% and 60% VO_{2max}) in healthy subjects. Amongst the most up-regulated genes in response to exercise were *HSP, IL-1RA*, and membrane metalloendopeptidase *(MME),* which is thought to control the bioavailability of pro-inflammatory peptides. On the other hand, amongst the most down-regulated genes was the natural killer receptor gene *BY55/CD160*, which is an important co-stimulator of the T-cell receptor possibly able to affect T-cell expansion and cytotoxicity (38). The authors suggested that these gene responses might be associated with the anti-inflammatory response to exercise.

In a series of studies, Radom-Aizik et al. (52, 54, 55) assessed gene expression in different leukocyte subpopulations (*i.e.*, monocytes, natural killer cells, and neutrophils) in response to acute exercise in adult healthy subjects (55). These authors observed that ten 2-min bouts of exercise (85% of VO_{2peak}) interspersed by 1-min intervals were able to modulate monocytes gene expression in healthy subjects (55). The main up-regulated genes in the monocytes following exercise were heparin-binding EGF-like growth factor *(HBEGF)*, amphiregulin *(AREG)*, and epiregulin *(EREG)*, which have been associated with vascular remodeling (70). Additionally, the main down-regulated genes in response to exercise were *TNF-α, TLR4*, and *CD36*, which have been associated with the anti-inflammatory effects of exercise (21, 61). Radom-Aizik et al. (52) also showed that an acute exercise protocol (2-min exercise at 77% of VO_{2peak}, interspersed by 1-min intervals) modulated pathways related to cancer, cell communication, and inflammation in circulating NK cells. This conclusion was based on the modulation of TNF family genes, with an increase in the gene expression of TNF-induced protein 3 *(TNFAIP3)*, and decreases in the gene expression of TNF ligand superfamily member 13b *(TNFSF13B)* and TNF ligand superfamily member 4 *(TNFSF4)*. Finally, because neutrophils remain elevated or continue to increase after exercise, unlike peripheral blood mononuclear cells, Radom-Aizik et al. (54) investigated the effects of a 30-min exercise bout (VO_{2peak}) on neutrophil gene expression in healthy young men. The authors showed that many of the altered genes were related to regulation of cell physiology, immune response, stress response, apoptosis, and signal transduction. A number of genes involved in growth and tissue repair were up-regulated (*e.g., ARE*, a member of the epidermal growth factor), as well as genes potentially involved in angiogenesis [*e.g.*, platelet-derived factor D *(PDGFD)* and fibroblast growth factor receptor-2 *(FGFR2)*]. Moreover, up-regulation of pro- and anti-apoptotic genes was also observed. Finally, as observed in peripheral blood mononuclear cells, both pro- and anti-inflammatory genes were up-regulated. For instance, granzyme A *(GZMA)* and perforin *(PRF1)* genes, which are known to control protein that promote cell lysing of pathogens, thereby allowing neutrophils to kill or damage these cells, were up-regulated. In addition, annexin 1 *(ANXA1)*, which is thought to inhibit the release of free radicals from activated neutrophils (48), and *HSP* genes were also up-regulated. The apparently "paradoxical response" to exercise led the authors to suggest that "the genomic response in neutrophils immediately following the perturbation of exercise might be characterized as a cellular 'wake up' call". These findings were further supported by another study (36), which observed an activation of the innate immunity characterized by an increase in gene expression of toll-like receptors in neutrophils after a single bout of exercise.

Therefore, in healthy individuals, a global gene expression analysis of circulating peripheral blood mononuclear cells and neutrophils shows that exercise stimulates an orchestrated up-regulation of both pro- and anti-inflammatory genes, which are normalized after at least one hour of recovery (16, 54). However, a concern remains that exercise may trigger an immunological "danger" type of stress and inflammatory response characterized by "pre-activated" circulating leukocytes (*e.g.*, asthma, musculoskeletal injury, and anaphylaxis), which could lead, at least in theory, to detrimental effects in inflammatory diseases (17). In contrast to this hypothesis, we did not observe exacerbated inflammation following both acute and chronic exercise in SLE patients (40, 41). Nonetheless, the influence of exercise on inflammation-related gene expression in blood cells of SLE patients has not been addressed. Therefore, this study aimed to investigate the effects of acute aerobic exercise on the modulation of immune-related gene expression in circulating leukocytes from $SLE_{INACTIVE}$ and SLE_{ACTIVE} patients and healthy individuals. Based on our previous findings (40, 41), we hypothesized that a single bout of exercise would similarly modulate leukocyte gene expression in SLE patients and controls, without inducing an exacerbated gene inflammatory response.

Table 1. Demographic, clinical and therapy data of patients ($SLE_{INACTIVE}$ and SLE_{ACTIVE}) and healthy controls (HC).

	SLE_{ACTIVE} (n = 4)	$SLE_{INACTIVE}$ (n = 4)	HC (n = 4)
Age (years)	32.5 ± 3.4	34.5 ± 3.4	29.3 ± 4.8
Body mass (kg)	69.8 ± 8.8	62.0 ± 5.3	58.0 ± 4.9
Height (cm)	164.8 ± 2.9	158.1 ± 3.6	157.8 ± 5.4
BMI (kg/m²)	25.7 ± 3.0	24.9 ± 3.1	23.3 ± 1.6
SLEDAI	6.3 ± 1.1*	1.3 ± 0.8	-
Disease duration (years)	6.8 ± 2.8	6.8 ± 1.8	-
Drugs [nº(%)]			
Glucocorticoid	4 (100%)	0 (0%)	-
Antimalarial	3 (75%)	3 (75%)	-
Azathioprine	1 (25%)	1 (25%)	-
Methotrexate	1 (25%)	0 (0%)	-
Mycophenolate mofetil	2 (50%)	1 (25%)	-

Data are presented as the mean ± standard deviation or n (%). BMI = body mass index; SLEDAI = systemic lupus erythematosus disease activity index; SLE: systemic lupus erythematosus; $SLE_{INACTIVE}$: women with inactive SLE; SLE_{ACTIVE}: women with active SLE. * denotes significant differences between SLE_{ACTIVE} versus $SLE_{INACTIVE}$ ($P < 0.05$).

MATERIAL AND METHODS

Ethical approval

This study was approved by the Local Ethical Committee (School of Medicine of University of Sao Paulo – nº 0185/11 and the subjects signed an informed consent. This study was registered at clinicaltrials.gov as NCT01515163. All of the procedures were in accordance with the Helsinki Declaration revised in 2008.

Participants and Experimental design

Four $SLE_{INACTIVE}$ and four SLE_{ACTIVE} women were consecutively selected and were regularly followed at the Outpatient Lupus Clinic of the Rheumatology Division of the School of Medicine at the University of Sao Paulo, Brazil. All patients fulfilled the American College of Rheumatology criteria for SLE diagnosis (25). Disease activity was determined by Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores. The inclusion criteria for both SLE groups were an age range of 20 to 40 years and physical inactivity for at least six months prior to the selection process. Exclusion criteria were secondary rheumatic disease (*e.g.*, Sjögren syndrome, antiphospholipid syndrome), body mass index (BMI) ≥ 30 kg/m², acute renal failure, cardiac and pulmonary involvement, fibromyalgia, and musculoskeletal and joint disorders precluding the exercise testing. For the $SLE_{INACTIVE}$ group, inclusion criteria were SLEDAI scores < 4 and the absence of glucocorticoid therapy for at least six months prior to entry. All patients in the SLE_{ACTIVE} group had SLEDAI scores of between 4 and 8 and received a daily glucocorticoid dose of ≤ 20 mg. Disease manifestations were defined as follows: cutaneous disease, articular involvement, neuropsychiatric disease, renal disease, cardiopulmonary disease, and hematologic complications. Four age- and BMI-matched healthy women were selected for this study as the healthy control (HC) group. The participants' demographic and laboratory parameters are demonstrated in Tables 1 and 2. Patients and controls were randomly selected from a larger group of participants described elsewhere (46).

All subjects (*i.e.*, $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC) performed a maximal graded treadmill cardiopulmonary exercise test to

Table 2. Laboratory parameters of patients ($SLE_{INACTIVE}$ and SLE_{ACTIVE}) and healthy controls (HC).

	SLE_{ACTIVE} (n = 4)	$SLE_{INACTIVE}$ (n = 4)	HC (n = 4)
C3 (90-180 mg/dL)	70.8 ± 14.4#	100.7 ± 9.0	130.8 ± 17.4
C4 (10-40 mg/dL)	6.3 ± 1.3#	13.1 ± 4.9	27.0 ± 7.6
CPK (26-192 U/L)	83.0 ± 15.0	111.7 ± 38.0	103.5 ± 22.0
Creatinine (0.50-0.90 mg/dL)	0.83 ± 0.18	0.77 ± 0.12	0.78 ± 0.08
Erythrocytes (4.0-5.4 million/mm³)	4.2 ± 0.4	4.1 ± 0.2	4.5 ± 0.3
Hematocrit (35-47%)	39.3 ± 2.9	38.9 ± 0.5	38.2 ± 1.0
Leukocytes (4.0-11.0 mil/mm³)	5.6 ± 0.5	4.3 ± 1.2	8.0 ± 3.6
Platelets (140-450 mil/mm³)	202.5 ± 45.2	259.5 ± 66.5	255.8 ± 50.4
CRP (< 5 mg/L)	3.2 ± 4.6	2.2 ± 2.5	3.3 ± 2.3
ESR (5.6-11.0 mm)	7.5 ± 9.0	12.0 ± 6.4	7.0 ± 5.0

Data are presented as the mean ± standard deviation. CPK: creatine phosphokinase; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate. # denotes significant differences between SLE_{ACTIVE} versus HC ($P < 0.05$).

determine their ventilatory anaerobic threshold (VAT) and RCP. Thereafter, the subjects in the $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups underwent a 30-min single bout of acute aerobic exercise, and a leukocyte gene expression analysis was performed prior to exercise (Baseline), immediately after exercise (End-ex), and after three hours of recovery (Recovery).

Cardiopulmonary exercise test

A maximal graded exercise test was performed on a treadmill (Centurion 200, Micromed, Brazil) with increments in velocity and grade each minute until volitional exhaustion. Oxygen consumption (VO_2) and carbon dioxide output were obtained through breath-by-breath sampling and expressed as a 30-s average using an indirect calorimetric system (Cortex - model Metalyzer IIIB, Leipzig, Germany). HR was continuously recorded at rest, during exercise and at recovery using a 12-lead electrocardiogram (Ergo PC Elite, Inc. Micromed, Brazil). Cardiopulmonary exercise test results were considered to be maximal when one of the following criteria was met: VO_2 plateau (*i.e.*, < 150 mL/min increase between two consecutive stages), HR of no less than 10 beats below age-predicted maximal HR and respiratory exchange ratio value of above 1.10 (49). The VO_2peak was calculated as the average value for the final 30 s of the test. The VAT was identified following previously described procedures (68). In brief, this value was determined when the ventilatory equivalent for VO_2 (VE/VO_2) increased without a concomitant increase in the ventilatory equivalent for carbon dioxide (VE/VCO_2). The RCP was determined when the VE/VO_2 and VE/VCO_2 increased simultaneously.

Single bout of acute aerobic exercise

At least 72 hours after the cardiopulmonary exercise test, a single bout of acute aerobic exercise was performed on a treadmill for the assessment of gene expression in the leukocytes.

The acute aerobic exercise bout was set at an intensity corresponding to 50% of the delta difference (Δ) between the VAT and the RCP ($SLE_{INACTIVE}$: 69.0 ± 11.3% of VO_{2peak}; SLE_{ACTIVE}: 67.6 ± 5.1% of VO_{2peak}; HC: 63.2 ± 6.6% of VO_{2peak}). The exercise bout was comprised of a 5-min warm-up and 30 min of exercise at the pre-determined exercise intensity.

Blood sampling and RNA isolation

Prior to exercise, the antecubital vein was cannulated for blood sampling. Approximately 10 mL of blood were collected with the patients in the seated position immediately before the acute exercise (Baseline), immediately after the acute exercise (End-ex), and 3 hours after the end of the acute exercise (Recovery). Blood was drawn into tubes containing anticoagulant (EDTA) for subsequent analysis. White blood cells were isolated using an erythrocyte lysis buffer (17 mM tris-HCl and 0.144 M ammonium chloride, pH 7.2) followed by two washing steps in PBS containing 2 mmol/L EDTA. The buffers were used at 4°C, and the tubes were placed on ice. Total RNA was isolated from the resulting cell pellet using the Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE). RNA quality was assessed using the Agilent Bioanalyzer 2100 System (Agilent Technologies, Palo Alto, CA), following the manufacturer's recommendations.

PCR array analysis

The PCR array analyses of the mRNA were performed in two sets of six 96-well plates per group, following the manufacturer's recommendations (The Human Innate & Adaptive Immune Responses RT^2 Profiler™ PCR Array Kit; Qiagen, South Korea). The data analysis and validation were performed using the free online software provided by the manufacturer's website (Qiagen, South Korea). Gene expression levels were considered to be altered (up- or down-regulated) if fold changes of > 2.0 were observed.

Bioinformatics analysis

A protein-interaction network analysis was performed to evaluate the network representations of the signaling pathways and the physical relationships between the proteins encoded by the differentially expressed (DE) genes. The Cytoscape plugin GeneMania (31) was employed to scan for physical and pathway interactions between the seed nodes. Network descriptive parameters (*i.e.*, number of nodes, number of edges, average node degree, diameter, characteristic path length, clustering coefficient, and network density) were calculated using the Cytoscape Plugin Network Analyzer (5). After the networks were constructed, the twenty percent most connected nodes in each network (considered as hubs) were marked with yellow borders for a better visualization.

Statistical analysis

Data are presented as mean ± standard deviation. The Gaussian distribution of the data was tested by Kolmogorov-Smirnov's test (with Lilliefor's correction). Demographic data of the three groups ($SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC) were compared using one way ANOVA followed by Bonferroni post hoc test. Drugs proportions of both SLE groups were compared with χ^2 test. All data analysis was performed using the Statistical Package for Social Sciences (SPSS), version 17.0 for Windows. The level of significance was set at $P \leq 0.05$.

RESULTS

Patients and healthy controls main characteristics' are presented in Table 1 and 2. Age, body mass, height, and BMI were comparable between the $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups ($P > 0.05$). The SLE_{ACTIVE} group showed higher SLEDAI score compared with the $SLE_{INACTIVE}$ group ($P < 0.05$) and lower C3 and C4 levels compared with the HC group ($P < 0.05$).

In the HC group, 46 DE genes (5 up-regulated and 41 down-regulated) were modulated at the end of the single bout of acute exercise, whereas 26 DE genes were up-regulated at Recovery compared with Baseline (Table 3). Additionally, the network analysis indicated that the main exercise-modulated genes were as follows: *IL2, IFNG, TNF, IL18, IL23A, IL1B,*

Table 3. Gene expression response to a single bout of exercise in healthy controls (HC).

Gene symbol	Gene name	End-ex *vs.* Baseline (FC)	Recovery *vs.* Baseline (FC)
	INNATE IMMUNITY		
C3	Complement component 3	-3.4826	NC
CASP1	Caspase 1	-2.0997	2.8927
CCL2	Chemokine (C-C motif) ligand 2	-4.1143	NC
CCL5	Chemokine (C-C motif) ligand 5	-3.1449	NC
CD14	CD14 molecule	NC	2.33
CD40LG	CD40 ligand	-2.467	NC
CD8A	CD8a molecule	-2.1748	NC
CRP	C-reactive protein	-4.1143	NC
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	-3.1182	NC
CXCL10	Chemokine (C-X-C motif) ligand 10	-3.9112	NC
CXCR3	Chemokine (C-X-C motif) receptor 3	-3.0706	NC
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-2.4561	2.3022
HLA-A	Major histocompatibility complex. class I, A	2.9903	NC
HLA-E	Major histocompatibility complex, class I, E	2.0173	2.0892
IFNA1	Interferon alpha 1	-4.1143	NC
IFNB1	Interferon beta 1	-3.9216	NC
IL18	Interleukin 18	-2.0383	NC
IL1A	Interleukin 1 alpha	-4.5095	NC
IL1B	Interleukin 1 beta	NC	4.6954
IL1R1	Interleukin 1 receptor, type I	NC	4.9493
IL2	Interleukin 2	-6.0684	NC
IRF3	Interferon regulatory factor 3	-2.1479	NC
ITGAM	Integrin, alpha	NC	3.2972
LY96	Lymphocyte antigen 96	-4.6578	NC
LYZ	Lysozyme	NC	2.0842
MBL2	Mannose-binding lectin (protein C) 2, soluble	-3.6605	NC
MX1	Interferon-inducible protein p78	-3.5097	NC
MYD88	Myeloid differentiation primary response gene (88)	-2.2664	NC
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NC	3.1521
NOD1	Nucleotide-binding oligomerization domain containing 1	NC	3.6606
RAG1	Recombination activating gene 1	-3.5375	NC
STAT1	Signal transducer and activator of transcription 1	NC	2.4584
TICAM1	Toll-like receptor adaptor molecule 1	-2.9017	NC
TLR1	Toll-like receptor 1	NC	2.6445
TLR3	Toll-like receptor 3	-2.9082	NC
TLR7	Toll-like receptor 7	-2.0592	NC
TLR8	Toll-like receptor 8	NC	2.7166
TNF	Tumor necrosis factor	-3.1592	NC
	ADAPTIVE IMMUNITY		
C3	Complement component 3	-3.4826	NC
CCL2	Chemokine (C-C motif) ligand 2	-4.1143	NC
CCL5	Chemokine (C-C motif) ligand 5	-3.1449	NC
CCR4	Chemokine (C-C motif) receptor 4	2.2592	NC
CCR5	Chemokine (C-C motif) receptor 5	2.023	2.3474
CCR6	Chemokine (C-C motif) receptor 6	2.0789	2.4998
CCR8	Chemokine (C-C motif) receptor 8	-8.4509	NC
CD40LG	CD40 ligand	-2.467	NC
CD80	CD80 molecule	-5.0934	NC
CD8A	CD8a molecule	-2.1748	NC
CRP	C-reactive protein	-4.1143	NC
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	-3.1182	NC

CXCL10	Chemokine (C-X-C motif) ligand 10	-3.9112	NC
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-2.4561	2.3022
FASLG	Fas ligand	-2.3892	NC
FOXP3	Forkhead box P3	-2.7225	NC
GATA3	GATA binding protein 3	-3.3916	NC
HLA-A	Major histocompatibility complex, class I, A	2.9903	NC
IFNA1	Interferon alpha 1	-4.1143	NC
IFNAR1	Interferon (alpha, beta and omega) receptor 1	NC	2.7179
IFNB1	Interferon beta 1	-3.9216	NC
IFNG	Interferon gamma	NC	2.1988
IL10	Interleukin 10	-4.0672	NC
IL13	Interleukin 13	-3.3514	3.2836
IL17A	Interleukin 17A	-4.1143	NC
IL18	Interleukin 18	-2.0383	NC
IL1A	Interleukin 1 alpha	-4.5095	NC
IL1B	Interleukin 1 beta	NC	4.6954
IL1R1	Interleukin 1 receptor, type I	NC	4.9493
IL2	Interleukin 2	-6.0684	NC
IL23A	Interleukin 23, alpha subunit p19	-3.1288	24.9473
IL4	Interleukin 4	-4.3607	NC
IL5	Interleukin 5	-4.1143	2.1612
IL6	Interleukin 6	-4.2226	NC
IRF3	Interferon regulatory factor 3	-2.1479	NC
ITGAM	Integrin, alpha	NC	3.2972
JAK2	Janus kinase 2	NC	2.8748
LYZ	Lysozyme	NC	2.0842
MAPK8	Mitogen-activated protein kinase 8	NC	2.524
MBL2	Mannose-binding lectin (protein C) 2, soluble	-3.6605	NC
MX1	Interferon-inducible protein p78	-3.5097	NC
MYD88	Myeloid differentiation primary response gene (88)	-2.2664	NC
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NC	3.1521
NOD1	Nucleotide-binding oligomerization domain containing 1	NC	3.6606
RAG1	Recombination activating gene 1	-3.5375	NC
RORC	RAR-related orphan receptor C	-2.726	NC
SLC11A1	Solute carrier family 11, member 1	NC	3.2821
STAT1	Signal transducer and activator of transcription 1	NC	2.4584
STAT3	Signal transducer and activator of transcription 3	NC	2.5869
STAT4	Signal transducer and activator of transcription 4	NC	2.9454
STAT6	Signal transducer and activator of transcription 6	-2.2238	NC
TICAM1	Toll-like receptor adaptor molecule 1	-2.9017	NC
TLR1	Toll-like receptor 1	NC	2.6445
TLR3	Toll-like receptor 3	-2.9082	NC
TLR7	Toll-like receptor 7	-2.0592	NC
TLR8	Toll-like receptor 8	NC	2.7166
TNF	Tumor necrosis factor	-3.1592	NC
TYK2	Tyrosine kinase 2	NC	2.0777

Table 4. Gene expression response to a single bout of exercise in inactive systemic lupus erythematosus (SLE$_{INACTIVE}$).

Gene symbol	Gene name	End-ex *vs.* Baseline (FC)	Recovery *vs.* Baseline (FC)
	INNATE IMMUNITY		
C3	Complement component 3	-2.6061	NC
CASP1	Caspase 1	-2.3041	NC
CCL2	Chemokine (C-C motif) ligand 2	-2.2908	NC
CCL5	Chemokine (C-C motif) ligand 5	-2.3139	2.0725
CD14	CD14 molecule	2.2325	NC
CD40	CD40 molecule	NC	2.1064
CD40LG	CD40 ligand	2.1076	NC
CD8A	CD8a molecule	NC	2.0703
CRP	C-reactive protein, pentraxin-related	-3.8309	NC
CXCL10	Chemokine (C-X-C motif) ligand 10	-4.3281	2.9765
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-3.2047	NC
HLA-A	Major histocompatibility complex, class I, A	3.9397	NC
IFNA1	Interferon alpha 1	-3.8309	NC
IFNB1	Interferon beta 1	-3.8309	NC
IL18	Interleukin 18	-3.8301	NC
IL1A	Interleukin 1 alpha	-3.021	2.8189
IL2	Interleukin 2	-3.5093	2.1392
IRF7	Interferon regulatory factor 7	2.2998	NC
LY96	Lymphocyte antigen 96	-4.9407	NC
MBL2	Mannose-binding lectin (protein C) 2, soluble	-3.8309	2.2082
NLRP3	NLR family, pyrin domain containing 3	2.1374	NC
NOD2	Nucleotide-binding oligomerization domain containing 2	2.4438	NC
TICAM1	Toll-like receptor adaptor molecule 1	-2.8619	2.0873
TLR3	Toll-like receptor 3	-3.0218	NC
TLR4	Toll-like receptor 4	NC	-2.3616
TLR6	Toll-like receptor 6	-2.1854	NC
TLR7	Toll-like receptor 7	NC	2.0258
TLR8	Toll-like receptor 8	NC	-2.2878
TLR9	Toll-like receptor 9	NC	2.4685
IFNB1	Interferon beta 1	-3.8309	NC
CCR4	Chemokine (C-C motif) receptor 4	2.4068	NC
CCR5	Chemokine (C-C motif) receptor 5	NC	2.1401
IL17A	Interleukin 17A	-3.8309	NC
IFNA1	Interferon alpha 1	-3.8309	NC
IL4	Interleukin 4	-2.9785	2.3649
IL6	Interleukin 6	-3.8309	2.1509
IL5	Interleukin 5	-5.684	NC
IL2	Interleukin 2	-3.5093	2.1392
IL18	Interleukin 18	-3.8301	NC
IL13	Interleukin 13	-3.7825	2.1634
IL10	Interleukin 10	-3.8309	2.0211
CXCL10	Chemokine (C-X-C motif) ligand 10	-4.3281	2.9765
IL1A	Interleukin 1 alpha	-3.021	2.8189
CCL2	Chemokine (C-C motif) ligand 2	-2.2908	NC
CCL5	Chemokine (C-C motif) ligand 5	-2.3139	2.0725
FASLG	Fas ligand	-3.07	2.0561
CXCR3	Chemokine (C-X-C motif) receptor 3	-3.9117	NC

	ADAPTIVE IMMUNITY		
C3	Complement component 3	-2.6061	NC
CCL2	Chemokine (C-C motif) ligand 2	-2.2908	NC
CCL5	Chemokine (C-C motif) ligand 5	-2.3139	2.0725
CCR4	Chemokine (C-C motif) receptor 4	2.4068	NC
CCR5	Chemokine (C-C motif) receptor 5	NC	2.1401
CCR8	Chemokine (C-C motif) receptor 8	-3.2653	NC
CD40	CD40 molecule	NC	2.1064
CD40LG	CD40 ligand	2.1076	NC
CD80	CD80 molecule	NC	3.0758
CRP	C-reactive protein, pentraxin-related	-3.8309	NC
CXCL10	Chemokine (C-X-C motif) ligand 10	-4.3281	2.9765
CXCR3	Chemokine (C-X-C motif) receptor 3	-3.9117	NC
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-3.2047	NC
FASLG	Fas ligand	-3.07	2.0561
FOXP3	Forkhead box P3	-3.2481	NC
GATA3	GATA binding protein 3	-2.7182	NC
HLA-A	Major histocompatibility complex, class I, A	3.9397	NC
IFNA1	Interferon alpha 1	-3.8309	NC
IFNB1	Interferon beta 1	-3.8309	NC
IFNG	Interferon gamma	-2.8439	2.4089
IL10	Interleukin 10	-3.8309	2.0211
IL13	Interleukin 13	-3.7825	2.1634
IL17A	Interleukin 17A	-3.8309	NC
IL18	Interleukin 18	-3.8301	NC
IL1A	Interleukin 1 alpha	-3.021	2.8189
IL2	Interleukin 2	-3.5093	2.1392
IL4	Interleukin 4	-2.9785	2.3649
IL5	Interleukin 5	-5.684	NC
IL6	Interleukin 6	-3.8309	2.1509
IRF7	Interferon regulatory factor 7	2.2998	NC
MBL2	Mannose-binding lectin (protein C) 2, soluble	-3.8309	2.2082
NLRP3	NLR family, pyrin domain containing 3	2.1374	NC
NOD2	Nucleotide-binding oligomerization domain containing 2	2.4438	NC
RAG1	Recombination activating gene 1	-5.7506	NC
RORC	RAR-related orphan receptor C	-2.8572	NC
STAT4	Signal transducer and activator of transcription 4	NC	2.0954
TICAM1	Toll-like receptor adaptor molecule 1	-2.8619	2.0873
TLR3	Toll-like receptor 3	-3.0218	NC
TLR4	Toll-like receptor 4	NC	-2.3616
TLR6	Toll-like receptor 6	-2.1854	NC
TLR7	Toll-like receptor 7	NC	2.0258
TLR8	Toll-like receptor 8	NC	-2.2878
TLR9	Toll-like receptor 9	NC	2.4685

Table 5. Gene expression response to a single bout of exercise in active systemic lupus erythematosus (SLE_{ACTIVE}).

Gene symbol	Gene name	End-ex *vs.* Baseline (FC)	Recovery *vs.* Baseline (FC)
	INNATE IMMUNITY		
C3	Complement component 3	-3.4223	NC
CASP1	Caspase 1	-2.0748	NC
CCL2	Chemokine (C-C motif) ligand 2	-2.0748	NC
CCL5	Chemokine (C-C motif) ligand 5	-2.6933	NC
CRP	C-reactive protein, pentraxin-related	-3.7408	NC
CXCL10	Chemokine (C-X-C motif) ligand 10	-3.7408	2.1367
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-2.3726	NC
HLA-A	Major histocompatibility complex, class I, A	2.6664	NC
IFNA1	Interferon alpha 1	-2.3368	NC
IFNB1	Interferon, beta 1	-3.0087	2.6837
IL18	Interleukin 18	-2.3353	NC
IL2	Interleukin 2	-3.5439	NC
IRF7	Interferon regulatory factor 7	2.4521	2.0215
LY96	Lymphocyte antigen 96	-4.7781	NC
MAPK8	Mitogen-activated protein kinase 8	NC	2.2523
MBL2	Mannose-binding lectin (protein C) 2, soluble	-3.7146	NC
TICAM1	Toll-like receptor adaptor molecule 1	-2.885	-2.0228
TLR3	Toll-like receptor 3	NC	3.0184
	ADAPTIVE IMMUNITY		
C3	Complement component 3	-3.4223	NC
CCL2	Chemokine (C-C motif) ligand 2	-2.0748	NC
CCL5	Chemokine (C-C motif) ligand 5	-2.6933	NC
CCR4	Chemokine (C-C motif) receptor 4	2.2639	3.5957
CCR5	Chemokine (C-C motif) receptor 5	NC	2.2158
CCR6	Chemokine (C-C motif) receptor 6	NC	2.1947
CCR8	Chemokine (C-C motif) receptor 8	-2.594	NC
CD80	CD80 molecule	-4.8174	3.6854
CRP	C-reactive protein, pentraxin-related	-3.7408	NC
CXCL10	Chemokine (C-X-C motif) ligand 10	-3.7408	2.1367
CXCR3	Chemokine (C-X-C motif) receptor 3	-2.1625	-2.3697
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-2.3726	NC
FASLG	Fas ligand	-2.3726	-2.4915
FOXP3	Forkhead box P3	-3.6605	-4.0451
GATA3	GATA binding protein 3	-2.3368	NC
HLA-A	Major histocompatibility complex, class I, A	2.6664	NC
IFNA1	Interferon alpha 1	-2.3368	NC
IFNB1	Interferon, beta 1	-3.0087	2.6837
IFNG	Interferon gamma	-4.8211	NC
IL10	Interleukin 10	-2.9632	NC
IL13	Interleukin 13	-2.1952	NC
IL17A	Interleukin 17A	-2.9683	NC
IL18	Interleukin 18	-2.3353	NC
IL2	Interleukin 2	-3.5439	NC
IL4	Interleukin 4	-2.5493	2.1261
IL5	Interleukin 5	-2.8056	NC
IL6	Interleukin 6	-3.7408	2.1817
IRF7	Interferon regulatory factor 7	2.4521	2.0215
MAPK8	Mitogen-activated protein kinase 8	NC	2.2523
MBL2	Mannose-binding lectin (protein C) 2, soluble	-3.7146	NC

RAG1	Recombination activating gene 1	-4.8882	NC
STAT4	Signal transducer and activator of transcription 4	NC	2.1524
TICAM1	Toll-like receptor adaptor molecule 1	-2.885	-2.0228
TLR3	Toll-like receptor 3	NC	3.0184

Table 6. Network descriptive parameters in patients ($SLE_{INACTIVE}$ and SLE_{ACTIVE}) and healthy controls (HC).

Network parameters	SLE_{ACTIVE}	$SLE_{INACTIVE}$	HC
Number of nodes	29	40	58
Number of edges	29	60	195
Average node degree	2.0	3.0	6.72
Diameter	9	8	6
Characteristic path length	3.64	3.40	2.62
Clustering coefficient	0.15	0.33	0.39
Network density	0.07	0.08	0.12

IFNAR1, IL1R1, JAK2, STAT1, STAT3, STAT4, TYK2, IRF3, NFKB1 and *MYD88* (Figure 1; genes with yellow borders).

In the $SLE_{INACTIVE}$ group, there were 39 DE genes (7 up-regulated and 32 down-regulated) at End-ex compared with Baseline, whereas 22 genes (20 up-regulated and 2 down-regulated) were modulated at Recovery compared with Baseline (Table 4). Additionally, the network analysis revealed that the main exercise-modulated hubs were as follows: *IL2, IL13, IL18, GATA3, STAT4, CCL5, LY96, TLR4, TLR7, TLR8* and *TLR9* (Figure 2; genes with yellow borders).

In the SLE_{ACTIVE} group, 32 DE genes (3 up-regulated and 29 down-regulated) were modulated at End-ex when compared with Baseline, while 17 (13 up-regulated and 4 down-regulated) were modulated at Recovery when compared with Baseline (Table 5). Network analysis revealed that the main exercise-modulated hubs were *IL2, IFNG, IL18, IL13, GATA3, STAT4,* and *CCL5* (Figure 3; genes with yellow borders).

The analysis of the network descriptive parameters is shown in Table 6. Interestingly, the lower number of nodes and edges, average node degree, and network density in the SLE_{ACTIVE} and $SLE_{INACTIVE}$ groups indicated that a single bout of exercise modulated less dense gene networks in the SLE groups when compared with the HC group. In addition, SLE_{ACTIVE} had lower network connectivity when compared with $SLE_{INACTIVE}$.

DISCUSSION

To the best of our knowledge, this is the first study to assess the effects of acute exercise on the gene expression profiles of circulating leukocytes from SLE patients. The main findings of this study were two-fold: *i)* the SLE patients and healthy individuals displayed changes in the expression of their leukocyte inflammation-related genes following acute aerobic exercise; and *ii)* exercise regulated fewer immune-related genes and less connected networks in the leukocytes from the SLE patients compared with their healthy peers.

Leukocytes from the HC group showed a down-regulation of genes related to innate immunity (*i.e.*, cytokines and their receptors) at End-ex, in contrast with previous findings (30, 66). Although these previous studies did not detect any alterations in cytokine-related gene expression, cytokine serum levels did increase after exercise in accordance with a recent study from our group (46). In addition, the expression of Toll-like receptor pathway-related genes *(i.e., TLR3, TLR7, MYD88, IRF3,* and *IFNB1)*, which are associated with the inflammatory process (3), were down-regulated at End-ex in the HC group, suggesting that an anti-inflammatory response occurred immediately after exercise as previously reported (60). Conversely, at Recovery, the leukocytes of the HC group showed the up-regulation of the expression of genes related to the JAK/STAT pathway (*i.e., JAK2, TYK2, STAT1, STAT3, STAT4, IFNG,* and *IFNAR1*) and the pro-inflammatory pathway (*i.e., IL1B, IL1R1, NFKB1, TLR1, TLR8,* and *CD14)*, cor-

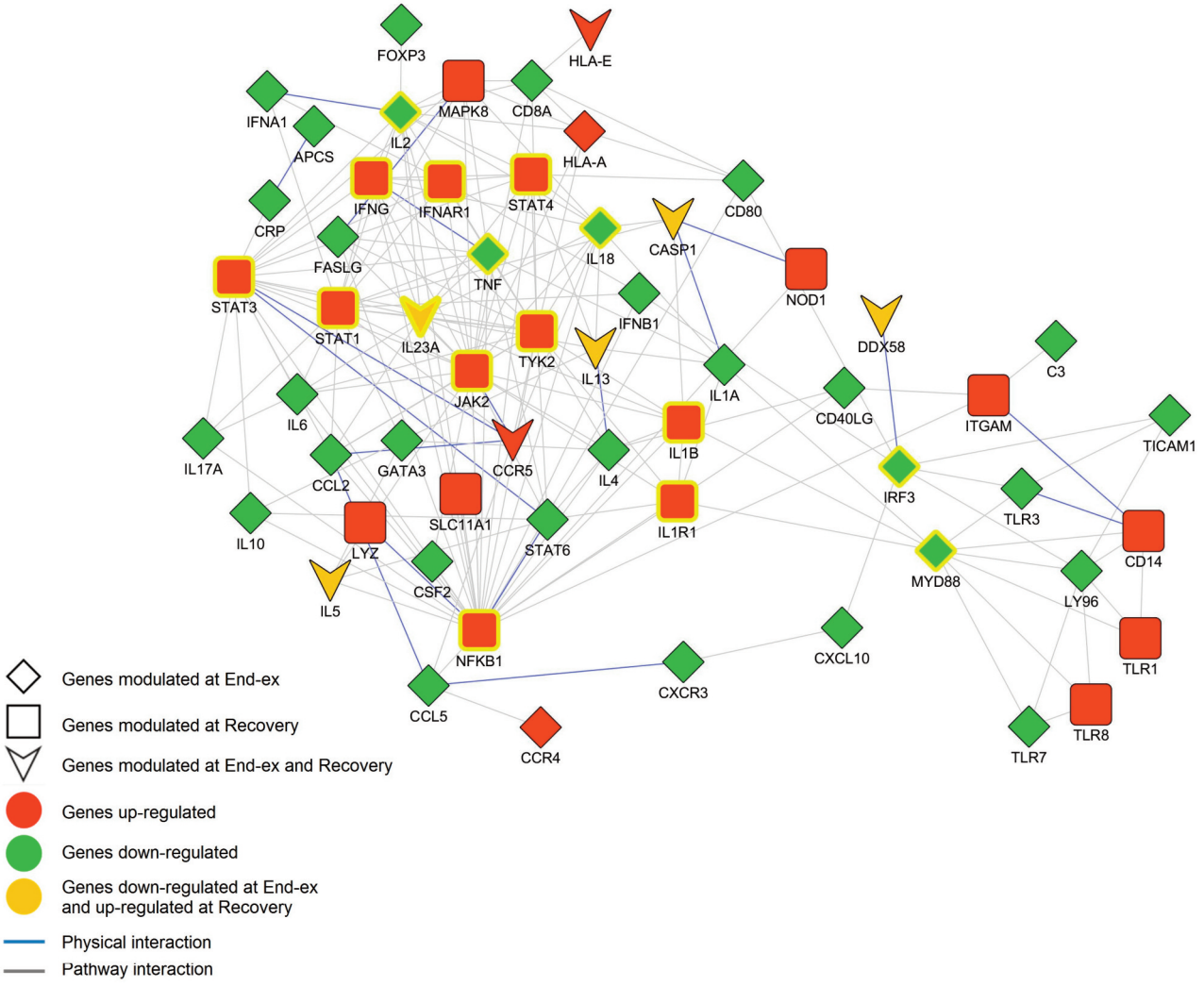


Figure 1. Network analysis of changes in gene expression obtained from comparisons between Baseline, End-ex and Recovery periods in healthy controls. Network analysis of the DE genes resulting from the comparisons of End-ex *vs.* Baseline and Recovery *vs.* Baseline in the HC. Networks were constructed using information obtained from the pathways (grey edges) and physical interactions (blue edges). Diamond and square nodes denote the DE genes regulated immediately after exercise (End-ex) and at Recovery, respectively. V-shaped nodes represent the genes regulated during both periods (End-ex and Recovery). The up- and down-regulated genes are depicted in red and green, respectively. The down-regulated genes at End-ex that were up-regulated at Recovery are depicted in orange.

roborating previous results (35, 54). The increased gene expression in relation to the JAK/STAT pathway has been associated with a variety of cellular functions (32); however, particularly in response to exercise, it has been linked to cell growth and the survival of neutrophils (54). Additionally, the increased expression of pro-inflammatory pathway-related genes at Recovery support previous findings (35). Neubauer et al. (35) suggested that this up-regulation might be explained by the appearance of muscle-derived damage-associated molecular mechanisms. Such molecules could be recognized by Toll-like receptors (TLR), triggering downstream pathways in a similar manner as the activation of sterile inflammation (14), but differing due to the transient nature of the exercise-induced inflammatory response (35).

The $SLE_{INACTIVE}$ group showed a similar response to the HC group, with the down-regulation of innate immunity genes such as cytokine-related *(i.e., IL13, IL2, IL18,* and *CCL5*) and TLR-related pathway (*i.e., TLR6, LY96*, and *TICAM1*) genes at End-ex. These genes were up-regulated at Baseline. Thus, it seems that exercise may negatively modulate the expression of genes over-expressed at baseline in SLE. However, while cytokine-related genes were altered in response to exercise, serum cytokine levels appeared to be not affected (46). The down-regulation observed at End-ex was not sustained during Recovery, where there was up-regulation of genes related to JAK/STAT pathway (*i.e., STAT4, IL13,* and *IL2*) and cytokines *(i.e., IL13, IL1A*, and *CCR5*). Although these genes have been implicated in SLE pathogenesis (4, 28), the same response has also been observed in healthy individuals (54) in a transitory manner (35). Moreover, adaptive immunity genes *(i.e., GATA3, RORC,* and *FOXP3*) were down-regulated at End-ex in $SLE_{INACTIVE}$, and *GATA3* was the highest connected gene in its corresponding network. *GATA3* is a transcription factor responsible for promoting the responses of T helper 2 cells and has been associated with the autoantibodies production in SLE patients (44), as well as autoimmune glomerulonephritis in mice (69). Therefore, it seems that a single bout of exercise could, at least transiently, down-regulate transcription factors associated with SLE disease pathogenesis.

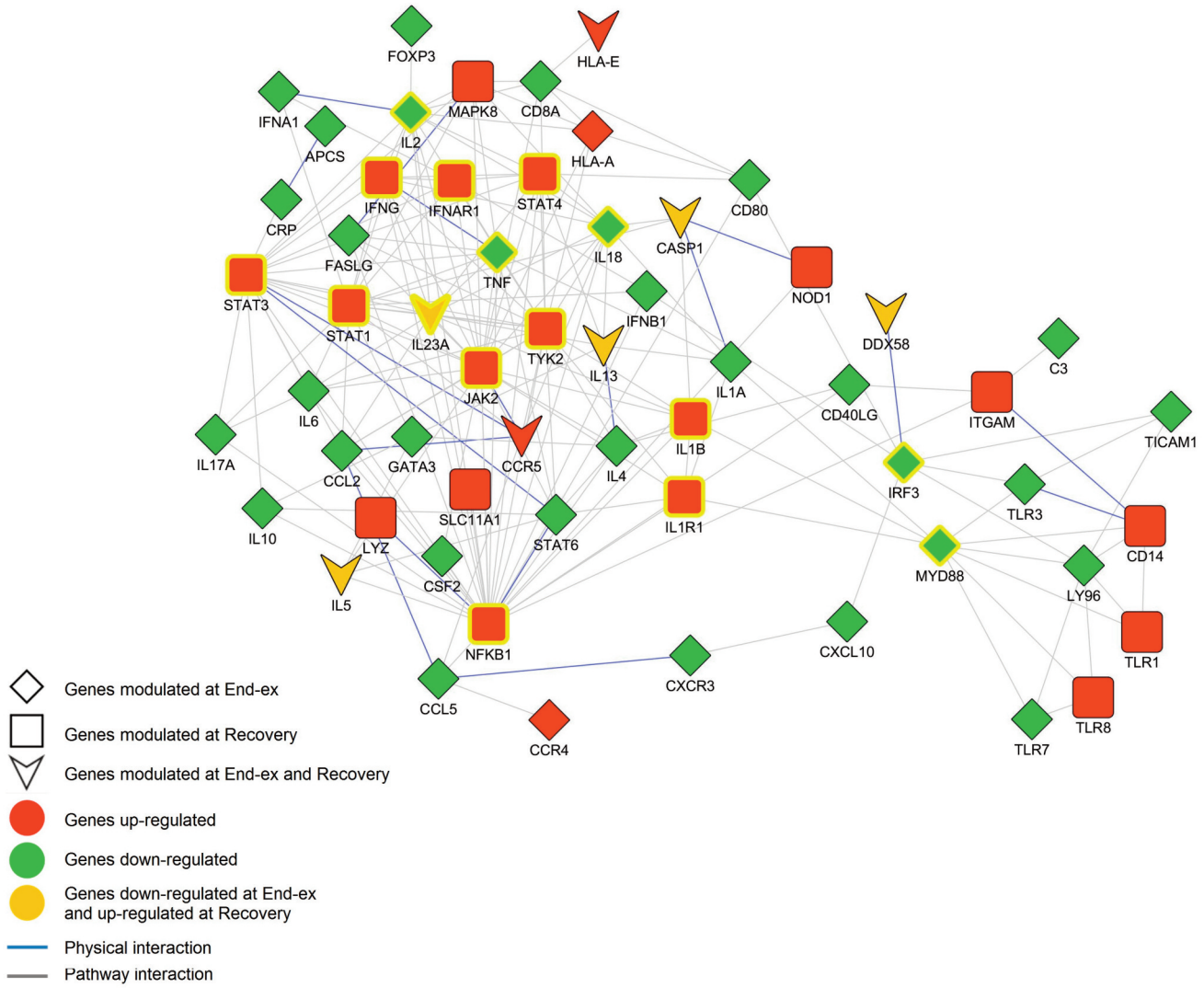


Figure 2. Network analysis of changes in gene expression obtained from comparisons between Baseline, End-ex and Recovery periods in $SLE_{INACTIVE}$ patients. Network analysis of DE genes resulting from the comparisons of End-ex *vs.* Baseline and Recovery *vs.* Baseline in the $SLE_{INACTIVE}$ patients. Networks were constructed using information obtained from the pathways (grey edges) and physical interactions (blue edges). Diamond and square nodes denote the DE genes regulated immediately after exercise (End-ex) and at Recovery, respectively. V-shaped nodes represent genes regulated during both periods (End-ex and Recovery). The up- and down-regulated genes are depicted in red and green, respectively. The down-regulated genes at End-ex phase that were up-regulated genes at Recovery are depicted in orange.

In the SLE_{ACTIVE} group, fewer genes were modulated by exercise, resulting in a less connected network. The main down-regulated network genes at End-ex were related to both innate and adaptive immunity, such as *IL2, IFNG, IL18, IL13, GATA3,* and *CCL5*. Interestingly, we observed a down-regulation of *IFNG*, which has been pointed out as a major effector molecule in SLE disease (64). Moreover, the reduction in *GATA3* gene expression was also observed in $SLE_{INACTIVE}$ at End-ex. At Recovery, fewer genes were up-regulated (*i.e., STAT4, TLR3, IRF7, CCR4,* and *CCR5*), and they possessed low degrees of connection, suggesting only the partial activation of the immune pathway.

Overall, we observed a similar response pattern of leukocyte gene expression in the $SLE_{INACTIVE}$, SLE_{ACTIVE} and HC groups following a single bout of acute exercise, since all groups showed a global down-regulation of blood leukocyte gene expression at End-ex compared with Baseline. These findings further support the notion that a single bout of aerobic exercise (up to ~70% VO_{2peak}) may not trigger a pro-inflammatory response in SLE patients (46, 47). Despite this, the analysis of network parameters (*i.e.,* number of nodes, number of edges, average node degree, and network density) suggested a lower network connectivity in the $SLE_{INACTIVE}$ and SLE_{ACTIVE} groups when compared with the HC group. Moreover, when compared the HC group, the SLE groups showed a higher network diameter with a lower clustering coefficient, which may suggest a less efficient and controlled flow of information (57). One hypothesis for the less connected networks and reduced number of modulated genes in SLE groups would be that an overexpression of inflammatory genes at Baseline might have precluded the additional exercise-induced transcriptional up-regulation in the leukocytes, as the expression of inflammatory genes might have approached maximum levels at Baseline. Alternatively, the drug therapies in SLE groups may have partially impeded the broader gene modulation observed in the HC group. In fact, treatment with immunosuppressive drugs (*e.g.*, glucocorticoid, chloroquine, and methotrexate) has been

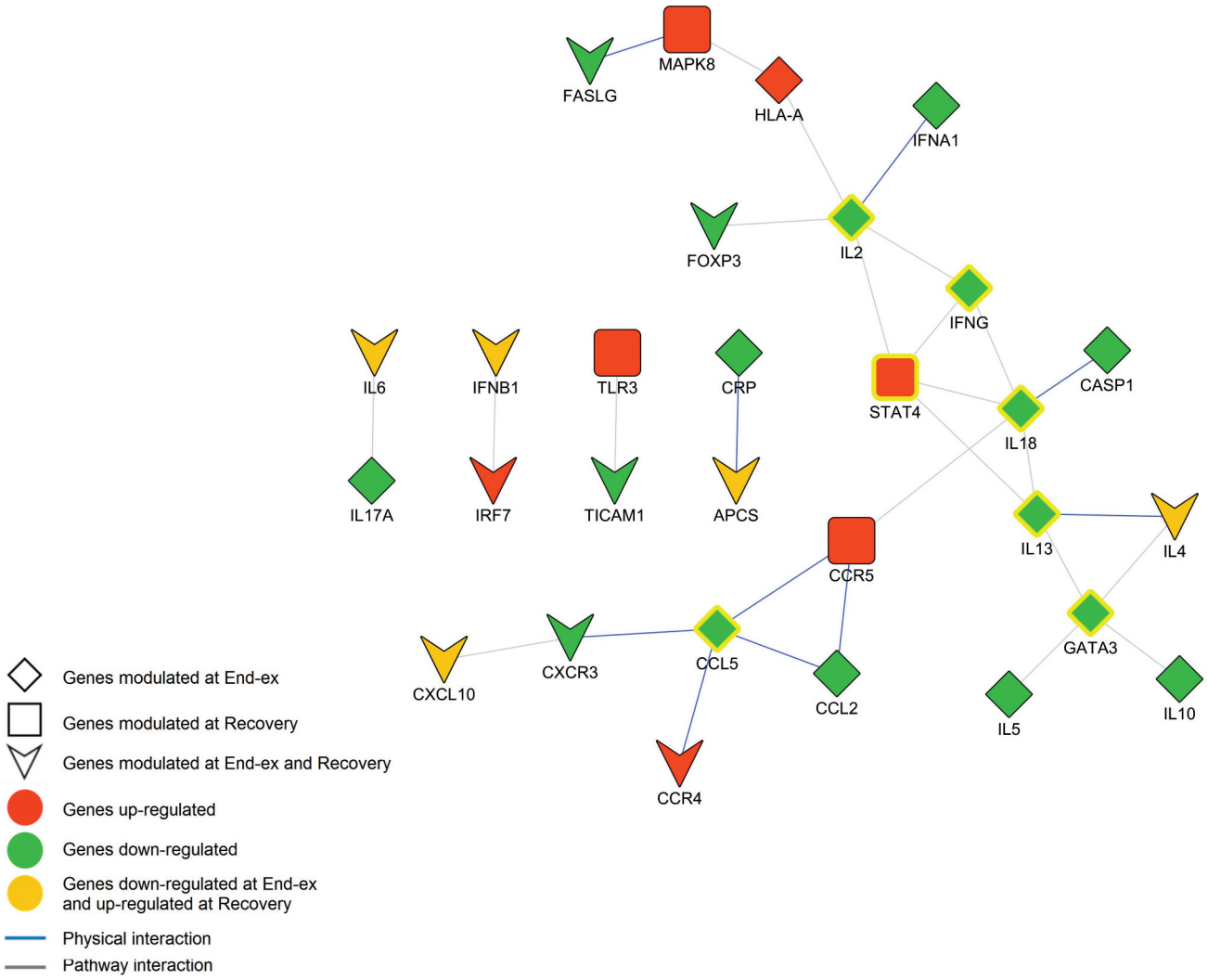


Figure 3. Network analysis of changes in gene expression obtained from comparisons between Baseline, End-ex and Recovery periods in SLE_{ACTIVE} patients. Network analysis of the DE genes resulting from the comparisons of End-ex *vs.* Baseline and Recovery *vs.* Baseline in the SLE_{ACTIVE} patients. Networks were constructed using information obtained from the pathways (grey edges) and physical interactions (blue edges). Diamond and square nodes denote the DE genes regulated immediately after exercise (End-ex) and at Recovery, respectively. V-shaped nodes represent genes regulated during both periods (End-ex and Recovery). The up- and down-regulated genes are depicted in red and green, respectively. Down-regulated genes at End-ex that were up-regulated at Recovery are depicted in orange.

shown to suppress the expression of inflammatory cytokine-related genes in human peripheral blood mononuclear cells (24, 67). Interestingly, lower network connectivity was observed in the SLE_{ACTIVE} group in comparison with the $SLE_{INACTIVE}$ group. Difference in drug therapy between the SLE groups may possibly account for this observation; although both SLE groups were receiving immunosuppressive therapy, only the SLE_{ACTIVE} group was treated with glucocorticoids, which may induce a very potent immunomodulatory response (24, 67).

Importantly, the gene expression changes in the leukocytes from the HC group formed more densely connected networks compared to those from the SLE groups, and some genes were exclusively regulated in the HC group, such as *IL23, JAK2, STAT1, STAT3,* and *TYK2*. The latter has been considered important in the maintenance of complex I-dependent mitochondrial respiration and ATP production. Notably, $Tyk2^{(-/-)}$ showed decreased exercise tolerance compared with wild-type mice, which was paralleled by deficiencies in Tyk2 kinase and other components of the JAK/STAT pathway, predisposing the animals to abnormal responses following inflammatory challenges (50).

One may argue that the main limitation of this study is the lack of gene expression assessment in specific sub-sets of leukocytes, such as neutrophils or peripheral blood mononuclear cells. However, our main purpose was to investigate the global effect of exercise on leucocyte gene response, rather than the specific response of each leukocyte subset. Furthermore, previous studies reported that a single bout of acute aerobic exercise elicited a similar gene expression in total leukocytes (10) and neutrophils (16), suggesting that leukocyte gene expression may be representative of its sub-populations. Notwithstanding, since we did not analyze possible changes in leukocyte number, which may have influenced gene expression, additional studies involving SLE patients are required to further clarify the role of acute aerobic exercise upon leukocyte sub-sets gene expression. Furthermore, the lack of biochemistry data (*e.g.*, cytokines and acute phase pro-

teins) at each time-point may be considered another limitation, thus a more integrative study comprising molecular, biochemical, and physiological responses remains necessary. Finally, caution should be exercised in generalizing the current findings since this is a relatively small-scale study (*i.e.*, n = 8 patients and 4 controls).

CONCLUSIONS

In conclusion, our results indicated that a single bout of acute aerobic exercise altered gene expression levels in circulating leukocytes from healthy individuals and SLE patients, irrespective of disease activity. Immediately after exercise, inflammatory genes were down-regulated in all groups compared with Baseline; at Recovery, an up-regulation was observed. Less connected networks were revealed in the SLE groups, suggesting that the leukocytes from these patients are deficient in triggering a normal exercise-induced immune transcriptional response. Further studies are needed to assess the association of exercise-induced molecular changes with the potential benefits and risks of exercise training in SLE management.

DISCLOSURE OF FUNDING

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) to LAP and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) to LAP, DS, SBVM, NOC, FBB, FRL, EB, EB, ALS, HR, BG.

COMPETING INTERESTS

The authors declare that they do not have conflict of interests.

AUTHOR CONTRIBUTIONS

- **Conception and design of the experiments:** L.A.P.; A.L.S..; E.B.; H.R.; B.G.
- **Collection, analysis and interpretation of data:** L.A.P.; D.S.; D.C.A.; H. A.; C.A.M.; M.A.C.; F.B.B.; F.R.L.; E.B.; E.B.; A.L.S.; R.H.; N.O.S.C.; B.G.
- **Drafting the article or revising it critically for important intellectual content:** L.A.P.; D.S.; D.C.A.; H. A.; C.A.M.; M.A.C.; F.B.B.; F.R.L.; E.B.; E.B.; A.L.S.; R.H.; N.O.S.C.; B.G.

All authors approved the final version of the manuscript

ACKNOWLEDGEMENTS

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2011/24093-2), Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

1. Adamopoulos S, Parissis J, Karatzas D, Kroupis C, Georgiadis M, Karavolias G, Paraskevaidis J, Koniavitou K, Coats AJ, and Kremastinos DT. Physical training modulates proinflammatory cytokines and the soluble Fas/soluble Fas ligand system in patients with chronic heart failure. J Am Coll Cardiol 39: 653-663, 2002.
2.. Akira S, Taga T, and Kishimoto T. Interleukin-6 in biology and medicine. Adv Immunol 54: 1-78, 1993.
3. Akira S, and Takeda K. Toll-like receptor signalling. Nat Rev Immunol 4: 499-511, 2004.
4. Arasappan D, Tong W, Mummaneni P, Fang H, and Amur S. Meta-analysis of microarray data using a pathway-based approach identifies a 37-gene expression signature for systemic lupus erythematosus in human peripheral blood mononuclear cells. BMC Med 9: 65, 2011.
5. Assenov Y, Ramírez F, Schelhorn SE, Lengauer T, and Albrecht M. Computing topological parameters of biological networks. Bioinformatics 24: 282-284, 2008.
6. Bansi J, Bloch W, Gamper U, and Kesselring J. Training in MS: influence of two different endurance training protocols (aquatic versus overland) on cytokine and neurotrophin concentrations during three week randomized controlled trial. Mult Scler 19: 613-621, 2013.
7. Baslund B, Lyngberg K, Andersen V, Halkjaer Kristensen J, Hansen M, Klokker M, and Pedersen BK. Effect of 8 wk of bicycle training on the immune system of patients with rheumatoid arthritis. J Appl Physiol 75: 1691-1695, 1993.
8. Bearne LM, Scott DL, and Hurley MV. Exercise can reverse quadriceps sensorimotor dysfunction that is associated with rheumatoid arthritis without exacerbating disease activity. Rheumatology (Oxford) 41: 157-166, 2002.
9. Benatti FB, and Pedersen BK. Exercise as an anti-inflammatory therapy for rheumatic diseases-myokine regulation. Nat Rev Rheumatol 2014.
10. Büttner P, Mosig S, Lechtermann A, Funke H, and Mooren FC. Exercise affects the gene expression profiles of human white blood cells. J Appl Physiol (1985) 102: 26-36, 2007.
11. Carroll MC. A protective role for innate immunity in systemic lupus erythematosus. Nat Rev Immunol 4: 825-831, 2004.
12. Carvalho MR, Sato EI, Tebexreni AS, Heidecher RT, Schenkman S, and Neto TL. Effects of supervised cardiovascular training program on exercise tolerance, aerobic capacity, and quality of life in patients with systemic lupus erythematosus. Arthritis Rheum 53: 838-844, 2005.
13. Castellano V, Patel DI, and White LJ. Cytokine responses to acute and chronic exercise in multiple sclerosis. J Appl Physiol 104: 1697-1702, 2008.
14. Chen GY, and Nuñez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol 10: 826-837, 2010.
15. Connolly AM, Keeling RM, Streif EM, Pestronk A, and Mehta S. Complement 3 deficiency and oral prednisolone improve strength and prolong survival of laminin alpha2-deficient mice. J Neuroimmunol 127: 80-87, 2002.
16. Connolly PH, Caiozzo VJ, Zaldivar F, Nemet D, Larson J, Hung SP, Heck JD, Hatfield GW, and Cooper DM. Effects of exercise on gene expression in human peripheral blood mononuclear cells. J Appl Physiol (1985) 97: 1461-1469, 2004.

17. Cooper DM, Radom-Aizik S, Schwindt C, and Zaldivar F. Dangerous exercise: lessons learned from dysregulated inflammatory responses to physical activity. J Appl Physiol (1985) 103: 700-709, 2007.
18. Crispín JC, Hedrich CM, and Tsokos GC. Gene-function studies in systemic lupus erythematosus. Nat Rev Rheumatol 9: 476-484, 2013.
19. dos Reis-Neto ET, da Silva AE, Monteiro CM, de Camargo LM, and Sato EI. Supervised physical exercise improves endothelial function in patients with systemic lupus erythematosus. Rheumatology (Oxford) 52: 2187-2195, 2013.
20. Gabay C. Interleukin-6 and chronic inflammation. Arthritis Res Ther 8 Suppl 2: S3, 2006.
21. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, and Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol 11: 607-615, 2011.
22. Goldhammer E, Tanchilevitch A, Maor I, Beniamini Y, Rosenschein U, and Sagiv M. Exercise training modulates cytokines activity in coronary heart disease patients. Int J Cardiol 100: 93-99, 2005.
23. Han GM, Chen SL, Shen N, Ye S, Bao CD, and Gu YY. Analysis of gene expression profiles in human systemic lupus erythematosus using oligonucleotide microarray. Genes Immun 4: 177-186, 2003.
24. Hobl EL, Mader RM, Erlacher L, Duhm B, Mustak M, Bröll H, Högger P, Kalipciyan M, and Jilma B. The influence of methotrexate on the gene expression of the pro-inflammatory cytokine IL-12A in the therapy of rheumatoid arthritis. Clin Exp Rheumatol 29: 963-969, 2011.
25. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 40: 1725, 1997.
26. Jara LJ, Medina G, Vera-Lastra O, and Amigo MC. Accelerated atherosclerosis, immune response and autoimmune rheumatic diseases. Autoimmun Rev 5: 195-201, 2006.
27. Joyeux M, Godin-Ribuot D, Yellon DM, Demenge P, and Ribuot C. Heat stress response and myocardial protection. Fundam Clin Pharmacol 13: 1-10, 1999.
28. Kawasaki M, Fujishiro M, Yamaguchi A, Nozawa K, Kaneko H, Takasaki Y, Takamori K, Ogawa H, and Sekigawa I. Possible role of the JAK/STAT pathways in the regulation of T cell-interferon related genes in systemic lupus erythematosus. Lupus 20: 1231-1239, 2011.
29. Miossi R, Benatti FB, Lúciade de Sá Pinto A, Lima FR, Borba EF, Prado DM, Perandini LA, Gualano B, Bonfá E, and Roschel H. Using exercise training to counterbalance chronotropic incompetence and delayed heart rate recovery in systemic lupus erythematosus: a randomized trial. Arthritis Care Res (Hoboken) 64: 1159-1166, 2012.
30. Moldoveanu AI, Shephard RJ, and Shek PN. Exercise elevates plasma levels but not gene expression of IL-1beta, IL-6, and TNF-alpha in blood mononuclear cells. J Appl Physiol (1985) 89: 1499-1504, 2000.
31. Montojo J, Zuberi K, Rodriguez H, Kazi F, Wright G, Donaldson SL, Morris Q, and Bader GD. GeneMANIA Cytoscape plugin: fast gene function predictions on the desktop. Bioinformatics 26: 2927-2928, 2010.
32. Murray PJ. The JAK-STAT signaling pathway: input and output integration. J Immunol 178: 2623-2629, 2007.
33. Muñoz LE, Janko C, Schulze C, Schorn C, Sarter K, Schett G, and Herrmann M. Autoimmunity and chronic inflammation - two clearance-related steps in the etiopathogenesis of SLE. Autoimmun Rev 10: 38-42, 2010.
34. Nader GA, Dastmalchi M, Alexanderson H, Grundtman C, Gernapudi R, Esbjörnsson M, Wang Z, Rönnelid J, Hoffman EP, Nagaraju K, and Lundberg IE. A longitudinal, integrated, clinical, histological and mRNA profiling study of resistance exercise in myositis. Mol Med 16: 455-464, 2010.
35. Neubauer O, Sabapathy S, Ashton KJ, Desbrow B, Peake JM, Lazarus R, Wessner B, Cameron-Smith D, Wagner KH, Haseler LJ, and Bulmer AC. Time-course dependent changes in the transcriptome of human skeletal muscle during recovery from endurance exercise: from inflammation to adaptive remodeling. J Appl Physiol (1985) 2013.
36. Neubauer O, Sabapathy S, Lazarus R, Jowett JB, Desbrow B, Peake JM, Cameron-Smith D, Haseler LJ, Wagner KH, and Bulmer AC. Transcriptome analysis of neutrophils after endurance exercise reveals novel signaling mechanisms in the immune response to physiological stress. J Appl Physiol (1985) 114: 1677-1688, 2013.
37. Niebauer J. Effects of exercise training on inflammatory markers in patients with heart failure. Heart Fail Rev 13: 39-49, 2008.
38. Nikolova M, Marie-Cardine A, Boumsell L, and Bensussan A. BY55/CD160 acts as a co-receptor in TCR signal transduction of a human circulating cytotoxic effector T lymphocyte subset lacking CD28 expression. Int Immunol 14: 445-451, 2002.
39. Ortega E, Bote ME, Giraldo E, and García JJ. Aquatic exercise improves the monocyte pro- and anti-inflammatory cytokine production balance in fibromyalgia patients. Scand J Med Sci Sports 22: 104-112, 2012.
40. Ortega E, García JJ, Bote ME, Martín-Cordero L, Escalante Y, Saavedra JM, Northoff H, and Giraldo E. Exercise in fibromyalgia and related inflammatory disorders: known effects and unknown chances. Exerc Immunol Rev 15: 42-65, 2009.
41. Ostrowski K, Rohde T, Asp S, Schjerling P, and Pedersen BK. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. J Physiol 515 (Pt 1): 287-291, 1999.
42. Ostrowski K, Schjerling P, and Pedersen BK. Physical activity and plasma interleukin-6 in humans--effect of intensity of exercise. Eur J Appl Physiol 83: 512-515, 2000.
43. Pedersen BK. Muscle as a secretory organ. Compr Physiol 3: 1337-1362, 2013.
44. Peng XB, and Deng Y. [Expression of transcription factors T-bet and GATA-3 in peripheral blood mononuclear cells from patients with systemic lupus erythematosus]. Nan Fang Yi Ke Da Xue Xue Bao 29: 2138-2139, 2009.
45. Perandini LA, de Sá-Pinto AL, Roschel H, Benatti FB, Lima FR, Bonfá E, and Gualano B. Exercise as a therapeutic tool to counteract inflammation and clinical symptoms in autoimmune rheumatic diseases. Autoimmun Rev 12: 218-224, 2012.
46. Perandini LA, Sales-de-Oliveira D, Mello S, Camara NO, Benatti FB, Lima FR, Borba E, Bonfa E, Roschel H, Sá-Pinto AL, and Gualano B. Inflammatory cytokine kinetics to single bouts of acute moderate and intense aerobic exercise in women with active and inactive systemic lupus erythematosus. Exerc Immunol Rev 21: 174-185, 2015.

47. Perandini LA, Sales-de-Oliveira D, Mello SB, Camara NO, Benatti FB, Lima FR, Borba E, Bonfa E, Sá-Pinto AL, Roschel H, and Gualano B. Exercise training can attenuate the inflammatory milieu in women with systemic lupus erythematosus. J Appl Physiol (1985) 117: 639-647, 2014.
48. Perretti M, and Flower RJ. Annexin 1 and the biology of the neutrophil. J Leukoc Biol 76: 25-29, 2004.
49. Poole DC, Wilkerson DP, and Jones AM. Validity of criteria for establishing maximal O2 uptake during ramp exercise tests. Eur J Appl Physiol 102: 403-410, 2008.
50. Potla R, Koeck T, Wegrzyn J, Cherukuri S, Shimoda K, Baker DP, Wolfman J, Planchon SM, Esposito C, Hoit B, Dulak J, Wolfman A, Stuehr D, and Larner AC. Tyk2 tyrosine kinase expression is required for the maintenance of mitochondrial respiration in primary pro-B lymphocytes. Mol Cell Biol 26: 8562-8571, 2006.
51. Rabinovich RA, Figueras M, Ardite E, Carbó N, Troosters T, Filella X, Barberà JA, Fernandez-Checa JC, Argilés JM, and Roca J. Increased tumour necrosis factor-alpha plasma levels during moderate-intensity exercise in COPD patients. Eur Respir J 21: 789-794, 2003.
52. Radom-Aizik S, Zaldivar F, Haddad F, and Cooper DM. Impact of brief exercise on peripheral blood NK cell gene and microRNA expression in young adults. J Appl Physiol (1985) 114: 628-636, 2013.
53. Radom-Aizik S, Zaldivar F, Leu SY, and Cooper DM. A brief bout of exercise alters gene expression and distinct gene pathways in peripheral blood mononuclear cells of early- and late-pubertal females. J Appl Physiol (1985) 107: 168-175, 2009.
54. Radom-Aizik S, Zaldivar F, Leu SY, Galassetti P, and Cooper DM. Effects of 30 min of aerobic exercise on gene expression in human neutrophils. J Appl Physiol (1985) 104: 236-243, 2008.
55. Radom-Aizik S, Zaldivar FP, Haddad F, and Cooper DM. Impact of brief exercise on circulating monocyte gene and microRNA expression: implications for atherosclerotic vascular disease. Brain Behav Immun 39: 121-129, 2014.
56. Rall LC, Roubenoff R, Cannon JG, Abad LW, Dinarello CA, and Meydani SN. Effects of progressive resistance training on immune response in aging and chronic inflammation. Med Sci Sports Exerc 28: 1356-1365, 1996.
57. Ruan J, Dean AK, and Zhang W. A general co-expression network-based approach to gene expression analysis: comparison and applications. BMC Syst Biol 4: 8, 2010.
58. Schulz KH, Gold SM, Witte J, Bartsch K, Lang UE, Hellweg R, Reer R, Braumann KM, and Heesen C. Impact of aerobic training on immune-endocrine parameters, neurotrophic factors, quality of life and coordinative function in multiple sclerosis. J Neurol Sci 225: 11-18, 2004.
59. Shoenfeld Y, Gerli R, Doria A, Matsuura E, Cerinic MM, Ronda N, Jara LJ, Abu-Shakra M, Meroni PL, and Sherer Y. Accelerated atherosclerosis in autoimmune rheumatic diseases. Circulation 112: 3337-3347, 2005.
60. Simpson RJ, and Bosch JA. Special issue on exercise immunology: Current perspectives on aging, health and extreme performance. Brain Behav Immun 39: 1-7, 2014.
61. Simpson RJ, McFarlin BK, McSporran C, Spielmann G, ó Hartaigh B, and Guy K. Toll-like receptor expression on classic and pro-inflammatory blood monocytes after acute exercise in humans. Brain Behav Immun 23: 232-239, 2009.
62. Starkie R, Ostrowski SR, Jauffred S, Febbraio M, and Pedersen BK. Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans. FASEB J 17: 884-886, 2003.
63. Steensberg A, Fischer CP, Keller C, Møller K, and Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. Am J Physiol Endocrinol Metab 285: E433-437, 2003.
64. Theofilopoulos AN, Koundouris S, Kono DH, and Lawson BR. The role of IFN-gamma in systemic lupus erythematosus: a challenge to the Th1/Th2 paradigm in autoimmunity. Arthritis Res 3: 136-141, 2001.
65. Tsokos GC. Systemic lupus erythematosus. N Engl J Med 365: 2110-2121, 2011.
66. Ullum H, Haahr PM, Diamant M, Palmø J, Halkjaer-Kristensen J, and Pedersen BK. Bicycle exercise enhances plasma IL-6 but does not change IL-1 alpha, IL-1 beta, IL-6, or TNF-alpha pre-mRNA in BMNC. J Appl Physiol (1985) 77: 93-97, 1994.
67. van de Garde MD, Martinez FO, Melgert BN, Hylkema MN, Jonkers RE, and Hamann J. Chronic exposure to glucocorticoids shapes gene expression and modulates innate and adaptive activation pathways in macrophages with distinct changes in leukocyte attraction. J Immunol 192: 1196-1208, 2014.
68. Wasserman K, Whipp BJ, Koyl SN, and Beaver WL. Anaerobic threshold and respiratory gas exchange during exercise. J Appl Physiol 35: 236-243, 1973.
69. Yoh K, Shibuya K, Morito N, Nakano T, Ishizaki K, Shimohata H, Nose M, Izui S, Shibuya A, Koyama A, Engel JD, Yamamoto M, and Takahashi S. Transgenic overexpression of GATA-3 in T lymphocytes improves autoimmune glomerulonephritis in mice with a BXSB/MpJ-Yaa genetic background. J Am Soc Nephrol 14: 2494-2502, 2003.
70. Zhang H, Sunnarborg SW, McNaughton KK, Johns TG, Lee DC, and Faber JE. Heparin-binding epidermal growth factor-like growth factor signaling in flow-induced arterial remodeling. Circ Res 102: 1275-1285, 2008.

Exercise, inflammation, and fatigue in cancer survivors

Emily C.P. LaVoy[1*], Christopher P. Fagundes[2,3], Robert Dantzer[3]

[1] Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, Houston, TX, USA
[2] Department of Psychology, Rice University, Houston, TX, USA
[3] Department of Symptom Research, Division of Internal Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX

ABSTRACT

Cancer-related fatigue significantly disrupts normal functioning and quality of life for a substantial portion of cancer survivors, and may persist for years following cancer treatment. While the causes of persistent fatigue among cancer survivors are not yet fully understood, accumulating evidence suggests that several pathways, including chronic inflammation, autonomic imbalance, HPA-axis dysfunction, and/or mitochondrial damage, could contribute towards the disruption of normal neuronal function and result in the symptom of cancer-related fatigue. Exercise training interventions have been shown to be some of the more successful treatment options to address cancer-related fatigue. In this review, we discuss the literature regarding the causes of persistent fatigue in cancer survivors and the mechanisms by which exercise may relieve this symptom. There is still much work to be done until the prescription of exercise becomes standard practice for cancer survivors. With improvements in the quality of studies, evidenced-based exercise interventions will allow exercise scientists and oncologists to work together to treat cancer-related fatigue.

INTRODUCTION

The National Comprehensive Cancer Network defines cancer-related fatigue as a "distressing, persistent, subjective sense of physical, emotional and/or cognitive tiredness or exhaustion related to cancer or cancer treatment that is not proportional to recent activity and interferes with usual function" [1]. Fatigue is thought to be the most widespread adverse side effect of cancer in adults and children [1], with some studies placing the percent of patients suffering from fatigue as high as 75-99% [2]. Fatigue lasts longer than other treatment side-effects [3], and is the symptom reported to interfere most substantially with activities of daily living [3-6]. While it is associated with cancer itself [1], fatigue frequently worsens during treatment and is recognized as a factor limiting patient adherence to cancer therapy [2, 7]. This may explain why patients reporting high levels of fatigue during treatment have shorter disease free intervals [8]. Although symptoms frequently improve following treatment completion, fatigue persists in a substantial number of cancer survivors. It has been estimated that 19-38% of survivors experience significant levels of fatigue following treatment [1, 7, 9], which is a much higher prevalence than in the population without a cancer history. In some instances, fatigue continues for years after the cancer treatment has ended. For example, in a longitudinal study of 763 breast cancer survivors, 35% were fatigued in the first 5 years after treatment, and 34% reported fatigue 5-10 years following treatment [10]. Similar results were found in a survey of 1294 breast, prostate, or colorectal cancer survivors, where approximately one third of survivors reported fatigue 6 years after treatment [11].

The diagnostic criteria for cancer-related fatigue are presented in Table 1. Fatigue is a complex multi-dimensional phenomenon that occurs across physical, cognitive, and emotional domains [2] and is comprised of both peripheral and central aspects [12]. Peripheral fatigue refers to events that occur in the muscles and at the neuromuscular junctions, while central fatigue refers to events that originate in the brain. Central fatigue includes physical *(I don't have the strength to do it)* as well as motivational *(I don't want to engage in the effort to do it)* components. In a qualitative study of cancer-related fatigue, 7 of 20 patients described motivational deficits during unstructured interviews [13]. Furthermore, in studies that directly asked patients about their level of motivation or interest, the rate of reported deficits increased to 50%-65% [14-16]. Fatigue is most commonly measured through self-reports. Standardized questionnaires ask individuals to rate fatigue on a numeric scale and frequently gather additional data, such as its temporal pattern and duration and its interference with daily function [17]. Several fatigue-specific questionnaires have been developed to assess dimensions of fatigue. In addition, fatigue is often rated by patients together with other symptoms on a single multi-symptom assessment measure, such as the M.D. Anderson Symptom Inventory [18]. Nevertheless, cancer-related fatigue is thought to be underreported and underestimated, and thus undertreated [7].

**CORRESPONDING AUTHOR:*
Emily C.P. LaVoy, Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, 3875 Holman Street, Houston, TX 77204, USA, eclavoy@uh.edu
Telephone: (713)743-0829, Fax: 713-743-9860

Factors leading to the development of persistent cancer-related fatigue are not well understood [1, 3]. The occurrence and severity of cancer-related fatigue is not related to the type of disease or treatment variables, making it difficult to identify populations with the greatest risk for fatigue. Specifically, no reliable associations have been found between fatigue and type of cancer, disease stage at diagnosis, tumor size, number of nodes involved, presence and site of metastases, time since diagnosis, the type, extent, and length of cancer treatment, or time since treatment [9]. Several processes have been proposed to play a role in fatigue, including anemia, inflammation [19], hypothalamic-pituitary-adrenal (HPA) axis dysfunction [20], disruption of circadian rhythms [21], disturbance of monoamine pathways that regulate neurotransmitters [12], and loss of skeletal muscle [22]. Psychological variables are also thought to play a role [7]. The contributions of these factors to cancer-related fatigue are discussed in more detail below. The lack of consensus on the underlying cause(s) of cancer-related fatigue has limited its treatment options [2]. No single pharmacological or behavioral intervention has been found to be completely effective at addressing cancer-related fatigue [19]; however exercise training interventions have been some of the most successful at alleviating this symptom [23]. As presented in the current review, the multifaceted effects of exercise training, which include improvements in inflammation, brain function, fitness, and self-efficacy, may help explain the beneficial effects of exercise on cancer-related fatigue.

Table 1. Diagnostic criteria for cancer-related fatigue

Symptoms present every day or nearly every day during the same 2-week period in the past month:

- Significant fatigue, diminished energy, or increased need to rest, disproportionate to recent change in activity level

And at least five of the following symptoms:

- Generalized weakness or limb heaviness
- Diminished concentration or attention
- Decreased motivation or interest to engage in usual activities
- Insomnia or hypersomnia
- Sleep is unrefreshing or non-restorative
- Perceived need to struggle to overcome inactivity
- Marked emotional reactivity (e.g., sadness, frustration, or irritability) to feeling fatigued
- Difficulty completing daily tasks attributed to feeling fatigued
- Perceived problems with short-term memory
- Post-exertional malaise lasting several hours

The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

There is evidence from the history, physical examination, or laboratory findings that the symptoms are a consequence of cancer or cancer therapy.

The symptoms are not primarily a consequence of comorbid psychiatric disorders such as major depression, somatization disorder, somatoform disorder, or delirium.

From Bennett et al (3).

Mechanisms of persistent fatigue in cancer survivors

Inflammation

Chronic inflammation has received much attention as a potential mechanism leading to persistent fatigue in cancer survivors, partially due to observed associations between inflammation and fatigue in cancer survivors. Compared to nonfatigued survivors, fatigued breast cancer survivors exhibit higher levels of neopterin [24], a biomarker of macrophage activation, and C reactive protein (CRP) [25-27], an acute phase protein that is the most commonly used biomarker of inflammation. Further signs of heightened immune activity in fatigued breast cancer survivors are elevated white blood cell counts [25, 27-29], elevated T-cell counts [30], and increased production of tumor necrosis factor (TNF)-α and interleukin (IL)-6 following lipopolysaccharide stimulation [31] in comparison to nonfatigued survivors. While most of the evidence linking inflammation and fatigue in cancer survivors has been collected in breast cancer survivors, immune activation has been implicated in fatigue among other groups as well. For example, in testicular cancer survivors measured a median of 11 years post-treatment, fatigue was associated with higher levels of CRP [28]. Additionally, ovarian cancer survivors whose symptoms of fatigue improved in the year following treatment had decreases in plasma IL-6 [32]. Genetic markers also support a link between inflammation and fatigue. Significant associations between single nucleotide polymorphisms for several cytokines and fatigue have been demonstrated in lung cancer survivors [33]. Although the sample size was small, Bower et al found that fatigued breast cancer survivors had heightened gene expression for activation of pro-inflammatory cytokines, chemokine signaling, vascular growth factor, and transcriptional activation in leukocytes [34]. The same study by Bower et al also reported increased signaling by transcription factor nuclear factor-kappa B (NF-κB), which is responsible for and responds to the upregulation of many pro-inflammatory genes, and also found decreased expression of glucocorticoid receptor transcription factor [34]. Decreased glucocorticoid signaling suggests decreased sensitivity to cortisol, the main endogenous brake on the production of pro-inflammatory cytokines [12].

Causal links between inflammation and central fatigue have been demonstrated using animal models in which the symptoms of fatigue and reduced motivation appear after induction of high levels of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) [12]. Further evidence has come from human studies where fatigue is induced by typhoid vaccination, activation of the immune system through administration of low dose endotoxin, or in patients receiving recombinant cytokines such as interferon (IFN)-α as treatment for hepatitis C [12]. Treatments which reduce inflammation, such as antagonists of TNF-α, have also been shown to reduce fatigue in patients with rheumatoid arthritis or psoriasis [35, 36].

Fatigue is one component of 'sickness behavior', the coordinated set of adaptive behavioral changes that occur in infected individuals to promote survival, and include lethargy and sleepiness. These changes are orchestrated in the brain following the release of inflammatory mediators that ultimately stimulate the brain to induce feelings of sickness. Mechanistically, elevated cytokines that mediate the inflammatory

response have been proposed to cause central fatigue and other symptoms by targeting the basal ganglia and dopamine function [37]. Inflammation originating in the periphery is communicated to the central nervous system (CNS) by several pathways, including activation of sensory nerves [19]. This results in the production of prostaglandins and pro-inflammatory cytokines such as IL-1β and TNF-α by endothelial cells, macrophages, and microglia in the CNS [38]. These CNS inflammatory mediators then influence neurons directly or indirectly by modifying astrocyte, oligodendrocyte, and endothelial cell functions [12], thereby contributing to instances of fatigue.

Systemic inflammation also affects the bioavailability of amino acid precursors of neurotransmitters. For example, an enzyme involved in the synthesis of neopterin by peripheral macrophages also leads to a relative deficit of an essential cofactor of aromatic amino acid hydroxylase enzymes used in the synthesis of dopamine, norepinephrine, and serotonin [39]. Serotonin neurotransmission can also be impaired during inflammation by cytokine-induced activation of indoleamine 2,3-dioxygenase, which metabolizes the serotonin precursor tryptophan into kynurenine [40-43]. Kynurenine is further metabolized into neurotoxic kynurenine metabolites that are thought to play a role in depression [44] and are associated with fatigue in lung cancer patients [45]. Thus, inflammation in the periphery impacts neural pathways that play a role in behavior, motivation, and central fatigue. The links between immune activation and changes in the central nervous system thought to contribute to fatigue are illustrated in Figure 1.

Autonomic nervous system and hypothalamic-pituitary-adrenal axis dysregulation

An imbalance in the autonomic nervous system may also play a role in cancer-related fatigue. Heightened sympathetic activity increases energy demands, whereas higher parasympathetic activity facilitates energy conservation [46]. Parasympathetic activity is also referred to as vagal tone, and can be assessed by measuring the fluctuation in time between consecutive heart beats, or heart rate variability (HRV). Low HRV indicates low vagal tone and thus parasympathetic underactivity [47]. In non-cancer populations, sympathetic overactivity and parasympathetic underactivity are linked to fatigue [48]. For example, healthy adults with lower HRV report more fatigue when performing cognitively demanding tasks than those with higher HRV [49], and lower HRV is associated with driver-related fatigue [50] and greater fatigue after exercise [51]. Autonomic dysfunction has been observed in breast cancer patients during and after treatment [52]. In breast cancer survivors, fatigue is associated with low HRV at rest [20, 53, 54] and in response to social stress [20]. Low vagal tone is also linked to an exaggerated pro-inflammatory profile due to a corresponding deficiency in the cholinergic anti-inflammatory pathway of the parasympathetic nervous system [55]. In a study of breast cancer survivors, low HRV was associated with greater plasma levels of IL-6 and CRP; these markers of inflammation did not however mediate the relationship between fatigue and HRV [54]. Fagundes et al also found elevated levels of norepinephrine in fatigued survivors indicating heightened sympathetic activity; this occurred independently of parasympathetic underactivity, suggesting that both aspects of the autonomic nervous system may be altered in cancer-related fatigue [20].

The HPA-axis is central to regulating inflammatory responses through glucocorticoids such as cortisol [56]. In healthy people, cortisol levels display a diurnal pattern corresponding to the rest-activity cycle. Altered diurnal cortisol secretion and disrupted circadian rhythms are indicative of HPA-axis dys-

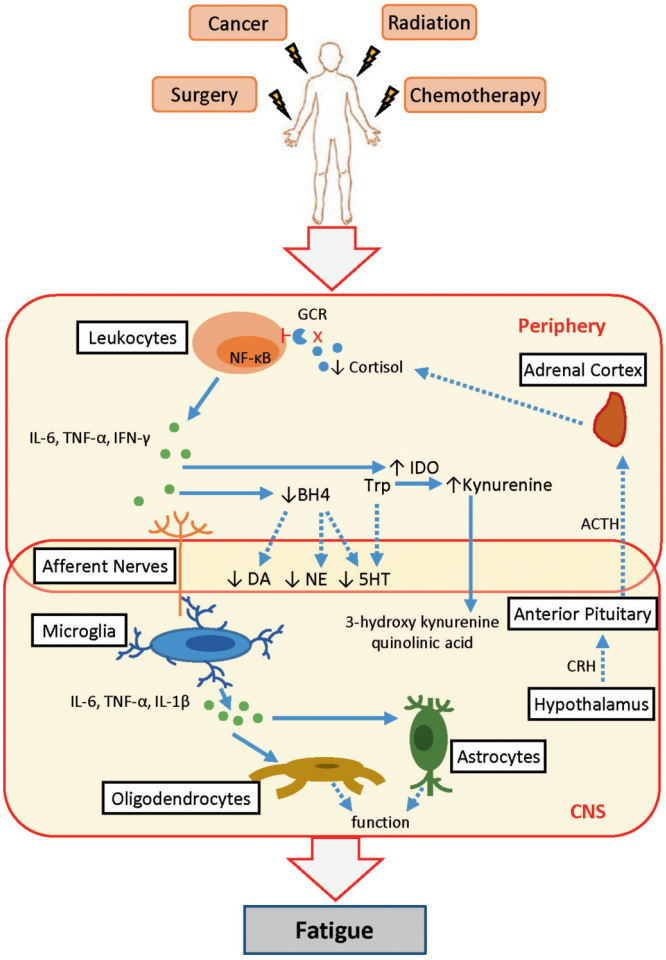


Figure 1. Systemic inflammation contributes to cancer-related fatigue. Cancer and its treatments activate leukocytes, resulting in increased expression of the transcription factor NF-κB and production of pro-inflammatory mediators, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ). Dysregulation of the hypothalamic-pituitary-adrenal (HPA)-axis leads to altered release of cortisol; glucocorticoid receptor (GCR) function on leukocytes is also decreased. Inflammation in the periphery leads to inflammation in the central nervous system (CNS) through several immune-to-brain communication pathways. Signals from sensory nerves activate CNS cells such as microglia to produce pro-inflammatory cytokines, including IL-6, TNF-α, and interlukin-1 beta (IL-1β). These inflammatory mediators then impact neurons directly or indirectly through altered oligodendrocyte, astrocyte, and endothelial cell functions. Inflammation in the periphery also decreases the availability of precursors, such as tetrahydrobiopterin (BH4) required for the synthesis of the neurotransmitters dopamine (DA), norepinephrine (NE), and serotonin (5HT). Deficits in serotonin production also occur as tryptophan (Trp) is converted into kynurenine from an inflammation-mediated increase in indoleamine 2,3-dioxygenase (IDO). Kynurenine is further metabolized in the brain into neurotoxic kynurenine metabolites such as 3-hydroxy kynurenine and quinolinic acid. Dashed lines indicate a disrupted pathway. CRH: corticotropin release hormone; ACTH: adrenocorticotropic hormone.

function and are linked to increased cytokine signaling and fatigue in a variety of conditions. For example, a flattened circadian cortisol cycle is observed in chronic fatigue syndrome [57]. Fatigued breast cancer survivors have been found to have lower morning serum cortisol levels [24], flattened diurnal cortisol cycles [58], and decreased cortisol responses to acute psychological stress [59]. Ovarian cancer survivors are also reported to have altered cortisol cycles, and a return to normal cortisol cycles are associated with improvements in fatigue [32]. HPA-axis dysregulation has been reported in other cancer survivor groups, including nasopharyngeal [60], prostate [61], and leukemia [62], although fatigue was not measured in these studies. Nonetheless, there is evidence suggesting that HPA-axis dysfunction is a common occurrence following cancer treatment and that it is associated with fatigue.

Alternative mechanisms

Additional explanations for fatigue among cancer survivors include loss of fitness. Sarcopenia is a common side effect of cancer and its treatments, where alterations in skeletal muscle metabolism lead to the loss of skeletal muscle contractile strength and mass [63]. The resulting loss of muscle strength likely contributes to the physical experience of fatigue [22]. Cancer patients also exhibit lower oxygen consumption, which combined with decreased muscle strength makes the relative intensity of daily living activities closer to the anaerobic threshold [64, 65]. Certain psychological traits are also risk factors for developing fatigue. Specifically, breast cancer survivors suffering from fatigue have poor self-efficacy [66], that is, the belief in their ability to execute behaviors necessary to produce a specific performance. Engaging in catastrophizing or negative thoughts is also strongly predictive of fatigue in breast cancer survivors [7].

Despite the fact that the majority of the literature to date supports a connection between inflammation and persistent fatigue in cancer survivors, no definite proof has yet been obtained for a causal role of inflammation in this population. In addition, a few studies report negative results [67, 68], or concomitant increases in anti-inflammatory mediators among fatigued cancer survivors [24, 28]. A closer analysis of the literature on toxicities of cancer therapy and inflammation shows that there are alternative to the inflammation hypothesis. In addition to their direct cytotoxic effects on highly proliferative cells, chemotherapeutic agents generate free radicals and superoxides that contribute to cellular DNA damage and cell death. These events take place not only at the level of the tumor but also in distant organs. There, radical oxygen species induce tissue injury that triggers inflammatory pathway cascades. This mechanism is responsible for the toxic effect of chemotherapeutic agents on the gastrointestinal tract, kidney, and heart [69, 70]. However, there is no evidence for propagation of the very localized inflammatory response affecting peripheral organs.

The situation is similar with radiation. Radiotherapy can also cause inflammation in target organs, such as the lung in patients with lung cancer. However, even in this case there is no correlation between the severity of radiation-induced pneumonitis and circulating biomarkers of inflammation, other than IL-6 and this in a non-consistent manner [71]. Most chemotherapeutic agents have a limited penetrability into the nervous system, and even when they can cross the blood-nerve or the blood-brain barrier they do not cause neuronal death since neurons are terminally differentiated cells. What is observed instead is evidence of neuronal mitochondrial damage that usually manifests in the form of swelling mitochondria [72]. In summary, there is no conclusive evidence for the occurrence of systemic inflammation in response to cancer therapy and subsequent activation of immune-to-brain communication pathways. A causal role for mitochondrial dysfunction has been demonstrated for chemotherapy-induced peripheral neuropathy [73-75]. This could also be involved in the pathophysiology of other cancer-related symptoms, including fatigue, by impairing neuronal energetic metabolism and function.

Exercise training interventions to reduce cancer-related fatigue

Several treatments have been proposed to address fatigue in cancer survivors, although so far no single treatment has been shown to be fully effective. A meta-analysis of randomized controlled trials of pharmaceutical therapies for cancer-related fatigue reveal a small effect size for all drug classes [76]. Psychosocial interventions, including educational, supportive, and behavioral interventions, have been more successful, with meta-analyses showing a small to moderate effect on cancer-related fatigue [77-79]. In particular, exercise interventions are significantly associated with improvements in fatigue, both during and after treatment, for a variety of cancers, including breast, colorectal, prostate, head and neck, gynecological, and hematological cancers [65, 80-86].

A significant reduction in cancer-related fatigue was reported in a systematic review of randomized controlled trials and controlled clinical trials that compared exercise interventions with non-exercise or standard-of-care controls on quality of life in adult cancer patients [83]. This analysis included 40 trials with 3694 cancer patients; 30 of the trials were conducted post-treatment. Despite the fact that a wide range of exercise interventions were employed (including resistance training, walking, cycling, yoga, Qigong, and Tai Chi), an overall significant reduction in fatigue was found after 12 weeks (standardized mean difference: -0.82, 95% confidence interval (95% CI): -1.50 to -0.14) and at a 6 month follow-up (standardized mean difference: -0.42; 95% CI -0.02 to -0.83) [83]. A meta-analysis of randomized controlled trials of exercise interventions among cancer survivors also reported an overall significant reduction in fatigue after the interventions [82]. Data from 44 studies enrolling a total of 3254 participants of different cancer types were included and demonstrated that the interventions reduced cancer-related fatigue to a greater extent than standard-of-care controls (weighted mean effect size: 0.31, 95% CI: 0.22 to 0.4). Characteristics of the exercise interventions from individual trials were also analyzed for moderating effects on change in fatigue. Greater reductions in cancer-related fatigue were noted with interventions that used moderate-to-high intensity resistance exercise (3-6 METs, 60-80% 1-Repetition Maximum). The length and number of each exercise session included in an intervention did not significantly impact results [82].

The importance of exercise intensity has also been demonstrated by a randomized controlled trial of exercise training in breast cancer survivors [84]. In this study, 25 postmenopausal breast cancer survivors trained 3 times a week for 15 weeks on a cycle ergometer; changes in peak oxygen consumption, peak power, and fatigue were compared to untrained controls. Statistically and clinically significant improvements in fatigue were observed in the trained group; these improvements were significantly correlated with increases in peak oxygen consumption and peak power output [84]. These results suggest that improvements in cardiopulmonary fitness mediate improvements in fatigue, although a causal relationship has not been established. A more recent meta-analysis of controlled studies involving cancer patients and survivors also found a statistically significant reduction in fatigue resulting from physical exercise interventions (weighted mean effect size: -0.54, 95% CI: -0.90 to -0.19) [86]. Of the 14 studies included in the analysis that used post treatment interventions, 13 report positive results and 7 were statistically significant. The authors of the meta-analysis caution that the overall moderate effect size should be interpreted bearing in mind that the effect sizes of the individual studies were heterogeneous [86].

Thus far, variability in the study populations, exercise prescription, outcome measures, and overall study quality make results hard to translate clinically. High quality, randomized controlled trials that prescribe and monitor exercise using the best available techniques and include multiple measurements of fatigue would add to the literature. It is also important for future studies to include various modes of exercise as it is unlikely that a one-size-fits-all approach in exercise training will be successful. Rather, taking into account factors such as age, cancer site, medical comorbidities, previous exercise training, and personal preference would help target exercise interventions to individuals. One note of caution in the interpretation of these studies discussed above is the existence of publication bias [78], meaning studies with minor or negative results could be missed. Another limitation is that most exercise interventions have not included fatigue as a criterion for study entry. Individuals not experiencing fatigue are unlikely to show change in this symptom, and so the effect of exercise on fatigue may actually be larger than has been reported. Alternatively, fatigue could be a barrier for entry into exercise trials. The randomized controlled trial by Courneya et al had a 14% recruitment rate which could limit the generalizability of the findings [84]. However, once enrolled, the exercise group completed 98.4% of the prescribed exercise sessions; similar adherence has been reported by others [86].

Exercise appears to be safe for cancer survivors, as similar numbers of adverse events have been reported in interventions and control groups [86]. Overall, exercise is well-tolerated by cancer survivors, and the American College of Sports Medicine guidelines recommend most cancer survivors accrue 150 minutes/week of moderate or 75 minutes/week of vigorous intensity aerobic activity, and engage in strength training twice a week, similar to recommendations for healthy populations [87]. Together, the literature support the feasibility of exercise training in cancer survivors, and the possibility that training yields improvements in both fitness and fatigue.

Mechanisms by which exercise may reduce fatigue

Psychological well-being and physical fitness

Just as the benefits of exercise training are multifaceted in healthy populations, exercise likely acts through a variety of mechanisms to improve the quality of life and reduce fatigue in cancer survivors. Even without addressing the underlying cause(s) of cancer-related fatigue, exercise training yields psychological benefits that can reduce the symptoms of fatigue. Attaining new skills and meeting physical activity goals can improve confidence, decrease catastrophizing behavior, and increase self-efficacy [88], thus reducing the contribution of these factors towards fatigue. McAuley et al have shown that self-efficacy mediates the relationship between increased physical activity levels and reduced fatigue in breast cancer survivors [66, 89]. Exercise training can also improve quality of sleep and decrease pain and mood disturbances [90].

Training-induced gains in physical fitness may alleviate fatigue by countering physical deconditioning through increased lean muscle mass and aerobic capacity. Cancer survivors participating in exercise interventions have by and large shown improvements in cardiopulmonary fitness and muscle strength [65, 84, 91], which could decrease the effort required to complete daily living activities and thus reduce fatigue. Of note, exercise interventions with pediatric patients have shown particular benefit for improving cardiopulmonary fitness and muscle strength [81]. Exercise training can also decrease fat mass, an important consideration as high body mass index (BMI) has been shown to be predictive of persistent fatigue in breast cancer survivors [29]. Reduction in BMI may also address chronic inflammation through decreased release of pro-inflammatory adipokines from visceral fat mass [92]. However, many studies have controlled for BMI when examining the effect of exercise interventions, and have found that exercise improves fatigue independently of changes in body composition [7, 84].

Anti-inflammatory effects

The anti-inflammatory effects of exercise training may also reduce cancer-related fatigue. In healthy populations, exercise training has been shown to increase the level of anti-inflammatory cytokines such as IL-10, reduce overall TNF-α expression, decrease CRP, decrease pro-inflammatory adipokines, and reduce expression of Toll-like receptors on monocytes and macrophages (reviewed in: [92-94]). Given the role that inflammation is proposed to play in the promotion of cancer-related fatigue, it is surprising that only a few studies have examined changes in inflammatory mediators following exercise interventions with cancer survivors. One study in leukemia patients undergoing chemotherapy reported that participants in an in-hospital aerobic and resistance training program had reductions in fatigue and a trend towards reduced IL-6 and increased IL-10 circulating levels [95]. A small 3-month randomized controlled trial in breast cancer survivors found that supervised aerobic and resistance exercise training led to decreases in TNF-α and the ratio of IL-6 to IL-10, although these did not reach significance [96]. Other studies in breast cancer survivors have reported a reduction in CRP after 15 weeks of aerobic training [97], and IL-6 after 6 months of aerobic training [98]. Unfortunately, these studies

did not measure fatigue, and so it is not known if the reduction in inflammation would translate to improvements in fatigue. Yoga interventions in fatigued breast cancer survivors have demonstrated decreased symptoms of fatigue and reduced markers of inflammation, such as decreased IL-6, TNF-α, IL-1β [99], as well as reduced NF-κB activity, and increased activity of glucocorticoid receptors [100]. However the effects of the physical exercise and mindfulness components of yoga are difficult to separate [100].

Conversely, a few studies have reported an increase in immune system activation in cancer survivors following an exercise intervention. A two-week moderate aerobic exercise intervention in colorectal cancer survivors found a more pro-inflammatory state in response to lipopolysaccharide, although this study lacked a control group [101]. Similarly, increased lymphocyte activation to *ex vivo* stimulation was observed in exercise-trained breast cancer survivors [102].

Autonomic nervous system balance

Cardiopulmonary fitness is associated with greater HRV in healthy adults, and exercise training has been shown to increase HRV [103-105]. Among cancer patients, a 16 week moderate exercise intervention during and after treatment improved HRV [106]. This suggests that exercise can restore a balance between sympathetic and parasympathetic activity. As low HRV has been linked with fatigue in cancer survivors, exercise-mediated increases in parasympathetic activity could be an additional mechanism by which exercise training addresses fatigue. However, a relationship between exercise-induced increases in HRV and fatigue has not been established in cancer survivors.

It has been proposed that cancer and its treatments accelerate aging processes, and that fatigued patients and survivors might be biologically older than their chronological age may suggest [7]. In support of this, the decreases in HRV observed in fatigued cancer survivors mimic the lower HRV found in older adults, and have been calculated to be equivalent of a 20 year difference compared to age-matched non-fatigued cancer survivors [20]. Another characteristic shared between cancer survivors and older adults is the accumulation of senescent cells. In healthy aging, this is largely driven by repeated exposure to infectious agents across the lifetime [107]. Cancer and its treatments cause DNA damage which can also induce cellular senescence, and large numbers of senescent cells have been observed in childhood cancer survivors [108]. One consequence of the accretion of senescent cells, particularly senescent immune cells, is chronic inflammation [109]. Increased numbers of senescent immune cells have been observed in other patient groups suffering from fatigue [110], but a relationship between senescent immune cells and fatigue in cancer-survivors has not yet been established. There is evidence that regular physical exercise can help prevent or possibly reverse aspects of immunosenescence including senescent immune cells [111, 112]. Reductions in pro-inflammatory senescent cells could therefore be an additional means by which fatigue is reduced through exercise training, although at this time this is speculative and an area that warrants further research.

Neurotrophic factors

Exercise also has protective effects on brain function. For example, exercise training reduces microglial activation [113, 114] and increases expression of neurotrophic factors such as brain derived neurotrophic factor [115] and adiponectin [116, 117]. As discussed above, elevated levels of the tryptophan metabolite kynurenine are associated with inflammation, depression, and fatigue [12] (Figure 1), so an exercise-induced reduction in kynurenine levels could benefit these conditions. While one study reported no exercise-induced changes in the concentration of IL-6, neopterin, tryptophan, and kynurenine with moderate-intensity exercise in depressed patients, the exercise intervention was unsupervised and lasted just one week [118].

A more recent publication has provided evidence for an exercise-induced protection of brain function via modification of kynurenine levels [119]. Agudelo et al demonstrate that per-

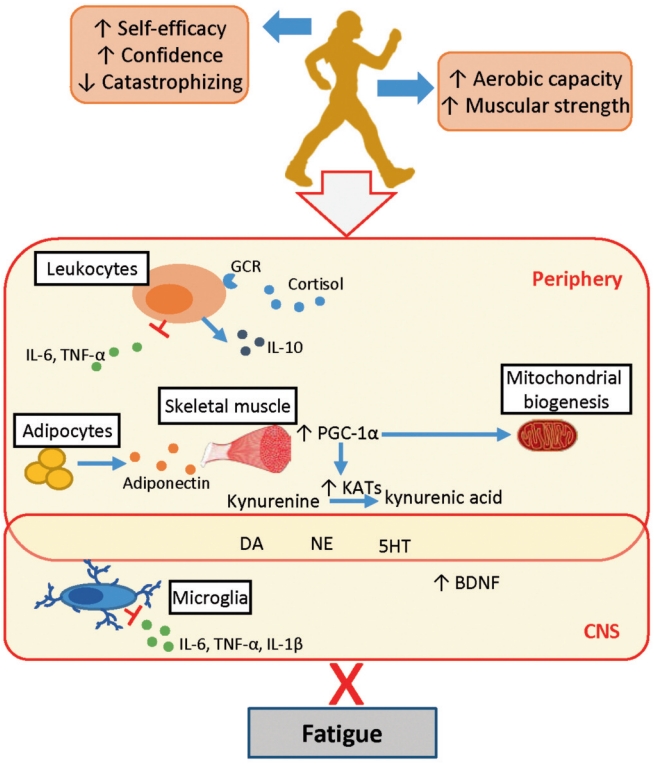


Figure 2. Pathways by which exercise may ameliorate cancer-related fatigue. Exercise training confers psychological benefits, such as increased self-efficacy, and leads to improved fitness which eases the effort required for daily life activities. Exercise training decreases chronic inflammation through reductions in inflammatory cytokines, increased production of interleukin-10 (IL-10), and increased glucocorticoid receptor (GCR) function. Reduced inflammation in the periphery prevents disruption of neurotransmitter production (including norepinephrine (NE) and dopamine (DA)) and inflammation in the central nervous system (CNS). Exercise is further associated with increased neurotrophic factors such as brain derived neurotrophic factor (BDNF) and adiponectin. Exercise-induced increases in skeletal muscle PGC-1α expression, perhaps mediated by adiponectin, helps maintain levels of tryptophan (Trp) and serotonin (5HT) through activation of kynurenine aminotransferases (KATs), which favor the formation of neuroprotective kynurenine metabolites (kynurenic acid). PGC-1α may also promote mitochondrial biogenesis. Dashed lines indicate a disrupted pathway.

oxisome proliferator-activated receptor-gamma co-activator (PGC)-1α1 overexpression in muscle promotes the expression of kynurenine aminotransferase (KAT), which prevents kynurenine from crossing the blood brain barrier [119]. Mice overexpressing PGC-1α1 also avoided the increase in neuro-inflammatory markers (such as macrophage inflammatory protein 1α (MIP1α)) following chronic stress exposure observed in wild type mice [119]. The authors further show that exercise training increased expression of both PGC-1α and KAT in mice as well as human skeletal muscle, corroborating several reports of increased PGC-1α expression in endurance-trained humans [120, 121]. Exercise-induced increases in adiponectin levels have been proposed to provide a stimulus for increased PGC-1α levels via AMPK/SIRT1-dependent pathways [122]. Thus, exercise-induced increases in adiponectin and PGC-1α1 may reduce neuro-inflammation and help maintain normal levels of tryptophan and serotonin. An alternative that still needs to be examined in the case of exercise and cancer-related symptoms is that PGC-1α activates mitochondrial biogenesis, therefore opposing the mitochondrial dysfunction induced by cancer therapy [123]. Pathways by which exercise training may reduce cancer related fatigue are summarized in Figure 2.

Areas for future research

Unfortunately, the translation of exercise interventions from the research lab to the clinic has been slow. Many fatigued cancer survivors are advised to conserve their energy expenditure and limit physical activity, which could actually worsen symptoms of fatigue through the synergistic effects of deconditioning and cancer cachexia [82, 124]. Further, some clinicians and patients fear that exercise could cause adverse events, such as lymphedema in breast cancer survivors [125]. While a meta-analysis has found no difference in adverse events including lymphedema between exercise and control groups [86], many studies have done a poor job describing adverse events [23]. Future investigations should carefully monitor and report these events so that conclusions can be accurately made about the safety of exercise. The recruitment rate and overall adherence should also be carefully described. Another difficulty in the implementation of exercise as standard practice is that although the American College of Sports Medicine recommends aerobic and resistance exercise for cancer survivors [87], there is no specific prescription for survivors suffering from fatigue [82]. This may be due in part to difficulties in comparing results across studies, as exercise interventions have differed in mode, intensity, frequency, the timing of the intervention relative to treatment, duration of each session and overall length of the intervention, and how and whether the exercise is monitored. Because associations have been found between improvements in cardiopulmonary fitness and fatigue [84], future exercise interventions should be designed using the best-available evidence from the exercise science literature to yield large fitness gains. Alternatively, the success that yoga interventions have had in reducing both inflammation and fatigue in breast cancer survivors [99, 100] suggests that yoga may provide additional options for patients who might otherwise decline to exercise.

Comparisons across different studies are also hindered by variability in outcome domains arising from the different definitions and measurements for cancer-related fatigue currently in use [2]. A key obstacle in mechanistic studies of cancer-related fatigue is the reliance on subjective feelings to assess fatigue instead of objective measurements of performance and fatigability. Besides the use of activity monitors and all their ambiguities this is an area of research that has been clearly left aside. Researchers should take note of the emergence of computerized tasks for assessing several aspects of motivated behavior in human subjects [126] and the increasing use of neuroimaging approaches in neurology, psychiatry, and cognitive psychology [127].

Future exercise intervention studies should also include outcome measures that aim to elucidate the mechanisms by which exercise yields its effect, such as longitudinal observations of changes in immune system activation and function. These measurements should go beyond measuring circulating cytokines, as serum or plasma levels of cytokines do not necessarily reflect levels in the brain where the symptoms of cancer-related fatigue likely originate. Inflammatory cytokines act as autocrine, juxtacrine, and paracrine communication signals in the microenvironment in which they are produced, and it is not clear if circulating cytokines reflect "spillover" of molecules released locally from the site of inflammation or if they arise from an inflammatory environment that influences tissue-specific activity. An alternative is to measure activation of intracellular cytokine signaling pathways in circulating immune cells or to assess the ability of these cells to produce cytokines in response to stimulation. Future exercise intervention studies could also consider measuring glucocorticoid receptor function as well as cortisol levels. In addition, it will be interesting for future studies to monitor training-induced changes in HRV in cancer survivors, and to determine if increases in HRV are linked to reductions in fatigue. Well-designed exercise interventions that measure factors known to act on central nervous system pathways involved in fatigue behavior, such as kynurenine, will also add to literature.

Overall, there is a need for higher quality randomized controlled trials with larger sample sizes and increased duration of follow-up measures. Other methodological issues in the existing literature include lack of control groups, failure to blind outcome assessments, and not controlling for potentially confounding variables such as age and previous fitness level. Inclusion criteria also need to be carefully considered. There is large inter-individual variability in the experience of cancer-related fatigue, as some patients develop high symptom levels whereas others experience very little. Using fatigue as an inclusion criterion would improve the translational potential of intervention studies, as highly fatigued individuals are likely to show the greatest improvements in this symptom following exercise training interventions. Finally, more studies should consider fatigue in pediatric cancer survivors, as their experience of fatigue and the underlying causes may differ from adult survivors. There are now fatigue measures available for use in children as young as 7 years, with ongoing efforts to validate common items in pediatric and adult questionnaires [2].

CONCLUSION

Awareness of the issue of cancer-related fatigue is growing, and its etiology and treatment are areas of active research. Symptom control during cancer treatment could enhance therapeutic outcomes by increasing adherence to treatment, and thus potentially increase survival. Reducing fatigue is also important following completion of cancer treatment, as persistent fatigue poses a barrier to the resumption of pre-cancer lifestyles. Research conducted in cancer survivors demonstrates that both psychological and biological factors contribute towards the development and persistence of fatigue. Although most work has focused on inflammation, the evidence is far from conclusive and there are alternatives that clearly deserve consideration. A large number of research studies support the ability of exercise training to alleviate cancer-related fatigue, especially among supervised interventions that also yield improvements in fitness parameters. While the exact mechanisms underlying this benefit are unknown, evidence is accumulating for several models. These include increases in functional capacity, reductions in chronic inflammation which can mediate sickness behavior, increases in parasympathetic nervous system activity, and the protection of neurotransmitters through mediators such as PGC-1α, all of which have been shown to accompany exercise training programs. There is still much work to be done until the prescription of exercise becomes standard practice for cancer survivors. With improvements in the quality of studies, evidenced-based exercise interventions will allow exercise scientists and oncologists to work together to treat cancer-related fatigue.

ACKNOWLEDGEMENTS

RD is supported by grants from the National Institute of Neurological Diseases and Stroke of the National Institutes of Health (Grants R01 NS073939; R01 NS074999) and the National Cancer Institute and the National Institute of Mental Health (Grants R01CA193522; R21CA183736). All research at The University of Texas MD Anderson Cancer Center is supported in part by the institution's Cancer Center Support Grant, NCI P30 016672. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute, the National Institutes of Health, or the other sponsors.

REFERENCES

1. Berger, A.M., et al., Cancer-Related Fatigue, Version 2.2015. J Natl Compr Canc Netw, 2015. 13(8): p. 1012-39.
2. Barsevick, A.M., et al., Recommendations for high-priority research on cancer-related fatigue in children and adults. J Natl Cancer Inst, 2013. 105(19): p. 1432-40.
3. Bennett, B., et al., The experience of cancer-related fatigue and chronic fatigue syndrome: a qualitative and comparative study. J Pain Symptom Manage, 2007. 34(2): p. 126-35.
4. Cleeland, C.S., et al., Are the symptoms of cancer and cancer treatment due to a shared biologic mechanism? A cytokine-immunologic model of cancer symptoms. Cancer, 2003. 97(11): p. 2919-25.
5. Ganz, P.A., et al., Quality of life in long-term, disease-free survivors of breast cancer: a follow-up study. J Natl Cancer Inst, 2002. 94(1): p. 39-49.
6. Lawrence, D.P., et al., Evidence report on the occurrence, assessment, and treatment of fatigue in cancer patients. J Natl Cancer Inst Monogr, 2004(32): p. 40-50.
7. Bower, J.E., Cancer-related fatigue--mechanisms, risk factors, and treatments. Nat Rev Clin Oncol, 2014. 11(10): p. 597-609.
8. Groenvold, M., et al., Psychological distress and fatigue predicted recurrence and survival in primary breast cancer patients. Breast Cancer Res Treat, 2007. 105(2): p. 209-19.
9. Prue, G., et al., Cancer-related fatigue: A critical appraisal. Eur J Cancer, 2006. 42(7): p. 846-63.
10. Bower, J.E., et al., Fatigue in long-term breast carcinoma survivors: a longitudinal investigation. Cancer, 2006. 106(4): p. 751-8.
11. Jones, J.M., et al., Cancer-related fatigue and associated disability in post-treatment cancer survivors. J Cancer Surviv, 2015.
12. Dantzer, R., et al., The neuroimmune basis of fatigue. Trends Neurosci, 2014. 37(1): p. 39-46.
13. Glaus, A., R. Crow, and S. Hammond, A qualitative study to explore the concept of fatigue/tiredness in cancer patients and in healthy individuals. European journal of cancer care, 1996. 5(2 Suppl): p. 8-23.
14. Curt, G.A., et al., Impact of cancer-related fatigue on the lives of patients: new findings from the Fatigue Coalition. The oncologist, 2000. 5(5): p. 353-60.
15. Sadler, I.J., et al., Preliminary evaluation of a clinical syndrome approach to assessing cancer-related fatigue. Journal of pain and symptom management, 2002. 23(5): p. 406-16.
16. Van Belle, S., et al., Comparison of proposed diagnostic criteria with FACT-F and VAS for cancer-related fatigue: proposal for use as a screening tool. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer, 2005. 13(4): p. 246-54.
17. Mendoza, T.R., et al., The rapid assessment of fatigue severity in cancer patients: use of the Brief Fatigue Inventory. Cancer, 1999. 85(5): p. 1186-96.
18. Cleeland, C.S., et al., Assessing symptom distress in cancer patients: the M.D. Anderson Symptom Inventory. Cancer, 2000. 89(7): p. 1634-46.
19. Dantzer, R., M.W. Meagher, and C.S. Cleeland, Translational approaches to treatment-induced symptoms in cancer patients. Nat Rev Clin Oncol, 2012. 9(7): p. 414-26.
20. Fagundes, C.P., et al., Sympathetic and parasympathetic activity in cancer-related fatigue: more evidence for a physiological substrate in cancer survivors. Psychoneuroendocrinology, 2011. 36(8): p. 1137-47.
21. Berger, A.M., et al., Patterns of circadian activity rhythms and their relationships with fatigue and anxiety/depression in women treated with breast cancer adjuvant chemotherapy. Support Care Cancer, 2010. 18(1): p. 105-14.
22. Courneya, K.S., Exercise in cancer survivors: an overview of research. Med Sci Sports Exerc, 2003. 35(11): p. 1846-52.
23. McNeely, M.L., et al., Effects of exercise on breast cancer patients and survivors: a systematic review and meta-analysis. CMAJ, 2006. 175(1): p. 34-41.
24. Bower, J.E., et al., Fatigue and proinflammatory cytokine activity in breast cancer survivors. Psychosom Med, 2002. 64(4): p. 604-11.

25. Alfano, C.M., et al., Fatigue, inflammation, and omega-3 and omega-6 fatty acid intake among breast cancer survivors. J Clin Oncol, 2012. 30(12): p. 1280-7.
26. Orre, I.J., et al., Higher levels of fatigue are associated with higher CRP levels in disease-free breast cancer survivors. J Psychosom Res, 2011. 71(3): p. 136-41.
27. Alexander, S., et al., A comparison of the characteristics of disease-free breast cancer survivors with or without cancer-related fatigue syndrome. Eur J Cancer, 2009. 45(3): p. 384-92.
28. Landmark-Hoyvik, H., et al., Alterations of gene expression in blood cells associated with chronic fatigue in breast cancer survivors. Pharmacogenomics J, 2009. 9(5): p. 333-40.
29. Reinertsen, K.V., et al., Predictors and course of chronic fatigue in long-term breast cancer survivors. J Cancer Surviv, 2010. 4(4): p. 405-14.
30. Bower, J.E., et al., T-cell homeostasis in breast cancer survivors with persistent fatigue. J Natl Cancer Inst, 2003. 95(15): p. 1165-8.
31. Collado-Hidalgo, A., et al., Inflammatory biomarkers for persistent fatigue in breast cancer survivors. Clin Cancer Res, 2006. 12(9): p. 2759-66.
32. Schrepf, A., et al., Cortisol and inflammatory processes in ovarian cancer patients following primary treatment: relationships with depression, fatigue, and disability. Brain Behav Immun, 2013. 30 Suppl: p. S126-34.
33. Rausch, S.M., et al., Relationship between cytokine gene single nucleotide polymorphisms and symptom burden and quality of life in lung cancer survivors. Cancer, 2010. 116(17): p. 4103-13.
34. Bower, J.E., et al., Fatigue and gene expression in human leukocytes: increased NF-kappaB and decreased glucocorticoid signaling in breast cancer survivors with persistent fatigue. Brain Behav Immun, 2011. 25(1): p. 147-50.
35. Yount, S., et al., Adalimumab plus methotrexate or standard therapy is more effective than methotrexate or standard therapies alone in the treatment of fatigue in patients with active, inadequately treated rheumatoid arthritis. Clin Exp Rheumatol, 2007. 25(6): p. 838-46.
36. Tyring, S., et al., Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet, 2006. 367(9504): p. 29-35.
37. Felger, J.C. and A.H. Miller, Cytokine effects on the basal ganglia and dopamine function: the subcortical source of inflammatory malaise. Front Neuroendocrinol, 2012. 33(3): p. 315-27.
38. Dantzer, R., Cytokine-induced sickness behavior: mechanisms and implications. Ann N Y Acad Sci, 2001. 933: p. 222-34.
39. Neurauter, G., et al., Chronic immune stimulation correlates with reduced phenylalanine turnover. Curr Drug Metab, 2008. 9(7): p. 622-7.
40. Dantzer, R., et al., From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci, 2008. 9(1): p. 46-56.
41. O'Connor, J.C., et al., Induction of IDO by bacille Calmette-Guerin is responsible for development of murine depressive-like behavior. J Immunol, 2009. 182(5): p. 3202-12.
42. O'Connor, J.C., et al., Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Mol Psychiatry, 2009. 14(5): p. 511-22.
43. Olsson, J., et al., Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. Mech Ageing Dev, 2000. 121(1-3): p. 187-201.
44. Walker, A.K., et al., NMDA receptor blockade by ketamine abrogates lipopolysaccharide-induced depressive-like behavior in C57BL/6J mice. Neuropsychopharmacology, 2013. 38(9): p. 1609-16.
45. Kurz, K., et al., Fatigue in patients with lung cancer is related with accelerated tryptophan breakdown. PLoS One, 2012. 7(5): p. e36956.
46. Thayer, J.F. and R.D. Lane, The role of vagal function in the risk for cardiovascular disease and mortality. Biol Psychol, 2007. 74(2): p. 224-42.
47. Stein, P.K. and R.E. Kleiger, Insights from the study of heart rate variability. Annu Rev Med, 1999. 50: p. 249-61.
48. Tak, L.M., et al., As good as it gets? A meta-analysis and systematic review of methodological quality of heart rate variability studies in functional somatic disorders. Biol Psychol, 2009. 82(2): p. 101-10.
49. Segerstrom, S.C. and L.S. Nes, Heart rate variability reflects self-regulatory strength, effort, and fatigue. Psychol Sci, 2007. 18(3): p. 275-81.
50. Egelund, N., Spectral analysis of heart rate variability as an indicator of driver fatigue. Ergonomics, 1982. 25(7): p. 663-72.
51. Hautala, A., et al., Changes in cardiac autonomic regulation after prolonged maximal exercise. Clin Physiol, 2001. 21(2): p. 238-45.
52. Lakoski, S.G., et al., Autonomic dysfunction in early breast cancer: Incidence, clinical importance, and underlying mechanisms. Am Heart J, 2015. 170(2): p. 231-41.
53. Vigo, C., et al., Evidence of altered autonomic cardiac regulation in breast cancer survivors. J Cancer Surviv, 2015.
54. Crosswell, A.D., et al., Low heart rate variability and cancer-related fatigue in breast cancer survivors. Psychoneuroendocrinology, 2014. 45: p. 58-66.
55. Tracey, K.J., Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest, 2007. 117(2): p. 289-96.
56. Nicolaides, N.C., et al., Circadian endocrine rhythms: the hypothalamic-pituitary-adrenal axis and its actions. Ann N Y Acad Sci, 2014. 1318: p. 71-80.
57. MacHale, S.M., et al., Diurnal variation of adrenocortical activity in chronic fatigue syndrome. Neuropsychobiology, 1998. 38(4): p. 213-7.
58. Bower, J.E., et al., Diurnal cortisol rhythm and fatigue in breast cancer survivors. Psychoneuroendocrinology, 2005. 30(1): p. 92-100.
59. Bower, J.E., P.A. Ganz, and N. Aziz, Altered cortisol response to psychologic stress in breast cancer survivors with persistent fatigue. Psychosom Med, 2005. 67(2): p. 277-80.
60. Ratnasingam, J., et al., Hypothalamic pituitary dysfunction amongst nasopharyngeal cancer survivors. Pituitary, 2015. 18(4): p. 448-55.
61. Hoyt, M.A., et al., Approach and avoidance coping: diurnal cortisol rhythm in prostate cancer survivors. Psychoneuroendocrinology, 2014. 49: p. 182-6.
62. Follin, C., et al., Moderate dose cranial radiotherapy causes central adrenal insufficiency in long-term survivors of childhood leukaemia. Pituitary, 2014. 17(1): p. 7-12.
63. Al-Majid, S. and H. Waters, The biological mechanisms of cancer-related skeletal muscle wasting: the role of progressive resistance exercise. Biol Res Nurs, 2008. 10(1): p. 7-20.

64. Davis, M.P. and D. Walsh, Mechanisms of fatigue. J Support Oncol, 2010. 8(4): p. 164-74.
65. McMillan, E.M. and I.J. Newhouse, Exercise is an effective treatment modality for reducing cancer-related fatigue and improving physical capacity in cancer patients and survivors: a meta-analysis. Appl Physiol Nutr Metab, 2011. 36(6): p. 892-903.
66. McAuley, E., et al., Physical activity and fatigue in breast cancer and multiple sclerosis: psychosocial mechanisms. Psychosom Med, 2010. 72(1): p. 88-96.
67. Knobel, H., et al., High level of fatigue in lymphoma patients treated with high dose therapy. J Pain Symptom Manage, 2000. 19(6): p. 446-56.
68. Dimeo, F., et al., Physical performance, depression, immune status and fatigue in patients with hematological malignancies after treatment. Ann Oncol, 2004. 15(8): p. 1237-42.
69. Vyas, D., G. Laput, and A.K. Vyas, Chemotherapy-enhanced inflammation may lead to the failure of therapy and metastasis. Onco Targets Ther, 2014. 7: p. 1015-23.
70. Lee, C.S., E.J. Ryan, and G.A. Doherty, Gastro-intestinal toxicity of chemotherapeutics in colorectal cancer: the role of inflammation. World J Gastroenterol, 2014. 20(14): p. 3751-61.
71. Sprung, C.N., et al., Immunological markers that predict radiation toxicity. Cancer Lett, 2015.
72. Bennett, G.J., T. Doyle, and D. Salvemini, Mitotoxicity in distal symmetrical sensory peripheral neuropathies. Nat Rev Neurol, 2014. 10(6): p. 326-36.
73. Bennett, G.J., Pathophysiology and animal models of cancer-related painful peripheral neuropathy. Oncologist, 2010. 15 Suppl 2: p. 9-12.
74. Krukowski, K., et al., Prevention of chemotherapy-induced peripheral neuropathy by the small-molecule inhibitor pifithrin-mu. Pain, 2015. 156(11): p. 2184-92.
75. Zheng, H., W.H. Xiao, and G.J. Bennett, Functional deficits in peripheral nerve mitochondria in rats with paclitaxel- and oxaliplatin-evoked painful peripheral neuropathy. Exp Neurol, 2011. 232(2): p. 154-61.
76. Minton, O., et al., A systematic review and meta-analysis of the pharmacological treatment of cancer-related fatigue. J Natl Cancer Inst, 2008. 100(16): p. 1155-66.
77. Jacobsen, P.B., et al., Systematic review and meta-analysis of psychological and activity-based interventions for cancer-related fatigue. Health Psychol, 2007. 26(6): p. 660-7.
78. Duijts, S.F., et al., Effectiveness of behavioral techniques and physical exercise on psychosocial functioning and health-related quality of life in breast cancer patients and survivors--a meta-analysis. Psychooncology, 2011. 20(2): p. 115-26.
79. Kangas, M., D.H. Bovbjerg, and G.H. Montgomery, Cancer-related fatigue: a systematic and meta-analytic review of non-pharmacological therapies for cancer patients. Psychol Bull, 2008. 134(5): p. 700-41.
80. Speed-Andrews, A.E. and K.S. Courneya, Effects of exercise on quality of life and prognosis in cancer survivors. Curr Sports Med Rep, 2009. 8(4): p. 176-81.
81. Wolin, K.Y., et al., Exercise in adult and pediatric hematological cancer survivors: an intervention review. Leukemia, 2010. 24(6): p. 1113-20.
82. Brown, J.C., et al., Efficacy of exercise interventions in modulating cancer-related fatigue among adult cancer survivors: a meta-analysis. Cancer Epidemiol Biomarkers Prev, 2011. 20(1): p. 123-33.
83. Mishra, S.I., et al., Exercise interventions on health-related quality of life for cancer survivors. Cochrane Database Syst Rev, 2012. 8: p. CD007566.
84. Courneya, K.S., et al., Randomized controlled trial of exercise training in postmenopausal breast cancer survivors: cardiopulmonary and quality of life outcomes. J Clin Oncol, 2003. 21(9): p. 1660-8.
85. Donnelly, C.M., et al., A randomised controlled trial testing the feasibility and efficacy of a physical activity behavioural change intervention in managing fatigue with gynaecological cancer survivors. Gynecol Oncol, 2011. 122(3): p. 618-24.
86. Speck, R.M., et al., An update of controlled physical activity trials in cancer survivors: a systematic review and meta-analysis. J Cancer Surviv, 2010. 4(2): p. 87-100.
87. Schmitz, K.H., et al., American College of Sports Medicine roundtable on exercise guidelines for cancer survivors. Med Sci Sports Exerc, 2010. 42(7): p. 1409-26.
88. McAuley, E. and B. Blissmer, Self-efficacy determinants and consequences of physical activity. Exerc Sport Sci Rev, 2000. 28(2): p. 85-8.
89. Phillips, S.M. and E. McAuley, Physical activity and fatigue in breast cancer survivors: a panel model examining the role of self-efficacy and depression. Cancer Epidemiol Biomarkers Prev, 2013. 22(5): p. 773-81.
90. Hartescu, I., K. Morgan, and C.D. Stevinson, Increased physical activity improves sleep and mood outcomes in inactive people with insomnia: a randomized controlled trial. J Sleep Res, 2015.
91. Al-Majid, S. and D.P. Gray, A biobehavioral model for the study of exercise interventions in cancer-related fatigue. Biol Res Nurs, 2009. 10(4): p. 381-91.
92. Gleeson, M., et al., The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol, 2011. 11(9): p. 607-15.
93. Petersen, A.M. and B.K. Pedersen, The anti-inflammatory effect of exercise. J Appl Physiol (1985), 2005. 98(4): p. 1154-62.
94. Plaisance, E.P. and P.W. Grandjean, Physical activity and high-sensitivity C-reactive protein. Sports Med, 2006. 36(5): p. 443-58.
95. Battaglini, C.L., et al., The effects of an exercise program in leukemia patients. Integr Cancer Ther, 2009. 8(2): p. 130-8.
96. Rogers, L.Q., et al., Effects of a physical activity behavior change intervention on inflammation and related health outcomes in breast cancer survivors: pilot randomized trial. Integr Cancer Ther, 2013. 12(4): p. 323-35.
97. Fairey, A.S., et al., Effect of exercise training on C-reactive protein in postmenopausal breast cancer survivors: a randomized controlled trial. Brain Behav Immun, 2005. 19(5): p. 381-8.
98. Jones, S.B., et al., Effect of exercise on markers of inflammation in breast cancer survivors: the Yale exercise and survivorship study. Cancer Prev Res (Phila), 2013. 6(2): p. 109-18.
99. Kiecolt-Glaser, J.K., et al., Yoga's impact on inflammation, mood, and fatigue in breast cancer survivors: a randomized controlled trial. J Clin Oncol, 2014. 32(10): p. 1040-9.
100. Bower, J.E., et al., Yoga reduces inflammatory signaling in fatigued breast cancer survivors: a randomized controlled trial. Psychoneuroendocrinology, 2014. 43: p. 20-9.
101. Allgayer, H., S. Nicolaus, and S. Schreiber, Decreased interleukin-1 receptor antagonist response following moderate exercise in patients with colorectal carcinoma after primary treatment. Cancer Detect Prev, 2004. 28(3): p. 208-13.

102. Hutnick, N.A., et al., Exercise and lymphocyte activation following chemotherapy for breast cancer. Med Sci Sports Exerc, 2005. 37(11): p. 1827-35.
103. Nolan, R.P., et al., Effects of drug, biobehavioral and exercise therapies on heart rate variability in coronary artery disease: a systematic review. Eur J Cardiovasc Prev Rehabil, 2008. 15(4): p. 386-96.
104. Seals, D.R. and P.B. Chase, Influence of physical training on heart rate variability and baroreflex circulatory control. J Appl Physiol (1985), 1989. 66(4): p. 1886-95.
105. Levy, W.C., et al., Effect of endurance exercise training on heart rate variability at rest in healthy young and older men. Am J Cardiol, 1998. 82(10): p. 1236-41.
106. Niederer, D., et al., Exercise effects on HRV in cancer patients. Int J Sports Med, 2013. 34(1): p. 68-73.
107. Pawelec, G., et al., Cytomegalovirus and human immunosenescence. Rev Med Virol, 2009. 19(1): p. 47-56.
108. Ness, K.K., et al., Frailty in childhood cancer survivors. Cancer, 2015. 121(10): p. 1540-7.
109. Tchkonia, T., et al., Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest, 2013. 123(3): p. 966-72.
110. Hassan, I.S., et al., A study of the immunology of the chronic fatigue syndrome: correlation of immunologic parameters to health dysfunction. Clin Immunol Immunopathol, 1998. 87(1): p. 60-7.
111. Spielmann, G., et al., Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. Brain Behav Immun, 2011. 25(8): p. 1521-9.
112. Simpson, R.J., Aging, persistent viral infections, and immunosenescence: can exercise "make space"? Exerc Sport Sci Rev, 2011. 39(1): p. 23-33.
113. Kohman, R.A., et al., Exercise reduces activation of microglia isolated from hippocampus and brain of aged mice. J Neuroinflammation, 2013. 10: p. 114.
114. Masson, G.S., et al., Aerobic training normalizes autonomic dysfunction, HMGB1 content, microglia activation and inflammation in hypothalamic paraventricular nucleus of SHR. Am J Physiol Heart Circ Physiol, 2015. 309(7): p. H1115-22.
115. Cotman, C.W. and C. Engesser-Cesar, Exercise enhances and protects brain function. Exerc Sport Sci Rev, 2002. 30(2): p. 75-9.
116. Yau, S.Y., et al., Physical exercise-induced hippocampal neurogenesis and antidepressant effects are mediated by the adipocyte hormone adiponectin. Proc Natl Acad Sci U S A, 2014. 111(44): p. 15810-5.
117. Kriketos, A.D., et al., Exercise increases adiponectin levels and insulin sensitivity in humans. Diabetes Care, 2004. 27(2): p. 629-30.
118. Hennings, A., et al., Exercise affects symptom severity but not biological measures in depression and somatization - results on IL-6, neopterin, tryptophan, kynurenine and 5-HIAA. Psychiatry Res, 2013. 210(3): p. 925-33.
119. Agudelo, L.Z., et al., Skeletal muscle PGC-1alpha1 modulates kynurenine metabolism and mediates resilience to stress-induced depression. Cell, 2014. 159(1): p. 33-45.
120. Handschin, C. and B.M. Spiegelman, The role of exercise and PGC1alpha in inflammation and chronic disease. Nature, 2008. 454(7203): p. 463-9.
121. Mahoney, D.J. and M.A. Tarnopolsky, Understanding skeletal muscle adaptation to exercise training in humans: contributions from microarray studies. Phys Med Rehabil Clin N Am, 2005. 16(4): p. 859-73, vii.
122. Sanchis-Gomar, F., C.P. Quilis, and A. Lucia, Antidepressant Effects of Exercise: A Role for the Adiponectin-PGC-1alpha-kynurenine Triad? J Cell Physiol, 2015. 230(10): p. 2328-9.
123. Villena, J.A., New insights into PGC-1 coactivators: redefining their role in the regulation of mitochondrial function and beyond. FEBS J, 2015. 282(4): p. 647-72.
124. Lucia, A., C. Earnest, and M. Perez, Cancer-related fatigue: can exercise physiology assist oncologists? Lancet Oncol, 2003. 4(10): p. 616-25.
125. Eickmeyer, S.M., et al., The role and efficacy of exercise in persons with cancer. PM R, 2012. 4(11): p. 874-81.
126. Treadway, M.T. and D.H. Zald, Reconsidering anhedonia in depression: lessons from translational neuroscience. Neurosci Biobehav Rev, 2011. 35(3): p. 537-55.
127. Dobryakova, E., et al., The dopamine imbalance hypothesis of fatigue in multiple sclerosis and other neurological disorders. Front Neurol, 2015. 6: p. 52.

Muscle atrophy in patients with Type 2 Diabetes Mellitus: roles of inflammatory pathways, physical activity and exercise

Ben D Perry[1,8], Marissa K Caldow[2], Tara C Brennan-Speranza[3], Melissa Sbaraglia[1], George Jerums[4], Andrew Garnham[5], Chiew Wong[6], Pazit Levinger[1], Muhammad Asrar ul Haq[7], David L Hare[7], S. Russ Price[8,9], Itamar Levinger[1,7]

[1] Clinical Exercise Science Program, Institute of Sport, Exercise and Active Living (ISEAL), Victoria University, Melbourne, Australia
[2] Basic and Clinical Myology Laboratory, Department of Physiology, The University of Melbourne, Melbourne, Victoria, Australia;
[3] Department of Physiology, Bosch Institute for Medical Research, University of Sydney, Sydney, Australia.
[4] University of Melbourne and the Department of Endocrinology, Austin Health, Melbourne, Australia
[5] School of Exercise & Nutrition Sciences, Deakin University, Melbourne, Australia
[6] University of Melbourne and the Northern Heart, The Northern Hospital, Melbourne, Australia
[7] University of Melbourne and the Department of Cardiology, Austin Health, Melbourne Australia.
[8] Renal Division, Department of Medicine, Emory University, Atlanta, Georgia, USA
[9] Atlanta Veterans Affairs Medical Centre, Decatur, Georgia, USA

ABSTRACT

Muscle atrophy is caused by an imbalance in contractile protein synthesis and degradation which can be triggered by various conditions including Type 2 Diabetes Mellitus (T2DM). Reduced muscle quality in patients with T2DM adversely affects muscle function, the capacity to perform activities of daily living, quality of life and ultimately may increase the risk of premature mortality. Systemic inflammation initiated by obesity and prolonged overnutrition not only contributes to insulin resistance typical of T2DM, but also promotes muscle atrophy via decreased muscle protein synthesis and increased ubiquitin-proteasome, lysosomal-proteasome and caspase 3-mediated protein degradation. Emerging evidence suggests that the inflammation-sensitive Nuclear Factor κ B (NF-κB) and Signal Transducer and Activator of Transcription 3 (STAT3) pathways may contribute to muscle atrophy in T2DM. In contrast, exercise appears to be an effective tool in promoting muscle hypertrophy, in part due to its effect on systemic and local (skeletal muscle) inflammation. The current review discusses the role inflammation plays in muscle atrophy in T2DM and the role of exercise training in minimising the effect of inflammatory markers on skeletal muscle. We also report original data from a cohort of obese patients with T2DM compared to age-matched controls and demonstrate that patients with T2DM have 60% higher skeletal muscle expression of the atrophy transcription factor FoxO1. This review concludes that inflammatory pathways in muscle, in particular, NF-κB, potentially contribute to T2DM-mediated muscle atrophy. Further in-vivo and longitudinal human research is required to better understand the role of inflammation in T2DM-mediated atrophy and the anti-inflammatory effect of exercise training under these conditions.

Key words: Skeletal muscle, inflammation, cytokines, training

ATROPHIC SIGNALLING IN SKELETAL MUSCLE

Muscle atrophy occurs in response to many insults, including prolonged disuse, ageing and chronic disease such as Type 2 Diabetes Mellitus (T2DM) (30, 73, 92). Muscle atrophy is the result of a negative balance between the rate of contractile protein synthesis and degradation. In catabolic conditions, muscle atrophy in combination with inactivity can decrease the capacity to perform activities of daily living, quality of life and subsequently increase mortality (84, 146). The ubiquitin-proteasome, autophagy-lysosome and caspase-3-mediated proteolytic pathways are responsible for protein degradation in muscle and thus contribute to muscle atrophy (Figure 1) (73, 110). In healthy muscle, the degradation of damaged or unfolded proteins is vital for the maintenance of cellular homeostasis (73, 77). In atrophic conditions such as disuse or diabetes, however, prolonged increased activity of these pathways increases the rate of contractile protein degradation, ultimately leading to muscle atrophy (30, 44, 73). In addition, decreased protein synthesis is apparent in T2DM and disuse, primarily through decreased activation of the mammalian target of rapamyacin (mTOR) pathway (Figure 1) (13, 34).

A variety of genes, collectively termed “atrogenes”, are involved in muscle atrophy (74, 111). Perhaps the most prominent of these muscle atrogenes are two E3 ubiquitin ligases, muscle RING finger 1 (MuRF1, or TRIM63) and Atrogin-1 (also known as MAFbx or FBXO32) (17, 48). These

Address for correspondence:
A/Prof Itamar Levinger, Institute for Sport, Exercise and Active Living (ISEAL), College of Sport and Exercise Science, Victoria University, PO Box 14428, Melbourne, VIC 8001, Australia.
Tel: (61-3) 9919 5343, Fax: (61-3) 9919 5532, E-mail: itamar.levinger@vu.edu.au

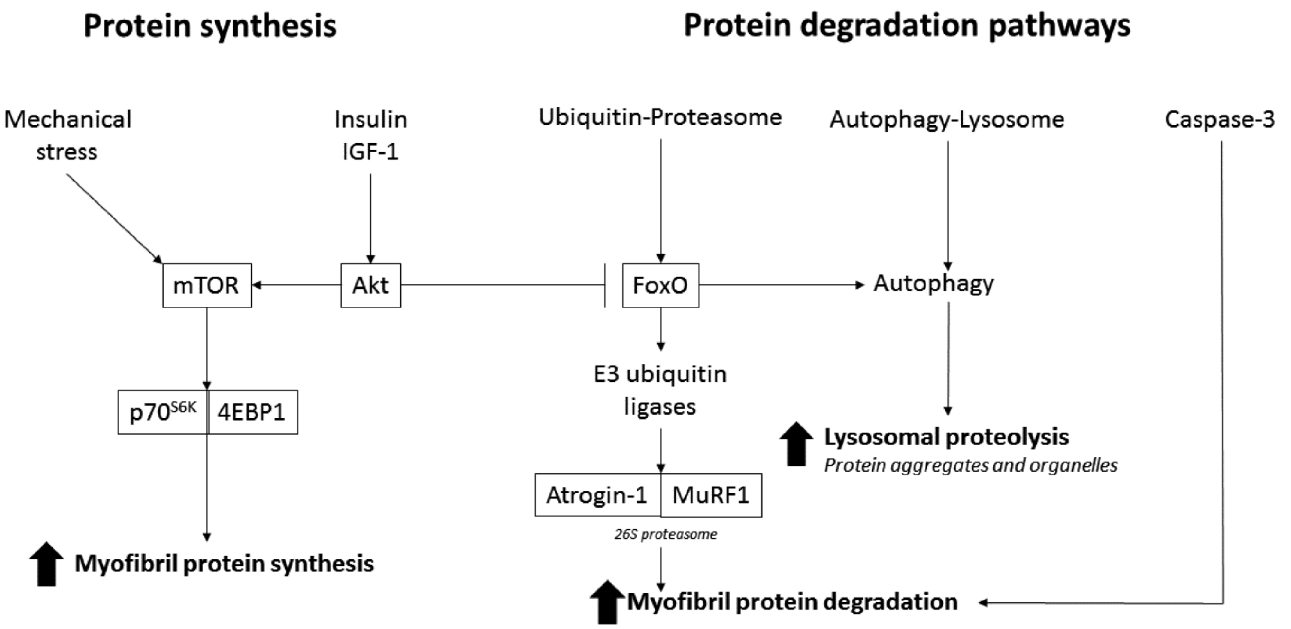


Figure 1: Protein synthesis and degradation pathways in skeletal muscle. Arrows represent activation, capped lines represent inhibition. Abbreviations: mTOR, mechanistic target of rapamycin; $p70^{S6k}$, $p70^{S6}$ kinase; IGF-1, insulin-like growth factor 1; FoxO, forkhead box O transcription factor; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1.

atrogenes are key components of the ubiquitin-proteasome system and are activated by the atrophy-related transcription factors, forkhead box O family transcription factors 1 and 3a (FoxO 1 and 3a) (85, 111, 144). In mice, global deletion of either Atrogin-1 or MuRF1 attenuated denervation-mediated atrophy (17), whereas Atrogin-1 and MuRF1 protein and mRNA were increased in hind-limb unloading (17), dexamethasone treated myotubes (145), and cancer cachexia (74). It is not yet clear, however, whether MuRF1 or Atrogin-1 are chronically upregulated in humans with catabolic conditions (38, 140). The activation of FoxOs, Atrogin-1 and MuRF1 may be an earlier and potentially transient maladaptation in some atrophic conditions. In streptozotocin (STZ)-induced type 1 diabetes, upregulation of Atrogin-1 and MuRF-1 mRNA is apparent only up to 3 weeks post injection in mice and rats (28, 36, 72). These findings suggest that the timing of the experiment is crucial for identifying atrophic markers and may explain disparities observed between studies. Similarly, short durations of disuse in humans (<10 days) resulted in elevated Atrogin-1 and MuRF1 mRNA (1, 22, 34, 122), whereas no change was seen after 2 weeks (1, 19, 34). Taken together, these results suggest that whilst the acute regulation of FoxO1 and 3a, Atrogin-1 and MuRF1 *in vivo* and *in vitro* is relatively well understood in atrophic conditions, the time-course of maladaptations to these important atrogenes in humans with catabolic conditions is not completely understood. This is likely due to the complex interactions between disease/condition duration, medication usage, physical activity levels, and muscle atrophy.

T2DM AS AN INFLAMMATORY DISEASE

Insulin resistance is defined by a reduction or inability of insulin stimulated glucose uptake in insulin target tissues (35). Insulin resistance in skeletal muscle, which is seen in T2DM and obesity, has substantial adverse effects on glucose metabolism as it is a major site of glucose uptake and disposal in response to insulin (16, 42). Insulin resistance may be caused by several mechanisms, including chronically increased production of reactive oxygen species and mitochondrial dysfunction (60, 108, 117), endoplasmic reticulum (ER) stress (40), lipotoxicity, and glucotoxicity (37). The mechanisms by which these factors lead to the development of T2DM may be in part mediated by the activation of inflammatory pathways, or exacerbated by inflammation (40, 59, 117). Indeed, higher circulating C-reactive protein (CRP), and various pro-inflammatory cytokines such as tumour necrosis factor α (TNF α) and interleukin 6 (IL-6) are observed in patients with T2DM (55, 102, 118). Furthermore, in muscle from obese T2DM patients, a range of pro-inflammatory pathways are upregulated (Figure 2), including chemokine (c-c motif) ligand (CCL2) (100), signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signalling 3 (SOCS3), and nuclear factor κ B (NF-κB) (87, 107, 119).

The role of cytokines in insulin resistance

T2DM promotes increased levels of pro-inflammatory cytokines such as TNFα and IL-6, which may be involved in the development of insulin resistance in skeletal muscle (41, 52, 114). Increased systemic levels of TNFα in T2DM reduces the level of inhibitor of NF-κB (IKB), that subsequently upregulates NF-κB and the c-Jun N-terminal kinases (JNK) pathways in muscle, resulting in insulin resistance through the inhibition of insulin receptor substrate 1 (IRS-1; Figure 2) (7, 52, 103). While increased TNFα has been shown to promote muscle insulin resistance acutely in some studies in humans and *in vitro* (33, 103), several studies contest the functional importance of TNFα independently on insulin resistance as pharmaceutical blockade of TNFα did not alter glycaemic control in humans (15, 39, 83). Another pro-inflammatory cytokine, IL-6, is elevated in obese individuals (40). Activation of the IL-6 receptor in muscle can activate the janus kinase (JAK)/STAT3 pathways that inhibits IRS-1 and subse-

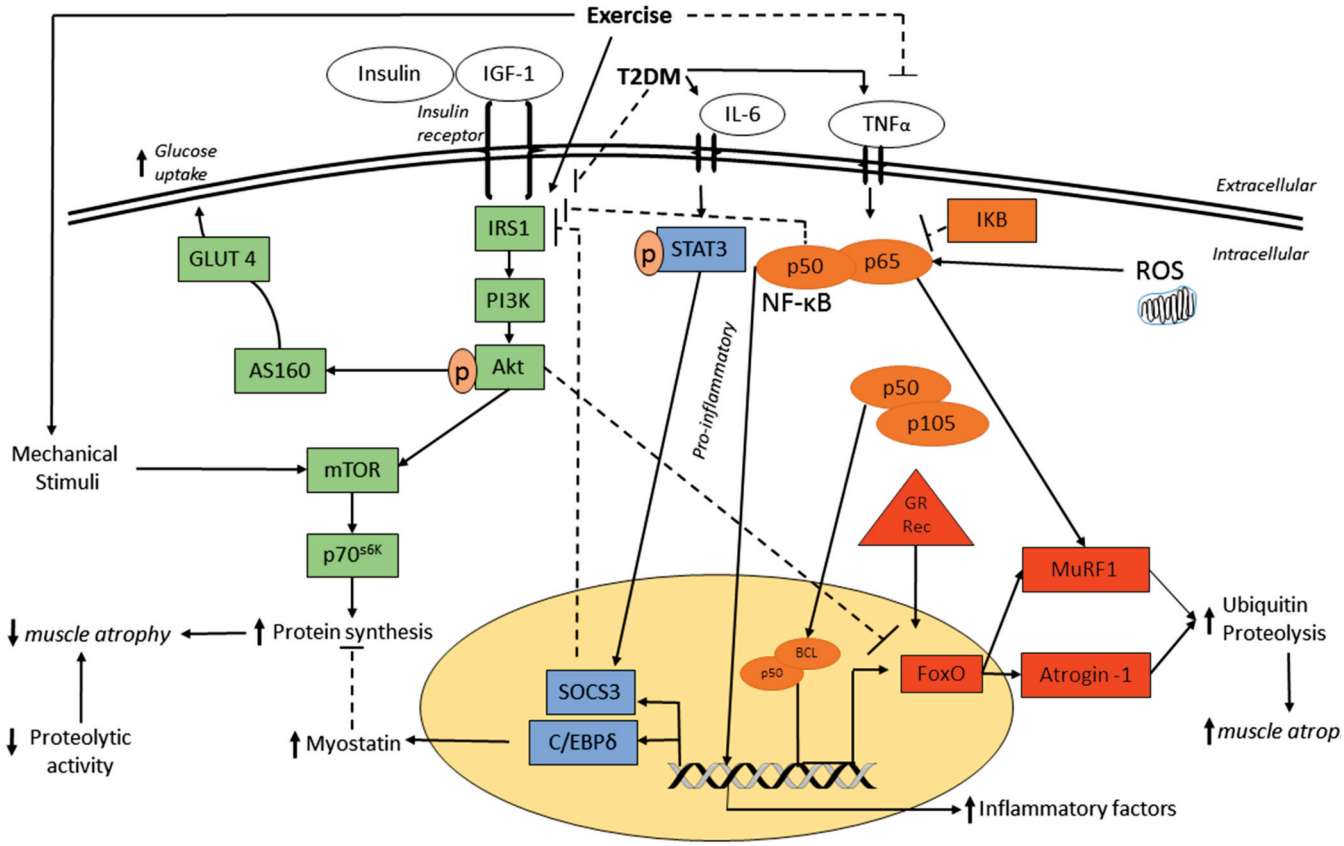


Figure 2: Insulin and inflammatory signalling and their potential signalling in protein synthesis and degradation in Type 2 Diabetes Mellitus (T2DM) and exercise training. Solid lines denote activation, dotted lines represent inhibitory effect. Some of these pathways, such as the p50-p105-BCL pathways are untested in T2DM. Abbreviations: IRS1, Insulin receptor substrate 1; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; SOCS3, Suppressor of cytokine signalling 3; C/EBPδ, CCAAT-enhancer-binding protein δ; STAT3, Signal transducer and activator of transcription 3; NF-κB, Nuclear factor κ B; IKB, Inhibitor of NF-κB; ROS, reactive oxygen species; GR Rec, Glucocorticoid receptor.

quent insulin-mediated glucose uptake through SOCS3 (Figure 2) (68). Initial studies investigating the role of IL-6 indicated that it reduced whole body insulin sensitivity and impaired muscle glucose uptake via reduced activity of the IRS-1 and Phosphatidylinositide 3-Kinase (PI3K) pathway (14, 67). The role of IL-6, however, remains unclear as several studies have reported there is no connection between IL-6 and insulin resistance in skeletal muscle (25, 68, 141). Furthermore, humans infused with IL-6 had increased muscle glucose uptake, possibly via activation of AMPK (25), a finding replicated in rodent muscle (141). The divergent and tissue specific actions of IL-6 might reflect secondary inflammatory signalling effects that are tissue-specific (141). The controversies surrounding whether IL-6 or TNFα are involved in the mechanism of insulin resistance in muscle suggests that changes in the level of individual cytokines may not be sufficient to cause insulin resistance. Further, secondary tissue-specific inflammatory signalling in response to cytokines along with the duration of elevations in systemic cytokines are important factors to be considered.

THE LINK BETWEEN INFLAMMATION AND MUSCLE ATROPHY IN T2DM

Patients with T2DM exhibit muscle atrophy that is initially mild in middle age (12, 124), and becomes more substantial with older age and diabetic neuropathy (6, 69, 75, 99). This loss of muscle leads to decreased strength, functional capacity and ultimately increased mortality in patients with T2DM (26, 75, 105). The following sections discuss the potential atrophic pathways in the muscle of patients with T2DM that are dysregulated via inflammatory processes, and conclude with novel data obtained from a small cohort of older age, obese patients with T2DM and age-matched controls

Insulin resistance promotes atrophy signalling in T2DM

Insulin resistance that is at least partially derived from systemic inflammation in T2DM and obesity is a key contributor to muscle atrophy signalling. The specific activity of Akt kinase in response to insulin was reduced by 34% in patients with T2DM compared to healthy controls (70). This impairment of the PI3K-Akt pathway has been implicated in decreasing both insulin mediated glucose uptake and protein synthesis in rodents and patients with T2DM (13, 18, 70, 121). The primary regulator of protein synthesis in skeletal muscle is the activation of mammalian target of rapamycin (mTOR), which is activated by Akt via insulin or insulin-like growth factor 1 (IGF1) and mechanical stimuli (18, 50, 131). However, the Akt-mTOR pathway also interacts with the ubiquitin-proteasome and autophagy-lysosome pathways (18, 85, 110, 111). Reduced activation of Akt decreases the phosphorylation of the FoxO transcription factors, which leads to their nuclear translocation and subsequent increase in the transcription of MuRF1 and Atrogin-1 (Figure 2) (9, 47, 70, 111). In non-diabetic haemodialysis patients, insulin resistance was

associated with an increased rate of muscle protein degradation (115). Further, obese *db/db* mice exhibit muscle atrophy, up to 43% increase in the rate of protein degradation, and insulin resistance compared to lean controls (95, 128). Administration of the insulin-sensitising drug, Rosiglitazone, to obese *db/db* mice recovered the maladaptations to the insulin signalling cascade and the ubiquitin-proteasome system, but only partly reversed the difference in muscle cross sectional area compared to controls (128). Rosiglitazone alleviates insulin resistance via several pathways, including increased circulating adiponectin (137, 138), and decreased levels of IL-6 and TNFα (71, 89). The reduction of these pro-inflammatory cytokines (IL-6 and TNFα) may attenuate muscle atrophy signalling through mechanisms independent of insulin signalling, such as reduced activation of the pro-inflammatory transcription factor NF-κB, which is discussed in later in this review (62).

Non-esterified fatty acids (NEFA) cause insulin resistance in muscle through elevations in intramuscular diacylglycerol (DAG) and ceramides (2, 57, 91, 123), which can lead to insulin resistance via upregulation of the NF-κB inflammatory pathway through activation of the JNK pathway (3, 56, 103, 132). The role of DAG and ceramides in promoting insulin resistance through the NF-κB and JNK pathways in muscle has been reliant on cell culture or animal models of T2DM and obesity, and although research in humans with T2DM and obesity is sparse, it appears to support the role of NF-κB and JNK in NEFA mediated insulin resistance (82). NEFA also promote muscle atrophy-related signalling and protein degradation *in vitro* through impaired activation of the PI3K-Akt pathway (21, 133). In cultured myotubes, Akt phosphorylation was reduced with incubation of 500 μM palmitate (NEFA), a concentration similar to that found in the plasma of patients with T2DM (112). The reduced Akt phosphorylation coincided with increased FoxO3a nuclear localisation, increased Atrogin-1 mRNA, and an increase in the rate of cellular protein degradation (21, 133). Further, the upregulation of FoxO3a in palmitate-treated cells increased the autophagy markers BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and microtubule-associated proteins 1A/1B light chain 3B (LC3a/b), suggesting that palmitate alone upregulates both the ubiquitin-proteasome and lysosome-autophagy systems through the impairment of Akt signalling. Only high levels of NEFA, like palmitate, induce atrophic signalling, whereas incubation of cells with non-saturated fats, such as docosahexaenoic acid and linoleic acid, prevent NEFA-induced muscle atrophy signalling (21, 27, 133). It is important to note that NEFA-induced muscle atrophy research is a relatively new area of research, and the direct role of palmitate-induced changes to the rate of protein synthesis, inflammatory pathways, autophagy, and induction of ER stress in regard to muscle atrophy signalling requires further investigation.

Whilst impaired insulin signalling in muscle is an important contributor to both decreased protein synthesis, via the Akt-mTOR pathway and activation of proteolysis, reduced insulin action alone may not be sufficient to cause muscle atrophy. Adrenalectomy prevented the increase in protein degradation caused by an acute STZ injection in rodents despite downregulation of the PI3K-Akt pathway (61). Although rodent models of diabetes, such as *db/db* mice, have substantial insulin resistance and muscle atrophy (128), other diabetic rodent models such as TallyHo mice become insulin resistant without muscle atrophy (95). This differential response between the *db/db* and TallyHo models may have occurred due to the higher circulating glucocorticoids and inflammatory cytokines in obese *db/db* mice; although this explanation is speculative and needs to be investigated further. Thus, whilst inflammation is a key mediator of insulin resistance which subsequently impairs protein synthesis and degradation signalling, T2DM induced atrophy is likely to involve other contributing signalling factors. Such factors could include increased circulating glucocorticoids, cytokines, NEFA, and upregulation of tissue-specific inflammatory pathways (23, 61, 62, 127, 143).

Pro-inflammatory muscle pathways and atrophy signalling in T2DM

In addition to their role in insulin resistance, increased circulating pro-inflammatory cytokines and NEFA directly upregulate several inflammatory pathways, such as the NF-κB and STAT3 pathways (Figure 2), leading to increased activation of the ubiquitin proteasome system (23, 96, 143). NF-κB is a protein complex comprising a family of proteins which share the Rel homology domain, allowing for nuclear translocation, DNA binding, binding to other NF-κB subunits and interaction with the inhibitor of NF-κB, IKB (10). Whilst NF-κB exists as mono- and hetero-dimer proteins, the p50/p65 heterodimer of NF-κB is the most important for transcription of canonical target genes (10). However, an alternative p105-p50-B-cell lymphoma 3-encoded protein (BCL3) transcription pathway has also recently been described that contributes to disuse atrophy, although its role in T2DM is unknown (Figure 2) (62). NF-κB can be activated through various pathways that are targeted by pro-inflammatory cytokines, including TNFα, and by circulating NEFA through the toll-like receptor 4 (TLR4) (40, 113). Whilst elevated TNFα by itself is not sufficient to cause muscle atrophy (90, 106), upregulation of NF-κB can cause muscle atrophy in rodents (23, 127). Mice overexpressing the inhibitor of NF-κB Kinase β (IKKβ) had a 15-fold increase in NF-κB, which reduced muscle fibre cross sectional area by 50-65%, depending on the muscle group (23). In other studies, transgenic overexpression of dominant negative IKKβ/α in rat muscle caused a 70% reduction in disuse-initiated muscle atrophy, a response that was presumed to be due to inhibition of NF-κB (127). Importantly, NF-κB can increase the degradation of specific muscle proteins via increasing expression of the E3 ubiquitin ligase MuRF1 (134), suggesting that the atrophic effects of increased NF-κB activity are not solely mediated by insulin resistance. Whilst NF-κB is increased in muscle during atrophic conditions in humans (94), including T2DM (125), its direct role in T2DM related atrophy is relatively unknown. In cancer cachexia, atrophy was attenuated with overexpression of dominant negative IKKβ, independent of the canonical p65 NF-κB pathway, suggesting that IKKβ may act through the NF-κB p50-p105-BCL3 pathway in cachexia-induced atrophy (32). Considering the elevated systemic inflammation and activation of the NF-κB pathway in T2DM skeletal muscle (40, 125), the NF-κB pathways are a promising, but relatively unexplored, area of T2DM mediated muscle atrophy.

STAT3 is a signalling protein that is activated by pro-inflammatory cytokines such as IL-6. Recently it was found to impair protein synthesis signalling in muscle in chronic kidney disease (CKD), cancer cachexia and STZ-induced diabetes in mice (116, 143). STAT3 is implicated in insulin resistance (Figure 2) (87), but only recently has the mechanism directly linking STAT3 and atrophy been described (143). In mice with CKD, genetic knockout of STAT3, or small molecular inhibition of STAT3, attenuated muscle atrophy in the gastrocnemius and tibialis anterior muscles (143). The mechanism of STAT3-dependent atrophy was elucidated *in vitro*, where phosphorylation of STAT3 upregulated myostatin transcription via CAAT/enhancer-binding protein δ (C/EBP δ), a finding confirmed using C/EBP δ knockout mice with CKD. Thus, activation of STAT3 via inflammatory factors such as IL-6 can reduce protein synthesis via the induction of insulin resistance through SOCS3 (142), and increased transcription of myostatin (143). However, the importance of this newly discovered STAT3-CEBP δ-myostatin pathway in T2DM needs to be investigated.

EXERCISE, INFLAMMATION AND ATROPHY SIGNALLING

Exercise and inflammation in T2DM

Evidence suggests chronic exercise, particularly endurance training or combined endurance and resistance training, in patients with T2DM can lower both systemic and muscle-based inflammation (11, 54, 66), although not all studies report consistent findings (104, 135, 148). A comprehensive study by Balducci et al., (11) investigated a range of systemic inflammatory markers over the course of 12 months in T2DM participants completing either low intensity endurance exercise, moderate-high intensity endurance exercise, or combined resistance and endurance training. While CRP decreased in all of the exercise groups, only the combined training group had lower circulating levels of both TNFα and IL-6 after 12 months, whereas the high intensity endurance group also had lower circulating IL-6 (11). In another study, frequent moderate intensity endurance exercise (4 times per week for 6 months) reduced CRP (66). Importantly, changes in IL-6, CRP and TNFα occurred independently of alterations in fat mass (11, 66, 139), implying that the reduced systemic inflammation was directly due to exercise rather than loss of adipose tissue. This may indicate that improvements in fitness through exercise, rather than changes in adiposity per se, decrease the levels of systemic inflammatory markers. Several groups reported no change to CRP or IL-6 with either resistance training or with moderate intensity endurance exercise (104, 135, 148). This could be due to a range of different factors, including insufficient program duration, exercise mode, frequency, intensity, and relatively small sample sizes. Further studies are needed to determine which exercise mode, intensity and duration is needed to influence pro-inflammatory markers.

In lean and obese non-diabetic patients, acute endurance exercise transiently increased muscle NF-κB DNA binding activity, whereas no change was found after exercise in patients with T2DM (125). This may, in part, be due to the already high basal levels of NF-κB activity in T2DM (125). Studies investigating the anti-inflammatory effects of chronic exercise (exercise training) in patients with T2DM are sparse. There is, however, evidence that chronic exercise attenuates muscle-based inflammation in T2DM (119). The inhibitor of NF-κB, IKB, was lower in T2DM muscle compared to controls and was increased in response to eight weeks of endurance training (119). In addition, muscle TNFα was also lower in muscle of T2DM patients after eight weeks of moderate intensity (70% of $\dot{V}O_{2peak}$) endurance training (119), suggesting that endurance exercise has anti-inflammatory properties in skeletal muscle in T2DM. However, a separate study in humans with T2DM found that a 16 week resistance training program caused no change in muscle-based markers of inflammation, including TNFα (51). Altogether, the data to date indicate that endurance training can reduce inflammation in T2DM, whilst the effect of resistance training is still unclear.

Exercise attenuates muscle atrophy

Exercise, particularly resistance training, is an effective method to promote muscle hypertrophy and attenuate muscle atrophy during various atrophic conditions (4, 5, 130). Indeed, 6-12 weeks of resistance training (three times per week, 70-80% of 1RM) increased knee extensor muscle size, and improved strength in patients with T2DM (20, 46, 58, 64). mTOR is the primary signalling pathway by which exercise training stimulates muscle hypertrophy and protein synthesis (50, 131). Mechanical stimuli, such as resistance training, have the potential to activate muscle mTOR complex 1 (mTORC1) through phosphorylation and subsequent lysosomal exclusion of the mTORC1 repressor tuberous sclerosis complex 2 (63, 131). Further, the extracellular-signal-regulated kinases 1/2 (ERK1/2) and the PI3K-Akt-mTOR pathways also increase mTORC1 activity (18, 50). The upregulation of mTORC1 phosphorylates several proteins vital for protein synthesis and hypertrophy, such as ribosomal S6 kinase 1 ($p70^{S6K1}$) and 4E-BP1 (50). Indeed, patients with T2DM exhibit a reduced capacity for protein synthesis, perhaps due to a reduced 4E-BP1 phosphorylation in muscle in response to protein feeding and insulin (101, 120). In an animal model, obese sarcopenic Zucker rats exhibit reduced contractile protein synthesis (15% of the protein synthesis rate of lean controls) in response to exercise compared to lean controls (93). The anabolic resistance to insulin and protein feeding in combination with increased ubiquitin-proteasome activation may contribute to the lower muscle mass with older age in T2DM (13). Resistance training is an effective therapy against anabolic resistance in T2DM, as mTOR can be activated independently of insulin or insulin-like growth factor -1 (IGF-1) with resistance training (8, 43, 49, 50, 131), and resistance training can increase *vastus lateralis* Type I and II muscle fibre CSA by 18-21% in T2DM patients (20, 46, 58, 64). Whether resistance training can attenuate muscle atrophy through reducing muscle-based inflammation is unclear, but presents one potential mechanism which requires further investigation.

In addition to the direct effect of mTORC1 activation on protein synthesis, upregulation of mTORC2 by training may also attenuate muscle atrophy. mTORC2 activates Akt, causing downregulation of the ubiquitin-proteasome system (53, 147).

In support of this premise, eight weeks of resistance training decreased muscle FoxO1 while increasing both Akt and mTOR phosphorylation in healthy humans, whilst detraining decreased Akt phosphorylation and increased FoxO1 (76). Further, muscle Atrogin-1 mRNA in muscle was reduced 48 hours after a single bout of resistance exercise (86). The decrease in Atrogin-1 mRNA reported by Mascher et al., (86) was accompanied by increased MuRF1 mRNA immediately post-exercise (86) and in a separate study, MuRF1 mRNA was increased after a bout of intense interval exercise (8). The divergent signalling response with the E3 ubiquitin ligases suggests exercise mode and intensity are important factors in the activation of the ubiquitin-proteasome system after exercise; with intense exercise potentially temporarily upregulating atrogene signalling, whilst resistance training may decrease ubiquitin proteasome signalling (8, 86). These divergent data further suggest that in healthy populations acute activation of the ubiquitin-proteasome system in response to exercise may be required for removal of damaged proteins and is unlikely to have a prominent role on muscle atrophy or hypertrophy, likely due to the substantial concurrent increase in protein synthesis (8, 43, 49). In inflammatory conditions, such as T2DM, however, the downregulation of the ubiquitin-proteasome system with training, potentially via improvements in inflammatory pathways, may be of greater importance. For instance, long term resistance training (12 months), increased thigh CSA muscle mass by 4.5% in older-aged patients with T2DM, and the increase in muscle mass was associated with a lower circulating CRP (88). A limitation of the study was that muscle samples were not taken, preventing the evaluation of muscle inflammation. To our knowledge, no *in vivo* evidence investigating the effects of T2DM and resistance training on both muscle atrophy and muscle-based inflammation exists; thus, the potential anti-inflammatory role of exercise training and its effect on the ubiquitin-proteasome system are still not understood in T2DM.

Exercise training increases insulin sensitivity via the IRS1-PI3K-Akt pathway in T2DM, which consequently may upregulate the mTOR pathway and protein synthesis (18, 31, 129, 131). As systemic inflammation substantially contributes to insulin resistance, the improvements in systemic and local (muscle) inflammation by exercise training may upregulate protein synthesis and reduce the ubiquitin-proteasome protein degradation via upregulation of the IRS1-PI3K-Akt pathway (18, 33, 40, 50, 111). As described previously, the NF-κB pathway is capable of directly upregulating atrophy signalling, and NF-κB is emerging as a potential inflammatory pathway which can promote atrophy signalling in T2DM (23, 134). As such, the NF-κB pathway may also be important in training adaptations to T2DM muscle, as NF-κB signalling is upregulated in humans with T2DM and decreased with training (52, 119). However, no studies that investigated NF-κB in muscle in humans with T2DM measured markers of the ubiquitin-proteasome system at baseline or after training.

CONCLUSIONS AND FUTURE DIRECTIONS

T2DM is characterised by systemic, low-grade inflammation that impairs insulin sensitivity and upregulates muscle atrophy signalling. Inflammation-induced insulin resistance decreases protein synthesis through the PI3K-Akt pathway, and upregulates the ubiquitin-proteasome system via FoxO family proteins and their downstream E3 ubiquitin ligases. There is emerging evidence that inflammation may also directly promote atrophy signalling through pathways such as NF-κB and STAT3. However, studies in T2DM muscle investigating inflammatory and atrophy pathways need to be performed. Exercise training, particularly resistance training, has proven particularly valuable in promoting increased muscle mass through mTOR signalling and potentially reducing inflammation in T2DM; although to what extent improvements in inflammatory pathways contribute to attenuating muscle atrophy signalling or improving hypertrophy is yet to be elucidated.

Future studies should explore the mechanism/s behind the effects of T2DM (and in particular hyperinsulinemia) on muscle atrophy as well as the mechanism by which exercise training can minimise or prevent the effects of diabetes on skeletal muscle. The NF-κB pathway is emerging as an important contributor to atrophy signalling in muscle; however the exact mechanisms behind this cause and effect are still unclear. It is possible that the newly discovered p50-p105 and BCL3-NF-κB pathway, which has not been investigated in T2DM-mediated muscle atrophy, is a promising area of future investigation. The recently described STAT3-C-EBPδ-myostatin pathway also requires investigation in models of T2DM such as high fat feeding, and interventional studies in humans. The muscle atrophy in T2DM is most severe with advanced age. Hence, muscle signalling involved in other atrophic conditions prevalent in T2DM, such as physical inactivity and sarcopenia, and their potential interaction with T2DM mediated atrophy is a clinically important area of future research. Most importantly, longitudinal interventional studies examining muscle cross sectional area, muscle atrophy signalling and muscle inflammatory pathways are required in human T2DM populations, ideally also with structured training and aging populations.

ORIGINAL RESEARCH

Muscle inflammation and atrophic signalling in humans with T2DM

The role of inflammation in muscle degradation pathways in humans remains largely unexplored. Patients with T2DM exhibit increased systemic inflammation (40), as evident through the chronic upregulation of several inflammatory pathways and markers, including CCL2, NF-κB and STAT3, which are associated with insulin resistance and/or muscle atrophy signalling *in vivo* and *in vitro* (23, 65, 87, 109, 143). The association between muscle inflammatory markers and the ubiquitin-proteasome system, however, has not been investigated in humans with T2DM. Therefore, we investigated markers of muscle inflammation (CCL2, NF-κB p65 subunit, STAT3 and SOCS3) and ubiquitin-proteasome signalling (FoxO1, Atrogin-1 and MuRF1) in *vastus lateralis* muscle of older age obese patients with T2DM and age-matched controls. We hypothesized that T2DM patients would have increased inflammatory markers and upregulation of the ubiquitin-proteasome system compared to the controls.

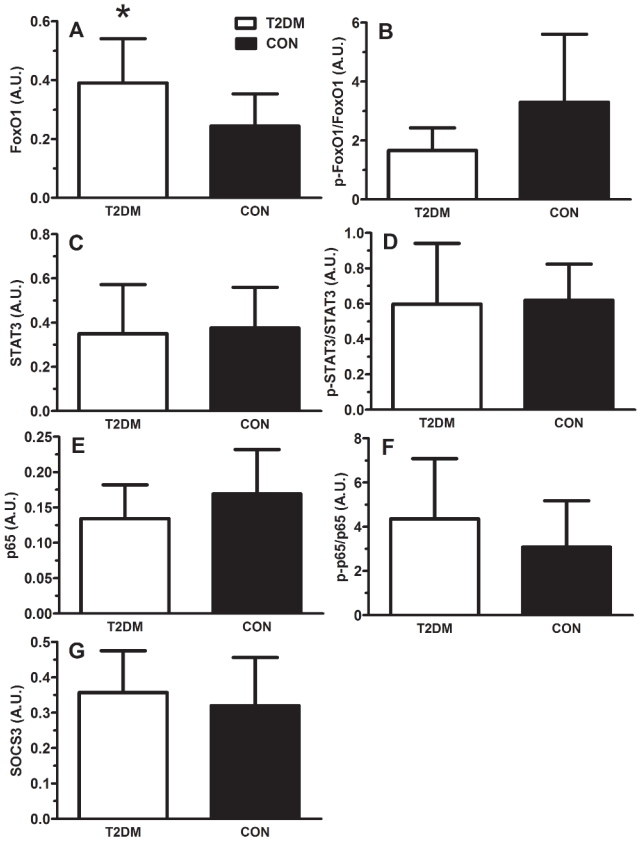


Figure 3: Skeletal muscle protein content of FoxO1 (A), p-FoxO1 relative to FoxO1 (B), STAT3 (C), p-STAT3 relative to STAT3 (D), p65 (E), p-p65 relative to p65 (F), and SOCS3 (G). All data was normalised to GAPDH abundance. Hollow bars denote participants with type 2 diabetes mellitus (T2DM), and filled bars represent age matched controls (CON). * different to CON ($p < 0.05$) with BMI as a covariate. For T2DM n = 12, and in CON n = 9. Data is presented as mean ± SD.

METHODS

Overall, 12 sedentary obese T2DM patients (T2DM; 5 females, 7 males, Age: 63.5 ± 13.8, BMI: 39.0 ± 5.5 kg.m^{-2}, mean ± SD) and 9 age matched, sedentary controls without insulin resistance (CON; 6 females, 3 males, Age: 67. 4 ± 8.0, BMI: 28.0 ± 5.9 kg.m^{-2}) participated in the study. A *vastus lateralis* muscle biopsy was performed for the assessment of markers of muscle inflammation and atrophy (CCL2, FoxO1, SOCS3, STAT3, p65 subunit of NF-κB, MuRF1 and Atrogin-1). Subject characteristics, including medications, are described in the Supplementary Methods.

RESULTS

Skeletal muscle FoxO1 protein abundance was 60% higher in T2DM ($p = 0.02$, Figure 3) compared to CON. There were no differences in protein abundance for STAT3, p65, SOCS3, p-STAT3/STAT3 and p-p65/p65 (Figure 4), or with mRNA expression of *MURF1, ATROGIN-1, CCL2, SOCS3, FOXO1* and *FOXO3* between groups (Table 1). In the pooled data, p-FoxO1/FoxO1 was negatively correlated to BMI ($r = -0.50$, $p = 0.02$, Figure 4), p65 was inversely related to BMI ($r = -0.50$, $p = 0.02$, Figure 4) and fasting glucose correlated with BMI ($r = 0.63$, $p = 0.01$). In regards to associations to physical fitness, there was a negative correlation between $\dot{V}O_{2peak}$ and the ratio of muscle p-STAT3/STAT3 in T2DM ($r = -0.60$, $p = 0.04$, Figure 5), but no other significant correlations to any other muscle inflammation or atrophy markers (*CCL2*, STAT3, p65 and FoxO1).

DISCUSSION OF ORIGINAL DATA

Our data indicate that atrophy signalling transcription factor FoxO1 (total protein) is increased in skeletal muscle in obese older-aged patients with T2DM. This is consistent with upregulation of the ubiquitin-proteasome proteolytic system despite finding no concomitant differences in several markers of muscle inflammation compared to age-matched controls. In T2DM, Akt activation is reduced (70), leading to increased FoxO transcription and upregulation of the E3 ubiquitin ligases MuRF1 and Atrogin-1 (111). Hence, in the absence of any difference in muscle inflammatory markers, our findings of increased FoxO1 reflect an increased catabolic state T2DM muscle, which may be in part caused by decreased Akt phosphorylation. Whilst we did not detect any differences in *ATROGIN-1* and *MURF1* mRNA expression, the expression of these proteins is transient and does not remain elevated during conditions of prolonged disuse- or glucocorticoid-induced atrophy (1, 24, 29). The ratio of p-FoxO1/FoxO1 was inverse-

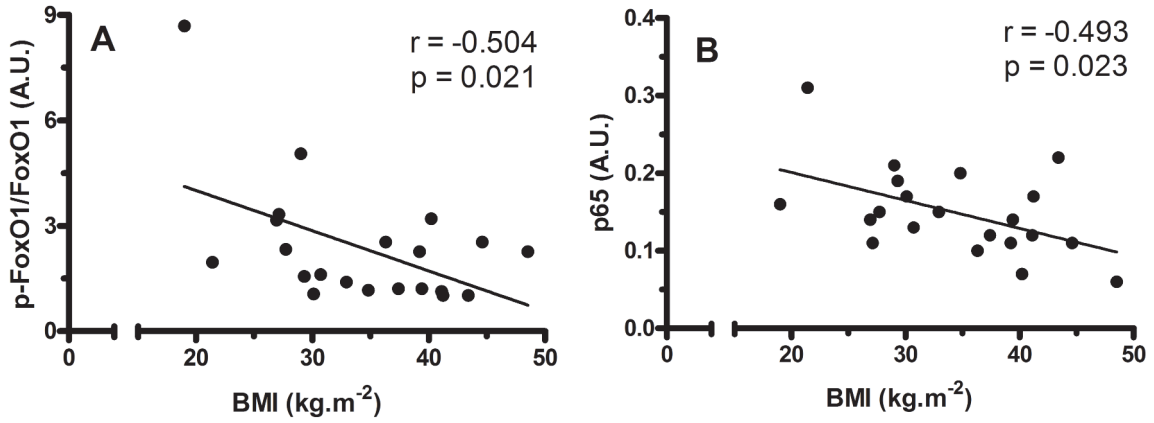


Figure 4: Correlations between p-FoxO1 relative to FoxO1 and BMI (A), and between p65 and BMI in all participants pooled (B).

Table 1: Gene expression of *MURF1, ATROGIN-1, CCL2, SOCS3, FOXO1* and *FOXO3* in T2DM and CON. Data represents n = 11 for T2DM and n = 8 for CON. Data is presented as mean ± SD. All data was normalised to *TBP*.

mRNA (A.U.)	**T2DM**	**CON**	**p-value**
MURF1	59.6 ± 57.3	45.1 ± 14.8	0.84
ATROGIN-1	134.2 ± 73.0	85.1 ± 34.5	0.11
CCL2	4.0 ± 5.3	2.0 ± 0.9	0.90
SOCS3	5.6 ± 6.8	6.3 ± 5.9	0.72
FOXO1	15.0 ± 12.4	12.9 ± 7.2	0.61
FOXO3	51.1 ± 59.9	36.1 ± 13.4	0.99

ly associated with BMI, suggesting that increased adiposity is related to an increase in FoxO1 activation; phosphorylation of FoxO1 inhibits FoxO nuclear localisation and transcription (17). Hence, the inverse association between p-FoxO1 and BMI may have been be due to obesity-induced inflammation and/or insulin resistance.

Despite the increased FoxO1 in T2DM muscle and the inverse relationship between p-FoxO1 and BMI, the p65 subunit of NF-κB was negatively correlated with BMI in the pooled data. Combined with the lack of difference in muscle inflammation in our T2DM patients, the findings from our study are incongruent with previous studies reporting obesity and T2DM promoting muscle-based inflammation signalling (87, 107, 119). It is important to note, however, that this study was cross sectional, and the sample size was relatively small. Thus, our data suggest that the relationship between inflammation and atrophy signalling is complex, and multiple factors are required to be taken into consideration. For example, the development of muscle inflammation may have been transient in these participants, altered by pharmaceutical treatment and other lifestyle factors, and complicated by the potential existing systemic inflammation in the age-matched control group (97, 98). Further, other inflammatory pathways not analysed in our study may have contributed to the higher FoxO1 in T2DM, such as through NF-κB via the p50-p105-BCL3 pathway, as is seen in disuse and cancer cachexia (32, 62). Finally, emerging evidence indicates that microRNA is important in proteolytic signalling; for example microRNA 486 inhibits FoxO1 expression (136). The inconsistencies between our findings in muscle inflammation and the few previous studies conducted in human skeletal muscle (87, 107, 119) indicate that further research is needed to elucidate the complex signalling between inflammatory and atrophic pathways in human T2DM muscle, and the extrinsic factors which may affect these signalling pathways.

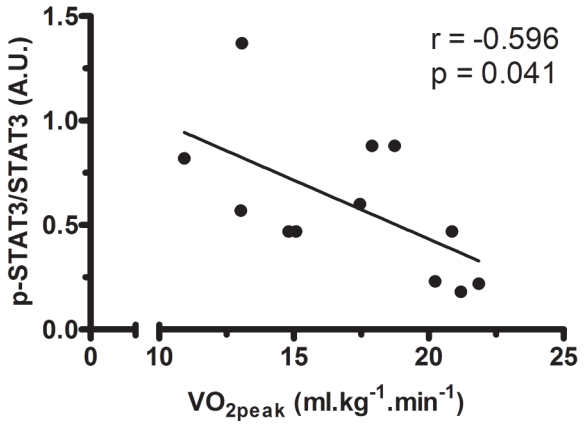


Figure 5: Correlation between p-STAT3 relative to STAT3 and $\dot{V}O_{2peak}$ in patients with type 2 diabetes mellitus.

In regards to the role of aerobic fitness in muscle inflammation and atrophy signalling in T2DM, we report a negative correlation between $\dot{V}O_{2peak}$ and the ratio of muscle p-STAT3/STAT3 ($r = -0.60$, $p = 0.04$, Figure 5); suggesting that lower aerobic fitness is associated with increased activation of inflammatory pathways in skeletal muscle of T2DM patients. This is consistent with previous research which reported both endurance and combined exercise training reduced systemic and muscle-based inflammation in T2DM (11, 119). However, the effects of exercise intensity, mode and frequency on systemic inflammation, muscle STAT3 signalling and other muscle-based inflammation with T2DM requires substantially more investigation *in-vivo*, particularly in humans.

CONCLUSIONS FROM ORIGINAL DATA

Our findings suggest that patients with T2DM exhibit upregulation of FoxO1 in skeletal muscle, suggesting greater muscle catabolism in muscle of patients with T2DM. In contrast to previous studies, concurrent upregulation of several inflammatory pathways including NF-κB p65, STAT3 and CCL2 was not observed, suggesting a complex relationship between muscle proteolytic and inflammatory pathways in humans that requires further investigation. Finally, we also found a negative relationship between $\dot{V}O_{2peak}$ and muscle p-STAT3/STAT3, suggesting that endurance exercise may be a useful intervention to reduce muscle inflammation in T2DM.

ACKNOWLEDGEMENTS

Associate Professor Itamar Levinger is a Heart Foundation Future Leader Fellow (ID: 100040) and Dr Tara C Brennan-Speranzais supported by an NHMRC Early. This work was supported by NIH T32DK007656 (B.D.P.), NIH RO1DK95610 (S.R.P.), and Merit Review Award I01BX001456 (S.R.P.) from the US Department of Veterans Affairs Biomedical Development Laboratory Research and Development Service.
B.D.P. and S.R.P.; no conflict of interest disclosures. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

SUPPLEMENTAL METHODS

Twelve patients diagnosed with type 2 diabetes mellitus (T2DM; 5 females, 7 males, Age: 63.5 ± 13.8, BMI: 39.0 ± 5.5 $kg.m^{-2}$, mean ± SD) and nine age matched non-diabetic controls (CON; 6 females, 3 males, Age: 67. 4 ± 8.0, BMI: 28.0 ± 5.9 $kg.m^{-2}$) participated. In the T2DM group partici-

pants were taking a range of medications, including oral hypoglycaemic agents (n = 8), insulin, (n = 5), statins (n = 7), anti-coagulants (n = 2), ACE inhibitors (n = 8), beta-blockers (n = 2), Calcium channel blockers (n = 3), diuretics (n = 1), corticosteroid inhaler (n = 1), dopamine agonist (n = 1), proton pump inhibitors (n = 2), xanthine oxidase inhibitors (n = 2), antidepressants (n = 1), norethisterone (n = 1), calcium supplementation (n = 2), fish oil supplementation (n = 1), vitamin B12 supplementation (n = 1) and anticonvulsants (Topiramate, n = 1). In the CON group, the medications taken included statins (n =2), anti-depressants (n = 1), non-steroidal anti-inflammatory medications (n = 1), anti-coagulants (n = 1), proton pump inhibitors (n = 1) and thyroid hormone replacement therapy (n = 1). In both groups, participants were over 18 years of age, and the following exclusion criteria were adhered to: known coronary artery disease or evidence of ischemia on baseline stress echocardiography, significant (moderate or severe) valvular heart disease medication changes over the past 3 months, unstable diabetes as evidenced by hypoglycaemic events in the past week or HbA1c >9.0%, and persistent or permanent atrial fibrillation. Weight ($p = 0.001$), BMI ($p = 0.001$) and fasting blood glucose ($p = 0.001$) were higher in T2DM, whilst total cholesterol ($p = 0.001$), LDL ($p = 0.001$) and HDL ($p = 0.004$) were lower in T2DM (Supplemental Table 1). In the T2DM, body fat percentage was 41.8 ±6.1%, lean mass 57.1 ± 9.4 kg, and HbA1c 7.3 ± 2.7%.

Supplemental table 1: Participant characteristics of the Type 2 diabetes mellitus group (T2DM) and age matched controls (CON).* different to CON ($p < 0.05$). For T2DM n = 12, for CON n = 9, except fasting glucose and blood lipid measurements where n = 10 for T2DM. SBP = systolic blood pressure, DBP = diastolic blood pressure. Values are represented as mean ± SD.

	T2DM	CON
Age (years)	63.5 ± 13.8	67.4 ± 8.0
Weight (kg)	104.5 ± 13.5*	74.1 ± 19.8
Height (cm)	164.5 ± 9.1	162.1 ±7.4
BMI ($kg.m^{-2}$)	39.0 ± 5.5*	28.0 ± 5.9
Fasting glucose ($mmol.L^{-1}$)	8.9 ± 2.4*	4.5 ± 1.4
HDL ($mmol.L^{-1}$)	1.1 ± 0.3*	1.7 ± 0.6
LDL ($mmol.L^{-1}$)	2.2 ± 0.8*	3.7 ± 0.6
Total cholesterol ($mmol.L^{-1}$)	3.9 ± 1.1*	6.0 ±0.6
Triglycerides ($mmol.L^{-1}$)	1.5 ± 0.9	1.2 ± 0.6
SBP (mmHg)	136 ± 22.9	110 ± 44.4
DBP (mmHg)	80.1 ± 17.3	66 ±25.2
$\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	17.1 ± 3.7	n/a

Each participant received both verbal and written explanations of the study before giving informed consent and the protocol was approved by the Human Research Ethics Committees of both Victoria University and Austin Health. Only T2DM participants performed the symptom-limited $\dot{V}O_{2peak}$ test with a 12 lead ECG (Model X-Scribe Stress Test 114 System, Mortara Instrument Inc., WI, USA) on a Cybex MET 100 exercise cycle, and had a DXA scan (78, 79). The $\dot{V}O_{2peak}$ test protocol comprised of an initial intensity of 25 W, with increments of 20 W/min for men and 10 W/min for women (78, 80). Control participants did not perform the $\dot{V}O_{2peak}$ test or DXA scan. Within the next three weeks, all participants then completed: a fasting blood sample for blood glucose, HbA1c% (T2DM only), and blood lipid profile (45). Blood pressure was measured using a mercury sphygmomanometer, and a resting *vastus lateralis* muscle biopsy (80, 81) was performed, with a minimum of 48 hours between each testing session.

For immunoblot analysis, homogenization of approximately 20 mg of skeletal muscle was performed using 300 µl of lysis buffer (20 mM Tris/HCl, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% Nonidet P40) including protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Australia) using the Precellys®24 tissue homgeniser (20 sec, setting 5500 rpm) (Sapphire Bioscience, NSW, Australia) and 1.0 mm zirconia/silica beads (Daintree Scientific, Tasmania, Australia). Protein content was determined using the Biorad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein (30 µg) was separated by 4-15% SDS-PAGE using Criterion TGX precast gels (Bio-Rad Laboratories, Hercules, CA) and transferred onto PVDF membranes (Trans-Blot® Turbo™ Transfer System, Bio-Rad Laboratories, Hercules, CA) and blocked with 5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, Australia). Primary antibodies, diluted in blocking buffer were applied and incubated overnight at 4°C; p-STAT3 (Tyr705), STAT3, p-p65 NF-KB (ser536), p65 NF-KB, p-FoxO1 (ser256), FoxO1 (Cell Signaling Technology Inc., Danvers, MA, USA), SOCS3 (Santa Cruz Biotechnology, CA, USA) and GAPDH (Sigma-Aldrich, Australia). Membranes were exposed to anti-rabbit HRP-conjugated secondary antibodies (GE Healthcare, NSW, Australia) and visualized by enhanced chemiluminescence (Super Signal West Femto Maximum Sensitivity Substrate, Thermo-Fisher Scientific, VIC, Australia). Blot images were captured using the Chemidoc MP Imaging system (Bio-Rad Laboratories, Hercules, CA) and the density of the bands was quantified using Image Lab 4.1 software (Bio-Rad Laboratories, Hercules, CA). Membranes were stripped (Restore Western Blot Stripping Buffer, Thermo-Fisher Scientific, VIC, Australia) before being re-probed for total STAT3, p65 NF-KB, FoxO1 and GAPDH.

Total cellular RNA was extracted as performed as described previously (126). RNA was transcribed into cDNA using the SuperScript™VILO cDNA Synthesis Kit (Life Technologies, VIC, Australia). qPCR was performed using the Bio-Rad CFX384 PCR system (Bio-Rad Laboratories, NSW, Australia) and PCR performed in duplicate with reaction volumes of 10 µl, containing SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories), forward and reverse primers and cDNA template. Data were analyzed using a comparative quantification cycle (Cq) method where the amount of target relative to NF is given by $2^{-\Delta\Delta Cq}$. Primers were designed using NCBI Primer BLAST from gene sequences obtained from GenBank and listed below: *ATROGIN-1* forward: 5'CATCCATATGTACACTGGTCCAAAGA; *ATROGIN -1* reverse: 5' – TCCGATACACCCACATGT-

TAATG; *MURF-1* forward: 5' – GGCGTGGCTCTCATTC-CTT; *MURF-1* reverse: 5' – TCTCCAAGTTCTCCAGTG-GATT; *CCL2* forward: 5' – CGCCTCCAGCATGAAAGTCT; *CCL2* reverse: 5' – GGAATGAAGGTGGCTGCTATG; *SOCS3* forward: 5' – GACCAGCGCCACTTCTTCA: *SOCS3* reverse: 5' – CTGGATGCGCAGGTTCTTG; *FOXO3A* forward: 5' – TGCAAACCTGCCCGTCAT; *FOXO3A* reverse: 5' – CTAAGCTCCCATTGAACATGT; *FOXO1* forward: 5'-CCGAACAGGATGATCTTGGAG; *FOXO1* reverse 5'-GCGGGTACACCATAGAATGCA; *TBP* forward: 5'-CGAATATAATCCCAAGCGGTTT; *TBP* reverse 5'-CCGTGGTTCGTGGCTCTCT.

STATISTICS

All data are presented as means ± SD and statistical significance was accepted at $p < 0.05$. Data was analysed using either an analysis of covariance (ANCOVA), with BMI as a covariate due to the difference in BMI between groups, or via independent samples t-tests. Correlations were performed using Pearson product moment coefficient correlations.

REFERENCES

1. Abadi A, Glover EI, Isfort RJ, Raha S, Safdar A, Yasuda N, Kaczor JJ, Melov S, Hubbard A, and Qu X. Limb immobilization induces a coordinate down-regulation of mitochondrial and other metabolic pathways in men and women. PLOS one 4: e6518, 2009.
2. Adams JM, Pratipanawatr T, Berria R, Wang E, DeFronzo RA, Sullards MC, and Mandarino LJ. Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. Diabetes 53: 25-31, 2004.
3. Aguirre V, Uchida T, Yenush L, Davis R, and White MF. The c-Jun NH2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser307. J Biol Chem 275: 9047-9054, 2000.
4. Akima H, Hotta N, Sato K, Ishida K, Koike T, and Katayama K. Cycle ergometer exercise to counteract muscle atrophy during unilateral lower limb suspension. Aviat Space Environ Med 80: 652-656, 2009.
5. Alkner BA, and Tesch PA. Knee extensor and plantar flexor muscle size and function following 90 days of bed rest with or without resistance exercise. Euro J Appl Physiol 93: 294-305, 2004.
6. Andersen H, Gjerstad MD, and Jakobsen J. Atrophy of Foot Muscles A measure of diabetic neuropathy. Diabetes Care 27: 2382-2385, 2004.
7. Andreasen AS, Kelly M, Berg RM, Moller K, and Pedersen BK. Type 2 diabetes is associated with altered NF-kappaB DNA binding activity, JNK phosphorylation, and AMPK phosphorylation in skeletal muscle after LPS. PLOS One 6: e23999, 2011.
8. Apro W, Moberg M, Hamilton DL, Ekblom B, van Hall G, Holmberg HC, and Blomstrand E. Resistance exercise-induced S6K1 kinase activity is not inhibited in human skeletal muscle despite prior activation of AMPK by high-intensity interval cycling. Am J Physiol Endocrinol Metab 308: E470-481, 2015.
9. Bailey JL, Zheng B, Hu Z, Price SR, and Mitch WE. Chronic kidney disease causes defects in signaling through the insulin receptor substrate/phosphatidylinositol 3-kinase/Akt pathway: implications for muscle atrophy. J Am Soc Nephrol 17: 1388-1394, 2006.
10. Bakkar N, and Guttridge DC. NF-kappaB signaling: a tale of two pathways in skeletal myogenesis. Physiol Rev 90: 495-511, 2010.
11. Balducci S, Zanuso S, Nicolucci A, Fernando F, Cavallo S, Cardelli P, Fallucca S, Alessi E, Letizia C, Jimenez A, Fallucca F, and Pugliese G. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. Nutr Metab Cardiovasc Dis 20: 608-617, 2010.
12. Baltadjiev AG, and Baltadjiev GA. Assessment of body composition of male patients with type 2 diabetes by bioelectrical impedance analysis. Folia Med (Plovdiv) 53: 52-57, 2011.
13. Bassil MS, and Gougeon R. Muscle protein anabolism in type 2 diabetes. Curr Opin Clin Nutr Metab Care 16: 83-88, 2013.
14. Bastard J-P, Maachi M, van Nhieu JT, Jardel C, Bruckert E, Grimaldi A, Robert J-J, Capeau J, and Hainque B. Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. J Clin Endocrinol Metab 87: 2084-2089, 2002.
15. Bernstein LE, Berry J, Kim S, Canavan B, and Grinspoon SK. Effects of etanercept in patients with the metabolic syndrome. Arch Intern Med 166: 902-908, 2006.
16. Bjornholm M, and Zierath JR. Insulin signal transduction in human skeletal muscle: identifying the defects in Type II diabetes. Biochem Soc Trans 33: 354-357, 2005.
17. Bodine SC, Latres E, Baumhueter S, Lai VK-M, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, and Dharmarajan K. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294: 1704-1708, 2001.
18. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, and Glass DJ. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nature Cell Biol 3: 1014-1019, 2001.
19. Brocca L, Cannavino J, Coletto L, Biolo G, Sandri M, Bottinelli R, and Pellegrino MA. The time course of the adaptations of human muscle proteome to bed rest and the underlying mechanisms. J Physiol 2012.
20. Brooks N, Layne JE, Gordon PL, Roubenoff R, Nelson ME, and Castaneda-Sceppa C. Strength training improves muscle quality and insulin sensitivity in Hispanic older adults with type 2 diabetes. Int J Med Sci 4: 19-27, 2007.
21. Bryner RW, Woodworth-Hobbs ME, Williamson DL, and Alway SE. Docosahexaenoic Acid protects muscle cells from palmitate-induced atrophy. ISRN Obes 2012: 647348, 2012.
22. Bunn JA, Buford TW, Serra MC, Kreider RB, and Willoughby DS. Protein and Amino Acid Supplementation Does Not Alter Proteolytic Gene Expression following Immobilization. J Nutr Metab 2011: 539690, 2011.
23. Cai D, Frantz JD, Tawa NE, Melendez PA, Oh B-C, Lidov HG, Hasselgren P-O, Frontera WR, Lee J, and Glass DJ. IKKβ/NF-κB activation causes severe muscle wasting in mice. Cell 119: 285-298, 2004.
24. Cannavino J, Brocca L, Sandri M, Bottinelli R, and Pellegrino MA. PGC1-α over-expression prevents metabolic alterations and soleus muscle atrophy in hindlimb unloaded mice. J Physiol 592: 4575-4589, 2014.

25. Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, Prelovsek O, Hohnen-Behrens C, Watt MJ, James DE, Kemp BE, Pedersen BK, and Febbraio MA. Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. Diabetes 55: 2688-2697, 2006.
26. Cetinus E, Buyukbese MA, Uzel M, Ekerbicer H, and Karaoguz A. Hand grip strength in patients with type 2 diabetes mellitus. Diabetes Res Clin Prac 70: 278-286, 2005.
27. Chen PY, Wang J, Lin YC, Li CC, Tsai CW, Liu TC, Chen HW, Huang CS, Lii CK, and Liu KL. 18-Carbon polyunsaturated fatty acids ameliorate palmitate-induced inflammation and insulin resistance in mouse C2C12 myotubes. J Nutr Biochem 2015.
28. Chen Y, Cao L, Ye J, and Zhu D. Upregulation of myostatin gene expression in streptozotocin-induced type 1 diabetes mice is attenuated by insulin. Biochem Biophys Res Com 388: 112-116, 2009.
29. Cho JE, Fournier M, Da X, and Lewis MI. Time course expression of Foxo transcription factors in skeletal muscle following corticosteroid administration. J Appl Physiol 108: 137-145, 2010.
30. Cohen S, Nathan JA, and Goldberg AL. Muscle wasting in disease: molecular mechanisms and promising therapies. Nature Rev Drug Discov 14: 58-74, 2015.
31. Conn VS, Koopman RJ, Ruppar TM, Phillips LJ, Mehr DR, and Hafdahl AR. Insulin Sensitivity Following Exercise Interventions: Systematic Review and Meta-Analysis of Outcomes Among Healthy Adults. J Prim Care Community Health 5: 211-222, 2014.
32. Cornwell EW, Mirbod A, Wu CL, Kandarian SC, and Jackman RW. C26 cancer-induced muscle wasting is IKKbeta-dependent and NF-kappaB-independent. PLOS One 9: e87776, 2014.
33. De Alvaro C, Teruel T, Hernandez R, and Lorenzo M. Tumor necrosis factor α produces insulin resistance in skeletal muscle by activation of inhibitor κB kinase in a p38 MAPK-dependent manner. J Biol Chem 279: 17070-17078, 2004.
34. De Boer MD, Selby A, Atherton P, Smith K, Seynnes OR, Maganaris CN, Maffulli N, Movin T, Narici MV, and Rennie MJ. The temporal responses of protein synthesis, gene expression and cell signalling in human quadriceps muscle and patellar tendon to disuse. J Physiol 585: 241-251, 2007.
35. DeFronzo RA, Gunnarsson R, Björkman O, Olsson M, and Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J Clin Invest 76: 149-155, 1985.
36. Dehoux M, Van Beneden R, Pasko N, Lause P, Verniers J, Underwood L, Ketelslegers J-M, and Thissen J-P. Role of the insulin-like growth factor I decline in the induction of atrogin-1/MAFbx during fasting and diabetes. Endocrinol 145: 4806-4812, 2004.
37. Del Prato S. Role of glucotoxicity and lipotoxicity in the pathophysiology of Type 2 diabetes mellitus and emerging treatment strategies. Diabet Med 26: 1185-1192, 2009.
38. den Kamp CMO, Langen RC, Snepvangers FJ, de Theije CC, Schellekens JM, Laugs F, Dingemans A-MC, and Schols AM. Nuclear transcription factor κ B activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia. Am J Clin Nutr 98: 738-748, 2013.
39. Dominguez H, Storgaard H, Rask-Madsen C, Steffen HT, Ihlemann N, Baunbjerg ND, Spohr C, Kober L, Vaag A, and Torp-Pedersen C. Metabolic and vascular effects of tumor necrosis factor-alpha blockade with etanercept in obese patients with type 2 diabetes. J Vasc Res 42: 517-525, 2004.
40. Donath MY, and Shoelson SE. Type 2 diabetes as an inflammatory disease. Nature Rev Immunol 11: 98-107, 2011.
41. Fain JN. Release of inflammatory mediators by human adipose tissue is enhanced in obesity and primarily by the nonfat cells: a review. Mediators Inflam 2010: 2010.
42. Ferrannini E, Simonson DC, Katz LD, Reichard G, Bevilacqua S, Barrett EJ, Olsson M, and DeFronzo RA. The disposal of an oral glucose load in patients with non-insulin-dependent diabetes. Metabolism 37: 79-85, 1988.
43. Figueiredo VC, Caldow MK, Massie V, Markworth JF, Cameron-Smith D, and Blazevich AJ. Ribosome biogenesis adaptation in resistance training-induced human skeletal muscle hypertrophy. Am J Physiol Endocrinol Metab ajpendo.00050.02015, 2015.
44. Franch HA, and Price SR. Molecular signaling pathways regulating muscle proteolysis during atrophy. Curr Opin Clin Nutr Metab Care 8: 271-275, 2005.
45. Friedewald WT, Levy RI, and Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 18: 499-502, 1972.
46. Geirsdottir OG, Arnarson A, Briem K, Ramel A, Jonsson PV, and Thorsdottir I. Effect of 12-week resistance exercise program on body composition, muscle strength, physical function, and glucose metabolism in healthy, insulin-resistant, and diabetic elderly Icelanders. J Gerontol A Biol Sci Med Sci 67: 1259-1265, 2012.
47. Glass DJ. Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. Nature Cell Biol 5: 87-90, 2003.
48. Gomes MD, Lecker SH, Jagoe RT, Navon A, and Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc Natl Acad Sci U S A 98: 14440-14445, 2001.
49. Gonzalez AM, Hoffman JR, Townsend JR, Jajtner AR, Wells AJ, Beyer KS, Willoughby DS, Oliveira LP, Fukuda DH, Fragala MS, and Stout JR. Association between myosin heavy chain protein isoforms and intramuscular anabolic signaling following resistance exercise in trained men. Physiol Rep 3: 2015.
50. Goodman CA. The role of mTORC1 in regulating protein synthesis and skeletal muscle mass in response to various mechanical stimuli. In: Reviews of Physiology, Biochemistry and Pharmacology 166Springer, 2014, p. 43-95.
51. Gordon PL, Vannier E, Hamada K, Layne J, Hurley BF, Roubenoff R, and Castaneda-Sceppa C. Resistance training alters cytokine gene expression in skeletal muscle of adults with type 2 diabetes. Int J Immunopathol Pharmacol 19: 739-749, 2006.
52. Green CJ, Pedersen M, Pedersen BK, and Scheele C. Elevated NF-kappaB activation is conserved in human myocytes cultured from obese type 2 diabetic patients and attenuated by AMP-activated protein kinase. Diabetes 60: 2810-2819, 2011.
53. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, and Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell 11: 859-871, 2006.

54. Hayashino Y, Jackson JL, Hirata T, Fukumori N, Nakamura F, Fukuhara S, Tsujii S, and Ishii H. Effects of exercise on C-reactive protein, inflammatory cytokine and adipokine in patients with type 2 diabetes: a meta-analysis of randomized controlled trials. Metabolism 63: 431-440, 2014.
55. Herder C, Baumert J, Thorand B, Koenig W, De Jager W, Meisinger C, Illig T, Martin S, and Kolb H. Chemokines as risk factors for type 2 diabetes: results from the MONICA/KORA Augsburg study, 1984–2002. Diabetol 49: 921-929, 2006.
56. Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, and Hotamisligil GS. A central role for JNK in obesity and insulin resistance. Nature 420: 333-336, 2002.
57. Holland WL, Brozinick JT, Wang L-P, Hawkins ED, Sargent KM, Liu Y, Narra K, Hoehn KL, Knotts TA, and Siesky A. Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. Cell Metab 5: 167-179, 2007.
58. Holten MK, Zacho M, Gaster M, Juel C, Wojtaszewski JF, and Dela F. Strength training increases insulin-mediated glucose uptake, GLUT4 content, and insulin signaling in skeletal muscle in patients with type 2 diabetes. Diabetes 53: 294-305, 2004.
59. Hotamisligil GS, and Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nature Rev Immunol 8: 923-934, 2008.
60. Houstis N, Rosen ED, and Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440: 944-948, 2006.
61. Hu Z, Wang H, Lee IH, Du J, and Mitch WE. Endogenous glucocorticoids and impaired insulin signaling are both required to stimulate muscle wasting under pathophysiological conditions in mice. J Clin Invest 119: 3059-3069, 2009.
62. Jackman RW, Cornwell EW, Wu CL, and Kandarian SC. Nuclear factor-kappaB signalling and transcriptional regulation in skeletal muscle atrophy. Exp Physiol 98: 19-24, 2013.
63. Jacobs BL, You JS, Frey JW, Goodman CA, Gundermann DM, and Hornberger TA. Eccentric contractions increase the phosphorylation of tuberous sclerosis complex□2 (TSC2) and alter the targeting of TSC2 and the mechanistic target of rapamycin to the lysosome. J Physiol 591: 4611-4620, 2013.
64. Jorge MLMP, de Oliveira VN, Resende NM, Paraiso LF, Calixto A, Diniz ALD, Resende ES, Ropelle ER, Carvalheira JB, and Espindola FS. The effects of aerobic, resistance, and combined exercise on metabolic control, inflammatory markers, adipocytokines, and muscle insulin signaling in patients with type 2 diabetes mellitus. Metabolism 60: 1244-1252, 2011.
65. Jorgensen SB, O'Neill HM, Sylow L, Honeyman J, Hewitt KA, Palanivel R, Fullerton MD, Öberg L, Balendran A, and Galic S. Deletion of skeletal muscle SOCS3 prevents insulin resistance in obesity. Diabetes 62: 56-64, 2013.
66. Kadoglou NP, Iliadis F, Angelopoulou N, Perrea D, Ampatzidis G, Liapis CD, and Alevizos M. The anti-inflammatory effects of exercise training in patients with type 2 diabetes mellitus. Euro J Cardio Prev Rehab 14: 837-843, 2007.
67. Kim H-J, Higashimori T, Park S-Y, Choi H, Dong J, Kim Y-J, Noh H-L, Cho Y-R, Cline G, and Kim Y-B. Differential effects of interleukin-6 and-10 on skeletal muscle and liver insulin action in vivo. Diabetes 53: 1060-1067, 2004.
68. Kim JH, Bachmann RA, and Chen J. Interleukin-6 and insulin resistance. Vitam Horm 80: 613-633, 2009.
69. Kim KS, Park KS, Kim MJ, Kim SK, Cho YW, and Park SW. Type 2 diabetes is associated with low muscle mass in older adults. Geriat Gerontol Int 14 Suppl 1: 115-121, 2014.
70. Krook A, Roth RA, Jiang XJ, Zierath JR, and Wallberg-Henriksson H. Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. Diabetes 47: 1281-1286, 1998.
71. Lagathu C, Bastard J-P, Auclair M, Maachi M, Capeau J, and Caron M. Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. Biochem Biophysiol Res Com 311: 372-379, 2003.
72. Lambertucci AC, Lambertucci RH, Hirabara SM, Curi R, Moriscot AS, Alba-Loureiro TC, Guimaraes-Ferreira L, Levada-Pires AC, Vasconcelos DA, and Sellitti DF. Glutamine supplementation stimulates protein-synthetic and inhibits protein-degradative signaling pathways in skeletal muscle of diabetic rats. PLOS One 7: e50390, 2012.
73. Lecker SH, Goldberg AL, and Mitch WE. Protein degradation by the ubiquitin–proteasome pathway in normal and disease states. J Am Soc Nephrol 17: 1807-1819, 2006.
74. Lecker SH, Jagoe RT, Gilbert A, Gomes M, Baracos V, Bailey J, Price SR, Mitch WE, and Goldberg AL. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. FASEB J 18: 39-51, 2004.
75. Leenders M, Verdijk LB, van der Hoeven L, Adam JJ, van Kranenburg J, Nilwik R, and van Loon LJ. Patients with type 2 diabetes show a greater decline in muscle mass, muscle strength, and functional capacity with aging. J Am Med Dir Assoc 14: 585-592, 2013.
76. Léger B, Cartoni R, Praz M, Lamon S, Dériaz O, Crettenand A, Gobelet C, Rohmer P, Konzelmann M, and Luthi F. Akt signalling through GSK-3β, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. J Physiol 576: 923-933, 2006.
77. Levine B, and Kroemer G. Autophagy in the pathogenesis of disease. Cell 132: 27-42, 2008.
78. Levinger I, Goodman C, Hare DL, Jerums G, and Selig S. The effect of resistance training on functional capacity and quality of life in individuals with high and low numbers of metabolic risk factors. Diabetes Care 30: 2205-2210, 2007.
79. Levinger I, Goodman C, Matthews V, Hare DL, Jerums G, Garnham A, and Selig S. BDNF, metabolic risk factors, and resistance training in middle-aged individuals. Med Sci Sports Exerc 40: 535-541, 2008.
80. Levinger I, Howlett KF, Peake J, Garnham A, Hare DL, Jerums G, Selig S, and Goodman C. Akt, AS160, metabolic risk factors and aerobic fitness in middle-aged women. Exerc Immunol Rev 16: 98-104, 2010.
81. Levinger I, Jerums G, Stepto NK, Parker L, Serpiello FR, McConell GK, Anderson M, Hare DL, Byrnes E, and Ebeling PR. The Effect of Acute Exercise on Undercarboxylated Osteocalcin and Insulin Sensitivity in Obese Men. J Bone Miner Res 29: 2571–2576, 2014.
82. Liang H, Tantiwong P, Sriwijitkamol A, Shanmugasundaram K, Mohan S, Espinoza S, Defronzo RA, Dube JJ, and Musi N. Effect of a sustained reduction in plasma free fatty acid concentration on insulin signalling and inflammation in skeletal muscle from human subjects. J Physiol 591: 2897-2909, 2013.

83. Lo J, Bernstein LE, Canavan B, Torriani M, Jackson MB, Ahima RS, and Grinspoon SK. Effects of TNF-α neutralization on adipocytokines and skeletal muscle adiposity in the metabolic syndrome. Am J Physiol Endocrinol Metab 293: E102-E109, 2007.
84. Lynch GS. Therapies for improving muscle function in neuromuscular disorders. Exerc Sport Sci Rev 29: 141-148, 2001.
85. Mammucari C, Schiaffino S, and Sandri M. Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle. Autophagy 4: 524-526, 2008.
86. Mascher H, Tannerstedt J, Brink-Elfegoun T, Ekblom B, Gustafsson T, and Blomstrand E. Repeated resistance exercise training induces different changes in mRNA expression of MAFbx and MuRF-1 in human skeletal muscle. Am J Physiol Endocrinol Metab 294: E43-E51, 2008.
87. Mashili F, Chibalin AV, Krook A, and Zierath JR. Constitutive STAT3 phosphorylation contributes to skeletal muscle insulin resistance in type 2 diabetes. Diabetes 62: 457-465, 2013.
88. Mavros Y, Kay S, Simpson KA, Baker MK, Wang Y, Zhao RR, Meiklejohn J, Climstein M, O'Sullivan AJ, de Vos N, Baune BT, Blair SN, Simar D, Rooney K, Singh NA, and Fiatarone Singh MA. Reductions in C-reactive protein in older adults with type 2 diabetes are related to improvements in body composition following a randomized controlled trial of resistance training. J Cachexia Sarcopenia Muscle 5: 111-120, 2014.
89. Mohanty P, Aljada A, Ghanim H, Hofmeyer D, Tripathy D, Syed T, Al-Haddad W, Dhindsa S, and Dandona P. Evidence for a potent antiinflammatory effect of rosiglitazone. J Clin Endocrinol Metab 89: 2728-2735, 2004.
90. Moldawer LL, Svaninger G, Gelin J, and Lundholm KG. Interleukin 1 and tumor necrosis factor do not regulate protein balance in skeletal muscle. Am J Physiol 253: C766-773, 1987.
91. Montell E, Turini M, Marotta M, Roberts M, Noé V, Macé K, and Gómez-Foix AM. DAG accumulation from saturated fatty acids desensitizes insulin stimulation of glucose uptake in muscle cells. Am J Physiol Endocrinol Metab 280: E229-E237, 2001.
92. Narici MV, and de Boer MD. Disuse of the musculo-skeletal system in space and on earth. European Journal of Applied Physiology 111: 403-420, 2011.
93. Nilsson MI, Dobson JP, Greene NP, Wiggs MP, Shimkus KL, Wudeck EV, Davis AR, Laureano ML, and Fluckey JD. Abnormal protein turnover and anabolic resistance to exercise in sarcopenic obesity. FASEB J 27: 3905-3916, 2013.
94. Op den Kamp CM, Langen RC, Snepvangers FJ, de Theije CC, Schellekens JM, Laugs F, Dingemans AM, and Schols AM. Nuclear transcription factor kappa B activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia. Am J Clin Nutr 98: 738-748, 2013.
95. Ostler JE, Maurya SK, Dials J, Roof SR, Devor ST, Ziolo MT, and Periasamy M. Effects of insulin resistance on skeletal muscle growth and exercise capacity in type 2 diabetic mouse models. Am J Physiol Endocrinol Metab 306: E592-605, 2014.
96. Palus S, von Haehling S, and Springer J. Muscle wasting: an overview of recent developments in basic research. Int J Cardiol 176: 640-644, 2014.
97. Pararasa C, Bailey CJ, and Griffiths HR. Ageing, adipose tissue, fatty acids and inflammation. Biogerontol 16: 235-238, 2015.
98. Park MH, Kim DH, Lee EK, Kim ND, Im DS, Lee J, Yu BP, and Chung HY. Age-related inflammation and insulin resistance: a review of their intricate interdependency. Arch Pharm Res 37: 1507-1514, 2014.
99. Park SW, Goodpaster BH, Lee JS, Kuller LH, Boudreau R, de Rekeneire N, Harris TB, Kritchevsky S, Tylavsky FA, Nevitt M, Cho YW, Newman AB, Health A, and Body Composition S. Excessive loss of skeletal muscle mass in older adults with type 2 diabetes. Diabetes Care 32: 1993-1997, 2009.
100. Patsouris D, Cao J-J, Vial G, Bravard A, Lefai E, Durand A, Durand C, Chauvin M-A, Laugerette F, and Debard C. Insulin Resistance is Associated with MCP1-Mediated Macrophage Accumulation in Skeletal Muscle in Mice and Humans. PLOS One 9: e110653, 2014.
101. Pereira S, Marliss EB, Morais JA, Chevalier S, and Gougeon R. Insulin resistance of protein metabolism in type 2 diabetes. Diabetes 57: 56-63, 2008.
102. Pickup J, Mattock M, Chusney G, and Burt D. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetol 40: 1286-1292, 1997.
103. Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, and Pedersen BK. Tumor necrosis factor-α induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. Diabetes 54: 2939-2945, 2005.
104. Praet SF, Jonkers RA, Schep G, Stehouwer CD, Kuipers H, Keizer HA, and van Loon LJ. Long-standing, insulin-treated type 2 diabetes patients with complications respond well to short-term resistance and interval exercise training. Eur J Endocrinol 158: 163-172, 2008.
105. Rantanen T. Muscle strength, disability and mortality. Scand J Med Sci Sports 13: 3-8, 2003.
106. Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, Cersosimo E, Defronzo RA, Coletta DK, Sriwijitkamol A, and Musi N. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes 57: 2595-2602, 2008.
107. Rieusset J, Bouzakri K, Chevillotte E, Ricard N, Jacquet D, Bastard J-P, Laville M, and Vidal H. Suppressor of cytokine signaling 3 expression and insulin resistance in skeletal muscle of obese and type 2 diabetic patients. Diabetes 53: 2232-2241, 2004.
108. Robertson RP, Harmon J, Tran POT, and Poitout V. β-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 53: S119-S124, 2004.
109. Rui L, Yuan M, Frantz D, Shoelson S, and White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 277: 42394-42398, 2002.
110. Sandri M. Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome. Int J Biochem Cell Biol 45: 2121-2129, 2013.
111. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, and Goldberg AL. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 117: 399-412, 2004.

112. Santomauro A, Boden G, Silva M, Rocha DM, Santos RF, Ursich M, Strassmann P, and Wajchenberg B. Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects. Diabetes 48: 1836-1841, 1999.
113. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, and Flier JS. TLR4 links innate immunity and fatty acid–induced insulin resistance. Journal of Clinical Investigation 116: 3015, 2006.
114. Shoelson SE, Lee J, and Goldfine AB. Inflammation and insulin resistance. J Clin Invest 116: 1793-1801, 2006.
115. Siew E, Pupim L, Majchrzak K, Shintani A, Flakoll P, and Ikizler T. Insulin resistance is associated with skeletal muscle protein breakdown in non-diabetic chronic hemodialysis patients. Kidney Int 71: 146-152, 2007.
116. Silva KA, Dong J, Dong Y, Dong Y, Schor N, Tweardy DJ, Zhang L, and Mitch WE. Inhibition of Stat3 Activation Suppresses Caspase-3 and the Ubiquitin-Proteasome System, Leading to Preservation of Muscle Mass in Cancer Cachexia. J Biol Chem 290: 11177-11187, 2015.
117. Souto Padron de Figueiredo A, Salmon AB, Bruno F, Jimenez F, Martinez HG, Halade GV, Ahuja SS, Clark RA, DeFronzo RA, Abboud HE, and El Jamali A. Nox2 Mediates Skeletal Muscle Insulin Resistance Induced by a High-Fat Diet. J Biol Chem 290: 13427-13439, 2015.
118. Spranger J, Kroke A, Möhlig M, Hoffmann K, Bergmann MM, Ristow M, Boeing H, and Pfeiffer AF. Inflammatory cytokines and the risk to develop type 2 diabetes results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Diabetes 52: 812-817, 2003.
119. Sriwijitkamol A, Christ-Roberts C, Berria R, Eagan P, Pratipanawatr T, DeFronzo RA, Mandarino LJ, and Musi N. Reduced Skeletal Muscle Inhibitor of κBβ Content Is Associated With Insulin Resistance in Subjects With Type 2 Diabetes Reversal by Exercise Training. Diabetes 55: 760-767, 2006.
120. Stephens FB, Chee C, Wall BT, Murton AJ, Shannon CE, van Loon LJ, and Tsintzas K. Lipid-induced insulin resistance is associated with an impaired skeletal muscle protein synthetic response to amino Acid ingestion in healthy young men. Diabetes 64: 1615-1620, 2015.
121. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, and Glass DJ. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 14: 395-403, 2004.
122. Suetta C, Frandsen U, Jensen L, Jensen MM, Jespersen JG, Hvid LG, Bayer M, Petersson SJ, Schrøder HD, and Andersen JL. Aging affects the transcriptional regulation of human skeletal muscle disuse atrophy. PLOS One 7: e51238, 2012.
123. Szendroedi J, Yoshimura T, Phielix E, Koliaki C, Marcucci M, Zhang D, Jelenik T, Muller J, Herder C, Nowotny P, Shulman GI, and Roden M. Role of diacylglycerol activation of PKC-theta in lipid-induced muscle insulin resistance in humans. Proc Natl Acad Sci U S A 111: 9597-9602, 2014.
124. Tajiri Y, Kato T, Nakayama H, and Yamada K. Reduction of skeletal muscle, especially in lower limbs, in Japanese type 2 diabetic patients with insulin resistance and cardiovascular risk factors. Metab Syndr Relat Disord 8: 137-142, 2010.
125. Tantiwong P, Shanmugasundaram K, Monroy A, Ghosh S, Li M, DeFronzo RA, Cersosimo E, Sriwijitkamol A, Mohan S, and Musi N. NF-kappaB activity in muscle from obese and type 2 diabetic subjects under basal and exercise-stimulated conditions. Am J Physiol Endocrinol Metab 299: E794-801, 2010.
126. Trenerry MK, Carey KA, Ward AC, and Cameron-Smith D. STAT3 signaling is activated in human skeletal muscle following acute resistance exercise. J Appl Physiol 102: 1483-1489, 2007.
127. Van Gammeren D, Damrauer JS, Jackman RW, and Kandarian SC. The IkappaB kinases IKKalpha and IKKbeta are necessary and sufficient for skeletal muscle atrophy. FASEB J 23: 362-370, 2009.
128. Wang X, Hu Z, Hu J, Du J, and Mitch WE. Insulin resistance accelerates muscle protein degradation: activation of the ubiquitin-proteasome pathway by defects in muscle cell signaling. Endocrinol 147: 4160-4168, 2006.
129. Wang Y, Simar D, and Fiatarone Singh MA. Adaptations to exercise training within skeletal muscle in adults with type 2 diabetes or impaired glucose tolerance: a systematic review. Diabetes Metab Res Rev 25: 13-40, 2009.
130. Watson EL, Greening NJ, Viana JL, Aulakh J, Bodicoat DH, Barratt J, Feehally J, and Smith AC. Progressive Resistance Exercise Training in CKD: A Feasibility Study. Am J Kidney Dis 2014.
131. Watson K, and Baar K. mTOR and the health benefits of exercise. Semin Cell Dev Biol 36: 130-139, 2014.
132. Werner ED, Lee J, Hansen L, Yuan M, and Shoelson SE. Insulin resistance due to phosphorylation of insulin receptor substrate-1 at serine 302. J Biol Chem 279: 35298-35305, 2004.
133. Woodworth-Hobbs ME, Hudson MB, Rahnert JA, Zheng B, Franch HA, and Price SR. Docosahexaenoic acid prevents palmitate-induced activation of proteolytic systems in C2C12 myotubes. J Nutr Biochem 25: 868-874, 2014.
134. Wu CL, Cornwell EW, Jackman RW, and Kandarian SC. NF-kappaB but not FoxO sites in the MuRF1 promoter are required for transcriptional activation in disuse muscle atrophy. Am J Physiol Cell Physiol 306: C762-767, 2014.
135. Wycherley TP, Noakes M, Clifton PM, Cleanthous X, Keogh JB, and Brinkworth GD. A high-protein diet with resistance exercise training improves weight loss and body composition in overweight and obese patients with type 2 diabetes. Diabetes Care 33: 969-976, 2010.
136. Xu J, Li R, Workeneh B, Dong Y, Wang X, and Hu Z. Transcription factor FoxO1, the dominant mediator of muscle wasting in chronic kidney disease, is inhibited by microRNA-486. Kidney Int 82: 401-411, 2012.
137. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, and Tsuboyama-Kasaoka N. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nature medicine 7: 941-946, 2001.
138. Yang W-S, Jeng C-Y, Wu T-J, Tanaka S, Funahashi T, Matsuzawa Y, Wang J-P, Chen C-L, Tai T-Y, and Chuang L-M. Synthetic peroxisome proliferator-activated receptor-γ agonist, rosiglitazone, increases plasma levels of adiponectin in type 2 diabetic patients. Diabetes Care 25: 376-380, 2002.

139. You T, and Nicklas BJ. Chronic inflammation: role of adipose tissue and modulation by weight loss. Curr Diabetes Rev 2: 29-37, 2006.
140. Yuan L, Han J, Meng Q, Xi Q, Zhuang Q, Jiang Y, Han Y, Zhang B, Fang J, and Wu G. Muscle-specific E3 ubiquitin ligases are involved in muscle atrophy of cancer cachexia: An in vitro and in vivo study. Oncol Rep 33: 2261-2268, 2015.
141. Yuen DY, Dwyer RM, Matthews VB, Zhang L, Drew BG, Neill B, Kingwell BA, Clark MG, Rattigan S, and Febbraio MA. Interleukin-6 attenuates insulin-mediated increases in endothelial cell signaling but augments skeletal muscle insulin action via differential effects on tumor necrosis factor-alpha expression. Diabetes 58: 1086-1095, 2009.
142. Zhang L, Du J, Hu Z, Han G, Delafontaine P, Garcia G, and Mitch WE. IL-6 and serum amyloid A synergy mediates angiotensin II–induced muscle wasting. J Am Sci Nephrol 20: 604-612, 2009.
143. Zhang L, Pan J, Dong Y, Tweardy DJ, Dong Y, Garibotto G, and Mitch WE. Stat3 activation links a C/EBPδ to myostatin pathway to stimulate loss of muscle mass. Cell Metab 18: 368-379, 2013.
144. Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, Lecker SH, and Goldberg AL. FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. Cell Metab 6: 472-483, 2007.
145. Zheng B, Ohkawa S, Li H, Roberts-Wilson TK, and Price SR. FOXO3a mediates signaling crosstalk that coordinates ubiquitin and atrogin-1/MAFbx expression during glucocorticoid-induced skeletal muscle atrophy. FASEB J 24: 2660-2669, 2010.
146. Zinna EM, and Yarasheski KE. Exercise treatment to counteract protein wasting of chronic diseases. Curr Opin Clin Nutrit Metab Care 6: 87-93, 2003.
147. Zoncu R, Efeyan A, and Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nature Rev Mol Cell Biol 12: 21-35, 2011.
148. Zoppini G, Targher G, Zamboni C, Venturi C, Cacciatori V, Moghetti P, and Muggeo M. Effects of moderate-intensity exercise training on plasma biomarkers of inflammation and endothelial dysfunction in older patients with type 2 diabetes. Nutr Metab Cardiovasc Dis 16: 543-549, 2006.

Emerging roles of pro-resolving lipid mediators in immunological and adaptive responses to exercise-induced muscle injury

James F. Markworth[1], Krishna Rao Maddipati[2] and David Cameron-Smith[1]

[1] Liggins Institute, University of Auckland, New Zealand.
[2] Department of Pathology, Lipidomics Core Facility, Wayne State University, and Karmanos Cancer Institute, Detroit USA

ABSTRACT

Lipid mediators are bioactive metabolites of the essential polyunsaturated fatty acids (PUFA) that play diverse roles in the initiation, self-limitation, and active resolution of inflammation. Prostaglandins, classical pro-inflammatory lipid metabolites of arachidonic acid, have long been implicated in immunological and adaptive muscle responses to acute injury and exercise-induced stress. More recently, PUFA metabolites have been discovered during the resolution phase of inflammation which collectively function as endogenous 'stop signals' to control inflammation whilst actively promoting the return to a non-inflamed state. The apparent self-resolving nature of inflammatory responses holds important implications for contexts of musculoskeletal injury, exercise recovery, and chronic inflammatory diseases originating in or impacting upon muscle. 'Anti-inflammatory' interventions that strive to control inflammation via antagonism of pro-inflammatory signals are currently commonplace in efforts to hasten muscle recovery from damaging or exhaustive exercise, as well as to relieve the pain associated with musculoskeletal injury. However, the scientific literature does not clearly support a benefit of this anti-inflammatory approach. Additionally, recent evidence suggests that strategies to block pro-inflammatory lipid mediator pathways (e.g. NSAIDs) may be counterintuitive and inadvertently derange or impair timely resolution of inflammation; with potentially deleterious implications on skeletal muscle remodelling. The current review will provide an overview of the current understanding of diverse roles of bioactive lipid mediators in the initiation, control, and active resolution of acute inflammation. The established and putative roles of lipid mediators in mediating immunological and adaptive skeletal muscle responses to acute muscle injury and exercise-induced muscle load/stress will be discussed.

Key words: Inflammation, resolution, PUFA, docosanoids, eicosanoids.

Corresponding Author:
Dr. James Markworth, The Liggins Institute, University of Auckland, 85 Park Road, Grafton, Auckland, New Zealand
Email: j.markworth@auckland.ac.nz
Phone: +64 9 9236547

CONTENTS

1. INTRODUCTION

Inflammation is an essential protective response to injury or infection to eliminate the harmful agent and promote tissue repair. Overwhelming and persistent inflammation, however, can result in secondary cellular damage, promote maladaptive tissue remodelling and lead to the onset of degenerative chronic inflammatory disease. In a typical self-limited acute inflammatory response, clearance of cellular debris and pathogen removal by phagocytic immune cells is followed by successful resolution of inflammatory infiltrates, involving clearance of invading leukotcytes, cellular repair, and restoration of tissue homeostasis. The resolution phase of the inflammatory response, although once thought to be a passive

process, has only more recently been recognised to be an active and biologically controlled event (1-3). The nature and extent of resolution of inflammation is an area of critical importance in the context of muscle and exercise physiology, both to understand the inherent self-resolving nature of skeletal muscle injury/altered use and gain insight into how this knowledge may be applied to contexts of exercise recovery, musculoskeletal injury management, and prevention/treatment of chronic inflammatory disease.

Skeletal muscle injury elicits an acute inflammatory response characterised by the secretion of soluble mediators within damaged tissue that promote influx of fluid and plasma proteins followed by the infiltration and local accumulation of blood leukocytes (4, 5) (Figure 1). Muscle inflammation occurs in response not only to traumatic injury, but also to a diverse range of loading stimuli including eccentric exercise and reloading following muscle disuse. Polymorphonuclear neutrophils (PMNs) constitute the first wave of host immune cells, infiltrating damaged muscle within minutes to hours (6-9) (Figure 1). PMNs are phagocytic granulocytes that ingest and eliminate damaged tissue, whilst releasing reactive molecules through degranulation which can potentially induce secondary muscle damage and exacerbate muscle injury (8, 10, 11). The early wave of PMNs is followed later by recruitment of blood monocytes which accumulate within muscle in the hours to days following mechanical insult and differentiate locally into tissue macrophages (MΦs) (6, 7, 12, 13) (Figure 1). Successful resolution of inflammation requires apoptosis of PMNs within the infiltrate, followed by their elimination by nonphlogistic MΦ phagocytosis. Systemic depletion of monocytes has been conclusively shown to delay resolution of muscle inflammation, leading to impaired myofiber regenerative/adaptive responses to both degenerative injury (13-17) and overload induced muscle hypertrophy (18). Early, following injury, circulating classically activated (M1) pro-inflammatory monocytes infiltrate muscle and phagocytose myofiber debris and apoptotic neutrophils, a process which triggers a switch to an alternatively activated anti-inflammatory (M2) phenotype (19). During the latter stages of inflammation these M2 populations are predominant and play an important active role in promoting muscle growth and regeneration (20-23) (Figure 1). Eosinophils, another key innate immune cell population active within the resolution phase of inflammation, were also recently shown to be indispensable in the muscle regenerative response to injury (24). Collectively these data show that resolution phase of inflammation plays an important role in skeletal muscle adaptation to injury.

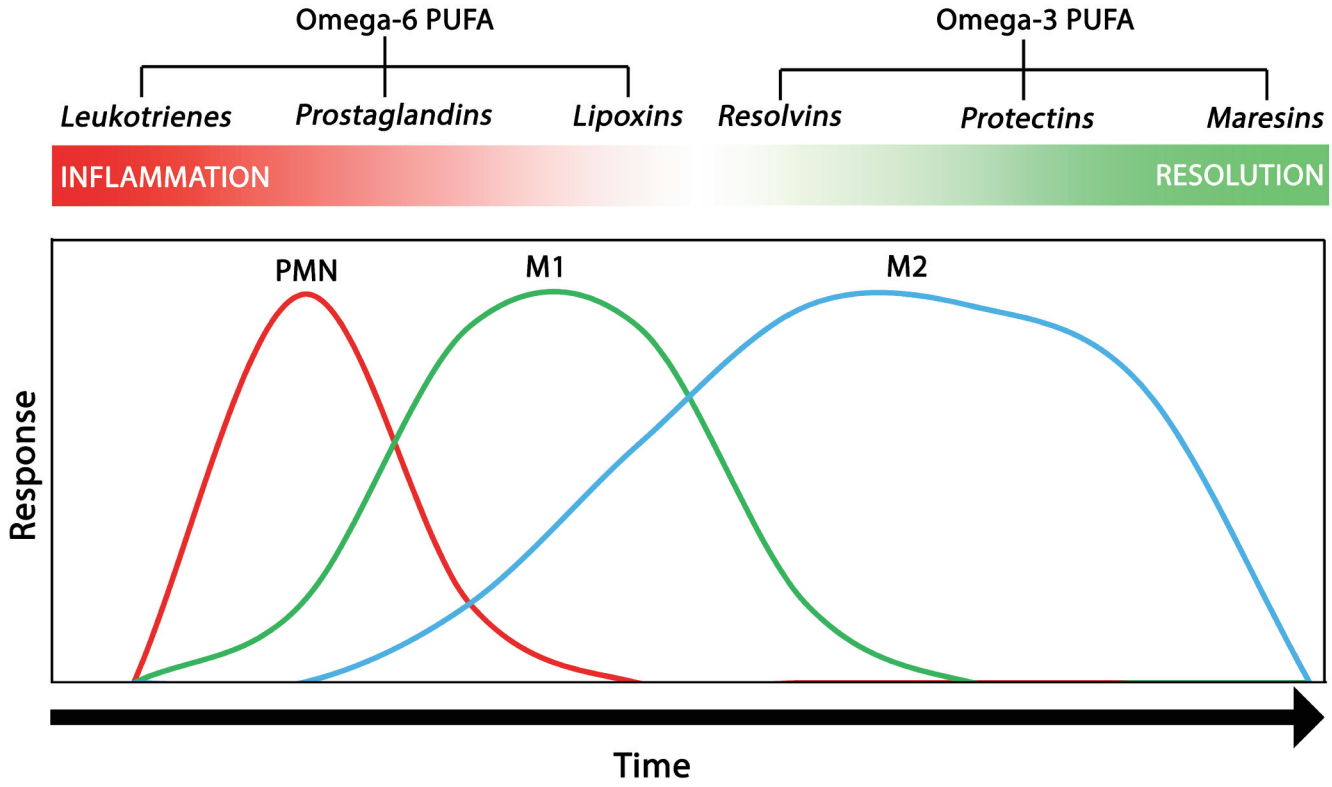


Figure 1: ***Time-course of leukocyte populations and bioactive lipid mediators in the inflammatory response to muscle injury/overload.*** Following tissue injury, neutrophils (PMNs) are rapidly mobilised, increase in circulation, and migrate from the blood stream into damaged tissue. Pro-inflammatory lipid mediators, the leukotrienes (LTs) and prostaglandins (PG) facilitate PMN trafficking via cellular effects on blood flow (vasodilation), vascular permeability, and PMN chemotaxis. Later in the inflammatory response there is a shift to biosynthesis of lipid mediators with anti-inflammatory and pro-resolving properties including n-6 PUFA derived lipoxins and n-3 PUFA derived resolvins, protectins and maresins. These signals function to limit and stop PMN trafficking, whilst promoting recruitment of blood monocytes/Mϕ which phagocytose and clear apoptopic PMNs. Classically-activated M1 monocyte populations initially are predominant but are later replaced by an alternatively activated M2 phenotype which play important roles in facilitating muscle growth and regeneration.

A complex signalling network exists between infiltrating immune cells and resident populations within the musculature including muscle cells (myofibers), myogenic stem cells (satellite cells), endothelial cells, and fibroblasts. A key, yet poorly understood, aspect of this regulation which is frequent-

Table 1:
Inflammatory and muscle roles of arachidonic acid metabolites of the COX-1 & 2 pathways

	Receptors	Major inflammatory role	Muscle role		References
			Effect	*Mechanisms*	
PGD2	DP1	Anti-inflammatory/pro-resolving	↑ Myoblast proliferation	↑ 15Δ-PGJ_2	(150)
	DP2		↓ Myoblast differentiation	↓ MyoD, ↓ myogenin	(97)
PGE2	EP1	Pro-inflammatory	↑ Myoblast proliferation	↑ ROS , ↑ cyclin E, ↓ myostatin	(98, 151)
	EP2	↑ *Vascular permeability*	↑ Myoblast differentiation	↑ MyoD & myogenin (Low dose)	(300)
	EP3	↑ *Leukocyte chemotaxis*	↓ Myoblast fusion	↓ Calcium (high dose)	(293)
	EP4	↑ *Vasodilation*	↑ Protein degradation	↑ MuRF/expression	(139, 301)
		↑*Algesia*		↑ IL-6 expression	(301)
PGF2α	FP	Pro-inflammatory	↑ Myoblast number	↓ Apoptosis, ↑ BRUCE	(152, 153)
		↑ *leukocyte chemotaxis*	↑ Myoblast fusion	↑ Survival,↑ NFATC2 signalling	(153, 154)
			↑ Protein synthesis		(139)
			↑ Myotube hypertrophy	↑ mTOR/ERK signalling	(149)
PGI2	IP	Pro-inflammatory/anti-aggregatory			
		↑ *vascular permeability*	↓ Myoblast migration	↓ Cell motility	(101)
		↓*platelet aggregation*	↑ Myoblast fusion		(101)
		↑*vasodilation*			
		↑*algesia*			
TXA2	TP	Pro-inflammatory/pro-Aggregatory	?	?	
		↑*platelet aggregation*			
		↑*vasoconstriction*			
		↑ *leukocyte chemotaxis*			

ly overlooked in the context of muscle and exercise is the role played by bioactive lipids. Lipid mediators are autocrine/paracrine signalling molecules derived from cellular polyunsaturated acids (PUFA). Under basal conditions, the majority of PUFA substrate remains esterified within cell membrane phospholipids, but a proportion is rapidly released by the action of phospholipase A2 (PLA2) in response to injurious or inflammatory stimuli. Lipid mediator biosynthesis involves oxidation of mobilised PUFA substrate by three major enzymatic pathways: (1) cyclooxygenase (COX), (2) lipoxygenase (LOX) and (3) epoxygenase catalysed by cytochrome P450 (CYP). Hundreds of distinct lipid mediator species can be synthesised via these pathways from numerous fatty acid (FA) precursors including major omega-6 (n-6) arachidonic acid (AA) and long-chain (LC) omega-3s (n-3) eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). During the early stages of tissue injury, classical pro-inflammatory lipid mediators are locally produced (e.g. prostaglandins and leukotrienes) which drive inflammation. Later in the inflammatory response, a shift in the profile of these bioactive lipids results in the predominant generation of mediators with anti-inflammatory and pro-resolving bioactivity which function to actively bring about the resolution phase of inflammation (e.g. lipoxins, resolvins, protectins and maresins) (25, 26) (Figure 1). The development of mass spectrometry (MS)-based lipidomic profiling methods has recently allowed for the simultaneous identification and quantification of large numbers of inflammatory and resolving lipid mediator metabolites in biological fluids. This approach sometimes referred in the literature to as "mediator lipidomics" or "metabololipidomics" enables the profiling of the "mediator lipidome" of biological samples encompassing the bioactive metabolites of the PUFAs (27-29). A large number of studies have focused on the systemic and intramuscular leukocyte and inflammatory cytokine immunological responses to exercise and these topics have been covered in several recent comprehensive reviews (e.g 30, 31). In contrast, the potential diverse role of bioactive lipid mediators in exercise immunology is yet to undergo systematic investigation and the topic has not been comprehensively reviewed previously.

The present review aims to provide an overview of the diverse role of bioactive lipid mediators in driving acute inflammation and bringing about its timely resolution, whilst discussing the established and putative roles of lipid mediators within muscle tissue. The current understanding of the role of lipid mediators in the adaptive responses to exercise is reviewed whilst highlighting the many gaps in our current knowledge and significant areas for future investigation.

2. CYCLOOXYGENASE PATHWAYS

The role of bioactive lipids in inflammation has classically focused on PUFA metabolites of the cyclooxygenase (COX) pathway. The COX pathway converts mobilised free intracellular AA to prostaglandin G_2 (PGG_2), and catalyses the subsequent reduction of PGG_2 to form prostaglandin H_2 (PGH_2) (Figure 2A). Specific synthase enzymes convert PGH_2 to the

five primary prostanoids; thromboxane A_2 (TXA_2), PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 (Figure 2A). Collectively these eicosanoid metabolites elicit the cardinal signs of inflammation; rubor (redness), calor (heat), tumor (swelling) and dollar (pain), via their cellular actions in vasodilation (32), vascular permeability (32-36), hyperalgesia (37-41), and PMN chemotaxis (42-45) (Table 1). Two COX isoforms are expressed in mammalian tissue. COX-1 is a constitutively expressed enzyme that allows for rapid PG synthesis following AA release which regulates a variety of homeostatic functions, whilst, COX-2 is transcribed by an inducible gene, which is up-regulated in the hours following exposure to inflammatory stimuli. The COX 1 & 2 pathways are well known as the molecular target of the non-steroidal anti-inflammatory class of drugs (NSAIDs), which chiefly function to prevent PG biosynthesis via inhibition of the cyclooxygenase activity of the COX-1 & 2 enzymes (46-48) (Figure 2A).

2.1 Prostanoid response to exercise:

The PG response to muscle loading has been studied for many years (49-51); however our understanding of the temporal changes in local and systemic PG concentrations in exercising humans *in-vivo* and their role in physiological exercise responses remains incomplete. Early human studies using experimental models of sub-maximal exercise consistently found heightened levels of PGs including PGE_2 (52-56), $PGF_{2\alpha}$ (52, 57), and PGI_2 (measured as 6-keto-$PGF_{1\alpha}$, a non-enzymatic hydrolysis product of PGI_2) (52, 53, 58-63) in

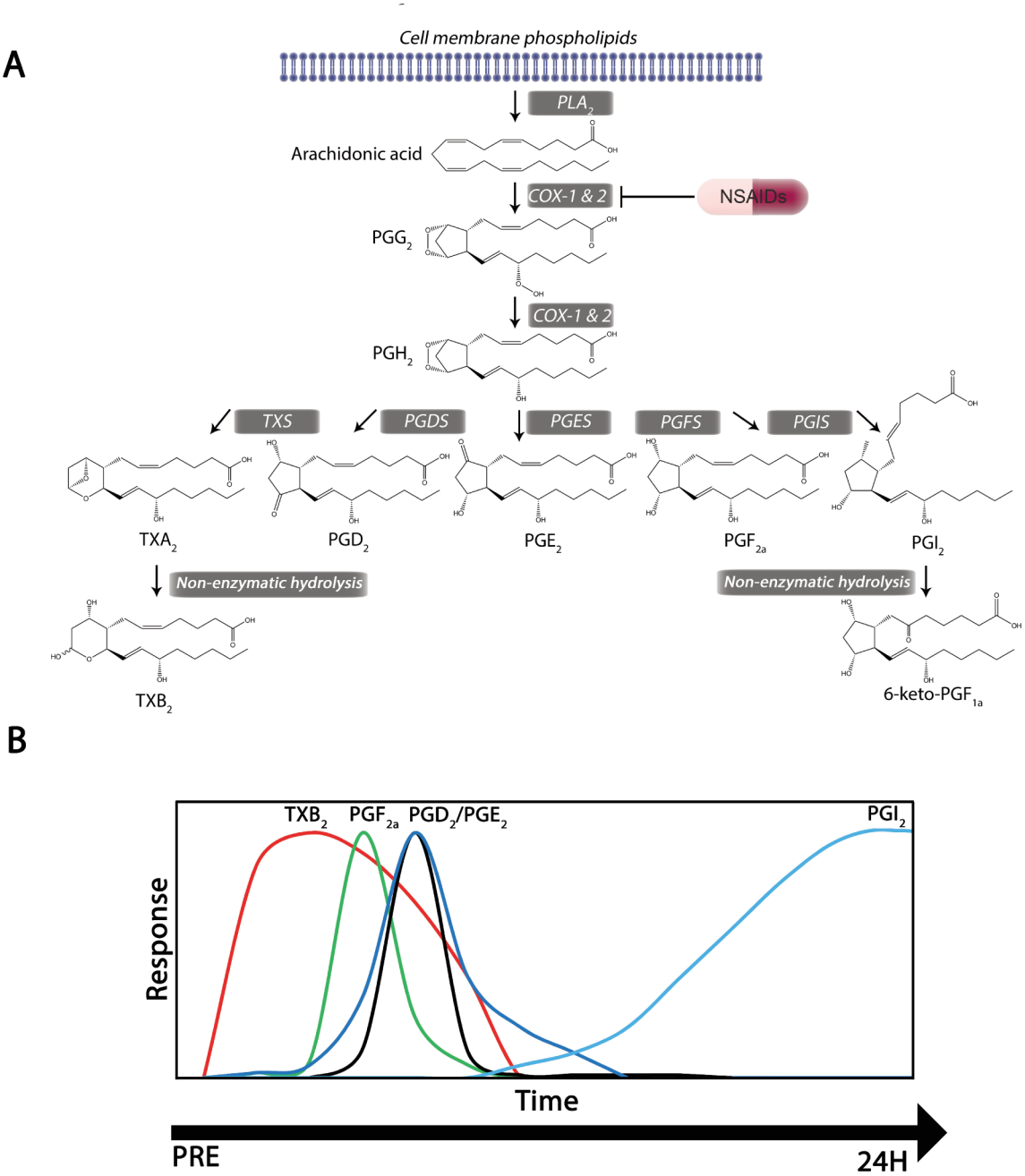


Figure 2: ***Cyclooxygenase (COX-1 & 2) pathways of lipid mediator biosynthesis during exercise recovery.*** A: COX-1 & 2 pathways of prostanoid biosynthesis. Free intracellular arachidonic acid (AA) substrate, mobilized from cell membrane phospholipids is converted to PGH_2 by the action of the COX-1 & 2 enzymes. PGH_2 is the precursor to the major bioactive PGs including TXA_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2 by action of specific PG synthases. B: Time-course of peripheral blood COX/PG responses during recovery from unaccustomed resistance exercise as determined by mediator lipidomic profiling (102).

human plasma/serum during exercise. Excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ (64-68), and tetranor-PGE_2 (69), urinary metabolites of PGI_2 and PGE_2 respectively, were also found to be elevated during exercise recovery. The effect of exercise on TXA_2 (measured as circulating TXB_2 a non-enzymatic hydrolysis product of TXA_2 or urinary 2,3-dinor-TXB_2 metabolites) is less clear. Low intensity exercise appears to predominantly increase the vasodilator/anti-platelet aggregator PGI2, with no (61, 64, 66, 68), or minimal (65), changes in the vasoconstrictor/platelet aggregator TXA_2. In contrast, maximal (53, 59, 60, 70) or high intensity (71) exercise has been reported to elevate TXB_2 in most, but not all (63) studies.

Fewer studies have investigated the PG response to potentially damaging muscular contractions with resistance or eccentric exercise, but available data suggests a relatively delayed or prolonged response. A bout of intense stretch-shortening cycle exercise was reported to elevate circulating PGE_2, peaking 2 h into recovery (72). Other studies have reported increased plasma/serum PG concentrations for 1-3 days following a bout of resistance exercise consisting of bench press (50-90% 1RM) (PGE_2) (73), barbell squat (70% 1RM) (13,14-dihydro-15-keto-$PGF_{2\alpha}$) (74) and step-up (body weight) (PGE_2) (75). In contrast, several studies failed to detect any changes in circulating PGE_2 following isolated eccentric contractions of the knee extensors (76, 77) or elbow flexors (78-80). Similarly, downhill running had no effect (81), or no greater effect than level surface high intensity running (55), on circulating PGE_2. The reasons for such conflicting findings across different exercise protocols is unclear, but may relate to the intensity of muscle load (downhill running (55, 81) vs. resistance exercise (73, 75)) or the volume of active muscle mass (unilateral isolation (76-80) vs. bilateral compound exercises (73, 75, 82, 83). Additionally, the sensitivity and specificity of immunological assays (e.g. ELISA) used by the majority of these studies is a likely contributing factor (84, 85).

In addition to studies measuring PGs in peripheral circulation, a limited number of studies have attempted to detect PGs within exercised muscle tissue (86-92). At 24 h of recovery from eccentric resistance exercise, $PGF_{2\alpha}$ was found to be increased in human muscle biopsy sample homogenates (90). A more traditional bout of resistance exercise, also increased $PGF_{2\alpha}$ in muscle microdialysates at 5-6 and 8-9 h post-exercise, but returned to basal levels by 24 h (86). Enzymatic activity of both COX-1 and 2 are elevated in muscle biopsies collected during exercise recovery (93), a response which appears to be driven by delayed elevation of the inducible COX-2 (but not COX-1) at both the mRNA (94) and protein (93) level. The expression of COX-2 also increases in human peripheral blood mononuclear cells during exercise recovery, suggesting that infiltrating leukocytes are likely a key source of PGs in exercised muscle (95). Interestingly, however, muscle cells themselves express both COX-1 and 2 (96) and are capable of synthesising and secreting a range of COX metabolites including PGD_2 (97), PGE_2/$PGF_{2\alpha}$ (14, 49, 98-100) and PGI_2 (101). Therefore PGs are likely to be important autocrine/paracrine signalling molecules produced locally within muscle tissue, and contracting muscle presumably contributes to elevated PGs and/or their metabolic degradation products in peripheral blood during post-exercise recovery.

The development of mediator lipidomic profiling methods has recently allowed for more comprehensive analysis of circulating COX metabolites during exercise recovery than previously available. By employing a human model of unaccustomed resistance exercise, we analysed the temporal changes in peripheral blood lipid mediators during post-exercise recovery using an unbiased metabolipidomic profiling approach (102). Immediately post-exercise we observed marked elevation in serum TXB_2, which preceded the other COX metabolites. This early response was followed by delayed elevation of, PGD_2, PGE_2, $PGF_{2\alpha}$ (and/or their circulating stable metabolic degradation products) between 1-2 h of recovery. Finally, peak elevation of PGI_2 (measured as the stable circulating product 6-keto-$PGF_{1\alpha}$) was not observed until 24 h of recovery. These results appear to show clear temporal differences in the systemic response to individual PG species throughout the time-course of post-exercise recovery (summarised in Figure 2B). Further studies using a metabolipidomic approach are needed to comprehensively address the local changes in prostanoids within exercise musculature throughout the time course of exercise recovery.

2.2 Role of the COX pathway in immunological and adaptive responses to exercise:

Classical NSAIDs (e.g. ibuprofen and indomethacin) administered at typically recommended doses have been shown to effectively block the exercise-induced elevation of PGs in both human muscle tissue (89-91, 103) and peripheral blood (74, 102). Acetaminophen (paracetamol), although not classically considered a NSAID and does not inhibit COX *in-vitro*, also appears to interfere with COX activity in muscle during exercise recovery (90). Therefore NSAID treatment can be effectively used to investigate the role of PGs in physiological responses to exercise.

The literature on the role of PGs in exercise-induced muscle injury and the purported benefit of NSAIDs to improve recovery or reduce symptoms of delayed onset muscle soreness (DOMs) is highly controversial. Many studies have reported positive short terms effects of NSAID treatment on muscle recovery including reduced swelling (104), DOMs (104-117), circulating creatine kinase (CK) (111, 113, 114, 116-118), and improved strength (104, 105, 108, 110, 115, 119). Conversely, numerous other studies have failed to find any significant influence of NSAIDs on muscle swelling (112, 120), DOMs (77, 82, 90, 118-132), circulating CK (77, 82, 107-110, 112, 119, 129, 130), or strength loss (77, 106, 112, 113, 117, 118, 123, 128, 131). Given their purported primary therapeutic role as anti-inflammatory, it also may be considered surprising that human studies to date have failed to find an effect of NSAIDs on systemic leukocytosis (117, 118, 133) or intramuscular leukocyte infiltration (112, 119, 134) responses to exercise stress. In fact, recent studies have found that acute NSAID treatment appears to augment exercise-induced increases in skeletal muscle inflammatory cytokine (e.g. IL-6 & MCP-1) (129, 135-137) and inducible COX-2 gene expression (136, 138). Although the underlying mechanisms remain unclear,

this response may be a compensatory response elicited secondary to reduced PG concentrations.

In addition to their immunoregulatory properties, a role of PGs in the control of muscle protein turnover has been proposed over three decades ago (139). Twenty years later, the first human study to investigate the effect of NSAID treatment on muscle protein turnover, showed that oral ingestion of the non-selective NSAID ibuprofen appeared to block the normal increase in the rate of muscle protein synthesis 24 h following a bout of maximal eccentric resistance exercise (132). Further key studies showed that a different non-selective NSAID (indomethacin) interferes with the muscle satellite cell proliferative response which occurred later (7 days) during exercise recovery (103, 128, 140, 141). However, subsequent follow up studies testing the effect of COX-2 selective NSAIDs on exercise recovery failed to show an effect on exercise-induced muscle protein synthesis (138) or muscle satellite cell responses (112). These findings suggest that despite the well-established indispensable role of COX-2 activity in rodent muscle reparative response to injury (14, 99, 142-146), COX-1 rather than COX-2, may be the primary isoform involved in human muscle adaptive responses to exercise. More recent studies have focused on the effect of NSAIDs on the molecular response to exercise stress have provided further mechanistic insight (136, 147, 148). Oral ibuprofen treatment was reported to blunt activation of anabolic signalling kinases early (3 h) during recovery from resistance exercise, showing that the early elevation in PG biosynthesis during post-exercise recovery (102) appear to be important for the anabolic signalling response in human muscle (148). On the other hand, impaired satellite cell proliferation in the presence of indomethacin treatment (103), was not found to be associated with any effect on the expression of growth factors and extracellular matrix-related genes (136), nor changes in the heat shock protein (HSP) response (147). Thus, the molecular mechanisms by which NSAIDs may impair satellite cell myogenesis in human muscle during post-exercise recovery remains to be elucidated.

A number of cell culture studies have also investigated the direct regulatory roles of specific PGs in various stages of muscle cell growth and development (summarised in Table 1). These studies suggest that individual PGs may play distinct and specific temporal roles in muscle tissue throughout exercise recovery. In the early hours post-exercise, myofiber protein turnover is rapidly increased and there is an expansion and migration of the myogenic stem cell (satellite cell) pool. This timeframe corresponds to elevated PGs with roles in stimulating muscle protein turnover (139, 149) and enhancing myoblast proliferation (98, 150-152)/survival (153, 154), whilst limiting myogenic differentiation (97) (Figure 2A, Table 1). During the latter stages of recovery, myoblasts must cease to migrate and divide, allowed to undergo myogenic differentiation and stimulated to fuse in order to regenerate damaged tissue and support cellular hypertrophy. This latter phase is associated with an increase in PGI_2 which has been shown to act a "brake" on myoblast migration to facilitate cell-cell contact and promote myogenic fusion events (101). These speculative roles of individual PG species in specific stages of the skeletal muscle growth and regeneration require further demonstration *in-vivo.*

3. LIPOXYGENASE PATHWAYS

The lipoxygenase (LOX) pathways oxygenate PUFA substrate to form hydroperoxy products (e.g. hydroperoxy-eicosatetraenoic acid (HpETE) from AA), which are rapidly reduced to corresponding monohydroxy fatty acid metabolites (e.g. hydroxy-eicosatetranoic acid (HETE) from HETE) (Figure 3A). The naming of LOX enzymes was originally based on their specificity with respect to 20-carbon AA. For example, 12-LOX oxygenates AA at carbon-12 to form 12-hydroxy-eicosatetranoic acid (12-HETE) (Figure 3A). In animal tissues, enzymes exist with specificity for three major oxidation sites on AA at C-5, C-12, and C-15 and the enzymes are named 5-LOX, 12-LOX, and 15-LOX, respectively (Figure 3A). However, certain LOX enzymes possess dual specificities and interspecies differences exist (155). The human genome includes six functional LOX genes *(ALOX5, ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3)* each encoding a distinct enzyme, often with cell-type specific expression (155) (summarised in Table 2). Signalling lipids produced by LOX pathways are involved in a range of physiological and pathological processes, one of the best characterised of which is their intricate role in the inflammatory response.

3.1 Human LOX enzymes:

3.1.1 5-LOX pathway: A single 5-LOX enzyme is encoded by the ALOX5 gene which is highly expressed in various leukocyte populations, especially PMNs (156, 157) (Table 2). The leukotrienes (LTs) are classical pro-inflammatory AA metabolites generated by the 5-LOX pathway with key roles in both the innate and adaptive immune system (158-160).

Table 2:
Arachidonate lipoxygenase (ALOX) genes and isoforms

Gene	Enzyme	Aliases	Activity		Expression pattern in humans
			Human	***Mouse***	
ALOX5	5-LOX	5-LO	5(S)-HETE	5(S)-HETE	Leukocytes
ALOX12	12-LOX	12-LO, 12S-LOX, Platelet-Type 12-LOX	12(S)-HETE	12(S)-HETE	Platelets, macrophages, dendritic cells
ALOX12B	12-LOXB	12-LO, 12R-LOX, Epidermis-Type 12-LOX	12(R)-HETE	12(R)-HETE	Skin
ALOX15	15-LOX-1	15-LO-1, Leukocyte type 12-LOX, 12/15-LOX	15(S)-HETE	12(S)-HETE	Eosinophils, epithelium, M2 macrophages
ALOX15B	15-LOX-2	15-LO-2, 15-LOX-B, 15S-LOX, Mouse 8-LOX	15(S)-HETE	8(S)-HETE	Epithelium, macrophages
ALOXE3	ELOX-3	Epidermal LOX-3, E-LOX			Skin

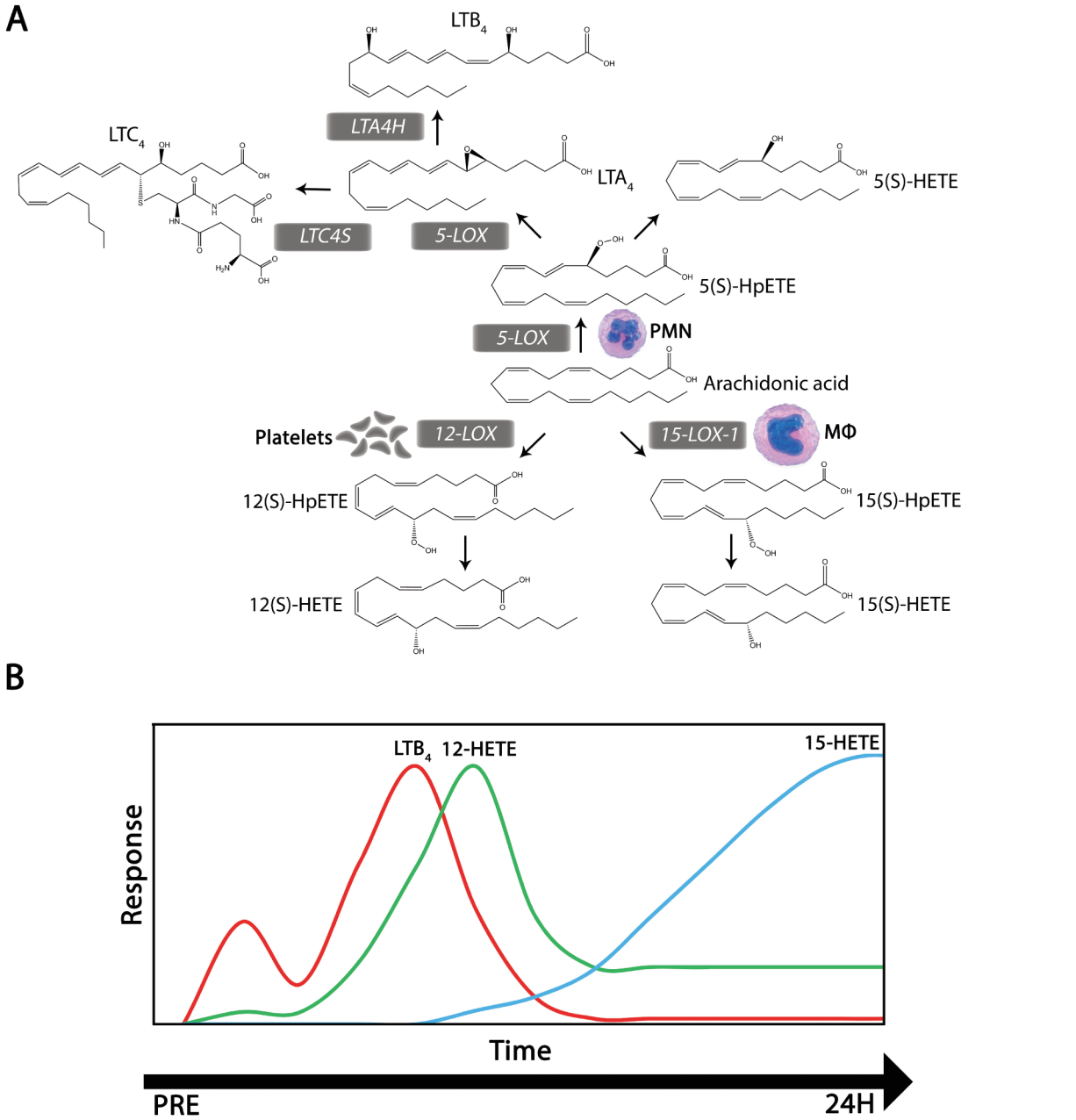


Figure 3: ***Human lipoxygenase (5-, 12-, and 15-LOX) pathways of lipid mediator biosynthesis during exercise recovery.*** A: The 5-, 12, and 15-LOX pathways of lipid mediator biosynthesis. Mobilised free intracellular arachidonic acid (AA) substrate derived from cell membrane phospholipids is converted to specific positional monohydroxy-AA isomers by the action of LOX enzymes expressed in a cell-type/tissue specific manner that oxygenate 20-carbon PUFA specifically at C-5 (5-LOX), C-12 (12-LOX), and C15 (15-LOX). B: Time-course of peripheral blood LOX metabolite responses during recovery from unaccustomed resistance exercise as determined by mediator lipidomic profiling (102).

Oxygenation of AA by 5-LOX forms the primary metabolite 5-HpETE, which can be reduced to 5-HETE (161), or further metabolised by the 5-LOX enzyme to generate leukotriene A_4 (LTA_4) (159, 162, 163) (Figure 3A). LTA_4 is the transient intermediate precursor to the major bioactive leukotrienes including LTB_4 (159, 164) and the cysteinyl LTs; LTC_4, LTD_4, LTE_4 (160). Most notably, LTB_4 is a powerful chemotactic lipid for PMNs (165-168), acting through BLT1 and BLT2 receptors to promote PMN chemotaxis and migration into tissues (169-171).

3.1.2 12-LOX pathway: Humans express two 12-LOX isoforms in a cell type specific manner; platelet-type 12-LOX (encoded by the *ALOX12* gene) (172, 173) and epidermis-type 12-LOX (encoded by the *ALOX12B* gene) (174, 175) (Table 2). Expression of the platelet type 12-LOX was also recently reported in human MΦ and dendritic cell populations at levels considerably higher than that present in their monocyte precursors (176). *ALOX12* metabolises AA to form 12-HETE carrying its hydroxyl group predominantly in the S-configuration (Figure 2A). In contrast, *ALOX12B*, which is expressed in the skin, primarily produces the 12(R)-HETE isomer. Although first discovered in human blood platelets four decades ago (173), the precise role of 12-LOX in platelet physiology has remained somewhat unclear (177). In addition to its purported role in blood thrombosis, 12(S)-HETE is a potent pro-inflammatory lipid mediator which stimulates PMN chemotaxis (178-183), monocyte adhesion (184), and inflammatory

cytokine expression (e.g. IL-6, TNFα, MCP1) in both macrophages (185, 186) and adipocytes (187, 188). Similarly, 12(R)-HETE plays a similar key pro-inflammatory role in the skin (189-194).

3.1.3 15-LOX pathway: Humans express two 15-LOX isoforms, 15-LOX-1 and 15-LOX-2, encoded by the *ALOX15* and *ALOX15B* genes respectively (Table 2). 15-LOX-1 is highly expressed in eosinophils and epithelial cell populations. Whereas expression of 15-LOX-1 is typically low in blood monocytes, it is markedly induced by the anti-inflammatory cytokines IL-4 (195, 196) and IL-13 (197-200). Therefore, 15-LOX-1 is highly expressed in alternatively activated M2, but not classically activated M1 monocytes/ MΦ (201-205). The 15-LOX-1 pathway results in the formation of both 12(S)-HETE and 15(S)-HETE from AA substrate, with the ratio varying from one species to another. Human 15-LOX-1 primarily synthesises 15(S)-HETE, together with smaller amounts of 12(S)-HETE (206) (Figure 2A). In contrast, the murine ALOX15 ortholog (often termed 12/15-LOX or leukocyte type 12-LOX), generates 12(S)-HETE as its primary product (207). Interestingly, 15(S)-HETE possesses primarily anti-inflammatory activity and counteracts the actions of the 5-LOX pathway by inhibiting PMN release of LTB_4 (208, 209) and dampening PMN chemotaxis to a LTB_4 gradient (209-212). In parallel, 15(S)-HETE has direct stimulatory effects on the migration of monocytes, indicative of a direct role in resolution phase of inflammation (213). A second 15-LOX isoform (15-LOX-2), encoded by the *ALOX15B* gene is expressed in human epidermis which also acts to convert AA almost exclusively to 15(S)-HETE (214). In contrast, the *ALOX15B* equivalent expressed in mouse skin primarily generates 8(S)-HETE from AA, and is thus often termed 8-LOX in the murine literature (215). In addition to its well-known role in the skin, 15-LOX-2 *(ALOX15B)* was recently reported to be a major 12/15-LOX isoform expressed during the differentiation of human monocytes to MΦs, a response which was further induced in response to M2 polarisation (204). Thus both 15-LOX-1 and 15-LOX-2 appear to play a key role in recruitment and polarisation of monocyte/ MΦ populations required for successful resolution of the inflammatory response.

3.2 LOX pathway responses to exercise:

Despite the well-established role of LOX pathway PUFA metabolites in the inflammatory milieu, few studies exist regarding their potential roles in the immunological and adaptive responses to exercise stress. Whole blood mRNA expressions of both 5-LOX and 5-LOX activating protein (FLAP) have been reported to increase during recovery from running exercise (216). Consistently, elevated plasma levels of the major 5-LOX product LTB_4 has been reported following high intensity running in some (216), but not all studies (55). 5-LOX and FLAP in human muscle appears to predominantly co-localise with immune cells (217), although 5-LOX immunoreactivity has also been reported within skeletal myofibers themselves (218). To our knowledge no study to date has investigated the effect of exercise on expression of LT biosynthetic machinery (e.g. 5-LOX or FLAP) within muscle tissue. Nevertheless, a bout of cycling exercise was recently reported to increase intramuscular concentrations of LTB_4 (a major 5-LOX product) in human myopathy patients (217), suggesting a possible local role of LTB_4 within the exercising musculature.

Metabolipidomic profiling has more recently provided a more detailed analysis of the response of LOX pathway products in human peripheral blood. We found that serum levels of the pro-inflammatory 5-LOX product LTB_4 were markedly elevated early (1-2 h) following an acute bout of unaccustomed resistance exercise (summarised in Figure 3B), a response which coincided with increased serum concentrations of inflammatory PGs (e.g. PGE_2) (102). This early transient response was followed by increases in 12-LOX pathway metabolites 12-HETE and its β oxidation product (termed tetranor 12-HETE), peaking later on at 3 h post-exercise (102). Finally, the 15-LOX pathway products 15-HETE and 15-Oxo-ETE were found to be elevated only during the latter stages of exercise recovery, achieving peak elevation 24 h post-exercise (102). This analysis suggests that specific PUFA metabolites of the LOX pathways as predominant at various stages of post-exercise recovery ranging from induction of pro-inflammatory metabolites early to a later switch to anti-inflammatory/pro-resolving lipids. It is important to note that this is the only study published to date to use a metabolipidomic approach to simultaneously profile PUFA metabolites of the LOX pathways in response to exercise stress. It is likely that exercise mode, duration, and intensity could lead to widely varying LOX pathway PUFA metabolite responses. Thus, future studies employing a metabolipidomic approach are needed to further characterise the PUFA LOX metabolic responses to exercise.

3.3 Role of LOX metabolites in immunological and adaptive responses to exercise:

Given their key roles in platelet and leukocyte biology, LOX metabolites may be hypothesised to play important and underappreciated roles in mediating the immunological and vascular responses to exercise stress. Elevated circulating levels pro-inflammatory lipid mediators LTB_4 and 12-HETE in the early hours of recovery likely promote PMN mobilisation from bone marrow and migration to skeletal muscle via effects to stimulate chemotaxis and enhance vascular permeability (169-171). Subsequent elevation of 15-HETE during the latter stages of recovery would potentially inhibit release of LTB_4 by PMNs (208, 209) and dampen PMN chemotaxis/migration (209-212) whilst promoting subsequent migration of monocytes required for timely resolution of inflammation (213).

In addition to their key role in mediating the local and systemic inflammatory response, LOX metabolites may potentially act as important signalling molecules locally within muscle tissue. The LT receptors BLT1 and BLT2 have been shown to be expressed by cultured muscle satellite cells (219) and the L6 myoblast cell line (169). Additionally, exogenous LTB_4 treatment was found to promote L6 cell myogenesis *in-vitro*, an action which is mediated by the BLT1 receptor (219). On this basis, increased LTB_4 biosynthesis following exercise stress (102, 216) may be potentially hypothesised to play a role in driving proliferation and differentiation of satellite cells involved in muscle growth/regeneration (219). However,

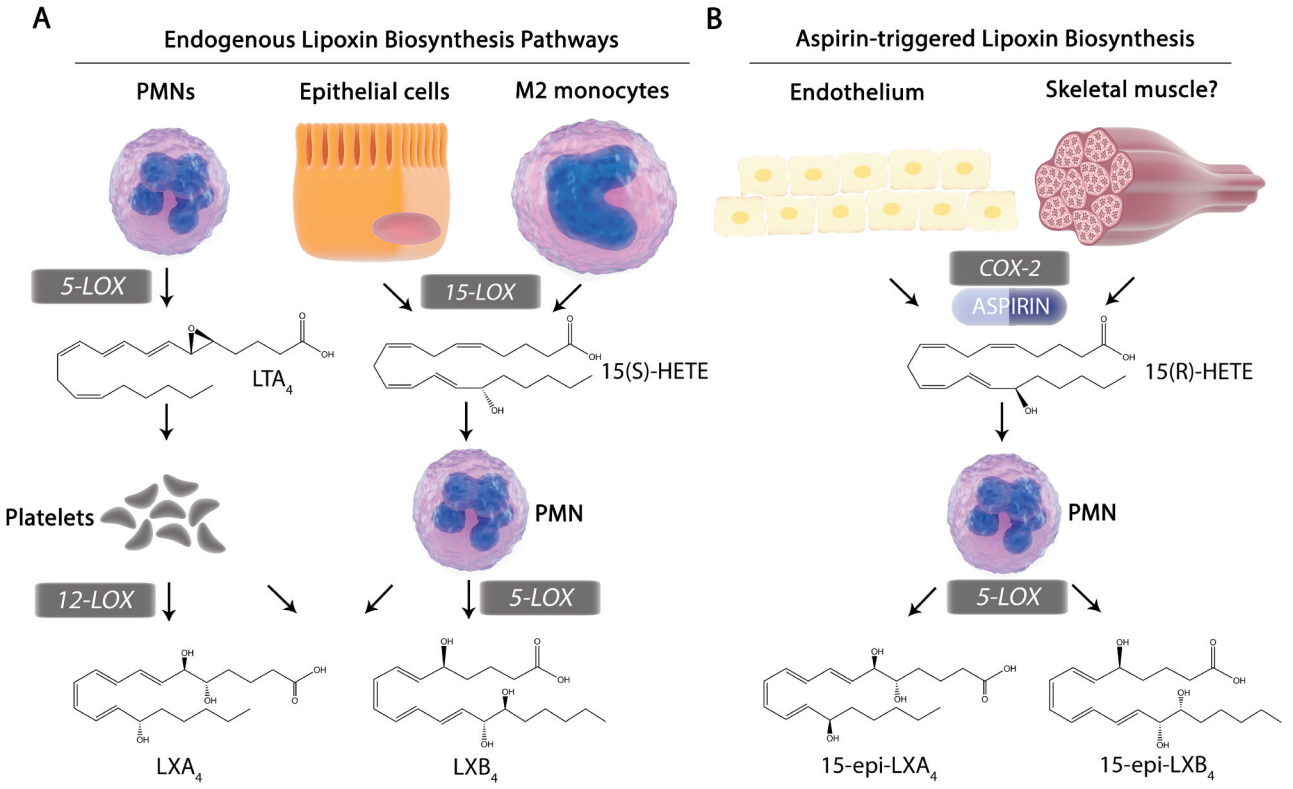


Figure 4: ***Lipoxin (LX) biosynthetic pathways in generating n-6 AA derived specialised pro-resolving lipid mediators.*** A: Endogenous pathways of LX biosynthesis involving cell-cell interactions of 5-LOX expressing cells (e.g. PMNs) with 12-LOX expressing cells (e.g. platelets), or 15-LOX expressing cells (epithelium or M2 monocytes/Mϕ) with 5-LOX expressing cells (PMNs). B Aspirin-triggered (AT) pathway in which aspirin acetylated COX-2 generates 15(R)-HETE which participates in transcellular LX biosynthesis via the subsequent action of PMN 5-LOX.

to our knowledge no study to date has directly investigated the role of the 5-LOX pathway in muscle growth and regeneration.

On the other hand evidence also exists to suggest that chronic activation of the 5-LOX pathway can have deleterious effects on muscle tissue. For example, LTB_4 has been found to be chronically elevated in muscle microdialysates from polymyositis or dermatomyositis patients and appears related to the extent of muscle weakness in this context (217). Recently, LTB_4 was also reported to be elevated in muscle, adipose, and liver tissue of obese mice (169). In this model, inhibition of the LTB_4 receptor BLT-1, via genetic or pharmacologic means, protected against high fat diet induced insulin resistance, suggesting a deleterious effect of chronically elevated muscle LTB_4 (169, 220). Interestingly, this appeared at least partially attributable to a direct effect of LTB_4 to induce inflammatory signalling cascades in muscle cells themselves via the BLT1 receptor, leading to impaired muscle insulin sensitivity (169). These data show that chronically elevated pro-inflammatory 5-LOX metabolites can have apparent deleterious effects on muscle physiology under certain circumstances

Some limited evidence suggests that 12/15-LOX metabolites might also have direct regulatory effects on muscle. For example, treatment of C2C12 myoblasts *in-vitro* with exogenous 12-HETE has been reported to result in dose dependent activation of peroxisome proliferator-activated receptor gamma (PPARγ) (221). Whilst platelets are a well-established source of 12-HETE, C2C12 skeletal myoblast cultures apparently synthesise and secrete 12-HETE, an action which can be modulated by treatment with the n-3 PUFA EPA (222). Additionally, 12-LOX immunoreactivity has also been reported within skeletal myofibres *in-vivo* in one study, further suggesting that muscle tissue may possess 12-lipoxygenase activity (223). In a recent study, *ALOX15* (12/15-LOX) knockout mice were reported to be protected from denervation induced skeletal muscle atrophy, suggesting a negative regulatory role of 12/15-HETEs in the maintenance of muscle mass in this model (224). This finding may be complicated by the fact that the murine enzyme encoded by the *ALOX15* gene generates 12-HETE, as its primary product, in contrast to the 15-lipoxygenase activity of the human enzyme encoded by the *ALOX15* gene (see section 3.1). Nevertheless, exogenous treatment with 15-HETE was also been found to increase rates of protein degradation in C2C12 myoblasts/myotubes *in-vitro* in a series of studies (222, 225, 226). Together these findings appear to be consistent with the potential negative regulatory role of 15-LOX metabolites on skeletal muscle mass.

4. TRANSCELLULAR LIPID MEDIATOR BIOSYNTHESIS: THE SPECIALISED PRO-RESOLVING MEDIATORS

In addition to generating their primary enzymatic products, the COX-2 and LOX pathways participate in transcellular biosynthetic pathways between two of more cell types which

express the required enzymatic machinery in a compartmentalised manner (227). During inflammation, cell-cell interactions between platelets, leukocytes, the vasculature and resident tissue cells facilitates local transcellular biosynthesis of unique lipid mediator species. Interestingly, whilst the actions of the COX and LOX pathways expressed by cells in isolation often generate products with pro-inflammatory properties, lipid mediators synthesised through transcellular routes are not only generally anti-inflammatory, but also actively function to promote resolution back to the non-inflamed state, i.e. 'pro-resolving'.

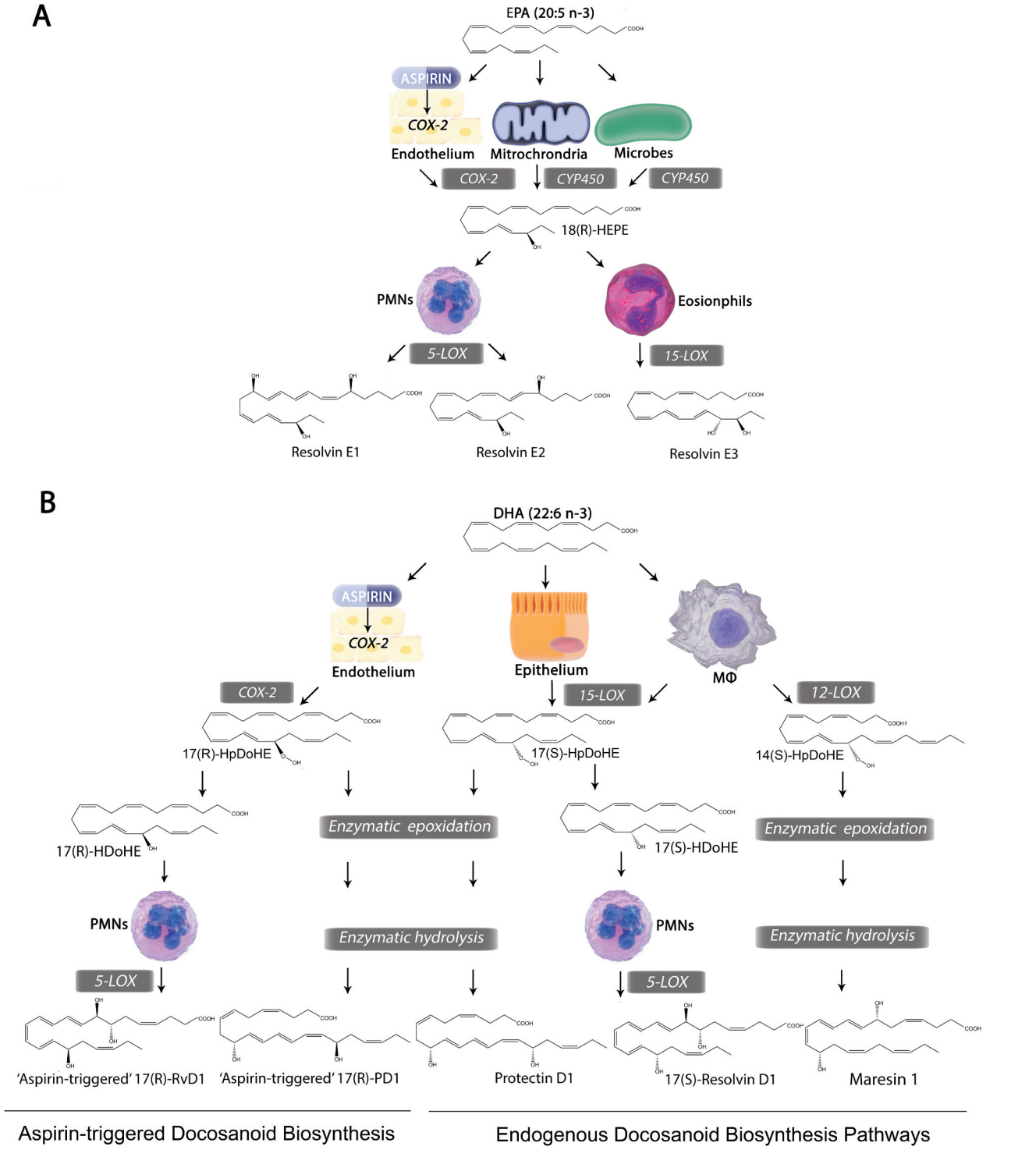


Figure 5: ***n-3 PUFA derived specialized pro-resolving lipid mediator pathways.*** A: Endogenous and aspirin-triggered routes of E-series resolvin (RvE) biosynthesis. EPA is converted to 18(R)-HEPE by aspirin acetylated COX-2 or alternatively CYP450 enzymes in host cells or resident microbes. 18(R)-HEPE is the primary intermediate in the biosynthesis of RvE1 and RvE2 by PMN 5-LOX, or RvE3 by eosinophil 15-LOX. B: Endogenous and aspirin triggered routes of DHA derived (D-series) resolvin (RvD), protectin (PD), and maresin (MaR) biosynthesis. DHA is converted to 17(R)-HpDoHE by aspirin acetylated COX-2 or 17(S)-HpDoHE by the endogenous 15-LOX pathway, precursors to the aspirin triggered and native PDs respectively. The corresponding 17-HDoHEs resulting from peroxidase catalysed reduction of the hydroperoxides serve as substrates for the RvDs by the action of the 5-LOX pathway. In an alternate pathway, DHA is converted by Mϕ 12-LOX to 14(S)-HpDoHE, which is the primary precursor to the MaR family of SPM.

4.1 Overview of the pro-resolving lipid mediators:

4.1.1 Lipoxins: The lipoxins (LXs or lipoxygenase interaction products) are bioactive metabolites of the n-6 PUFA AA originally isolated from human leukocytes (228), formed via transcellular biosynthetic routes (229) (Figure 4). In contrast to the classical AA derived eicosanoids (PGs and LTs), LXs possess potent anti-inflammatory actions by inhibiting the chemotaxis (230-233) and degranulation of PMNs (234). Furthermore, LXs also have direct pro-resolving bioactivity as they actively stimulate the migration/adherence of blood monocytes (233, 235-237) and eosinophils (238), whilst promoting MΦ nonphlogistic phagocytosis/clearance of apoptotic PMNs (239). Therefore LXs were the first lipid mediators identified to function as endogenous "braking signals" during the time course of inflammation to limit PMN infiltration and actively promoting their clearance from inflammatory infiltrates. Furthermore, LXs have direct analgesic properties which oppose that of the classical eicosanoids in the regulation of pain (240).

Two endogenous routes of LX biosynthesis exist in humans (Figure 4A). The first pathway involves leukocyte-platelet interactions during which the leukotriene biosynthesis intermediate LTA_4, secreted from 5-LOX expressing PMNs, is taken up by adherent platelets for conversion to lipoxin A_4 (LXA_4; 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid) or lipoxin B_4 (LXB_4; 5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid) by the action of platelet-type 12-LOX (241-245). A second major route involves initial secretion of the 15-LOX product 15(S)-HETE by epithelial cells, eosinophils or M2 monocytes/MΦs, which is then taken up by 5-LOX expressing cells (e.g. PMNs) and converted to LXA_4 and LXB_4 (246-248). A third unorthodox pathway of LX biosynthesis operates only in the presence of aspirin (acetylsalicylic acid) (Figure 4B). Aspirin is unique amongst the NSAIDS in that it achieves inhibition of PG biosynthesis via irreversible acetylation of the COX-1 & 2 enzymes (249). Acetylation renders COX-1 entirely inactive, however, acetylated COX-2 obtains 15-lipoxygenase activity, generating 15(R)-HETE (rather than PGG_2) as the primary product of AA (250-253). Secreted 15(R)-HETE participates in transcellular LX biosynthetic pathways, forming metabolites termed 15-epi-LXs (15(R)-LXs), or aspirin-triggered LXs (ATL) (254) which carry their C-15 hydroxyl group in a R-configuration, rather than the S-configuration characteristic of the native LX (254) (Figure 4B). The ATL and native LXs share their anti-inflammatory/pro-resolving (231, 233, 236, 254) and analgesic properties (240, 255, 256). On this basis, ATLs have in recent years been purported to account for a major mechanism underlying certain unique therapeutic effects of aspirin amongst the NSAID class.

4.1.2 Resolvins: The first pro-resolving metabolites of n-3 PUFAs identified were found to be synthesised from EPA (257), and later termed the E-series resolvins (Rv or resolving phase interaction products) (258). In the presence of aspirin, EPA is converted to 18(R)-hydroxy-EPA (18(R)-HEPE) by acetylated COX-2, in a similar manner to conversion of AA to 15(R)-HETE during AT-LX biosynthesis (257, 259) (Figure 5). In a second intriguing endogenous route, resident microbes expressing cytochrome p450 (CYP450) can generate and provide 18(R)-HEPE to their human host (260). Secreted 18(R)-HEPE obtained from endogenous or AT sources is taken up into PMNs and converted to resolvin E1 (RvE1; 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid) (257, 259, 260) and resolvin E2 (RvE2; 5S,18R-dihydroxy-6E,8Z,11Z,14Z,16E-eicosapentaenoic acid) (261-263) by the action of the 5-LOX pathway (Figure 5). RvE1 and RvE2 both possess potent multilevel anti-inflammatory and pro-resolving actions including inhibiting PMN migration and enhancing MΦ phagocytosis of apoptotic PMNs (257, 259, 264). The originally identified RvEs carry their C-18 hydroxyl group in the R-confirmation, although equivalent 18(S)-RvEs (e.g. 18(S)-RvE1; 5S,12R,18S-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid) were later identified (265). In a distinct pathway, involving the 15-LOX pathway in human eosinophils, 18(S/R)-HEPE are converted to resolvin E3 (RvE3; 17R,18R-dihydroxy-5Z,8Z,11Z,13E,15E-eicosapentaenoic acid and 18(S)-RvE3; 17R,18S-dihydroxy-5Z,8Z,11Z,13E,15E-eicosapentaenoic acid) (266-268) (Figure 5A). Both 18S-and 18R-RvE3 exhibit direct inhibitory activity on PMN chemotaxis (266, 267).

Parallel biosynthetic pathways metabolise the 22-carbon DHA to generate resolvins of the D-series (docosanoids) (Figure 6). In aspirin triggered routes, during which the D-series resolvins were originally discovered, aspirin acetylated COX-2 generates 17(R)-hydroxy-DHA (17(R)-HDoHE) from DHA substrate (258). In an alternate endogenous route not requiring aspirin, DHA is metabolised by the 15-LOX pathway to 17-HDoHE carrying the C-17 hydroxyl group in an S-confirmation (17(S)-HDoHE) (269). Human PMNs take up and transform secreted 17(S/R)-HDoHE to the native 17(S)-D-series resolvins (RvD1-RvD6) or their aspirin-triggered 17(R)-counterparts (AT-RvD1-RvD6) by action of the 5-LOX pathway (258, 269-271) (Figure 5B). The DHA derived resolvins are potent anti-inflammatory and pro-resolving lipid mediators which counteract inflammation and actively promote restoration of a non-inflamed state by suppressing PMN chemotaxis and promoting MΦ recruitment and phagocytosis (272-275).

4.1.3 Protectins: In addition to contributing to RvD biosynthesis, the primary DHA metabolite of 15-LOX, 17(S)-HpDoHE, is also a precursor to a distinct group of 10,17-dihydroxy docosanoids (with conjugated trienes) termed the protectins (PD) or neuroprotectins (NPD) (269, 270, 276) (Figure 5B). The major bioactive PD synthesised endogenously by human cells has been shown to be 10R,17S-dihydroxy-4Z,7Z,11E,13E,15Z,19Z-docosahexaenoic acid, termed protectin D1 (PD1) (277). Additionally, in the presence of aspirin, an aspirin-triggered PD1 isomer carrying its 17-hydroxyl group in the R-confirmation is formed termed AT-PD1 (278) or AT-NPD1 (279) (10R,17R-dihydroxy-4Z,7Z,11E,13E,15Z,19Z-docosahexaenoic acid). Native PD1/NPD1 and AT-PD1/NPD1 are both potent anti-inflammatory pro-resolving molecules that inhibit PMN chemotaxis/migration and enhance MΦ efferocytosis of apoptotic PMNs.

4.1.4 Maresins: In a third and most recently identified alternate pathway of DHA metabolism, a new class of SPMs were

identified as products produced by human MΦ and named 'macrophage mediators in resolving inflammation' or Maresins (MaR) (280) (Figure 5B). Maresin biosynthesis involves the initial 14-lipoxygenation of DHA by the human MΦ 12-LOX pathway to form 14(S)-HpDoHE. Subsequent epoxidation of 14(S)-HpDoHE by the same enzyme within MΦ forms an 13S,14S-epoxy-DHA intermediate (recently termed 13(S),14(S)-epoxy-maresin or 13,14-eMaR). 13(S),14(S)-epoxy-DHA possesses its own bioactivity, as well functioning as a key intermediate in downstream MaR biosynthesis (281). Further enzymatic hydrolysis of 13(S),14(S)-epoxy-DHA by an as yet to be determined pathway yields Maresin 1 (MaR1; 7R,14S-dihydroxy-4Z,8E,10E,12E,16Z,19Z-docosahexaenoic acid) (281). Alternatively, 13,14-eMaR can be converted by the action of soluble epoxide hydrolase (sEH) enzyme to the recently identified Maresin 2 (MaR2; 13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-hexaenoic acid) (176). MaR1 and MaR2 both possess potent anti-inflammatory, pro-resolving, tissue regenerative and anti-nociceptive actions (176, 280-282).

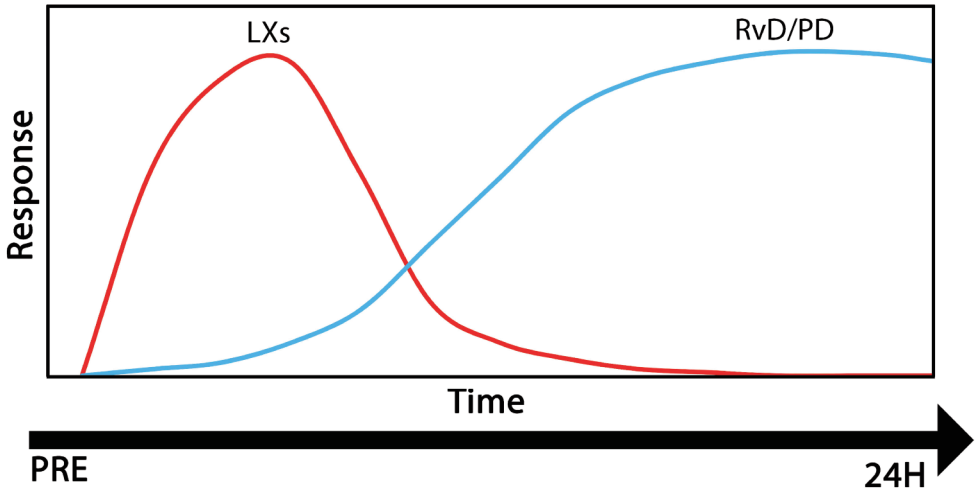


Figure 6: ***Specialised pro-resolving lipid mediator response to exercise.*** A: Time-course of human peripheral blood anti-inflammatory/pro-resolving lipid mediator responses during recovery from unaccustomed resistance exercise as determined by mediator lipidomic profiling (102).

4.2 Human pro-resolving lipid mediator responses to exercise

Appreciation of pro-resolving lipid mediator circuits in human acute inflammation has generally been limited to *in-vitro* assays utilizing blood immune cell population obtained from human volunteers. However, a small number of recent studies have suggested that exercise stress may be a human *in-vivo* model of self-limited inflammation during which resolving lipid mediator circuits play important physiological role and this process can be actively studied.

In the first study to address whether exercise might not only transiently increase concentrations of the pro-inflammatory eicosanoids, but also modulate bioactive lipids with anti-inflammatory and pro-resolving actions, maximal physical exertion was found to result in a rapid increase in the urinary excretion of LXA_4 post-exercise (283). Additionally, a second more modest elevation in urinary LXA_4 was observed at 24 h or recovery (283). This led the authors to speculate that increased lipoxin biosynthesis may be reflective of cell-cell immunological interactions occurring during exercise and represent an endogenous anti-inflammatory and pro-resolving defence mechanism against exercise-induced stress (283).

In order to more comprehensively characterise the role of pro-resolving lipid mediators in exercise responses, we undertook unbiased metabololipidomic profiling of human peripheral blood samples collected throughout 24 h recovery from a single bout of unaccustomed intense resistance exercise (102). We found that pro-resolving AA metabolites, the lipoxins, were increased early following exercise, with LXA_4 peaking immediately post-exercise and LXB_4 at 1 h of recovery. Similarly, the EPA derived pro-resolving lipid mediator, RvE1, was transiently increased in human serum 0-1 h post-exercise, rapidly decaying thereafter (102) (although we cannot discount that instability of highly labile RvE1 may have contributed to the latter response). Finally, docosanoids including resolvins (RvD1) and protectins (measured as the PD1 isomer (10S,17S-DiHDoHE) were increased in human peripheral blood later during recovery (2-3 h post) and remained elevated in circulation following 24 h of recovery (102). These results show that pro-resolving lipid mediator biosynthesis is increased in humans in response to a bout of unaccustomed resistance exercise and distinct classes of the resolving lipid mediators appear to exhibit specific temporal responses throughout the time-course of post-exercise recovery (Figure 6).

The utility of exercise as a model of self-resolving inflammation was further demonstrated in a recent study in which an exhaustive human exercise model was used alongside murine and cell-based experiments to demonstrate *in-vivo* detection and modulation of a new class of pro-resolving lipid mediators, termed the T-series resolvins (RvTs) in human peripheral

blood (284). This novel family of anti-inflammatory/pro-resolving lipid mediators synthesised from n-3 DPA (an intermediary PUFA between EPA and DHA) via the endogenous COX-2 pathway, were increased in human peripheral blood early (≤15 min) post-exercise (284). Furthermore, this study confirmed previously reported findings of elevated DHA derived docosanoids in human peripheral blood in response to acute exercise stress (102, 284).

The cellular source and local changes in pro-resolving lipid mediator concentrations during post-exercise recovery is of interest. Lipid mediators typically act in an autocrine/paracrine action within tissue micro-environments and further studies are needed to profile the local changes in inflammatory and resolving lipid mediators within the exercised muscle tissue by utilising the skeletal muscle biopsy or microdialysis techniques. On the basis of apparent capacity of exercise to elevate circulating pro-resolving lipid mediators throughout recovery, an interesting question that remains unanswered is whether exercised skeletal muscle contributes to pro-resolving lipid mediator biosynthesis, either directly or via cell-cell interactions with the vasculature or infiltrating immune cell populations. In one early study, cultured skeletal myoblasts have been reported to secrete 5-HETE, 12-HETE and 15-HETE, suggesting they may possess various lipoxygenating activities (222). Future studies should address the possible role of skeletal muscle in transcellular lipid mediator biosynthetic pathways by interactions with infiltrating leukocytes.

4.3 Lipid mediator class switching during post-exercise recovery

The time course of induction of inflammatory and resolving bioactive lipid mediators in peripheral blood of exercising humans appears to match well with common murine experimental models of self-limited inflammation (e.g. peritonitis and dorsal air pouch). In these spontaneously resolving murine models of inflammation, AA derived LTs increase rapidly after induction, followed by elevated PGs and LXs (within hours), and subsequently more delayed elevation of the docosanoids (e.g. RvD and PD1) ~12-24 h later (273, 285, 286). Interestingly, recent work using these models has suggested that the biosynthesis of specific resolving mediators such as resolvin D3 (RvD3) are uniquely positioned within the inflammatory response, appearing late (24-72 h) in the resolution time-frame (273). The time course of exercise recovery is known to be dependent on the extent of muscle injury inflicted, and in more extreme cases the inflammatory responses can persist for days to weeks (30). Thus, future studies that explore the latter time-frame of post-exercise recovery employ more diverse exercise models should provide valuable insight into the inflammatory and resolving lipid mediator response to exercise stress in humans.

Lipid mediator class-switching is a process first demonstrated by Levy et al. 2001 (286) in which increased concentrations of inflammatory COX-derived PGs early following exposure to an inflammatory stimuli actively triggers the subsequent induction on the enzymatic machinery of the pro-resolving lipid biosynthetic pathways, thereby bringing about resolution to a non-inflamed state (286-288). By employing a group of human volunteers that received treatment with the NSAID ibuprofen, we were able to demonstrate that active lipid mediator class switching occurs during human exercise recovery. Subjects ingesting ibuprofen pre-exercise showed no early elevation in COX derived prostanoids during post-exercise recovery, but also displayed a complete lack of the subsequent elevation in pro-resolving lipid mediators (LXs, RvEs/RvDs & PDs) that was observed in subjects receiving placebo control (102). Therefore, blockade of COX-1 and 2 activities led to deleterious secondary downstream effects on the induction of resolution phase lipid mediator pathways. This data shows that lipid mediator class-switching pathways appear to be operational *in-vivo* in humans throughout the time course of post-exercise recovery. Furthermore, the use of NSAIDs (a common treatment of muscle injury and soreness), can have deleterious effects on induction of pro-resolving lipid mediator circuits.

4.4 Putative roles of pro-resolving lipid mediators in the response to exercise

Several lines of evidence suggest that pro-resolving lipid mediators may play an important physiological role in the successful muscle regenerative and adaptive response following injury. The ability of PMNs to inflict secondary myofibre injury is well established (8, 10, 11). Thus, the activity of SPMs to limit PMN infiltration and actively promote their clearance by nonphlogistic MΦs phagocytosis is likely to play an important role in injured muscle. Additionally, a hallmark of the SPMs is their ability to actively promote monocyte recruitment whilst stimulating to MΦ function and M2 polarization. Monocyte/MΦ recruitment (13-18) and in particular induction of a M2 phenotype (18, 20-23) are processes which are indispensable for successful muscle growth and regeneration. On this basis, we hypothesise that pro-resolving lipid mediators may play an important role in exercise recovery as well as in muscle healing/regeneration following injury. Nevertheless, to date no studies have been undertaken to test this hypothesis.

In addition to the potential effects secondary to their effect on immune cell population, SPMs may play more direct roles in muscle cell growth and development. For example, the lipoxin receptor (ALX), (also known as the formyl peptide receptor 2 (FPR2)) is expressed by the C2C12 skeletal myoblast cell line (289). The peptide annexin A1, another established ALX ligand, was shown to promote migration of muscle satellite cells and consequently enhances skeletal muscle cell differentiation (289, 290). RvD1 and AT-RvD1 also act via the ALX receptor (291). Whether LXs and RvDs can influence muscle satellite cell migration and differentiation via the muscle ALX remains to be determined, but appears a likely scenario. The RvE1 receptor, chemokine-like receptor 1 (chemR23 or CMKLR) (264) is also expressed in muscle, where it has been shown to play an important positive role in myogenesis (292). Despite these associations, to date no studies have directly investigated the effect of pro-resolving lipid mediators on muscle cell growth and development or the role of pro-resolving lipid mediator circuits in the context of skeletal muscle injury and adaptation. Therefore these are key potential areas for further research.

Whereas PGs/LTs play stimulatory roles on muscle cell growth and development, chronic activation of the COX and 5-LOX pathways can have deleterious effects on muscle (169, 217, 220, 293, 294). An intriguing hypothesis is whether failed pro-resolving lipid mediator circuits may underlie chronic non-resolving muscle inflammation and if targeting pro-resolving lipid mediator pathways could be of therapeutic benefit in conditions of rampant muscle inflammation. In support of this hypothesis, recently the DHA derived pro-resolving mediator PD1 was found to be lacking in muscle tissue of high fat-fed mice in association with chronic non-resolving inflammation and insulin resistance (295). In this model, administration of the PD1 isomer 10S,17S-dihydroxy-DHA (also known as protectin DX (PDX)) was found to exert unanticipated glucoregulatory activity by directly stimulating the release of the myokine interleukin-6 (IL-6) from skeletal muscle tissue (296). In this context, PDX acted to upregulate a myokine-liver signalling axis leading to an improved whole body insulin sensitivity (296). These results show for the first time that pro-resolving lipid mediators can exert direct regulatory effects on skeletal muscle tissue. Further studies are needed investigating the role of pro-resolving lipid mediators in the context of muscle responses to injury and mechanical overload, as well as the potential theurapeutic value of resolving lipid mediators in settings of chronic muscle inflammation.

4.5 Anti-inflammatory vs. pro-resolving approaches to acute muscle injury and exercise recovery?

The specialized pro-resolving lipid mediators possess bioactivity that simultaneously limits PMN trafficking (i.e. anti-inflammatory) and actively promotes monocyte/MΦ recruitment and function (i.e. pro-resolving). However, these actions are not one and the same, and it is important to distinguish between them. For example, common mainstay approaches to exercise recovery as well as the clinical treatment of musculoskeletal injury (e.g. NSAIDs) are anti-inflammatory in nature in that they strive to dampen inflammation via antagonism of pro-inflammatory mediators (reviewed in 297). In this context, however, classical anti-inflammatory agents such as the NSAIDS appear to prevent or delay resolution of inflammation by interfering with endogenous lipid mediator class switching circuits and can thus also considered to be anti-resolving, or "resolution toxic" (286, 288, 298). The use of anti-inflammatory drugs (and potentially other interventions that are anti-inflammatory in nature e.g. cold exposure (299)) in an effort to provide analgesia and limit the cardinal signs of inflammation following muscle injury, may inadvertently derange or impair timely or complete resolution of muscle inflammation. Interestingly, this mechanisms may help to explain the apparently deleterious effects of NSAIDs reported in numerous studies in rodent models of muscle growth and regeneration (14, 99, 142-146), and human adaptive responses to exercise (90, 128, 132, 140, 141, 148). An ideal strategy to enhance exercise recovery and manage musculoskeletal injury would perhaps be one that is analgesic and anti-inflammatory (in terms of effects on PMNs), yet simultaneously pro-resolving (by being conducive or even stimulatory in terms of effects on monocyte/MΦ recruitment and function). Interestingly, aspirin is distinct from other non-selective and COX-2 selective NSAIDs, in that whilst it inhibits PG biosynthesis (potentially a deleterious outcome for endogenous SPM circuits); it simultaneously compensates by producing aspirin triggered pro-resolving lipid mediators (e.g. AT-LXs, AT-RvDs, & AT-PDs) from PUFA substrate by acetylated COX-2. Thus, future studies investigating the effect of aspirin on myogenesis and skeletal muscle growth/regeneration should help to delineate the roles of pro-inflammatory (e.g. PG and LT) vs. pro-resolving lipid mediators in successful muscle adaptation and whether certain interventions can be employed or developed which may potentially relieve pain and improve function whilst simultaneously ensuring endogenous regenerative mechanisms remain intact.

5. PERSPECTIVES AND FUTURE DIRECTIONS

Bioactive lipid mediators play diverse role in mediating the initiation, limitation, and active resolution of inflammation. Vigorous or unaccustomed exercise elicits a self-limited inflammatory response, involving cytokines/chemokines, blood leukocyte populations, and muscle-immune cell interactions. Classical pro-inflammatory lipid mediators (e.g. PGs and LTs) have long been implicated as playing a potential role in immunological and adaptive responses elicited following muscle loading. More recently, exercise has been found to be coupled to induction of a biologically active resolution of inflammation program involving the production of lipid mediators via cell-cell interactions which possess anti-inflammatory and pro-resolving bioactivity. These lipid mediators likely play important roles in the limitation and clearance of potentially myofiber damaging PMNs and the recruitment and polarisation of monocytes/Φs which restore tissue homeostasis and facilitate muscle growth and regeneration. Additionally, emerging data suggest that resolution phase lipid mediators may have direct effects on resident muscle cells including myofibers and satellite cells. Anti-inflammatory strategies targeted at relieving symptoms of exercise-induced muscle injury may perturb the resolution phase of inflammation by blocking key signals of resolution early in the inflammatory response (e.g. PGs), leading to deleterious effects on muscle remodelling. A paradigm shift away from current focus on anti-inflammatory approaches, towards interventions which are conducive or even facilitative to natural resolution of inflammation may lead to the development of novel approaches and therapeutics in order to enhance exercise recovery and more effectively manage and treat acute musculoskeletal injuries.

REFERENCES

1. Buckley, C. D., D. W. Gilroy, C. N. Serhan, B. Stockinger, and P. P. Tak. 2013. The resolution of inflammation. Nature Reviews Immunology 13: 59-66.
2. Headland, S. E., and L. V. Norling. 2015. The resolution of inflammation: Principles and challenges. Seminars in Immunology 27: 149-160.
3. Serhan, C. N. 2014. Pro-resolving lipid mediators are leads for resolution physiology. Nature 510: 92-101.

4. Tidball, J. G. 1995. Inflammatory cell response to acute muscle injury. / Reponse cellulaire inflammatoire suite a une blessure musculaire. Medicine & Science in Sports & Exercise 27: 1022-1032.
5. Smith, C., M. J. Kruger, R. M. Smith, and K. H. Myburgh. 2008. The Inflammatory Response to Skeletal Muscle Injury. Sports Medicine 38: 947-969.
6. Orimo, S., E. Hiyamuta, K. Arahata, and H. Sugita. 1991. Analysis of inflammatory cells and complement C3 in bupivacaine-induced myonecrosis. Muscle & Nerve 14: 515-520.
7. Tidball, J. G., E. Berchenko, and J. Frenette. 1999. Macrophage invasion does not contribute to muscle membrane injury during inflammation. Journal Of Leukocyte Biology 65: 492-498.
8. Pizza, F. X., J. M. Peterson, J. H. Baas, and T. J. Koh. 2005. Neutrophils contribute to muscle injury and impair its resolution after lengthening contractions in mice. The Journal Of Physiology 562: 899-913.
9. Fielding, R. A., and T. J. Manfredi. 1993. Acute phase response in exercise III. Neutrophil and IL-1beta accumulation in skeletal muscle. American Journal of Physiology 265: R166.
10. Dumont, N., P. Bouchard, and J. Frenette. 2008. Neutrophil-induced skeletal muscle damage: a calculated and controlled response following hindlimb unloading and reloading. American Journal Of Physiology. Regulatory, Integrative And Comparative Physiology 295: R1831-R1838.
11. Pizza, F. X., T. J. McLoughlin, S. J. McGregor, E. P. Calomeni, and W. T. Gunning. 2001. Neutrophils injure cultured skeletal myotubes. American Journal Of Physiology. Cell Physiology 281: C335-C341.
12. Frenette, J., M. St-Pierre, C. H. Côté, E. Mylona, and F. X. Pizza. 2002. Muscle impairment occurs rapidly and precedes inflammatory cell accumulation after mechanical loading. American Journal Of Physiology. Regulatory, Integrative And Comparative Physiology 282: R351-R357.
13. Dumont, N., and J. Frenette. 2010. Macrophages protect against muscle atrophy and promote muscle recovery in vivo and in vitro: a mechanism partly dependent on the insulin-like growth factor-1 signaling molecule. The American Journal Of Pathology 176: 2228-2235.
14. Shen, W., Y. Li, J. Zhu, R. Schwendener, and J. Huard. 2008. Interaction between macrophages, TGF-beta1, and the COX-2 pathway during the inflammatory phase of skeletal muscle healing after injury. Journal Of Cellular Physiology 214: 405-412.
15. Summan, M., G. L. Warren, R. R. Mercer, R. Chapman, T. Hulderman, N. Van Rooijen, and P. P. Simeonova. 2006. Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study. American Journal Of Physiology. Regulatory, Integrative And Comparative Physiology 290: R1488-R1495.
16. Tidball, J. G., and M. Wehling-Henricks. 2007. Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. The Journal Of Physiology 578: 327-336.
17. Bryer, S. C., G. Fantuzzi, N. Van Rooijen, and T. J. Koh. 2008. Urokinase-type plasminogen activator plays essential roles in macrophage chemotaxis and skeletal muscle regeneration. Journal Of Immunology (Baltimore, Md.: 1950) 180: 1179-1188.
18. DiPasquale, D. M., M. Cheng, W. Billich, S. A. Huang, N. van Rooijen, T. A. Hornberger, and T. J. Koh. 2007. Urokinase-type plasminogen activator and macrophages are required for skeletal muscle hypertrophy in mice. American Journal Of Physiology. Cell Physiology 293: C1278-C1285.
19. Arnold, L., A. Henry, F. Poron, Y. Baba-Amer, N. van Rooijen, A. Plonquet, R. K. Gherardi, and B. Chazaud. 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. The Journal Of Experimental Medicine 204: 1057-1069.
20. Deng, B., M. Wehling-Henricks, S. A. Villalta, Y. Wang, and J. G. Tidball. 2012. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. Journal Of Immunology (Baltimore, Md.: 1950) 189: 3669-3680.
21. Dumont, N. A., and J. Frenette. 2013. Macrophage colony-stimulating factor-induced macrophage differentiation promotes regrowth in atrophied skeletal muscles and C2C12 myotubes. The American Journal Of Pathology 182: 505-515.
22. Hammers, D. W., V. Rybalko, M. Merscham-Banda, H. Pei-Ling, L. J. Suggs, and R. P. Farrar. 2015. Anti-inflammatory macrophages improve skeletal muscle recovery from ischemia-reperfusion. Journal of Applied Physiology 118: 1067-1074.
23. Ruffell, D., F. Mourkioti, A. Gambardella, P. Kirstetter, R. G. Lopez, N. Rosenthal, and C. Nerlov. 2009. A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. Proceedings Of The National Academy Of Sciences Of The United States Of America 106: 17475-17480.
24. Heredia, J. E., L. Mukundan, F. M. Chen, A. A. Mueller, R. C. Deo, R. M. Locksley, T. A. Rando, and A. Chawla. 2013. Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. Cell 153: 376-388.
25. Serhan, C. N., N. Chiang, and J. Dalli. 2015. The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution. Seminars in Immunology 27: 200-215.
26. Crean, D., and C. Godson. 2015. Specialised lipid mediators and their targets. Seminars in Immunology 27: 169-176.
27. Astarita, G., A. C. Kendall, E. A. Dennis, and A. Nicolaou. 2015. Targeted lipidomic strategies for oxygenated metabolites of polyunsaturated fatty acids. Biochimica Et Biophysica Acta 1851: 456-468.
28. Kasuga, K., T. Suga, and N. Mano. 2015. Bioanalytical insights into mediator lipidomics. Journal of Pharmaceutical & Biomedical Analysis 113: 151-162.
29. Willenberg, I., A. I. Ostermann, and N. H. Schebb. 2015. Targeted metabolomics of the arachidonic acid cascade: current state and challenges of LC-MS analysis of oxylipins. Analytical And Bioanalytical Chemistry 407: 2675-2683.
30. Paulsen, G., U. Ramer Mikkelsen, T. Raastad, and J. M. Peake. 2012. Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? Exercise Immunology Review 18: 42-97.
31. Peake, J. M., P. Delia Gatta, S. Katsuhiko, and D. C. Nieman. 2015. Cytokine expression and secretion by skeletal muscle cells: regulatory mechanisms and exercise effects. Exercise Immunology Review 21: 8-25.
32. Williams, T. J. 1979. Prostaglandin E2, prostaglandin I2 and the vascular changes of inflammation. British Journal of Pharmacology 65: 517-524.

33. Ikeda, K., K. Tanaka, and M. Katori. 1975. Potentiation of bradykinin-induced vascular permeability increase by prostaglandin E2 and arachidonic acid in rabbit skin. Prostaglandins 10: 747-758.
34. Murata, T. F. 1997. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. Nature 388: 678.
35. Williams, T. J., and P. J. Jose. 1981. Mediation of increased vascular permeability after complement activation. Histamine-independent action of rabbit C5a. The Journal Of Experimental Medicine 153: 136-153.
36. Williams, T. J., and J. Morley. 1973. Prostaglandins as potentiators of increased vascular permeability in inflammation. Nature 246: 215-217.
37. Ferreira, S. H., M. Nakamura, and M. S. de Abreu Castro. 1978. The hyperalgesic effects of prostacyclin and prostaglandin E2. Prostaglandins 16: 31-37.
38. Higgs, E. A., S. Moncada, and J. R. Vane. 1978. Inflammatory effects of prostacyclin (PGI2) and 6-oxo-PGF1alpha in the rat paw. Prostaglandins 16: 153-162.
39. Lin, C.-R., F. Amaya, L. Barrett, H. Wang, J. Takada, T. A. Samad, and C. J. Woolf. 2006. Prostaglandin E2 receptor EP4 contributes to inflammatory pain hypersensitivity. The Journal Of Pharmacology And Experimental Therapeutics 319: 1096-1103.
40. Minami, T., J. Sugatani, K. Sakimura, M. Abe, M. Mishina, and S. Ito. 1997. Absence of prostaglandin E2-induced hyperalgesia in NMDA receptor epsilon subunit knockout mice. British Journal of Pharmacology 120: 1522-1526.
41. Zhang, Y., A. Shaffer, J. Portanova, K. Seibert, and P. C. Isakson. 1997. Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E2 production. The Journal Of Pharmacology And Experimental Therapeutics 283: 1069-1075.
42. Diaz-Perez, J. L., M. E. Goldyne, and R. K. Winkelmann. 1976. PROSTAGLANDIINS AND CHEMOTAXIS: ENHANCEMENT OF POLYMORHONUCLEAR LEUKOCYTE CHEMOTAXIS BY PROSTAGLANDIN F2α. Journal of Investigative Dermatology 66: 149-152.
43. Higgs, G. A., E. McCall, and L. J. Youlten. 1975. A chemotactic role for prostaglandins released from polymorphonuclear leucocytes during phagocytosis. British Journal of Pharmacology 53: 539-546.
44. Kitchen, E. A., J. R. Boot, and W. Dawson. 1978. Chemotactic activity of thromboxane B2, prostaglandins and their metabolites for polymorphonuclear leucocytes. Prostaglandins 16: 239-244.
45. McClatchey, W., and R. Snyderman. 1976. Prostaglandins and inflammation: enhancement of monocyte chemotactic responsiveness by prostaglandin E2. Prostaglandins 12: 415-426.
46. Ferreira, S. H., S. Moncada, and J. R. Vane. 1971. Indomethacin and aspirin abolish prostaglandin release from the spleen. Nature: New Biology 231: 237-239.
47. Smith, J. B., and A. L. Willis. 1971. Aspirin selectively inhibits prostaglandin production in human platelets. Nature: New Biology 231: 235-237.
48. Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature: New Biology 231: 232-235.
49. Vandenburgh, H. H., S. Hatfaludy, I. Sohar, and J. Shansky. 1990. Stretch-induced prostaglandins and protein turnover in cultured skeletal muscle. The American Journal Of Physiology 259: C232-C240.
50. Palmer, R. M., P. J. Reeds, T. Atkinson, and R. H. Smith. 1983. The influence of changes in tension on protein synthesis and prostaglandin release in isolated rabbit muscles. The Biochemical Journal 214: 1011-1014.
51. Smith, R. H., R. M. Palmer, and P. J. Reeds. 1983. Protein synthesis in isolated rabbit forelimb muscles. The possible role of metabolites of arachidonic acid in the response to intermittent stretching. The Biochemical Journal 214: 153-161.
52. Demers, L. M., T. S. Harrison, D. R. Halbert, and R. J. Santen. 1981. Effect of prolonged exercise on plasma prostaglandin levels. Prostaglandins And Medicine 6: 413-418.
53. Laustiola, K., E. SeppÃ¤lÃ¤, T. Nikkari, and H. Vapaatalo. 1984. Exercise-induced increase in plasma arachidonic acid and thromboxane B2 in healthy men: effect of beta-adrenergic blockade. Journal Of Cardiovascular Pharmacology 6: 449-454.
54. Nowak, J., and A. Wennmalm. 1978. Effect of exercise on human arterial and regional venous plasma concentrations of prostaglandin E. Prostaglandins And Medicine 1: 489-497.
55. Peake, J. M., K. Suzuki, M. Hordern, G. Wilson, K. Nosaka, and J. S. Coombes. 2005. Plasma cytokine changes in relation to exercise intensity and muscle damage. European Journal Of Applied Physiology 95: 514-521.
56. Venkatraman, J. T., X. Feng, and D. Pendergast. 2001. Effects of dietary fat and endurance exercise on plasma cortisol, prostaglandin E2, interferon-gamma and lipid peroxides in runners. Journal Of The American College Of Nutrition 20: 529-536.
57. Donovan, D. C., C. A. Jackson, P. T. Colahan, N. Norton, and D. J. Hurley. 2007. Exercise-induced alterations in pro-inflammatory cytokines and prostaglandin F2alpha in horses. Veterinary Immunology And Immunopathology 118: 263-269.
58. Carter, J. W., A. E. Ready, S. Singhroy, E. Duta, and J. M. Gerrard. 1989. The effect of exercise on bleeding time and local production of prostacyclin and thromboxane. European Journal Of Applied Physiology And Occupational Physiology 59: 355-359.
59. Feng, D. L., J. Murillo, P. Jadhav, C. McKenna, O. C. Gebara, I. Lipinska, J. E. Muller, and G. H. Tofler. 1999. Upright posture and maximal exercise increase platelet aggregability and prostacyclin production in healthy male subjects. British Journal Of Sports Medicine 33: 401-404.
60. Mehta, J., P. Mehta, and C. Horalek. 1983. The significance of platelet-vessel wall prostaglandin equilibrium during exercise-induced stress. American Heart Journal 105: 895-900.
61. Mourits-Andersen, T., I. W. Jensen, P. N. Jensen, J. Ditzel, and J. Dyerberg. 1987. Plasma 6-keto-PGF1 alpha, thromboxane B2 and PGE2 in type 1 (insulin-dependent) diabetic patients during exercise. Diabetologia 30: 460-463.
62. Ritter, J. M., S. E. Barrow, I. A. Blair, and C. T. Dollery. 1983. Release of prostacyclin in vivo and its role in man. Lancet 1: 317-319.
63. Viinikka, L., J. Vuori, and O. Ylikorkala. 1984. Lipid peroxides, prostacyclin, and thromboxane A2 in runners during acute exercise. Medicine And Science In Sports And Exercise 16: 275-277.

64. Böger, R. H., S. M. Bode-Böger, E. P. Schröder, D. Tsikas, and J. C. Frölich. 1995. Increased prostacyclin production during exercise in untrained and trained men: effect of low-dose aspirin. Journal Of Applied Physiology (Bethesda, Md.: 1985) 78: 1832-1838.
65. Lemne, C., O. Vesterqvist, N. Egberg, K. Green, T. Jogestrand, and U. de Faire. 1992. Platelet activation and prostacyclin release in essential hypertension. Prostaglandins 44: 219-235.
66. Numminen, H., M. Hillbom, H. Vapaatalo, E. Seppälä, K. Laustiola, G. Benthin, A. Muuronen, and M. Kaste. 1991. Effects of exercise and ethanol ingestion on platelet thromboxane release in healthy men. Metabolism: Clinical And Experimental 40: 695-701.
67. Piret, A., G. Niset, E. Depiesse, W. Wyns, J. M. Boeynaems, J. Poortmans, and S. Degre. 1990. Increased platelet aggregability and prostacyclin biosynthesis induced by intense physical exercise. Thrombosis Research 57: 685-695.
68. Wennmalm, A., and G. A. Fitzgerald. 1988. Excretion of prostacyclin and thromboxane A2 metabolites during leg exercise in humans. The American Journal Of Physiology 255: H15-H18.
69. Blatnik, M., and R. C. Steenwyk. 2010. Quantification of urinary PGEm, 6-keto PGF(1alpha) and 2,3-dinor-6-keto PGF(1alpha) by UFLC-MS/MS before and after exercise. Prostaglandins & Other Lipid Mediators 93: 8-13.
70. McGill, D., J. McGuiness, J. Lloyd, and N. Ardlie. 1989. Platelet function and exercise-induced myocardial ischaemia in coronary heart disease patients. Thrombosis Research 56: 147-158.
71. Todd, M. K., A. H. Goldfarb, and B. T. Boyer. 1992. Effect of exercise intensity on 6-keto-PGF1 alpha, TXB2, and 6-keto-PGF1 alpha/TXB2 ratios. Thrombosis Research 65: 487-493.
72. Dousset, E., J. Avela, M. Ishikawa, J. Kallio, S. Kuitunen, H. Kyröläinen, V. Linnamo, and P. V. Komi. 2007. Bimodal recovery pattern in human skeletal muscle induced by exhaustive stretch-shortening cycle exercise. Medicine & Science in Sports & Exercise 39: 453-460.
73. Uchida, M. C., K. Nosaka, C. Ugrinowitsch, A. Yamashita, E. Martins, Jr., A. S. Moriscot, and M. S. Aoki. 2009. Effect of bench press exercise intensity on muscle soreness and inflammatory mediators. Journal Of Sports Sciences 27: 499-507.
74. Boatwright, D., R. Byrd, and M. Mangum. 1991. Relationship between serum prostaglandin formation, creatine kinase activity, and ratings of perceived soreness. Sports Training, Medicine & Rehabilitation 2: 85-88.
75. Tartibian, B., B. H. Maleki, and A. Abbasi. 2011. Omega-3 Fatty Acids Supplementation Attenuates Inflammatory Markers After Eccentric Exercise in Untrained Men. Clinical Journal of Sport Medicine 21: 131-137.
76. Croisier, J. L., G. Camus, G. Deby-Dupont, F. Bertrand, C. Lhermerout, J. M. Crielaard, A. JuchmÃ¨s-Ferir, C. Deby, A. Albert, and M. Lamy. 1996. Myocellular enzyme leakage, polymorphonuclear neutrophil activation and delayed onset muscle soreness induced by isokinetic eccentric exercise. Archives Of Physiology And Biochemistry 104: 322-329.
77. Croisier, J. L., G. Camus, T. Monfils, G. Deby-Dupon, M. Fafchamps, I. Venneman, J. M. Crielaard, A. JuchmÃ¨s-Ferir, C. Lhermerout, M. Lamy, and C. Deby. 1996. Piroxicam fails to reduce myocellular enzyme leakage and delayed onset muscle soreness induced by isokinetic eccentric exercise. Mediators Of Inflammation 5: 230-234.
78. Black, C. D., M. P. Herring, D. J. Hurley, and P. J. O'Connor. 2010. Ginger (Zingiber officinale) reduces muscle pain caused by eccentric exercise. The Journal Of Pain: Official Journal Of The American Pain Society 11: 894-903.
79. Braun, W. A., M. G. Flynn, W. J. Armstrong, and D. D. Jacks. 2005. The effects of chondroitin sulfate supplementation on indices of muscle damage induced by eccentric arm exercise. The Journal Of Sports Medicine And Physical Fitness 45: 553-560.
80. Hirose, L., K. Nosaka, M. Newton, A. Laveder, M. Kano, J. Peake, and K. Suzuki. 2004. Changes in inflammatory mediators following eccentric exercise of the elbow flexors. Exercise Immunology Review 10: 75-90.
81. Buford, T. W., M. B. Cooke, L. L. Redd, G. M. Hudson, B. D. Shelmadine, and D. S. Willoughby. 2009. Protease supplementation improves muscle function after eccentric exercise. Medicine And Science In Sports And Exercise 41: 1908-1914.
82. Boatwright, S., R. Byrd, and M. Mangum. 1991. Relationship between serum prostaglandin formation, creatine kinase activity, and ratings of perceived soreness. Sports Medicine, Training and Rehabilitation: An International Journal 2: 85-88.
83. Dousset, E., J. Avela, M. Ishikawa, J. Kallio, S. Kuitunen, H. Kyröläinen, V. Linnamo, and P. V. Komi. 2007. Bimodal recovery pattern in human skeletal muscle induced by exhaustive stretch-shortening cycle exercise. Medicine And Science In Sports And Exercise 39: 453-460.
84. Glass, M., J. Hong, T. A. Sato, and M. D. Mitchell. 2005. Misidentification of prostamides as prostaglandins. Journal of lipid research 46: 1364-1368.
85. Maddipati, K. R., R. Romero, T. Chaiworapongsa, S. L. Zhou, Z. Xu, A. L. Tarca, J. P. Kusanovic, H. Munoz, and K. V. Honn. 2014. Eicosanomic profiling reveals dominance of the epoxygenase pathway in human amniotic fluid at term in spontaneous labor. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 28: 4835-4846.
86. Trappe, T., U. Raue, R. Williams, J. Carrithers, and R. Hickner. 2006. Effects of age and resistance exercise on skeletal muscle interstitial prostaglandin F(2alpha). Prostaglandins, Leukotrienes, And Essential Fatty Acids 74: 175-181.
87. Karamouzis, M., I. Karamouzis, E. Vamvakoudis, G. Ampatzidis, K. Christoulas, N. Angelopoulou, and K. Mandroukas. 2001. The response of muscle interstitial prostaglandin E(2)(PGE(2)), prostacyclin I(2)(PGI(2)) and thromboxane A(2)(TXA(2)) levels during incremental dynamic exercise in humans determined by in vivo microdialysis. Prostaglandins, Leukotrienes, And Essential Fatty Acids 64: 259-263.
88. Karamouzis, M., H. Langberg, D. Skovgaard, J. Bülow, M. Kjaer, and B. Saltin. 2001. In situ microdialysis of intramuscular prostaglandin and thromboxane in contracting skeletal muscle in humans. Acta Physiologica Scandinavica 171: 71-76.
89. Boushel, R., H. Langberg, C. Gemmer, J. Olesen, R. Crameri, C. Scheede, M. Sander, and M. Kjaer. 2002. Combined inhibition of nitric oxide and prostaglandins reduces human skeletal muscle blood flow during exercise. The Journal Of Physiology 543: 691-698.
90. Trappe, T. A., J. D. Fluckey, F. White, C. P. Lambert, and W. J. Evans. 2001. Skeletal muscle PGF(2)(alpha) and PGE(2) in response to eccentric resistance exercise: influence of ibuprofen acetaminophen. The Journal Of Clinical Endocrinology And Metabolism 86: 5067-5070.

91. Burian, A., V. Frangione, S. Rovati, G. Mautone, C. Leuratti, A. Vaccani, R. Crevenna, M. Keilani, B. Burian, M. Brunner, and M. Zeitlinger. 2013. An exploratory microdialysis study investigating the effect of repeated application of a diclofenac epolamine medicated plaster on prostaglandin concentrations in skeletal muscle after standardized physical exercise. British Journal Of Clinical Pharmacology 76: 880-887.
92. Tegeder, L., J. Zimmermann, S. T. Meller, and G. Geisslinger. 2002. Release of algesic substances in human experimental muscle pain. Inflammation Research: Official Journal Of The European Histamine Research Society ... [Et Al.] 51: 393-402.
93. Carroll, C. C., D. T. O'Connor, R. Steinmeyer, J. D. Del Mundo, D. R. McMullan, J. A. Whitt, J. E. Ramos, and R. J. Gonzales. 2013. The influence of acute resistance exercise on cyclooxygenase-1 and -2 activity and protein levels in human skeletal muscle. American Journal Of Physiology. Regulatory, Integrative And Comparative Physiology 305: R24-R30.
94. Weinheimer, E., B. Jemiolo, C. C. Carroll, M. P. Harber, J. M. Haus, N. A. Burd, J. K. LeMoine, S. W. Trappe, and T. A. Trappe. 2007. Resistance exercise and cyclooxygenase (COX) expression in human skeletal muscle: Implications for COX-inhibiting drugs and protein synthesis. FASEB Journal 21: A937-A937.
95. Kim, S.-Y., T.-W. Jun, Y.-S. Lee, H.-K. Na, Y.-J. Surh, and W. Song. 2009. Effects of exercise on cyclooxygenase-2 expression and nuclear factor-kappaB DNA binding in human peripheral blood mononuclear cells. Annals Of The New York Academy Of Sciences 1171: 464-471.
96. Testa, M., B. Rocca, L. Spath, F. O. Ranelletti, G. Petrucci, G. Ciabattoni, F. Naro, S. Schiaffino, M. Volpe, and C. Reggiani. 2007. Expression and activity of cyclooxygenase isoforms in skeletal muscles and myocardium of humans and rodents. Journal of Applied Physiology 103: 1412-1418.
97. Veliça, P., F. L. Khanim, and C. M. Bunce. 2010. Prostaglandin D2 inhibits C2C12 myogenesis. Molecular And Cellular Endocrinology 319: 71-78.
98. Otis, J. S., T. J. Burkholder, and G. K. Pavlath. 2005. Stretch-induced myoblast proliferation is dependent on the COX2 pathway. Experimental Cell Research 310: 417-425.
99. Shen, W., V. Prisk, Y. Li, W. Foster, and J. Huard. 2006. Inhibited skeletal muscle healing in cyclooxygenase-2 gene-deficient mice: the role of PGE2 and PGF2alpha. Journal of Applied Physiology 101: 1215-1221.
100. Markworth, J. F., and D. Cameron-Smith. 2013. Arachidonic acid supplementation enhances in vitro skeletal muscle cell growth via a COX-2-dependent pathway. American Journal Of Physiology. Cell Physiology 304: C56-C67.
101. Bondesen, B. A., K. A. Jones, W. C. Glasgow, and G. K. Pavlath. 2007. Inhibition of myoblast migration by prostacyclin is associated with enhanced cell fusion. The FASEB Journal: Official Publication Of The Federation Of American Societies For Experimental Biology 21: 3338-3345.
102. Markworth, J. F., L. Vella, B. S. Lingard, D. L. Tull, T. W. Rupasinghe, A. J. Sinclair, K. R. Maddipati, and D. Cameron-Smith. 2013. Human inflammatory and resolving lipid mediator responses to resistance exercise and ibuprofen treatment. American Journal Of Physiology. Regulatory, Integrative And Comparative Physiology 305: R1281-R1296.
103. Mikkelsen, U. R., I. C. Helmark, M. Kær, and H. Langberg. 2008. Prostaglandin synthesis can be inhibited locally by infusion of NSAIDS through microdialysis catheters in human skeletal muscle. Journal of Applied Physiology 104: 534-537.
104. Dudley, G. A., J. Czerkawski, A. Meinrod, G. Gillis, A. Baldwin, and M. Scarpone. 1997. Efficacy of naproxen sodium for exercise-induced dysfunction muscle injury and soreness. Clinical Journal Of Sport Medicine: Official Journal Of The Canadian Academy Of Sport Medicine 7: 3-10.
105. Baldwin, A. C., S. W. Stevenson, and G. A. Dudley. 2001. Nonsteroidal anti-inflammatory therapy after eccentric exercise in healthy older individuals. The Journals Of Gerontology. Series A, Biological Sciences And Medical Sciences 56: M510-M513.
106. Cannavino, C. R., J. Abrams, L. A. Palinkas, A. Saglimbeni, and M. D. Bracker. 2003. Efficacy of transdermal ketoprofen for delayed onset muscle soreness. Clinical Journal Of Sport Medicine: Official Journal Of The Canadian Academy Of Sport Medicine 13: 200-208.
107. Donnelly, A. E., K. McCormick, R. J. Maughan, P. H. Whiting, and P. M. Clarkson. 1988. Effects of a non-steroidal anti-inflammatory drug on delayed onset muscle soreness and indices of damage. British Journal Of Sports Medicine 22: 35-38.
108. Hasson, S. M., J. C. Daniels, J. G. Divine, B. R. Niebuhr, S. Richmond, P. G. Stein, and J. H. Williams. 1993. Effect of ibuprofen use on muscle soreness, damage, and performance: a preliminary investigation. Medicine And Science In Sports And Exercise 25: 9-17.
109. Hill, D. W., and J. D. Richardson. 1989. Effectiveness of 10% trolamine salicylate cream on muscular soreness induced by a reproducible program of weight training. The Journal Of Orthopaedic And Sports Physical Therapy 11: 19-23.
110. Lecomte, J. M., V. J. Lacroix, and D. L. Montgomery. 1998. A randomized controlled trial of the effect of naproxen on delayed onset muscle soreness and muscle strength. Clinical Journal Of Sport Medicine: Official Journal Of The Canadian Academy Of Sport Medicine 8: 82-87.
111. O'Grady, M., A. C. Hackney, K. Schneider, E. Bossen, K. Steinberg, J. M. Douglas, Jr., W. J. Murray, and W. D. Watkins. 2000. Diclofenac sodium (Voltaren) reduced exercise-induced injury in human skeletal muscle. Medicine And Science In Sports And Exercise 32: 1191-1196.
112. Paulsen, G., I. M. Egner, M. Drange, H. Langberg, H. B. Benestad, J. G. Fjeld, J. Hallén, and T. Raastad. 2010. A COX-2 inhibitor reduces muscle soreness, but does not influence recovery and adaptation after eccentric exercise. Scandinavian Journal of Medicine & Science in Sports 20: 1-13.
113. Rahnama, N., F. Rahmani-Nia, and K. Ebrahim. 2005. The isolated and combined effects of selected physical activity and ibuprofen on delayed-onset muscle soreness. Journal Of Sports Sciences 23: 843-850.
114. Riasati, S., M. Moghadasi, A. Torkfar, R. Shirazinejad, and H. Arvin. 2010. Aspirin may be an effective treatment for exercise-induced muscle soreness. Brazilian Journal of Biomotricity 4: 206-213.
115. Sayers, S. P., C. A. Knight, P. M. Clarkson, E. H. Van Wegen, and G. Kamen. 2001. Effect of ketoprofen on muscle function and sEMG activity after eccentric exercise. Medicine And Science In Sports And Exercise 33: 702-710.
116. Tartibian, B., and K. Azizbeigi. 2009. The effect of taking naproxen drug on the level of perceived pain and changes of CPK serum after eccentric exercise. World Journal of Sport Sciences 2: 165-170.

117. Tokmakidis, S. P., E. A. Kokkinidis, I. Smilios, and H. Douda. 2003. The effects of ibuprofen on delayed muscle soreness and muscular performance after eccentric exercise. Journal of Strength & Conditioning Research. 17: 53-59.
118. Pizza, F. X., D. Cavender, A. Stockard, H. Baylies, and A. Beighle. 1999. Anti-inflammatory doses of ibuprofen: effect on neutrophils and exercise-induced muscle injury. International Journal of Sports Medicine 20: 98-102.
119. Bourgeois, J., D. MacDougall, J. MacDonald, and M. Tarnopolsky. 1999. Naproxen does not alter indices of muscle damage in resistance-exercise trained men. Medicine And Science In Sports And Exercise 31: 4-9.
120. Reynolds, J. F., T. D. Noakes, M. P. Schwellnus, A. Windt, and P. Bowerbank. 1995. Non-steroidal anti-inflammatory drugs fail to enhance healing of acute hamstring injuries treated with physiotherapy. South African Medical Journal 85: 517-522.
121. Barlas, P., J. A. Craig, J. Robinson, D. M. Walsh, G. D. Baxter, and J. M. Allen. 2000. Managing delayed-onset muscle soreness: lack of effect of selected oral systemic analgesics. Archives Of Physical Medicine And Rehabilitation 81: 966-972.
122. Donnelly, A. E., R. J. Maughan, and P. H. Whiting. 1990. Effects of ibuprofen on exercise-induced muscle soreness and indices of muscle damage. British Journal Of Sports Medicine 24: 191-195.
123. Grossman, J. M., B. A. Arnold, D. H. Perrin, and D. M. Kahler. 1995. Effect of ibuprofen on pain, decreased range of motion, and decreased strength associated with delayed onset muscle soreness of the elbow flexors. Journal of Sport Rehabilitation 4: 253-263.
124. Hyldahl, R. D., J. Keadle, P. A. Rouzier, D. Pearl, and P. M. Clarkson. 2010. Effects of ibuprofen topical gel on muscle soreness. Medicine And Science In Sports And Exercise 42: 614-621.
125. Kuipers, H., H. A. Keizer, F. T. Verstappen, and D. L. Costill. 1985. Influence of a prostaglandin-inhibiting drug on muscle soreness after eccentric work. International Journal of Sports Medicine 6: 336-339.
126. Krentz, J. R., B. Quest, J. P. Farthing, D. W. Quest, and P. D. Chilibeck. 2008. The effects of ibuprofen on muscle hypertrophy, strength, and soreness during resistance training. Applied Physiology, Nutrition, And Metabolism 33: 470-475.
127. Loram, L. C., D. Mitchell, and A. Fuller. 2005. Rofecoxib and tramadol do not attenuate delayed-onset muscle soreness or ischaemic pain in human volunteers. Canadian Journal Of Physiology And Pharmacology 83: 1137-1145.
128. Mikkelsen, U. R., H. Langberg, I. C. Helmark, D. Skovgaard, L. L. Andersen, M. Kjaer, and A. L. Mackey. 2009. Local NSAID infusion inhibits satellite cell proliferation in human skeletal muscle after eccentric exercise. Journal Of Applied Physiology (Bethesda, Md.: 1985) 107: 1600-1611.
129. Nieman, D. C., D. A. Henson, C. L. Dumke, K. Oley, S. R. McAnulty, J. M. Davis, E. A. Murphy, A. C. Utter, R. H. Lind, L. S. McAnulty, and J. D. Morrow. 2006. Ibuprofen use, endotoxemia, inflammation, and plasma cytokines during ultramarathon competition. Brain, Behavior & Immunity 20: 578-584.
130. Semark, A., T. D. Noakes, A. St Clair Gibson, and M. I. Lambert. 1999. The effect of a prophylactic dose of flurbiprofen on muscle soreness and sprinting performance in trained subjects. Journal Of Sports Sciences 17: 197-203.
131. Stone, M. B., M. A. Merrick, C. D. Ingersoll, and J. E. Edwards. 2002. Preliminary comparison of bromelain and Ibuprofen for delayed onset muscle soreness management. Clinical Journal Of Sport Medicine: Official Journal Of The Canadian Academy Of Sport Medicine 12: 373-378.
132. Trappe, T. A., F. White, C. P. Lambert, D. Cesar, M. Hellerstein, and W. J. Evans. 2002. Effect of ibuprofen and acetaminophen on postexercise muscle protein synthesis. American Journal Of Physiology. Endocrinology And Metabolism 282: E551-E556.
133. Khoshkhahesh, F., M. Siahkuhain, G. Fisher, and B. Nakhostin-Rooh. 2013. INFLUENCE OF A LOW-DOSE COX-2 INHIBITOR DRUG ON EXERCISE-INDUCED INFLAMMATION, MUSCLE DAMAGE AND LIPID PEROXIDATION. Biology of Sport 30: 61-65.
134. Peterson, J. M., T. A. Trappe, E. Mylona, F. White, C. P. Lambert, W. J. Evans, and F. X. Pizza. 2003. Ibuprofen and acetaminophen: effect on muscle inflammation after eccentric exercise. . Medicine & Science in Sports & Exercise 35: 892-896.
135. Gump, B. S., D. R. McMullan, D. J. Cauthon, J. A. Whitt, J. D. Del Mundo, T. Letham, P. J. Kim, G. N. Friedlander, J. Pingel, H. Langberg, and C. C. Carroll. 2013. Short-term acetaminophen consumption enhances the exercise-induced increase in Achilles peritendinous IL-6 in humans. Journal Of Applied Physiology (Bethesda, Md.: 1985) 115: 929-936.
136. Mikkelsen, U. R., P. Schjerling, I. C. Helmark, S. Reitelseder, L. Holm, D. Skovgaard, H. Langberg, M. Kjær, and K. M. Heinemeier. 2011. Local NSAID infusion does not affect protein synthesis and gene expression in human muscle after eccentric exercise. Scandinavian Journal of Medicine & Science in Sports 21: 630-644.
137. Enos, R. T., J. M. Davis, J. L. McClellan, and E. A. Murphy. 2013. Indomethacin in combination with exercise leads to muscle and brain inflammation in mice. Journal Of Interferon & Cytokine Research: The Official Journal Of The International Society For Interferon And Cytokine Research 33: 446-451.
138. Burd, N. A., J. M. Dickinson, J. K. Lemoine, C. C. Carroll, B. E. Sullivan, J. M. Haus, B. Jemiolo, S. W. Trappe, G. M. Hughes, C. E. Sanders, Jr., and T. A. Trappe. 2010. Effect of a cyclooxygenase-2 inhibitor on postexercise muscle protein synthesis in humans. American Journal Of Physiology. Endocrinology And Metabolism 298: E354-E361.
139. Rodemann, H. P., and A. L. Goldberg. 1982. Arachidonic acid, prostaglandin E2 and F2 alpha influence rates of protein turnover in skeletal and cardiac muscle. The Journal Of Biological Chemistry 257: 1632-1638.
140. Mackey, A. L., M. Kjaer, S. Dandanell, K. H. Mikkelsen, L. Holm, S. D□ssing, K. Fawzi, S. O. Koskinen, C. H. Jensen, Schr, x00F, H. D. der, and H. Langberg. 2007. The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. Journal of Applied Physiology 103: 425-431.
141. Mackey, A. L. 2013. Does an NSAID a day keep satellite cells at bay? Journal Of Applied Physiology (Bethesda, Md.: 1985) 115: 900-908.
142. Bondesen, B. A., S. T. Mills, K. M. Kegley, and G. K. Pavlath. 2004. The COX-2 pathway is essential during early stages of skeletal muscle regeneration. American Journal Of Physiology. Cell Physiology 287: C475-483.

143. Bondesen, B. A., S. T. Mills, and G. K. Pavlath. 2006. The COX-2 pathway regulates growth of atrophied muscle via multiple mechanisms. American Journal Of Physiology. Cell Physiology 290: C1651-1659.
144. Novak, M. L., W. Billich, S. M. Smith, K. B. Sukhija, T. J. McLoughlin, T. A. Hornberger, and T. J. Koh. 2009. COX-2 inhibitor reduces skeletal muscle hypertrophy in mice. American Journal Of Physiology. Regulatory, Integrative And Comparative Physiology 296: R1132-1139.
145. Shen, W., Y. Li, Y. Tang, J. Cummins, and J. Huard. 2005. NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. The American Journal Of Pathology 167: 1105-1117.
146. Soltow, Q. A., J. L. Betters, J. E. Sellman, V. A. Lira, J. H. Long, and D. S. Criswell. 2006. Ibuprofen inhibits skeletal muscle hypertrophy in rats. Medicine And Science In Sports And Exercise 38: 840-846.
147. Mikkelsen, U., G. Paulsen, P. Schjerling, I. Helmark, H. Langberg, M. Kjær, and K. Heinemeier. 2013. The heat shock protein response following eccentric exercise in human skeletal muscle is unaffected by local NSAID infusion. European Journal of Applied Physiology 113: 1883-1893.
148. Markworth, J. F., L. D. Vella, V. C. Figueiredo, and D. Cameron-Smith. 2014. Ibuprofen treatment blunts early translational signaling responses in human skeletal muscle following resistance exercise. Journal of Applied Physiology 117: 20-28.
149. Markworth, J. F., and D. Cameron-Smith. 2011. Prostagiandin F2α stimulates PI3K/ERK/mTOR signaling and skeletal myotube hypertrophy. American Journal of Physiology: Cell Physiology 69: C671-C682.
150. Duchesne, E., M.-H. Tremblay, and C. H. Côté. 2011. Mast cell tryptase stimulates myoblast proliferation; a mechanism relying on protease-activated receptor-2 and cyclooxygenase-2. BMC Musculoskeletal Disorders 12: 235-235.
151. Mo, C., R. Zhao, J. Vallejo, O. Igwe, L. Bonewald, L. Wetmore, and M. Brotto. 2015. Prostaglandin E2 promotes proliferation of skeletal muscle myoblasts via EP4 receptor activation. Cell Cycle (Georgetown, Tex.) 14: 1507-1516.
152. Zalin, R. J. 1987. The role of hormones and prostanoids in the in vitro proliferation and differentiation of human myoblasts. Exp Cell Res 172: 265-281.
153. Jansen, K. M., and G. K. Pavlath. 2008. Prostaglandin F2α promotes muscle cell survival and growth through upregulation of the inhibitor of apoptosis protein BRUCE. Cell Death & Differentiation 15: 1619-1628.
154. Horsley, V., and G. K. Pavlath. 2003. Prostaglandin F2(alpha) stimulates growth of skeletal muscle cells via an NFATC2-dependent pathway. The Journal Of Cell Biology 161: 111-118.
155. Kuhn, H., S. Banthiya, and K. van Leyen. 2015. Mammalian lipoxygenases and their biological relevance. Biochimica Et Biophysica Acta 1851: 308-330.
156. Dixon, R. A., R. E. Jones, R. E. Diehl, C. D. Bennett, S. Kargman, and C. A. Rouzer. 1988. Cloning of the cDNA for human 5-lipoxygenase. Proceedings Of The National Academy Of Sciences Of The United States Of America 85: 416-420.
157. Rouzer, C. A., C. D. Bennett, R. E. Diehl, R. E. Jones, S. Kargman, E. Rands, and R. A. Dixon. 1989. Cloning and expression of human leukocyte 5-lipoxygenase. Advances In Prostaglandin, Thromboxane, And Leukotriene Research 19: 474-477.
158. Samuelsson, B. 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science (New York, N.Y.) 220: 568-575.
159. Samuelsson, B., P. Borgeat, S. Hammarström, and R. C. Murphy. 1979. Introduction of a nomenclature: leukotrienes. Prostaglandins 17: 785-787.
160. Samuelsson, B., and S. Hammarström. 1980. Nomenclature for leukotrienes. Prostaglandins 19: 645-648.
161. Borgeat, P., M. Hamberg, and B. Samuelsson. 1976. Transformation of arachidonic acid and homo-gamma-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases. The Journal Of Biological Chemistry 251: 7816-7820.
162. Borgeat, P., and B. Samuelsson. 1979. Arachidonic acid metabolism in polymorphonuclear leukocytes: unstable intermediate in formation of dihydroxy acids. Proceedings Of The National Academy Of Sciences Of The United States Of America 76: 3213-3217.
163. Rouzer, C. A., T. Matsumoto, and B. Samuelsson. 1986. Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. Proceedings Of The National Academy Of Sciences Of The United States Of America 83: 857-861.
164. Borgeat, P., and B. Samuelsson. 1979. Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes. Formation of a novel dihydroxyeicosatetraenoic acid. The Journal Of Biological Chemistry 254: 2643-2646.
165. Ford-Hutchinson, A. W., M. A. Bray, M. V. Doig, M. E. Shipley, and M. J. Smith. 1980. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature 286: 264-265.
166. Goetzl, E. J., and W. C. Pickett. 1980. The human PMN leukocyte chemotactic activity of complex hydroxy-eicosatetraenoic acids (HETEs). Journal Of Immunology (Baltimore, Md.: 1950) 125: 1789-1791.
167. Palmer, R. M., R. J. Stepney, G. A. Higgs, and K. E. Eakins. 1980. Chemokinetic activity of arachidonic and lipoxygenase products on leuocyctes of different species. Prostaglandins 20: 411-418.
168. Goetzl, E. J., and W. C. Pickett. 1981. Novel structural determinants of the human neutrophil chemotactic activity of leukotriene B. The Journal Of Experimental Medicine 153: 482-487.
169. Li, P., D. Y. Oh, G. Bandyopadhyay, W. S. Lagakos, S. Talukdar, O. Osborn, A. Johnson, H. Chung, R. Mayoral, M. Maris, J. M. Ofrecio, S. Taguchi, M. Lu, and J. M. Olefsky. 2015. LTB4 promotes insulin resistance in obese mice by acting on macrophages, hepatocytes and myocytes. Nature Medicine 21: 239-247.
170. Afonso, P. V., M. Janka-Junttila, Y. J. Lee, C. P. McCann, C. M. Oliver, K. A. Aamer, W. Losert, M. T. Cicerone, and C. A. Parent. 2012. LTB4 is a signal-relay molecule during neutrophil chemotaxis. Developmental Cell 22: 1079-1091.
171. Lämmermann, T. P. V. A. B. R. W. J. M. W. C. A. G. R. N. 2013. Neutrophil swarms require LTB4 and integrins at sites of cell death in vivo. Nature 498: 371-375.
172. Funk, C. D., L. Furci, and G. A. FitzGerald. 1990. Molecular cloning, primary structure, and expression of the human platelet/erythroleukemia cell 12-lipoxygenase. Proceedings Of The National Academy Of Sciences Of The United States Of America 87: 5638-5642.

173. Hamberg, M., and B. Samuelsson. 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. Proceedings Of The National Academy Of Sciences Of The United States Of America 71: 3400-3404.
174. Boeglin, W. E., R. B. Kim, and A. R. Brash. 1998. A 12R-lipoxygenase in human skin: mechanistic evidence, molecular cloning, and expression. Proceedings Of The National Academy Of Sciences Of The United States Of America 95: 6744-6749.
175. Meruvu, S., M. Walther, I. Ivanov, S. Hammarström, G. Fürstenberger, P. Krieg, P. Reddanna, and H. Kuhn. 2005. Sequence determinants for the reaction specificity of murine (12R)-lipoxygenase: targeted substrate modification and site-directed mutagenesis. The Journal Of Biological Chemistry 280: 36633-36641.
176. Deng, B., C.-W. Wang, H. H. Arnardottir, Y. Li, C.-Y. C. Cheng, J. Dalli, and C. N. Serhan. 2014. Maresin Biosynthesis and Identification of Maresin 2, a New Anti-Inflammatory and Pro-Resolving Mediator from Human Macrophages. Plos One 9: 1-9.
177. Porro, B., P. Songia, I. Squellerio, E. Tremoli, and V. Cavalca. 2014. Analysis, physiological and clinical significance of 12-HETE: a neglected platelet-derived 12-lipoxygenase product. Journal Of Chromatography. B, Analytical Technologies In The Biomedical And Life Sciences 964: 26-40.
178. Wiggins, R. E., M. S. Jafri, and A. D. Proia. 1990. 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid is a more potent neutrophil chemoattractant than the 12(R) epimer in the rat cornea. Prostaglandins 40: 131-141.
179. Dowd, P. M., A. K. Black, P. W. Woollard, and M. W. Greaves. 1987. Cutaneous responses to 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and 5,12-dihydroxyeicosatetraenoic acid (leukotriene B4) in psoriasis and normal human skin. Archives Of Dermatological Research 279: 427-434.
180. Dowd, P. M., A. K. Black, P. M. Woollard, R. D. R. Camp, and M. W. Greaves. 1985. Cutaneous Responses to 12-Hydroxy-5,8,10,14-eicosatetraenoic Acid (12-HETE). Journal of Investigative Dermatology 84: 537-541.
181. Goetzl, E. J., and F. F. Sun. 1979. Generation of unique monohydroxy-eicosatetraenoic acids from arachidonic acid by human neutrophils. The Journal Of Experimental Medicine 150: 406-411.
182. Goetzl, E. J., J. M. Woods, and R. R. Gorman. 1977. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid. The Journal Of Clinical Investigation 59: 179-183.
183. Turner, S. R., J. A. Tainer, and W. S. Lynn. 1975. Biogenesis of chemotactic molecules by the arachidonate lipoxygenase system of platelets. Nature 257: 680-681.
184. Patricia, M. K., J. A. Kim, C. M. Harper, P. T. Shih, J. A. Berliner, R. Natarajan, J. L. Nadler, and C. C. Hedrick. 1999. Lipoxygenase products increase monocyte adhesion to human aortic endothelial cells. Arteriosclerosis, Thrombosis, And Vascular Biology 19: 2615-2622.
185. Wen, Y., J. Gu, S. K. Chakrabarti, K. Aylor, J. Marshall, Y. Takahashi, T. Yoshimoto, and J. L. Nadler. 2007. The role of 12/15-lipoxygenase in the expression of interleukin-6 and tumor necrosis factor-alpha in macrophages. Endocrinology 148: 1313-1322.
186. Wen, Y., J. Gu, G. E. Vandenhoff, X. Liu, and J. L. Nadler. 2008. Role of 12/15-lipoxygenase in the expression of MCP-1 in mouse macrophages. American Journal Of Physiology. Heart And Circulatory Physiology 294: H1933-H1938.
187. Chakrabarti, S. K., B. K. Cole, W. Yeshao, S. R. Keller, and J. L. Nadler. 2009. 12/15-Lipoxygenase Products Induce Inflammation and Impair Insulin Signaling in 3T3-L1 Adipocytes. Obesity (19307381) 17: 1657-1663.
188. Chakrabarti, S. K., Y. Wen, A. D. Dobrian, B. K. Cole, Q. Ma, H. Pei, M. D. Williams, M. H. Bevard, G. E. Vandenhoff, S. R. Keller, J. Gu, and J. L. Nadler. 2011. Evidence for activation of inflammatory lipoxygenase pathways in visceral adipose tissue of obese Zucker rats. American Journal Of Physiology. Endocrinology And Metabolism 300: E175-E187.
189. Fretland, D. J., D. L. Widomski, R. L. Shone, T. D. Penning, J. M. Miyashiro, and S. W. Djuric. 1989. SC-41930 inhibits neutrophil infiltration of the cavine dermis induced by 12(R)-hydroxyeicosatetraenoic acid. Prostaglandins, Leukotrienes, And Essential Fatty Acids 38: 169-172.
190. Fretland, D. J., D. L. Widomski, J. M. Zemaitis, B. S. Tsai, S. W. Djuric, T. D. Penning, J. M. Miyashiro, and R. F. Bauer. 1989. 12(R)-hydroxyeicosatetraenoic acid is a neutrophil chemoattractant in the cavine, lapine, murine and canine dermis. Prostaglandins, Leukotrienes, And Essential Fatty Acids 37: 79-81.
191. Fretland, D. J., C. P. Anglin, M. Bremer, P. Isakson, D. L. Widomski, S. K. Paulson, S. H. Docter, S. W. Djuric, T. D. Penning, S. Yu, and a. et. 1995. Antiinflammatory effects of second-generation leukotriene B4 receptor antagonist, SC-53228: impact upon leukotriene B4- and 12(R)-HETE-mediated events. Inflammation 19: 193-205.
192. Wollard, P. M., F. M. Cunnigham, G. M. Murphy, R. D. Camp, F. F. Derm, and M. W. Greaves. 1989. A comparison of the proinflammatory effects of 12(R)- and 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid in human skin. Prostaglandins 38: 465-471.
193. Cunningham, F. M., and P. M. Woollard. 1987. 12(R)-hydroxy-5,8,10,14-eicosatetraenoic acid is a chemoattractant for human polymorphonuclear leucocytes in vitro. Prostaglandins 34: 71-78.
194. Cunningham, F. M., P. M. Woollard, and R. D. Camp. 1985. Proinflammatory properties of unsaturated fatty acids and their monohydroxy metabolites. Prostaglandins 30: 497-509.
195. Conrad, D. J., H. Kuhn, M. Mulkins, E. Highland, and E. Sigal. 1992. Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. Proceedings Of The National Academy Of Sciences Of The United States Of America 89: 217-221.
196. Profita, M., A. Sala, L. Siena, P. M. Henson, R. C. Murphy, A. Paternò, A. Bonanno, L. Riccobono, A. Mirabella, G. Bonsignore, and A. M. Vignola. 2002. Leukotriene B4 production in human mononuclear phagocytes is modulated by interleukin-4-induced 15-lipoxygenase. The Journal Of Pharmacology And Experimental Therapeutics 300: 868-875.
197. Nassar, G. M., J. D. Morrow, L. J. Roberts, 2nd, F. G. Lakkis, and K. F. Badr. 1994. Induction of 15-lipoxygenase by interleukin-13 in human blood monocytes. The Journal Of Biological Chemistry 269: 27631-27634.
198. Roy, B., and M. K. Cathcart. 1998. Induction of 15-lipoxygenase expression by IL-13 requires tyrosine phosphorylation of Jak2 and Tyk2 in human monocytes. The Journal Of Biological Chemistry 273: 32023-32029.

199. Xu, B., A. Bhattacharjee, B. Roy, G. M. Feldman, and M. K. Cathcart. 2004. Role of protein kinase C isoforms in the regulation of interleukin-13-induced 15-lipoxygenase gene expression in human monocytes. The Journal Of Biological Chemistry 279: 15954-15960.
200. Bhattacharjee, A., B. Xu, D. A. Frank, G. M. Feldman, and M. K. Cathcart. 2006. Monocyte 15-lipoxygenase expression is regulated by a novel cytosolic signaling complex with protein kinase C delta and tyrosine-phosphorylated Stat3. Journal Of Immunology (Baltimore, Md.: 1950) 177: 3771-3781.
201. Chaitidis, P., V. O'Donnell, R. J. Kuban, A. Bermudez-Fajardo, U. Ungethuem, and H. Kühn. 2005. Gene expression alterations of human peripheral blood monocytes induced by medium-term treatment with the TH2-cytokines interleukin-4 and -13. Cytokine 30: 366-377.
202. Bhattacharjee, A., M. Shukla, V. P. Yakubenko, A. Mulya, S. Kundu, and M. K. Cathcart. 2013. IL-4 and IL-13 employ discrete signaling pathways for target gene expression in alternatively activated monocytes/macrophages. Free Radical Biology & Medicine 54: 1-16.
203. Abrial, C., S. Grassin-Delyle, H. Salvator, M. Brollo, E. Naline, and P. Devillier. 2015. 15-Lipoxygenases regulate the production of chemokines in human lung macrophages. British Journal of Pharmacology.
204. Wuest, S. J. A., M. Crucet, C. Gemperle, C. Loretz, and M. Hersberger. 2012. Expression and regulation of 12/15-lipoxygenases in human primary macrophages. Atherosclerosis 225: 121-127.
205. Heydeck, D., L. Thomas, K. Schnurr, F. Trebus, W. E. Thierfelder, J. N. Ihle, and H. Kühn. 1998. Interleukin-4 and -13 induce upregulation of the murine macrophage 12/15-lipoxygenase activity: evidence for the involvement of transcription factor STAT6. Blood 92: 2503-2510.
206. Kühn, H., J. Barnett, D. Grunberger, P. Baecker, J. Chow, B. Nguyen, H. Bursztyn-Pettegrew, H. Chan, and E. Sigal. 1993. Overexpression, purification and characterization of human recombinant 15-lipoxygenase. Biochimica Et Biophysica Acta 1169: 80-89.
207. Chen, X. S., U. Kurre, N. A. Jenkins, N. G. Copeland, and C. D. Funk. 1994. cDNA cloning, expression, mutagenesis of C-terminal isoleucine, genomic structure, and chromosomal localizations of murine 12-lipoxygenases. The Journal Of Biological Chemistry 269: 13979-13987.
208. Brezinski, M. E., and C. N. Serhan. 1990. Selective incorporation of (15S)-hydroxyeicosatetraenoic acid in phosphatidylinositol of human neutrophils: agonist-induced deacylation and transformation of stored hydroxyeicosanoids. Proceedings Of The National Academy Of Sciences Of The United States Of America 87: 6248-6252.
209. Profita, M., A. Sala, L. Riccobono, E. Pace, A. Paternò, S. Zarini, L. Siena, A. Mirabella, G. Bonsignore, and A. M. Vignola. 2000. 15(S)-HETE modulates LTB(4) production and neutrophil chemotaxis in chronic bronchitis. American Journal Of Physiology. Cell Physiology 279: C1249-C1258.
210. Ternowitz, T., K. Fogh, and K. Kragballe. 1988. 15-Hydroxyeicosatetraenoic acid (15-HETE) specifically inhibits LTB4-induced chemotaxis of human neutrophils. Skin Pharmacology: The Official Journal Of The Skin Pharmacology Society 1: 93-99.
211. Fischer, D. B., J. W. Christman, and K. F. Badr. 1992. Fifteen-S-hydroxyeicosatetraenoic acid (15-S-HETE) specifically antagonizes the chemotactic action and glomerular synthesis of leukotriene B4 in the rat. Kidney International 41: 1155-1160.
212. Takata, S., M. Matsubara, P. G. Allen, P. A. Janmey, C. N. Serhan, and H. R. Brady. 1994. Remodeling of neutrophil phospholipids with 15(S)-hydroxyeicosatetraenoic acid inhibits leukotriene B4-induced neutrophil migration across endothelium. The Journal Of Clinical Investigation 93: 499-508.
213. Sultana, C., Y. Shen, V. Rattan, and V. K. Kalra. 1996. Lipoxygenase metabolites induced expression of adhesion molecules and transendothelial migration of monocyte-like HL-60 cells is linked to protein kinase C activation. Journal Of Cellular Physiology 167: 477-487.
214. Brash, A. R., W. E. Boeglin, and M. S. Chang. 1997. Discovery of a second 15S-lipoxygenase in humans. Proceedings Of The National Academy Of Sciences Of The United States Of America 94: 6148-6152.
215. Jisaka, M., R. B. Kim, W. E. Boeglin, L. B. Nanney, and A. R. Brash. 1997. Molecular cloning and functional expression of a phorbol ester-inducible 8S-lipoxygenase from mouse skin. The Journal Of Biological Chemistry 272: 24410-24416.
216. Hilberg, T., H.-P. Deigner, E. Möller, R. A. Claus, A. Ruryk, D. Gläser, J. Landre, F. M. Brunkhorst, K. Reinhart, H. H. W. Gabriel, and S. Russwurm. 2005. Transcription in response to physical stress--clues to the molecular mechanisms of exercise-induced asthma. FASEB Journal: Official Publication Of The Federation Of American Societies For Experimental Biology 19: 1492-1494.
217. Loell, I., L. Alemo Munters, J. Pandya, M. Zong, H. Alexanderson, A. E. Fasth, C. Ståhl Hallengren, O. Rådmark, I. E. Lundberg, P.-J. Jakobsson, and M. Korotkova. 2013. Activated LTB4 pathway in muscle tissue of patients with polymyositis or dermatomyositis. Annals Of The Rheumatic Diseases 72: 293-299.
218. Zuo, L., F. L. Christofi, V. P. Wright, S. Bao, and T. L. Clanton. 2004. Lipoxygenase-dependent superoxide release in skeletal muscle. Journal Of Applied Physiology (Bethesda, Md.: 1985) 97: 661-668.
219. Sun, R., X. Ba, L. Cui, Y. Xue, and X. Zeng. 2009. Leukotriene B4 regulates proliferation and differentiation of cultured rat myoblasts via the BLT1 pathway. Molecules And Cells 27: 403-408.
220. Spite, M., J. Hellmann, Y. Tang, S. P. Mathis, M. Kosuri, A. Bhatnagar, V. R. Jala, and B. Haribabu. 2011. Deficiency of the leukotriene B4 receptor, BLT-1, protects against systemic insulin resistance in diet-induced obesity. Journal Of Immunology (Baltimore, Md.: 1950) 187: 1942-1949.
221. Almeida, M., E. Ambrogini, L. Han, S. C. Manolagas, and R. L. Jilka. 2009. Increased Lipid Oxidation Causes Oxidative Stress, Increased Peroxisome Proliferator-activated Receptor-γ Expression, and Diminished Pro-osteogenic Wnt Signaling in the Skeleton. Journal of Biological Chemistry 284: 27438-27448.
222. Smith, H. J., M. J. Lorite, and M. J. Tisdale. 1999. Effect of a cancer cachectic factor on protein synthesis/degradation in murine C2C12 myoblasts: modulation by eicosapentaenoic acid. Cancer Research 59: 5507-5513.

223. Li, Z., F. L. Christofi, V. P. Wright, B. Shengying, and T. L. Clanton. 2004. Lipoxygenase-dependent superoxide release in skeletal muscle. Journal of Applied Physiology 97: 661-668.
224. Bhattacharya, A., R. Hamilton, A. Jernigan, Y. Zhang, M. Sabia, M. M. Rahman, Y. Li, R. Wei, A. Chaudhuri, and H. Van Remmen. 2014. Genetic ablation of 12/15-lipoxygenase but not 5-lipoxygenase protects against denervation-induced muscle atrophy. Free Radical Biology & Medicine 67: 30-40.
225. Whitehouse, A. S., J. Khal, and M. J. Tisdale. 2003. Induction of protein catabolism in myotubes by 15(S)-hydroxyeicosatetraenoic acid through increased expression of the ubiquitin-proteasome pathway. British Journal of Cancer 89: 737.
226. Wyke, S. M., J. Khal, and M. J. Tisdale. 2005. Signalling pathways in the induction of proteasome expression by proteolysis-inducing factor in murine myotubes. Cellular Signalling 17: 67-75.
227. Capra, V., G. E. Rovati, P. Mangano, C. Buccellati, R. C. Murphy, and A. Sala. 2015. Transcellular biosynthesis of eicosanoid lipid mediators. BBA - Molecular & Cell Biology of Lipids 1851: 377-382.
228. Serhan, C. N., M. Hamberg, and B. Samuelsson. 1984. Trihydroxytetraenes: a novel series of compounds formed from arachidonic acid in human leukocytes. Biochemical And Biophysical Research Communications 118: 943-949.
229. Serhan, C. N., M. Hamberg, and B. Samuelsson. 1984. Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. Proceedings Of The National Academy Of Sciences Of The United States Of America 81: 5335-5339.
230. Colgan, S. P., C. N. Serhan, C. A. Parkos, C. Delp-Archer, and J. L. Madara. 1993. Lipoxin A4 modulates transmigration of human neutrophils across intestinal epithelial monolayers. The Journal Of Clinical Investigation 92: 75-82.
231. Fierro, I. M., S. P. Colgan, G. Bernasconi, N. A. Petasis, C. B. Clish, M. Arita, and C. N. Serhan. 2003. Lipoxin A4 and aspirin-triggered 15-epi-lipoxin A4 inhibit human neutrophil migration: comparisons between synthetic 15 epimers in chemotaxis and transmigration with microvessel endothelial cells and epithelial cells. Journal Of Immunology (Baltimore, Md.: 1950) 170: 2688-2694.
232. Lee, T. H., C. E. Horton, U. Kyan-Aung, D. Haskard, A. E. Crea, and B. W. Spur. 1989. Lipoxin A4 and lipoxin B4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B4 and N-formyl-L-methionyl-L-leucyl-L-phenylalanine. Clinical Science (London, England: 1979) 77: 195-203.
233. Maddox, J. F., S. P. Colgan, C. B. Clish, N. A. Petasis, V. V. Fokin, and C. N. Serhan. 1998. Lipoxin B4 regulates human monocyte/neutrophil adherence and motility: design of stable lipoxin B4 analogs with increased biologic activity. FASEB Journal: Official Publication Of The Federation Of American Societies For Experimental Biology 12: 487-494.
234. Gewirtz, A. T., V. V. Fokin, N. A. Petasis, C. N. Serhan, and J. L. Madara. 1999. LXA4, aspirin-triggered 15-epi-LXA4, and their analogs selectively downregulate PMN azurophilic degranulation. The American Journal Of Physiology 276: C988-C994.
235. Maddox, J. F., and C. N. Serhan. 1996. Lipoxin A4 and B4 are potent stimuli for human monocyte migration and adhesion: selective inactivation by dehydrogenation and reduction. The Journal Of Experimental Medicine 183: 137-146.
236. Maddox, J. F., M. Hachicha, T. Takano, N. A. Petasis, V. V. Fokin, and C. N. Serhan. 1997. Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein-linked lipoxin A4 receptor. The Journal Of Biological Chemistry 272: 6972-6978.
237. Romano, M., J. F. Maddox, and C. N. Serhan. 1996. Activation of human monocytes and the acute monocytic leukemia cell line (THP-1) by lipoxins involves unique signaling pathways for lipoxin A4 versus lipoxin B4: evidence for differential Ca2+ mobilization. Journal Of Immunology (Baltimore, Md.: 1950) 157: 2149-2154.
238. Soyombo, O., B. W. Spur, and T. H. Lee. 1994. Effects of lipoxin A4 on chemotaxis and degranulation of human eosinophils stimulated by platelet-activating factor and N-formyl-L-methionyl-L-leucyl-L-phenylalanine. Allergy 49: 230-234.
239. Godson, C., S. Mitchell, K. Harvey, N. A. Petasis, N. Hogg, and H. R. Brady. 2000. Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. Journal Of Immunology (Baltimore, Md.: 1950) 164: 1663-1667.
240. Svensson, C. I., M. Zattoni, and C. N. Serhan. 2007. Lipoxins and aspirin-triggered lipoxin inhibit inflammatory pain processing. The Journal Of Experimental Medicine 204: 245-252.
241. Romano, M., X. S. Chen, Y. Takahashi, S. Yamamoto, C. D. Funk, and C. N. Serhan. 1993. Lipoxin synthase activity of human platelet 12-lipoxygenase. The Biochemical Journal 296 (Pt 1): 127-133.
242. Romano, M., and C. N. Serhan. 1992. Lipoxin generation by permeabilized human platelets. Biochemistry 31: 8269-8277.
243. Serhan, C. N., and K. A. Sheppard. 1990. Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro. The Journal Of Clinical Investigation 85: 772-780.
244. Edenius, C., J. Haeggström, and J. A. Lindgren. 1988. Transcellular conversion of endogenous arachidonic acid to lipoxins in mixed human platelet-granulocyte suspensions. Biochemical And Biophysical Research Communications 157: 801-807.
245. Fiore, S., and C. N. Serhan. 1990. Formation of lipoxins and leukotrienes during receptor-mediated interactions of human platelets and recombinant human granulocyte/macrophage colony-stimulating factor-primed neutrophils. The Journal Of Experimental Medicine 172: 1451-1457.
246. Serhan, C. N. 1989. On the relationship between leukotriene and lipoxin production by human neutrophils: evidence for differential metabolism of 15-HETE and 5-HETE. Biochimica Et Biophysica Acta 1004: 158-168.
247. Serhan, C. N., M. Hamberg, B. Samuelsson, J. Morris, and D. G. Wishka. 1986. On the stereochemistry and biosynthesis of lipoxin B. Proceedings Of The National Academy Of Sciences Of The United States Of America 83: 1983-1987.
248. Serhan, C. N., K. C. Nicolaou, S. E. Webber, C. A. Veale, S. E. Dahlén, T. J. Puustinen, and B. Samuelsson. 1986. Lipoxin A. Stereochemistry and biosynthesis. The Journal Of Biological Chemistry 261: 16340-16345.
249. Roth, G. J., N. Stanford, and P. W. Majerus. 1975. Acetylation of prostaglandin synthase by aspirin. Proceedings Of The National Academy Of Sciences Of The United States Of America 72: 3073-3076.

250. Lecomte, M., O. Laneuville, C. Ji, D. L. DeWitt, and W. L. Smith. 1994. Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. The Journal Of Biological Chemistry 269: 13207-13215.
251. Patrignani, P., M. R. Panara, M. G. Sciulli, G. Santini, G. Renda, and C. Patrono. 1997. Differential inhibition of human prostaglandin endoperoxide synthase-1 and -2 by nonsteroidal anti-inflammatory drugs. Journal Of Physiology And Pharmacology: An Official Journal Of The Polish Physiological Society 48: 623-631.
252. Holtzman, M. J., J. Turk, and L. P. Shornick. 1992. Identification of a pharmacologically distinct prostaglandin H synthase in cultured epithelial cells. The Journal Of Biological Chemistry 267: 21438-21445.
253. Mancini, J. A., G. P. O'Neill, C. Bayly, and P. J. Vickers. 1994. Mutation of serine-516 in human prostaglandin G/H synthase-2 to methionine or aspirin acetylation of this residue stimulates 15-R-HETE synthesis. FEBS Letters 342: 33-37.
254. Clària, J., and C. N. Serhan. 1995. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. Proceedings Of The National Academy Of Sciences Of The United States Of America 92: 9475-9479.
255. Hu, S., Q.-L. Mao-Ying, J. Wang, Z.-F. Wang, W.-L. Mi, X.-W. Wang, J.-W. Jiang, Y.-L. Huang, G.-C. Wu, and Y.-Q. Wang. 2012. Lipoxins and aspirin-triggered lipoxin alleviate bone cancer pain in association with suppressing expression of spinal proinflammatory cytokines. Journal Of Neuroinflammation 9: 278-278.
256. Li, Q., Y. Tian, Z. F. Wang, S. B. Liu, W. L. Mi, H. J. Ma, G. C. Wu, J. Wang, J. Yu, and Y. Q. Wang. 2013. Involvement of the spinal NALP1 inflammasome in neuropathic pain and aspirin-triggered-15-epi-lipoxin A4 induced analgesia. Neuroscience 254: 230-240.
257. Serhan, C. N., C. B. Clish, J. Brannon, S. P. Colgan, N. Chiang, and K. Gronert. 2000. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. The Journal Of Experimental Medicine 192: 1197-1204.
258. Serhan, C. N., S. Hong, K. Gronert, S. P. Colgan, P. R. Devchand, G. Mirick, and R.-L. Moussignac. 2002. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. The Journal Of Experimental Medicine 196: 1025-1037.
259. Arita, M., F. Bianchini, J. Aliberti, A. Sher, N. Chiang, S. Hong, R. Yang, N. A. Petasis, and C. N. Serhan. 2005. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. The Journal Of Experimental Medicine 201: 713-722.
260. Arita, M., C. B. Clish, and C. N. Serhan. 2005. The contributions of aspirin and microbial oxygenase to the biosynthesis of anti-inflammatory resolvins: novel oxygenase products from omega-3 polyunsaturated fatty acids. Biochemical And Biophysical Research Communications 338: 149-157.
261. Ogawa, S., D. Urabe, Y. Yokokura, H. Arai, M. Arita, and M. Inoue. 2009. Total synthesis and bioactivity of resolvin E2. Organic Letters 11: 3602-3605.
262. Oh, S. F., M. Dona, G. Fredman, S. Krishnamoorthy, D. Irimia, and C. N. Serhan. 2012. Resolvin E2 formation and impact in inflammation resolution. Journal Of Immunology (Baltimore, Md.: 1950) 188: 4527-4534.
263. Tjonahen, E., S. F. Oh, J. Siegelman, S. Elangovan, K. B. Percarpio, S. Hong, M. Arita, and C. N. Serhan. 2006. Resolvin E2: identification and anti-inflammatory actions: pivotal role of human 5-lipoxygenase in resolvin E series biosynthesis. Chemistry & Biology 13: 1193-1202.
264. Arita, M., T. Ohira, Y.-P. Sun, S. Elangovan, N. Chiang, and C. N. Serhan. 2007. Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. Journal Of Immunology (Baltimore, Md.: 1950) 178: 3912-3917.
265. Oh, S. F., P. S. Pillai, A. Recchiuti, R. Yang, and C. N. Serhan. 2011. Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. The Journal Of Clinical Investigation 121: 569-581.
266. Isobe, Y., M. Arita, R. Iwamoto, D. Urabe, H. Todoroki, K. Masuda, M. Inoue, and H. Arai. 2013. Stereochemical assignment and anti-inflammatory properties of the omega-3 lipid mediator resolvin E3. Journal of Biochemistry 153: 355-360.
267. Isobe, Y., M. Arita, S. Matsueda, R. Iwamoto, T. Fujihara, H. Nakanishi, R. Taguchi, K. Masuda, K. Sasaki, D. Urabe, M. Inoue, and H. Arai. 2012. Identification and structure determination of novel anti-inflammatory mediator resolvin E3, 17,18-dihydroxyeicosapentaenoic acid. The Journal Of Biological Chemistry 287: 10525-10534.
268. Urabe, D., H. Todoroki, K. Masuda, and M. Inoue. 2012. Total syntheses of four possible stereoisomers of resolvin E3. Tetrahedron 68: 3210-3219.
269. Hong, S., K. Gronert, P. R. Devchand, R.-L. Moussignac, and C. N. Serhan. 2003. Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. The Journal Of Biological Chemistry 278: 14677-14687.
270. Marcheselli, V. L., S. Hong, W. J. Lukiw, X. H. Tian, K. Gronert, A. Musto, M. Hardy, J. M. Gimenez, N. Chiang, C. N. Serhan, and N. G. Bazan. 2003. Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. The Journal Of Biological Chemistry 278: 43807-43817.
271. Serhan, C. N., K. Gotlinger, S. Hong, and M. Arita. 2004. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis. Prostaglandins & Other Lipid Mediators 73: 155-172.
272. Chiang, N., J. Dalli, R. A. Colas, and C. N. Serhan. 2015. Identification of resolvin D2 receptor mediating resolution of infections and organ protection. The Journal Of Experimental Medicine 212: 1203-1217.
273. Dalli, J., J. W. Winkler, R. A. Colas, H. Arnardottir, C.-Y. C. Cheng, N. Chiang, N. A. Petasis, and C. N. Serhan. 2013. Resolvin D3 and aspirin-triggered resolvin D3 are potent immunoresolvents. Chemistry & Biology 20: 188-201.
274. Sun, Y.-P., S. F. Oh, J. Uddin, R. Yang, K. Gotlinger, E. Campbell, S. P. Colgan, N. A. Petasis, and C. N. Serhan. 2007. Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation. The Journal Of Biological Chemistry 282: 9323-9334.
275. Spite, M., L. V. Norling, L. Summers, R. Yang, D. Cooper, N. A. Petasis, R. J. Flower, M. Perretti, and C. N. Serhan. 2009. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. Nature 461: 1287-1291.

276. Mukherjee, P. K., V. L. Marcheselli, C. N. Serhan, and N. G. Bazan. 2004. Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. Proceedings Of The National Academy Of Sciences Of The United States Of America 101: 8491-8496.
277. Serhan, C. N., K. Gotlinger, S. Hong, Y. Lu, J. Siegelman, T. Baer, R. Yang, S. P. Colgan, and N. A. Petasis. 2006. Anti-inflammatory actions of neuroprotectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes. Journal Of Immunology (Baltimore, Md.: 1950) 176: 1848-1859.
278. Serhan, C. N., G. Fredman, R. Yang, S. Karamnov, L. S. Belayev, N. G. Bazan, M. Zhu, J. W. Winkler, and N. A. Petasis. 2011. Novel proresolving aspirin-triggered DHA pathway. Chemistry & Biology 18: 976-987.
279. Bazan, N. G., T. N. Eady, L. Khoutorova, K. D. Atkins, S. Hong, Y. Lu, C. Zhang, B. Jun, A. Obenaus, G. Fredman, M. Zhu, J. W. Winkler, N. A. Petasis, C. N. Serhan, and L. Belayev. 2012. Novel aspirin-triggered neuroprotectin D1 attenuates cerebral ischemic injury after experimental stroke. Experimental Neurology 236: 122-130.
280. Serhan, C. N., R. Yang, K. Martinod, K. Kasuga, P. S. Pillai, T. F. Porter, S. F. Oh, and M. Spite. 2009. Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. The Journal Of Experimental Medicine 206: 15-23.
281. Dalli, J., Z. Min, N. A. Vlasenko, D. Bin, J. Z. Haeggström, N. A. Petasis, and C. N. Serhan. 2013. The novel 13S,14S-epoxy-maresin is converted by human macrophages to maresin 1 (MaR1), inhibits leukotriene A4 hydrolase (LTA4H), and shifts macrophage phenotype. FASEB Journal 27: 2573-2583.
282. Serhan, C. N., J. Dalli, S. Karamnov, A. Choi, P. Chul-Kyu, X. Zhen-Zhong, J. Ru-Rong, Z. Min, and N. A. Petasis. 2012. Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain. FASEB Journal 26: 1755-1765.
283. Gangemi, S., G. Luciotti, E. D'Urbano, A. Mallamace, D. Santoro, G. Bellinghieri, G. Davi, and M. Romano. 2003. Physical exercise increases urinary excretion of lipoxin A4 and related compounds. Journal Of Applied Physiology (Bethesda, Md.: 1985) 94: 2237-2240.
284. Dalli, J., N. Chiang, and C. N. Serhan. 2015. Elucidation of novel 13-series resolvins that increase with atorvastatin and clear infections. Nature Medicine.
285. Bannenberg, G. L., N. Chiang, A. Ariel, M. Arita, E. Tjonahen, K. H. Gotlinger, S. Hong, and C. N. Serhan. 2005. Molecular circuits of resolution: formation and actions of resolvins and protectins. Journal Of Immunology (Baltimore, Md.: 1950) 174: 4345-4355.
286. Levy, B. D., C. B. Clish, B. Schmidt, K. Gronert, and C. N. Serhan. 2001. Lipid mediator class switching during acute inflammation: signals in resolution. Nature Immunology 2: 612.
287. Norris, P. C., D. Gosselin, D. Reichart, C. K. Glass, and E. A. Dennis. 2014. Phospholipase A2 regulates eicosanoid class switching during inflammasome activation. Proceedings Of The National Academy Of Sciences Of The United States Of America 111: 12746-12751.
288. Schwab, J. M., N. Chiang, M. Arita, and C. N. Serhan. 2007. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. Nature 447: 869-874.
289. Bizzarro, V., R. Belvedere, F. Dal Piaz, L. Parente, and A. Petrella. 2012. Annexin A1 Induces Skeletal Muscle Cell Migration Acting through Formyl Peptide Receptors. Plos One 7: 1-11.
290. Bizzarro, V., B. Fontanella, S. Franceschelli, M. Pirozzi, H. Christian, L. Parente, and A. Petrella. 2010. Role of Annexin A1 in mouse myoblast cell differentiation. Journal Of Cellular Physiology 224: 757-765.
291. Krishnamoorthy, S., A. Recchiuti, N. Chiang, G. Fredman, and C. N. Serhan. 2012. Resolvin D1 receptor stereoselectivity and regulation of inflammation and proresolving microRNAs. The American Journal Of Pathology 180: 2018-2027.
292. Issa, M. E., S. Muruganandan, M. C. Ernst, S. D. Parlee, B. A. Zabel, E. C. Butcher, C. J. Sinal, and K. B. Goralski. 2012. Chemokine-like receptor 1 regulates skeletal muscle cell myogenesis. American Journal of Physiology: Cell Physiology 302: C1621-C1631.
293. Beaulieu, D., P. Thebault, R. Pelletier, P. Chapdelaine, M. Tarnopolsky, D. Furling, and J. Puymirat. 2012. Abnormal prostaglandin E2 production blocks myogenic differentiation in myotonic dystrophy. Neurobiology Of Disease 45: 122-129.
294. Melin, E., E. Lindroos, I. E. Lundberg, K. Borg, and M. Korotkova. 2014. Elevated expression of prostaglandin E2 synthetic pathway in skeletal muscle of prior polio patients. Journal Of Rehabilitation Medicine 46: 67-72.
295. White, P. J., M. Arita, R. Taguchi, J. X. Kang, and A. Marette. 2010. Transgenic Restoration of Long-Chain n-3 Fatty Acids in Insulin Target Tissues Improves Resolution Capacity and Alleviates Obesity-Linked Inflammation and Insulin Resistance in High-Fat-Fed Mice. Diabetes 59: 3066-3073.
296. White, P. J., P. St-Pierre, A. Charbonneau, P. L. Mitchell, E. St-Amand, B. Marcotte, and A. Marette. 2014. Protectin DX alleviates insulin resistance by activating a myokine-liver glucoregulatory axis. Nature Medicine 20: 664-669.
297. Peake, J. M., J. F. Markworth, K. Nosaka, T. Raastad, G. D. Wadley, and V. G. Coffey. 2015. Modulating exercise-induced hormesis: Does less equal more? Journal of Applied Physiology 119: 172-189.
298. Gilroy, D. W., P. R. Colville-Nash, D. Willis, J. Chivers, M. J. Paul-Clark, and D. A. Willoughby. 1999. Inducible cyclooxygenase may have anti-inflammatory properties. Nature Medicine 5: 698.
299. Roberts, L. A., T. Raastad, J. F. Markworth, V. C. Figueiredo, I. M. Egner, A. Shield, D. Cameron-Smith, J. S. Coombes, and J. M. Peake. 2015. Post-exercise cold water immersion attenuates acute anabolic signalling and long-term adaptations in muscle to strength training. J Physiol 593: 4285-4301.
300. Mo, C., S. Romero-Suarez, L. Bonewald, M. Johnson, and M. Brotto. 2012. Prostaglandin E2: from clinical applications to its potential role in bone- muscle crosstalk and myogenic differentiation. Recent patents on biotechnology 6: 223-229.
301. Standley, R. A., S. Z. Liu, B. Jemiolo, S. W. Trappe, and T. A. Trappe. 2013. Prostaglandin E2 induces transcription of skeletal muscle mass regulators interleukin-6 and muscle RING finger-1 in humans. Prostaglandins Leukot Essent Fatty Acids 88: 361-364.

Instructions for authors of EIR

EIR usually solicits papers from authors with acknowledged expertise in the field to be covered. Unsolicited papers will be considered and can also be accepted. All papers are subject to a peer review process.

Usually the manuscripts will fit into one of two major categories: i. a review which thoroughly covers the area indicated in the heading and includes structuring and critical discussion of existing knowledge and, if possible, the ideas of the authors about potential practical consequences and future developments. Mere mentioning and listing of existing literature is not considered to be a good review. The review can be long, if necessary, or short, if the field covered by the heading is relatively new or very focussed. ii. a paper showing original data accompanied by an extended, review-type discussion.

The general format of the review is somewhat flexible. A review must however have an abstract, an introduction and a conclusion around the main sections. Reviews with three or more sections should list the headings of the sections in form of a bullet point table at the end of the introduction. Longer sections should also give a short interim summary at their end. If substantial amounts of the authors‘ own new data are to be shown, a section on methods and on results must be included. Data will only be accepted, if methods are stated clearly and appropriate statistical evaluation of results is given.

Other types of papers, eg true meta-analyses of a circumscribed sector of literature or papers focussing on new ideas or hypotheses may also be considered. Interested authors, please contact the editorial board.

For reference style use the one as applied by J. Appl. Physiol., with references listed in alphabetical order. In text use ref. numbers in brackets. When giving more than 1 reference in one bracket, use numerical order. The maximum number of references is normally 150.

A short running head should appear after the title, followed by the authors and their respective affiliations. The full address of correspondence should include an e-mail address of the correspondent author. Up to five key words should be added after the abstract.

Please use e-mail for all communications including manuscript submission (word or pdf-file) if possible and paste "EIR” in the subject field of your mailing program. Generally, submission deadline for the next issue of EIR is July, 31th.

Send manuscripts to:
Justus-Liebig- Universität Gießen
Abteilung für Sportmedizin
Dr. Karsten Krüger
Kugelberg 62
35394 Gießen
E-mail: karsten.krueger@sport.uni-giessen.de

TOMsystemdruck

ISSN 1077-5552