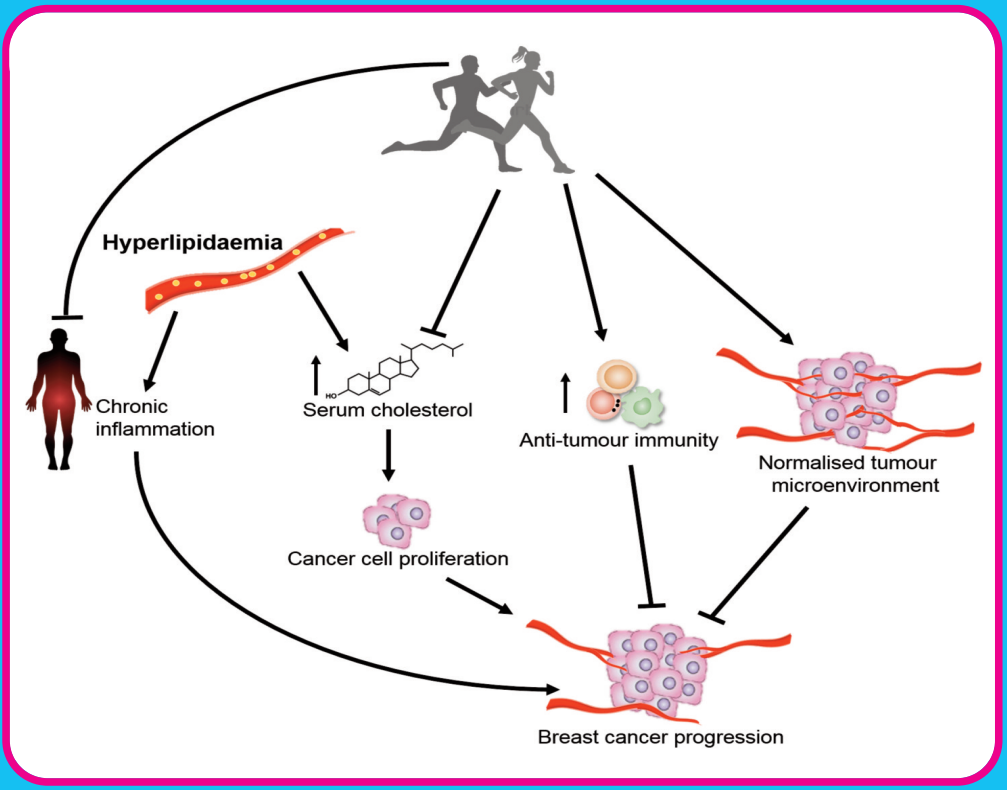
EXERCISE IMMUNOLOGY REVIEW



VOLUME 24 • 2018

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 24 • 2018

Contents

Exercise Immunology Review

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:
Prof. Dr. Karsten Krüger, Institute of Sport Science, Dept. of Exercise and Health, University of Hannover, Am Moritzwinkel 6, 30167 Hannover, Germany, karsten.krueger@sportwiss.uni-hannover.de

Editor-in-Chief: Prof. Dr. Karsten Krüger
Past Editor: Prof. Dr. Hinnak Northoff

Send editorial correspondence to:
Prof. Dr. Karsten Krüger
Institute of Sport Science, Dept. of Exercise and Health,
University of Hannover
Am Moritzwinkel 6,
30167 Hannover, Germany.
karsten.krueger@sportwiss.uni-hannover.de

Copyright © 2002 by Hinnak Northoff. *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.

Notice: authorization to photocopy items is granted for internal or personal use only. All other cases contact Karsten Krüger.

To subscribe or renew subscription go to http://www.isei.dk

From the Editors

This year's issue of EIR contains 6 articles. Buss and Dachs present an article about the alleviating influence of exercise on the pro-tumorigenic effects of hyperlipidaemia via modulation of cytokine release and via improved immune responses. Edwards et al. demonstrate a highly relevant data that the state-anxiety and perceived psychological stress levels before exercise play an important role for the immune response after exercise. The results might be important for patients, athletes and military personnel experiencing higher levels of psychological stress. Minuzzo et al. present a study which indicate that the maintenance of high levels of aerobic fitness during of aging delays immunosenescence possibly by preventing the accumulation of senescent T-cells. An original study by Rigonato-Oliveira et al. demonstrates that exercise inhibited LPS-induced lung inflammation by attenuating inflammatory cytokines and oxidative stress markers in mice and human cell culture via enhanced IL-10 production. The last two manuscripts are review articles. Highton et al. focused on the role of microparticles in clinical populations and comment on the use of exercise and dietary manipulation as therapeutic strategies. Finally, the systematic review of Rada et al. about the effect of exercise on TLR expression during obesity concluded that animal studies showed a marked tendency towards TLR2 and 4 down-regulation after endurance exercise, which is not sufficiently robust to conclude for humans.

For EIR25 and the future, as always, we would like 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 EIR25 is 31st July 2018. We hope you enjoy reading the new issue. Thank you (again), 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 Ana Teixeira and the other organizers, for the fantastic ISEI meeting in July 2017 in Coimbra, Portugal.

Thank you all for your ongoing support of EIR. A special thanks to all the authors of EIR24.

On behalf of the Editors,

Karsten Krüger

The Role of Exercise and Hyperlipidaemia in Breast Cancer Progression

Linda A. Buss and Gabi U. Dachs*

Mackenzie Cancer Research Group, Department of Pathology, University of Otago, Christchurch, New Zealand

ABSTRACT

Exercise reduces the risk of breast cancer development and improves survival in breast cancer patients. However, the underlying mechanisms of this protective effect remain to be fully elucidated. It is unclear whether exercise can attenuate or modify the pro-tumour effects of obesity and related conditions, such as hyperlipidaemia. This review summarises how hyperlipidaemia and exercise contribute to or reduce breast cancer risk and progression, respectively, and highlights the possible mechanisms behind each. In particular, the effects of exercise and hyperlipidaemia on the immune microenvironment of tumours is analysed. The potential value of commonly investigated circulating factors as exercise-modulated, prognostic biomarkers is also discussed. We propose that exercise may alleviate some of the pro-tumorigenic effects of hyperlipidaemia through the reduction of blood lipid levels and modulation of cytokine release to induce beneficial changes in the tumour microenvironment.

Key words: Breast cancer, cholesterol, physical activity, immunity, tumour microenvironment

INTRODUCTION

Breast cancer is a global health concern. It is the most common cancer diagnosed in women (193) and the fifth largest contributor to cancer deaths worldwide (23). As such, understanding the underlying mechanisms of disease progression is vital to providing better therapies and prevention.

The purpose of this review is to explore the association between exercise and (breast) cancer progression, and to highlight potential mechanisms underlying the exercise-prognosis relationship. Types of exercise include resistance training, aerobic exercise and training that includes a mindfulness component such as Tai chi or yoga. The role of obesity and associated hyperlipidaemia (abnormal elevation of serum lipid levels) in breast cancer risk and progression will be examined, and the value of some commonly investigated circulating factors as exercise-modulated, prognostic biomarkers will be discussed.

OBESITY, HYPERLIPIDAEMIA AND BREAST CANCER

Obesity is an increasing health problem in developed countries worldwide. It has been associated with an increased risk of developing breast cancer, as well as with a poorer prognosis (11, 141, 158). It has been shown that adipocytes in the immediate vicinity of the tumour (cancer associated adipocytes) interact with breast cancer cells, causing them to become more invasive and providing them with metabolites (145). In addition, obese adipose tissue is characterised by chronic, low-grade inflammation. Hypertrophic adipocytes in obese adipose tissue can grow to a size of 150-200 μm in diameter, thus reaching or exceeding the maximum oxygen diffusion distance (192). This results in a hypoxic state, leading to the activation of hypoxia inducible factors (HIF), subsequent tissue fibrosis and the increased secretion of inflammatory adipokines, as well as macrophage infiltration (192). This low-grade, chronic inflammatory state has been associated with further metabolic dysregulation (192).

Hyperlipidaemia is commonly comorbid with obesity, but its implications as an independent risk or prognostic factor in breast cancer are much less clear. Preclinical studies show that hyperlipidaemia increases breast tumour growth rate, incidence and metastasis (3, 100, 112, 153, 163). In addition, breast tumours from hyperlipidaemic mice are more proliferative (100, 153, 163), have reduced apoptosis (153) and show increased microvessel density (100, 112, 153).
However, epidemiological studies have produced contradictory results regarding the effect of hyperlipidaemia, particularly hypercholesterolaemia, on breast cancer risk and progression, with some studies showing that hyperlipidaemia increases risk and/or progression (10, 43, 97, 101, 157, 164), and others showing that it reduces or does not change risk and/or rate of progression (42, 58, 65, 177, 183). In connection with this, the role of statins and other lipid lowering drugs on breast cancer risk and progression is unclear (26, 42, 97, 104, 138, 159, 186). However, two recent meta-analyses of observational studies found that statin use was associated with reduced breast cancer recurrence and/or mortality (121, 204). These discrepancies may be due to the inherent limitations of epidemiological studies, as well as differences in methodology, influences of different cancer treatments and possible differ-

* *Corresponding author:*
Associate Professor G.U. Dachs,
University of Otago Christchurch, 2 Riccarton Ave, Christchurch 8011, New Zealand; gabi.dachs@otago.ac.nz

ences in the roles of high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C). Indeed, a recent study found that breast cancer patients with pre-operatively high levels of serum HDL-C had improved overall survival, while abnormal LDL-C and total cholesterol were not significantly correlated with prognosis (107). Furthermore, serum cholesterol may be reduced in cancer patients, as it is rapidly utilised by proliferating tumour cells (37, 63). It is also possible that some results from studies examining the role of serum cholesterol in breast cancer risk may be confounded by undiagnosed malignancies (83). Hence, the role of hyperlipidaemia and specifically the role of different types of cholesterol in breast cancer patients requires further investigation.

Proposed Molecular Mechanisms of Cholesterol in Cancer

A number of mechanisms to explain the observed pro-tumorigenic effect of hyperlipidaemia in rodent studies have been proposed, mainly focussing on the effect of hypercholesterolaemia (Figure 1). Firstly, Alikhani *et al.* demonstrated that

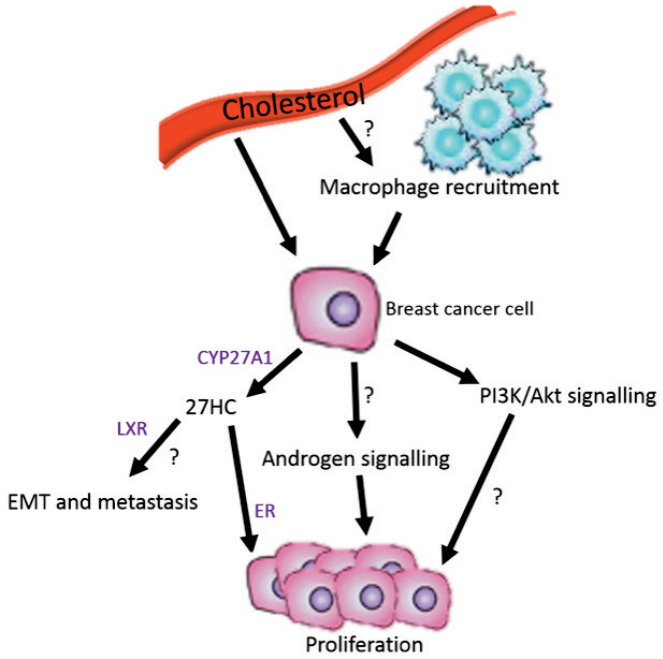


Figure 1: Potential Mechanisms of Cholesterol in Breast Cancer Progression.
It has been postulated that cholesterol exerts a pro-tumorigenic effect in a number of ways. It promotes proliferation through the action of its metabolite, 27-hydroxycholesterol (27HC), on the estrogen receptor (ER). In addition, it may increase macrophage recruitment, activate the liver X receptor (LXR) through 27HC, induce androgen signalling and/or induce PI3K/Akt signalling. ? denotes unverified or, in the case of PI3K/Akt signalling, potentially non-physiological mechanisms; CYP, cytochrome P450 enzyme; EMT, epithelial to mesenchymal transition. Schematic summarises findings or hypotheses of published works (3, 143, 153).

cholesterol induces protein kinase B (Akt) signalling, and suggested that cholesterol-mediated activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway may be causative for the increased proliferation of breast tumour cells (3). Secondly, it has been reported that 27-hydroxycholesterol (27HC), an abundant primary cholesterol metabolite generated by the cytochrome P450 oxidase, sterol 27-hydroxylase (CYP27A1), exerts pro-tumorigenic effects (40, 142). Two independent studies have shown that 27HC promotes MCF-7 breast cancer cell xenograft growth in mice (143, 194). Two mechanisms have been proposed to explain this. First, 27HC can function as an oestrogen receptor (ER) agonist (41). ER signalling is an important driver of ER-positive breast cancer growth, and as such activation of this pathway by 27HC could provide an explanation for the increased growth rate of ER-positive breast tumours in a hyperlipidaemic environment (41). Second, Nelson *et al.* postulate that 27HC stimulates liver X receptor (LXR) signalling, thereby promoting epithelial–mesenchymal transition (EMT) and metastasis (143). However, their data is inconclusive (and as yet unconfirmed), as a synthetic LXR agonist did not promote metastasis to the same extent as 27HC in ER-negative breast tumour xenografts (143). In addition, 25HC has been implicated in a similar way to 27HC in increasing breast cancer cell proliferation through the activation of ER-signalling (105).

The ER-mediated effect of 27HC and 25HC does not provide an explanation for the observed pro-tumorigenic effect of cholesterol on ER-negative breast tumours (153). The authors of that study suggest that cholesterol-induced tumour progression could be mediated by androgen signalling or monocyte/macrophage recruitment (153); however both hypotheses are yet to be validated.

Taken together, 27HC-mediated ER-signalling provides the most solid mechanism for increased proliferation of ER-positive breast tumour cells in a hyperlipidaemic environment, but does not explain cholesterol-mediated proliferation of ER-negative breast tumour cells. Thus, further studies are necessary to elucidate the mechanism(s) behind the more rapid tumour growth and increased metastasis occurring in a hyperlipidaemic host.

Hyperlipidaemia and Immunity

The impact of hyperlipidaemia on host immunity is complex, with studies indicating both an impairment of immunity resulting in increased susceptibility to infection (102, 114), and a reduction in tolerance through reduced functionality of regulatory T cells (T_{reg} cells) (9). Oxysterols, formed during cholesterol metabolism, are well known to play a role in regulating immune responses and can also directly act on tumour cells to influence tumour cell growth (reviewed in (184)). However, work to date has focussed on tumour-derived oxysterols and *in vitro* experiments with synthetic oxysterols. It is therefore unknown what effect hyperlipidaemia has on the abundances of oxysterols in the cancer setting, and what the impact of hyperlipidaemia on the intratumoral immune phenotype may be.

Hyperlipidaemia is often characterised by high levels of LDL-C (144). Oxidised LDL can bind to toll-like receptors (TLR) on macrophages and other phagocytes, initialising a pro-inflammatory signalling cascade (74). Thus, an abnormal elevation of LDL-C can lead to chronic inflammation and the development of atherosclerosis through TLR signalling on macrophages (74). Moreover, chronic inflammation has been linked to cancer development and progression (17), while

exercise has been shown to reduce chronic inflammation (110). In this regard, exercise may inhibit cancer progression and development in hyperlipidaemic individuals through the reduction of chronic inflammation.

EXERCISE AND CANCER

Physical activity is important for physical and mental well-being. It can prevent excess weight gain or aid in weight loss and reduce the risk of cardiovascular disease (140). In addition, it can lower serum lipid levels (120). As hyperlipidaemia, and high cholesterol in particular, promote breast tumour growth and progression (discussed in the previous section), this lowering of serum lipids by exercise is likely to slow tumour growth. Furthermore, exercise is increasingly recognised as an effective, well-tolerated adjunct to cancer therapy (15). It has been associated with reduced breast cancer risk and improved survival of breast cancer patients (11, 141). In addition to survival benefits, exercise can improve the quality of life of breast cancer patients by attenuating or inhibiting cachexia, fatigue, cardiotoxicity of chemotherapy, weight gain, bone loss and by improving mental health (reviewed in (2)).

Exercise has a role to play in all phases of breast cancer evolution, including prevention, treatment and aftercare. The exercise-induced reduction in the risk of developing breast cancer ranges from 15-80% (134). During treatment, exercise may be beneficial both by alleviating chemotherapy side effects (reviewed in (2)) and by altering the tumour microenvironment to improve drug delivery and reduce tumour aggressiveness (discussed in detail in the following sections). Finally, mental and physical quality of life can be significantly improved by exercise in breast cancer survivors during aftercare (reviewed in (2)). Furthermore, exercise may help to prevent disease recurrence (25, 73).

Epidemiological Studies

Numerous epidemiological studies have investigated the association between exercise and cancer prognosis (including breast, colorectal, prostate, ovarian, non-small cell lung cancer and glioma), with the majority reporting that exercise improves survival (14, 25, 72, 73, 77, 78, 91, 99, 128-131, 136, 137, 156, 162, 166, 176). Two studies showed no association (19, 31). The magnitude of this decrease in mortality ranged from 15-57% for all-cause mortality and 20-67% for cancer-specific mortality. Both studies that found no association between exercise and survival were observational studies (19, 31), which are prone to bias by over-reporting of exercise frequency/intensity (20); this may have skewed the results. In addition, the volume and intensity of physical activity required for the improved prognosis vary between studies. As such, the optimal therapeutic 'exercise dose' remains to be elucidated and may vary according to cancer type, although a recent meta-analysis suggests that the current WHO guidelines of at least 2.5 hours of moderate intensity exercise or 1.25 hours of vigorous intensity exercise per week (3-6 metabolic equivalents of task (MET)) are sufficient to improve survival by 24% for breast cancer (106). Survival was further improved with increasing level of exercise and up to 40% survival improvement at 20 MET-h/week, at which point no further survival benefit was seen with increasing exercise levels (106). With respect to exercise type, resistance and aerobic training have been shown to have quality of life benefits in breast cancer patients, both when used separately (32, 49, 149) or together (49, 133).

Pre and post-diagnosis exercise have both been shown to have survival benefits for breast cancer patients (25, 72, 73, 77, 78, 113). It seems that post-diagnosis exercise has a larger effect on survival than pre-diagnosis exercise, although the wide range of reported values makes it difficult to say with certainty (pre-diagnosis: 12-39% improved survival, post-diagnosis: 20-67%) (25, 72, 73, 77, 78, 113). However, it may be important to maintain pre-diagnosis exercise levels, as decreased physical activity after diagnosis was associated with a four-fold greater risk of death (78).

Emerging evidence indicates that cardiorespiratory fitness (CRF) may be an important prognostic marker (34, 89, 91, 93, 96, 166). A single study has specifically investigated the CRF-prognosis relationship in breast cancer patients, using VO_{2peak} which is the maximum rate of oxygen consumption during exercise (89). This study reported a non-significant improvement in survival in patients with metastatic disease with $VO_{2peak} > 1.09$ L/min compared to those with $VO_{2peak} < 1.09$ L/min (89). Multiple studies have found that breast cancer patients have reduced CRF compared to healthy individuals, placing them at increased risk of cardiovascular disease (reviewed in (152)). Moreover, CRF can be improved by exercise training, thereby reducing this risk (152). Therefore, it seems clear that improvement of CRF by exercise training can improve survival by reduction of the risk of cardiovascular events, but the relationship between CRF and breast cancer-specific mortality warrants further research.

Exercise Biomarkers in Cancer Patients

Exercise is known to modulate levels of blood-based biomarkers. As such, it is of interest whether an association can be found between biomarkers modulated by exercise and cancer prognosis. A number of studies have found changes in the levels of metabolic and/or inflammatory biomarkers in breast cancer survivors following exercise training, as discussed below.

The main metabolic biomarkers that have been investigated to date are factors of the insulin-glucose axis. Insulin-like growth factor 1 (IGF-1) has mitogenic and anti-apoptotic effects, while IGF binding protein 3 (IGFBP-3) regulates the activity and bioavailability of IGF-1 (170). As such, high serum levels of IGF-1 and/or low levels of IGFBP-3 have been associated with an increased risk of developing breast cancer, as well as aiding breast cancer progression (22, 62, 181). Therefore, modifying levels of these factors could be important for cancer outcome. Studies investigating the effect of exercise on levels of these factors have indicated that exercise reduces serum IGF-1 and/or increases IGFBP-3 in breast cancer patients (44, 76, 79, 85), but some studies reported opposite effects or no change in one or both markers (76, 79, 171). Nevertheless, a meta-analysis of randomised controlled trials in breast cancer survivors concluded that exercise was

significantly associated with a reduction in circulating IGF-1 levels (49), despite non-significant results in some of the primary studies. In addition, high levels of IGF-1 and an increased IGF-1:IGFBP-3 ratio (indicative of bioavailable IGF-1) have been correlated with decreased survival in breast cancer patients (39, 60). Conversely, in healthy individuals, exercise can increase serum IGF-1 levels, although this is not consistently reported (reviewed in (52)). In addition, a recent study suggests that elevated serum IGF-1 improves overall survival of breast cancer patients (180). Taken together, the effect of exercise on IGF-1 levels remains unclear, although the vast majority of studies indicate that high levels of serum IGF-1 are associated with poor prognosis in breast cancer patients.

Total serum IGF-1 has been negatively associated with hyperlipidaemia (119). In addition, human growth hormone (hGH) has been shown to lower plasma cholesterol and increase IGF-1 in hyperlipidaemic patients (168). This may be mediated by IGF-1 and hGH dependent stimulation of macrophages to take up LDL (66). Together, this suggests that the exercise-mediated reduction of plasma lipids may be mediated, at least in part, through an exercise-induced increase in IGF-1. In the context of cancer, this relationship may be more complex due to the mitogenic effect of IGF-1 on cancer cells.

Leptin is another metabolic biomarker that has been shown to be modulated by exercise. Leptin is an adipokine that has a wide array of physiological roles and is secreted by white adipose tissue, which functions as an energy storage site and endocrine organ (170). Leptin is present in higher levels in obese or overweight individuals (76, 86, 172). High levels of leptin are associated with an increased breast cancer risk (146, 170). In addition, leptin has been shown to promote breast cancer cell growth both *in vitro* and *in vivo* (38, 202). A number of studies have found that exercise decreases circulating leptin levels in breast cancer survivors (13, 76, 172, 187). However, some studies have found no reduction from baseline in circulating leptin levels following an exercise intervention (108, 165, 179). This could be explained by no or only a small decrease in body mass index (BMI), suggesting that weight loss rather than exercise itself is more important for the reduction of serum leptin. This is supported by a significant decrease in circulating leptin in three studies where weight loss was the goal (13, 86, 187). In addition, a recent study has demonstrated that alteration of leptin levels by exercise training is dependent on changes in body fat (178).

C reactive protein (CRP) is a common marker of systemic inflammation and is associated with an increased risk of cardiovascular disease (132). In addition, CRP has been associated with decreased overall and disease-free survival in breast cancer patients (154). The majority of studies investigating the effect of exercise on CRP levels in breast cancer survivors found a decrease in CRP levels in exercise groups (45, 53, 56, 59, 155, 187). Two studies found no difference, but of these one had a baseline level of CRP comparative to that of healthy individuals (179), suggesting that CRP levels may not have been sufficiently elevated for exercise to cause a reduction, and the other suggested that their exercise dose may not have been high enough to elicit a response (94). Taken together, there is evidence that exercise reduces CRP in breast cancer survivors, and therefore, that CRP may be an important prognostic biomarker modulated by exercise training.

Interleukin 6 (IL-6) is a myokine released from skeletal muscle during exercise, resulting in up to a 100-fold increase in its serum levels (150). It is postulated to mediate some exercise-induced anti-inflammatory effects by inhibiting tumour necrosis factor α (TNF-α) and IL-1 production, as well as inducing IL-1 receptor antagonist (IL-1ra) and IL-10 (150). On the other hand, increased serum IL-6 has also been associated with a poor prognosis in breast cancer (109). Studies investigating the effect of exercise on serum IL-6 levels in breast cancer survivors have reported no significant changes (59, 85, 94, 165, 179, 187). Two recent meta-analyses have investigated the effect of exercise on serum IL-6 in breast cancer survivors (95, 127). One of these indicates that exercise reduces serum IL-6 (127), despite non-significant results in a number of the initial studies, while the other shows no change (95). Taken together, the association between exercise, breast cancer and IL-6 is unclear, making it unlikely to have a use as an exercise-modulated prognostic biomarker.

Monocyte chemoattractant protein 1 (MCP-1), also known as CC chemokine ligand 2 (CCL2), is the primary chemokine responsible for attracting monocytes and immature macrophages to peripheral sites (36). As such, it plays an important role in the recruitment of monocytes and macrophages to the tumour, where they are programmed by factors in the microenvironment to take on either a pro-tumour M2 phenotype or an anti-tumour M1 phenotype (54). In general, a large number of tumour associated macrophages (TAMs) is associated with poor prognosis in breast cancer as the tumour microenvironment promotes an M2 phenotype (54). Similarly, intratumoral MCP-1 expression is linked to increased macrophage infiltration and poor prognosis (reviewed in (175)). Data on serum MCP-1 levels are inconclusive, with some reporting an increase in breast cancer patients and others reporting no change (reviewed in (175)). However, recent preclinical mouse studies indicate that serum MCP-1 is significantly elevated in tumour-bearing animals compared to non-tumour-bearing controls (139, 195) and exercise may attenuate this increase (139). To our knowledge, no epidemiological studies have investigated the influence of exercise on serum levels of MCP-1 in breast cancer patients. Taken together, preliminary data suggest that MCP-1 shows promise as a prognostic biomarker that may be modulated by exercise, but further studies, in both animal models and humans, are required to confirm this.

The abovementioned biomarkers are among those most commonly investigated with regards to exercise and cancer, and may prove to be of prognostic value. However, none of these were robust predictors of survival, and as such it would be of value to identify other biomarkers that are regulated by exercise and also play a role in breast cancer outcome.

Effect of Exercise on Tumour Growth in Preclinical Studies

Numerous preclinical studies have attempted to elucidate the role of exercise in tumour progression and the impact on the tumour microenvironment. However, these studies have pro-

duced conflicting results with regards to the effect of exercise on tumour growth, with some reporting inhibited tumour growth (12, 16, 55, 64, 70, 80, 151, 173, 203, 205), some reporting mixed results (118, 185, 206) and others reporting no inhibition of growth (24, 28, 46, 48, 88, 90, 92, 124, 125, 167, 188). These discrepancies may be explained by differences in animal model (immunocompetent versus immunodeficient), mode of exercise (forced versus voluntary), and other confounding factors, such as stress caused by individual housing or excessive tumour burden.

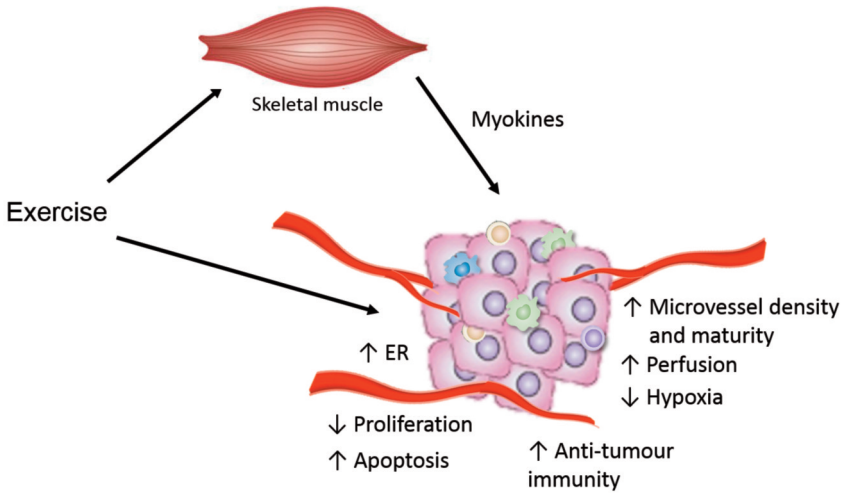


Figure 2: Proposed Mechanisms for the Effects of Exercise on the Tumour Microenvironment.
Exercise has been reported to have multiple effects on tumour growth and the tumour microenvironment. It induces increased perfusion through increased microvessel density and maturity, which results in decreased hypoxia and a less aggressive tumour as well as improved drug delivery. In addition, it promotes anti-tumour immunity and stimulates the release of myokines from skeletal muscle. This reduces proliferation and increases apoptosis of tumour cells. Moreover, exercise may upregulate the estrogen receptor (ER). Taken together, exercise induces favourable effects in the tumour microenvironment, resulting in inhibited tumour growth and metastasis. Figure summarises results from published studies (16, 46, 71, 122, 151).

Forced exercise paradigms, such as treadmill running, have been identified as a source of stress for rodents, increasing levels of corticosteroids and changing normal circadian rhythm (7, 21, 98, 196). This may be a confounding factor in studies investigating the effect of exercise on tumour progression. Indeed, of the 14 studies using forced exercise (12, 28, 46, 70, 80, 90, 118, 124, 126, 167, 173, 185, 188, 206), only 4 reported an inhibition of tumour growth (12, 70, 80, 173), whereas of the 10 studies using voluntary exercise (16, 28, 48, 55, 64, 88, 92, 151, 203, 205), 6 reported an inhibition of tumour growth (16, 55, 64, 151, 203, 205).

The majority of the abovementioned studies have investigated exercise as a monotherapy. The discrepant results in these studies suggest that the ability of exercise on its own to limit tumour growth is relatively small. However, it may reduce the incidence of metastasis (88, 200) and induce changes in the tumour microenvironment resulting in a less aggressive tumour phenotype (discussed in detail below). Furthermore, in a clinical setting, exercise would be utilised as an adjuvant therapy to surgery, chemotherapy, radiation and/or targeted therapies. It is therefore important that future exercise oncology studies include these aspects of cancer treatment and investigate how exercise influences tumour growth in conjunction with standard therapy.

Effect of Exercise on the Tumour Microenvironment

Despite the discrepant results regarding tumour growth, progress has been made towards determining changes in the tumour microenvironment following exercise training (Figure 2). The tumor microenvironment is the cellular, chemical and physical environment within the tumour, which includes immune and stromal cells, acidification due to increased glycolysis, and an aberrant blood supply leading to areas of low oxygen (hypoxia).

Jones *et al.* have shown that exercise increases intratumoral perfusion (16, 88, 92) and reduces hypoxia (16), thereby normalising the tumour microenvironment. Normalisation is here defined as remodelling of the microenvironment to more closely resemble that of normal tissue, and perfusion refers to blood flow, which should result in improved delivery of oxygen and therapeutics. Perfusion results were consistent across different cancer types (breast and prostate), immunocompetent and immunodeficient mice, and despite varying impact on tumour growth (16, 88, 92). In addition, McCullough *et al.* have reported increased intratumoral perfusion in prostate tumours of Copenhagen rats during acute exercise (126). However, in another study by the same group, tumours from rats that had been treadmill-trained for 5-7 weeks and sacrificed 48 h after the last exercise bout showed no increase in perfusion (125). Despite this lack of change in intratumoral perfusion, they observed a significant decrease in hypoxia in tumours from exercising compared to sedentary animals. Together, this suggests that even a transient increase in perfusion may aid in reducing hypoxia and in reducing an aggressive tumour phenotype (189).

The effect of exercise on intratumoral perfusion may differ depending on tumour location. Garcia *et al.* conducted a recent study in which they compared the effect of exercise on intratumoral blood flow in orthotopic (correct anatomical site) and ectopic (subcutaneous) prostate tumours, and found directly opposing effects in that blood flow was increased to the orthotopic tumour, but decreased to the ectopic tumour, during exercise (51). Indeed, blood flow to the skin and subcutaneous adipose tissue was also reduced during exercise (51). This raises important considerations for the study design of preclinical exercise studies, as results from studies using subcutaneous models may not reflect true physiological results.

Increased perfusion should lead to a reduction in hypoxia and an associated reduction in hypoxia factors, including the transcription factor hypoxia inducible factor-1 (HIF-1). However,

two studies by the same group have shown an increase in HIF-1α protein levels in tumours of exercising animals (88, 92), while a further study from a different group indicates a decrease in intratumoral HIF-1α mRNA following exercise training (80). Unfortunately, none of these studies measured intratumoral hypoxia directly, and as such it cannot be conclusively stated that the expression of HIF-1α reflected tumour hypoxia, as HIF-1α expression can also be regulated independently of oxygen tension (27). As such, the effect of exercise training on intratumoral hypoxia and HIF-1α expression remains to be confirmed.

Other modifications of the tumour microenvironment caused by exercise training include increased apoptosis and increased microvessel density and maturity, providing potential mechanisms for reduced tumour growth and improved perfusion, respectively (16, 64, 88, 118). One study reports contradictory results, showing a reduction in apoptosis and blood vessel density in the tumours of exercising mice (206). However, this study used forced treadmill running to exhaustion in order to investigate the effect of intense, prolonged exercise on tumour growth and the tumour microenvironment, and the method of detection of apoptosis and vessel density was suboptimal (quantification of haematoxylin and eosin (H&E) stained slides without a specific marker for the structures of interest). Thus, these results may not accurately reflect the effect of therapeutic exercise on the tumour microenvironment.

A recent study in Sprague-Dawley rats has found that exercise increases expression of the oestrogen receptor in breast tumours (46). The authors suggest that this may be a favourable change as it would make tumours easier to treat (via endocrine therapy). However, this is an isolated report that requires validation and further research into its implications.

Secretion of exercise-induced myokines from skeletal muscle may impact tumour growth (Figure 2). It has been demonstrated that a number of different myokines reduce tumour growth or tumour cell proliferation by as yet unidentified mechanisms (5, 50, 71), which include secreted protein acidic and rich in cysteine (SPARC), oncostatin M (OSM) and irisin.

Taken together, exercise impacts the tumour microenvironment in a number of ways: by increasing intratumoral perfusion, increasing vessel patency and reducing hypoxia, which leads to increased apoptosis and reduced proliferation of tumour cells. This may be mediated through the release of myokines. However, a number of questions remained unanswered. It is not known whether these effects are true for all cancer types, whether location on/in the body plays a role, or how these microenvironmental effects may change with chemotherapy, radiation or targeted therapies.

Effect of Exercise on the Immune Tumour Microenvironment

Exercise is known to influence immunity. In the context of cancer, increasing evidence over the last few years indicates that exercise induces a favourable change in the immune microenvironment of the tumour, making it less immunosuppressive and enhancing anti-tumour immunity (Figure 3; (4, 55, 123, 151, 199)). Key immunological cell types that are known to influence tumour progression include tumour associated macrophages (TAMs), natural killer (NK) cells and T cells.

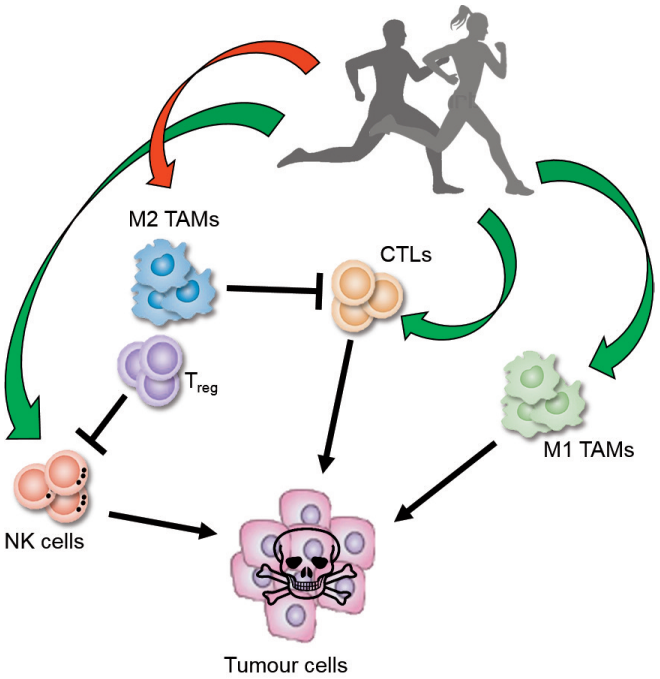


Figure 3: Overview of Exercise-Mediated Effects on the Immune Tumour Microenvironment.
Exercise has been reported to enhance anti-tumour immunity by alleviating the immunosuppressive microenvironment found within tumours and by enhancing the cytotoxic capabilities of tumour-killing cell types. Specifically, exercise increases cytotoxic activity of natural killer (NK) cells, induces a phenotypic shift from M2 to M1 macrophages and may alter proportions of T cells to include more cytotoxic T-cells (CTLs) and fewer regulatory T (T_{reg}) cells. Together, this results in improved killing of tumour cells. Green arrows denote stimulation and red arrow denotes inhibition. Figure summarises results from published studies (54, 55, 123, 151).

NK cells are the immune cells that respond most strongly to acute exercise, and as such they may be among the most important initial responders in the exercise-mediated anti-tumour response (75). It was first shown by Hoffman-Goetz *et al.* that NK cells from moderately exercised mice showed enhanced anti-tumour cytotoxicity against transformed fibroblasts, lymphoma cells and breast tumour metastases (67-69, 84, 116, 117). This has been substantiated by more recent publications in mice (151) and clinical studies ((18), reviewed in (103)). Pedersen *et al.* showed that exercise increases NK cell mobilisation to the tumour site, which was mediated by epinephrine (151). Idorn and Hojman suggest that it is this epinephrine-mediated mobilisation that is key, and that as long as exercise intensity is sufficient to elicit an epinephrine response, the duration of exercise is less important (75). In addition to their cytotoxic capabilities, NK cells influence the activity of many other immune cell types, including T cells, B cells and dendritic cells (30, 82, 87).

Macrophages can be broadly classified into two main phenotypic subtypes: M1 and M2. M1 macrophages are inflammatory, and responsible for the elimination of pathogens by

phagocytosis and the stimulation of adaptive immunity by the secretion of pro-inflammatory cytokines (81). M2 macrophages are immunoregulatory and play a role in wound healing (81). TAMs generally exhibit an M2 phenotype, which promotes tumour progression through the inhibition of cytotoxic immune cells and the secretion of angiogenic factors (191). Because of this, high numbers of TAMs have been associated with a poorer prognosis in breast cancer patients (57, 198, 201). Studies have shown that exercise training may cause a phenotypic shift in TAMs from a pro-tumour M2 to an anti-tumour M1 phenotype (reviewed in (54)). This is demonstrated by the increased secretion of M1-associated cytokines from *ex vivo* stimulated peritoneal macrophages from exercising compared to sedentary mice bearing mammary tumours (1), increased cytotoxicity/phagocytosis of macrophages from exercising mice (33, 174) and decreased expression of M2-associated genes (123). Therefore, some of the anti-tumour effects of exercise are likely mediated through the repolarisation of TAMs.

The adaptive immune system also plays an important role in the tumour microenvironment. T cells in the lymph nodes must be activated by antigen-presenting cells holding tumour-specific antigens, following which they travel to the site of the cancer and infiltrate the tumour (29). Cytotoxic T cells (CTLs) are responsible for tumour cell killing; however, their response can be switched off by inhibitory molecules on the tumour cell surface (29). In addition, T_{reg} cells and other immunoregulatory cell types can suppress CTL-mediated cell killing through the secretion of inhibitory cytokines, cytolysis and metabolic disruption (190). Exercise may increase the proportion of intratumoral CTLs and reduce T_{reg} cells, as evidenced by increased *Cd8* and reduced *Foxp3* gene expression in mucosal scrapings from a transgenic mouse model for colorectal cancer, the $Apc^{Min/+}$ mouse (123). However, the immune microenvironment in the gut is unique in that it tends to be highly immunosuppressive due to the need for tolerance to food and commensal microbiota-derived antigens, and thus these results may not be translatable to other cancer types. In breast cancer, it has been shown that exercise decreases intratumoral gene expression of *Ccl22*, a chemokine known to attract T_{reg} cells (55). However, none of these studies have investigated the functionality of intratumoral T cell subtypes following exercise, which would be an important next step in determining how exercise affects T cell immunity in tumours.

The role of hypoxia in anti-tumour immunity is fairly well established, in that it inhibits anti-tumour immune responses and promotes immunosuppression (6, 148). Specifically, hypoxia has been shown to decrease tumour cell susceptibility to CTL and NK cell mediated lysis (47, 61, 147, 169), increase T_{reg} cell attraction, induction and immunosuppressive function (35, 111, 160), and promote an immunosuppressive phenotype in myeloid cells (reviewed in (148)). Furthermore, a recent study has demonstrated that type 1 T helper (T_H1) cells can contribute to intratumoral vessel normalisation (182). On the other hand, T_{reg} cells and M2 macrophages can induce (pathological) angiogenesis (160, 161). Therefore, exercise-induced modulation of the immune phenotype within the tumour may be a contributing factor to the vessel normalisation and reduced pathological angiogenesis seen in some of the studies discussed in previous sections.

Although much progress has been made over the last few years in determining the effects of exercise on the immune microenvironment of the tumour, much is still unknown. The bulk of research thus far has focussed on NK cells and macrophages, with some work beginning to emerge on T cells. Future work will focus on how exercise affects other immune cell types within the tumour microenvironment (and systemically), such as B cells and neutrophils, as well as specific subtypes (e.g. T helper cell subtypes such as T_H1, T_H2 and T_H17).

Taken together, current data shows that exercise increases anti-tumour immunity and alleviates the immunosuppressive microenvironment found in solid tumours, which may also improve vessel patency and reduce hypoxia.

INTERPLAY OF HYPERLIPIDAEMIA AND EXERCISE IN BREAST CANCER PROGRESSION

Hyperlipidaemia is an inflammatory disease. This is at least partly attributable to the activation of TLR signalling by oxidised LDL, which induces a pro-inflammatory phenotype in innate immune cells (74). In turn, chronic inflammation has a well-established pro-tumour effect (17). Thus, we propose that hyperlipidaemia contributes to breast cancer progression through the induction of chronic inflammation. Conversely, exercise can reduce chronic inflammation (110, 197). This is likely through the action of anti-inflammatory myokines (197). In addition, exercise has been shown to reduce TLR expression on monocytes and reduce the number of circulating pro-inflammatory monocytes (197). Therefore, exercise may inhibit breast cancer progression in hyperlipidaemic individuals by dampening TLR activation and by the action of anti-inflammatory myokines, thereby reducing chronic inflammation.

Hyperlipidaemia is characterised by elevated serum cholesterol levels. This increase in serum cholesterol should result in an increased exposure of tumour cells to cholesterol. As cholesterol and some of its metabolites have been shown to directly increase breast cancer cell proliferation *in vitro* and breast tumour growth *in vivo* ((3, 143), reviewed in (8)), it is likely that hyperlipidaemia also exerts pro-tumour effects through the direct effect of cholesterol. On the other hand, exercise can lower serum lipid levels, possibly by augmenting the ability of skeletal muscles to utilise lipids rather than glycogen or by increases in lecithin-cholesterol acyltransferase and lipoprotein lipase activity (reviewed in (120)). Therefore, a further mechanism by which exercise may inhibit tumour growth in hyperlipidaemic individuals is the reduction of blood cholesterol, thereby reducing the amount of cholesterol that tumour cells are exposed to.

Taken together, exercise may inhibit breast cancer progression in hyperlipidaemic individuals by reducing hyperlipidaemia-

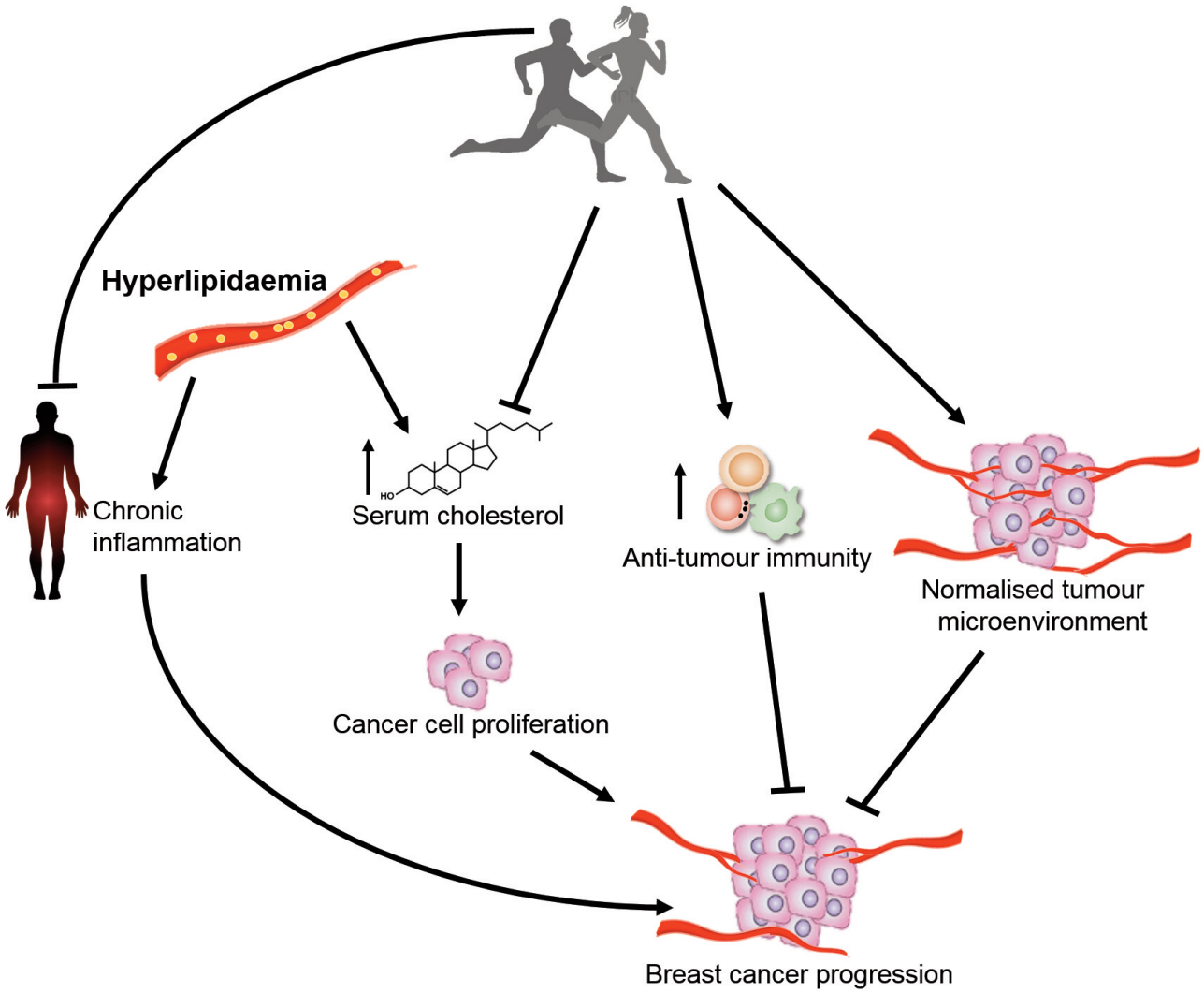


Figure 4: Hypothesised interplay of exercise and hyperlipidaemia in breast cancer progression.
Hyperlipidaemia can promote breast cancer through the induction of chronic inflammation and the mitogenic action of cholesterol on tumour cells. Exercise may work against the pro-tumour effect of hyperlipidaemia by reducing chronic inflammation, lowering serum lipids, increasing the ability of the immune system to recognise and destroy tumour cells and by normalising the vascular network of the tumour, thereby reducing hypoxia.

associated chronic inflammation, by lowering serum cholesterol and thereby reducing the exposure of tumour cells to cholesterol, by enhancing anti-tumour immunity and by normalising the tumour vasculature (Figure 4, discussed in the previous section).

CONCLUSION

Obesity-associated hyperlipidaemia and exercise have opposing effects on breast cancer growth and development, and as such it is possible that exercise could be utilised in hyperlipidaemic patients to lower lipid levels and mitigate the pro-tumour effect of hyperlipidaemia. To our knowledge, no published work is available which specifically investigates this issue. To date, substantial clinical and preclinical work has been undertaken separately to investigate the effects of hyperlipidaemia and exercise on breast cancer. However, it is unknown how hyperlipidaemia influences the anti-tumour immune response and the immune microenvironment of the tumour.

In terms of exercise, the optimal therapeutic 'dose' of exercise remains unclear, as few studies are available which specifically investigate this. It seems likely that a minimum level is required (it has been shown that the current WHO guidelines appear to be adequate (106)), and that excessive exercise may be detrimental, as overtraining is known to weaken the immune system in healthy individuals (115).

Furthermore, most studies to date have focussed on exercise as a monotherapy, whereas in the clinic it would function as an adjunct to surgery, chemotherapy, radiation and/or targeted therapies. Therefore, it is essential that future work determines how exercise and other therapies together influence tumour growth and the tumour microenvironment. In addition, it is unknown whether the effects of exercise on the tumour microenvironment differ according to tumour type and location. It needs to be further explored how exercise modulates perfusion to the skin and internal organs, and how this can have different effects on different cancer types. In this context, a recent study investigating the association between cancer risk and leisure time physical

activity in over one million adults reported that exercise was associated with decreased risk of developing lung cancer, but increased risk of developing melanoma (135).

Further work on the immune microenvironment of the tumour is also required to determine how cell types or subsets, such as neutrophils or T helper cell subsets, are affected by exercise. Functionality assays on T cell subsets would provide information as to whether exercise alters the cytotoxic or immunosuppressive activity of these cells, or whether it only influences recruitment to the tumour site.

In summary, work to date indicates that exercise can slow tumour growth and effect changes in the tumour microenvironment which make it less aggressive, more susceptible to treatment and more likely to be recognised and attacked by the immune system. In contrast, obesity-associated hyperlipidaemia makes tumours more aggressive. Joint analysis of diet and exercise will provide further insight into how the two modalities, which are two of the largest lifestyle factors responsible for a person's individual health and well-being, can affect the progression of cancer. We suggest that exercise may alleviate some of the pro-tumorigenic effects of hyperlipidaemia through the reduction of chronic inflammation, blood lipid levels and by exerting specific anti-cancer effects on the tumour microenvironment.

REFERENCES

1. Abdalla DR, Aleixo AAR, Murta EF, and Michelin MA. Innate immune response adaptation in mice subjected to administration of DMBA and physical activity. Oncology Letters 7: 886-890, 2014.
2. Adraskela K, Veisaki E, Koutsilieris M, and Philippou A. Physical Exercise Positively Influences Breast Cancer Evolution. Clinical Breast Cancer 17: 408-417, 2017.
3. Alikhani N, Ferguson RD, Novosyadlyy R, Gallagher EJ, Scheinman EJ, Yakar S, and LeRoith D. Mammary tumor growth and pulmonary metastasis are enhanced in a hyperlipidemic mouse model. Oncogene 32: 961-967, 2013.
4. Almeida PWM, Gomes-Filho A, Ferreira AJ, Rodrigues CEM, Dias-Peixoto MF, Russo RC, Teixeira MM, Cassali GD, Ferreira E, Santos IC, Garcia AMC, Silami-Garcia E, Wisløff U, and Pussieldi GA. Swim training suppresses tumor growth in mice. Journal of Applied Physiology 107: 261-265, 2009.
5. Aoi W, Naito Y, Takagi T, Tanimura Y, Takanami Y, Kawai Y, Sakuma K, Hang LP, Mizushima K, Hirai Y, Koyama R, Wada S, Higashi A, Kokura S, Ichikawa H, and Yoshikawa T. A novel myokine, secreted protein acidic and rich in cysteine (SPARC), suppresses colon tumorigenesis via regular exercise. Gut 62: 882-889, 2013.
6. Aouali N, Bosseler M, Sauvage D, Moer KV, Berchem G, and Janji B. The Critical Role of Hypoxia in Tumor-Mediated Immunosuppression. In: Hypoxia and Human Diseases, edited by Zheng J, and Zhou C. Rijeka: InTech, 2017, p. Ch. 18.
7. Ashcraft KA, Peace RM, Betof AS, Dewhirst MW, and Jones LW. Efficacy and Mechanisms of Aerobic Exercise on Cancer Initiation, Progression, and Metastasis: A Critical Systematic Review of In Vivo Preclinical Data. Cancer Research 76: 4032-4050, 2016.
8. Baek AE, and Nelson ER. The Contribution of Cholesterol and Its Metabolites to the Pathophysiology of Breast Cancer. Hormones and Cancer 7: 219-28, 2016.
9. Bagley J, Yuan J, Chandrakar A, and Iacomini J. Hyperlipidemia Alters Regulatory T Cell Function and Promotes Resistance to Tolerance Induction Through Costimulatory Molecule Blockade. American Journal of Transplantation 15: 2324-2335, 2015.
10. Bahl M, Ennis M, Tannock IF, Hux JE, Pritchard KI, Koo J, and Goodwin PJ. Serum Lipids and Outcome of Early-stage Breast Cancer: Results of a Prospective Cohort Study. Breast Cancer Research and Treatment 94: 135-144, 2005.
11. Ballard-Barbash R, Hunsberger S, Alciati MH, Blair SN, Goodwin PJ, McTiernan A, Wing R, and Schatzkin A. Physical Activity, Weight Control, and Breast Cancer Risk and Survival: Clinical Trial Rationale and Design Considerations. Journal of the National Cancer Institute 101: 630-643, 2009.
12. Baracos VE. Exercise inhibits progressive growth of the Morris hepatoma 7777 in male and female rats. Canadian Journal of Physiology and Pharmacology 67: 864-870, 1989.
13. Befort CA, Klemp JR, Austin H, Perri MG, Schmitz KH, Sullivan DK, and Fabian CJ. Outcomes of a Weight Loss Intervention among Rural Breast Cancer Survivors. Breast Cancer Research and Treatment 132: 631-639, 2012.
14. Bertram LAC, Stefanick ML, Saquib N, Natarajan L, Patterson RE, Bardwell W, Flatt SW, Newman VA, Rock CL, Thomson CA, and Pierce JP. Physical activity, additional breast cancer events, and mortality among early-stage breast cancer survivors: findings from the WHEL Study. Cancer Causes & Control 22: 427-435, 2010.
15. Betof AS, Dewhirst MW, and Jones LW. Effects and potential mechanisms of exercise training on cancer progression: a translational perspective. Brain Behav Immun 30 Suppl: S75-87, 2013.
16. Betof AS, Lascola CD, Weitzel D, Landon C, Scarbrough PM, Devi GR, Palmer G, Jones LW, and Dewhirst MW. Modulation of murine breast tumor vascularity, hypoxia and chemotherapeutic response by exercise. Journal of the National Cancer Institute 107: djv 040, 2015.
17. Bhatelia K, Singh K, and Singh R. TLRs: linking inflammation and breast cancer. Cellular Signalling 26: 2350-2357, 2014.
18. 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, Behavior and Immunity 39: 160-171, 2014.
19. Borugian MJ, Sheps SB, Kim-Sing C, Van Patten C, Potter JD, Dunn B, Gallagher RP, and Hislop TG. Insulin, Macronutrient Intake, and Physical Activity: Are Potential Indicators of Insulin Resistance Associated with Mortality from Breast Cancer? Cancer Epidemiology Biomarkers & Prevention 13: 1163-1172, 2004.
20. Brenner PS, and DeLamater JD. Social Desirability Bias in Self-reports of Physical Activity: Is an Exercise Identity the Culprit? Social Indicators Research 117: 489-504, 2013.
21. Brown DA, Johnson MS, Armstrong CJ, Lynch JM, Caruso NM, Ehlers LB, Fleshner M, Spencer RL, and Moore RL. Short-term treadmill running in the rat: what kind of stressor is it? Journal of Applied Physiology 103: 1979-1985, 2007.

22. Bruning PF, Van Doorn J, Bonfrèr JMG, Van Noord PAM, Korse CM, Linders TC, and Hart AAM. Insulin-like growth-factor-binding protein 3 is decreased in early-stage operable pre-menopausal breast cancer. International Journal of Cancer 62: 266-270, 1995.
23. Cancer Research UK. Worldwide Cancer Mortality Statistics 2017]. http://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer/mortality; accessed 8/12/2017
24. Cao L, Liu X, Lin E-JD, Wang C, Choi EY, Riban V, Lin B, and During MJ. Environmental and genetic activation of a brain-adipocyte BDNF/leptin axis causes cancer remission and inhibition. Cell 142: 52-64, 2010.
25. Chen X, Lu W, Zheng W, Gu K, Matthews CE, Chen Z, Zheng Y, and Shu XO. Exercise After Diagnosis of Breast Cancer in Association with Survival. Cancer Prevention Research 4: 1409-1418, 2011.
26. Cholesterol Treatment Trialists C. Lack of Effect of Lowering LDL Cholesterol on Cancer: Meta-Analysis of Individual Data from 175,000 People in 27 Randomised Trials of Statin Therapy. PloS One 7: e29849, 2012.
27. Chun Y-S, Kim M-S, and Park J-W. Oxygen-dependent and -independent regulation of HIF-1alpha. Journal of Korean Medical Science 17: 581-588, 2002.
28. Colbert LH, Westerlind KC, Perkins SN, Haines DC, Berrigan D, Donehower LA, Fuchs-Young R, and Hursting SD. Exercise Effects on Tumorigenesis in a p53-deficient Mouse Model of Breast Cancer. Medicine and Science in Sports and Exercise 41: 1597-1605, 2009.
29. Cronin SJF, and Penninger JM. From T-cell activation signals to signaling control of anti-cancer immunity. Immunological Reviews 220: 151-168, 2007.
30. Crouse J, Xu HC, Lang PA, and Oxenius A. NK cells regulating T cell responses: mechanisms and outcome. Trends in Immunology 36: 49-58, 2015
31. Dal Maso L, Zucchetto A, Talamini R, Serraino D, Stocco CF, Vercelli M, Falcini F, and Franceschi S. Effect of obesity and other lifestyle factors on mortality in women with breast cancer. International Journal of Cancer 123: 2188-2194, 2008.
32. Daley AJ, Crank H, Saxton JM, Mutrie N, Coleman R, and Roalfe A. Randomized Trial of Exercise Therapy in Women Treated for Breast Cancer. Journal of Clinical Oncology 25: 1713-1721, 2007.
33. de Lima C, Alves LE, Iagher F, Machado AF, Bonatto SJ, Kuczera D, de Souza CF, Pequito DC, Muritiba AL, Nunes EA, and Fernandes LC. Anaerobic exercise reduces tumor growth, cancer cachexia and increases macrophage and lymphocyte response in Walker 256 tumor-bearing rats. European Journal of Applied Physiology 104: 957-64, 2008.
34. Denehy L, Hornsby WE, Herndon Ii JE, Thomas S, Ready NE, Granger CL, Valera L, Kenjale AA, Eves ND, and Jones LW. Prognostic Validation of the Body Mass Index, Airflow Obstruction, Dyspnea, and Exercise Capacity (BODE) Index in Inoperable Non–Small-Cell Lung Cancer. Journal of Thoracic Oncology 8: 1545-1550, 2013.
35. Deng B, Zhu J-M, Wang Y, Liu T-T, Ding Y-B, Xiao W-M, Lu G-T, Bo P, and Shen X-Z. Intratumor Hypoxia Promotes Immune Tolerance by Inducing Regulatory T Cells via TGF-β1 in Gastric Cancer. PloS One 8: e63777, 2013.
36. Deshmane SL, Kremlev S, Amini S, and Sawaya BE. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. Journal of Interferon & Cytokine Research 29: 313-326, 2009.
37. Dessi S, Batetta B, Pulisci D, Spano O, Cherchi R, Lanfranco G, Tessitore L, Costelli P, Baccino F, Anchis C, and Pani P. Altered Pattern of Lipid Metabolism in Patients with Lung Cancer. Oncology 49: 436-441, 1992.
38. Dieudonne M-N, Machinal-Quelin F, Serazin-Leroy V, Leneveu M-C, Pecquery R, and Giudicelli Y. Leptin mediates a proliferative response in human MCF7 breast cancer cells. Biochemical and Biophysical Research Communications 293: 622-628, 2002.
39. Duggan C, Wang C-Y, Neuhouser ML, Xiao L, Smith AW, Reding KW, Baumgartner RN, Baumgartner KB, Bernstein L, Ballard-Barbash R, and McTiernan A. Associations of insulin-like growth factor and insulin-like growth factor binding protein-3 with mortality in women with breast cancer. International journal of cancer 132: 1191-1200, 2013.
40. DuSell CD, and McDonnell DP. 27-Hydroxycholesterol: a potential endogenous regulator of estrogen receptor signaling. Trends in Pharmacological Sciences 29: 510-514, 2008.
41. DuSell CD, Umetani M, Shaul PW, Mangelsdorf DJ, and McDonnell DP. 27-Hydroxycholesterol Is an Endogenous Selective Estrogen Receptor Modulator. Molecular Endocrinology 22: 65-77, 2008.
42. Eliassen A, Colditz GA, Rosner B, Willett WC, and Hankinson SE. Serum lipids, lipid-lowering drugs, and the risk of breast cancer. Archives of Internal Medicine 165: 2264-2271, 2005.
43. Emaus A, Veierød MB, Tretli S, Finstad SE, Selmer R, Furberg A-S, Bernstein L, Schlichting E, and Thune I. Metabolic profile, physical activity, and mortality in breast cancer patients. Breast Cancer Research and Treatment 121: 651-660, 2009.
44. Fairey AS, Courneya KS, Field CJ, Bell GJ, Jones LW, and Mackey JR. Effects of Exercise Training on Fasting Insulin, Insulin Resistance, Insulin-like Growth Factors, and Insulin-like Growth Factor Binding Proteins in Postmenopausal Breast Cancer Survivors: A Randomized Controlled Trial. Cancer Epidemiology Biomarkers & Prevention 12: 721-727, 2003.
45. Fairey AS, Courneya KS, Field CJ, Bell GJ, Jones LW, Martin BS, and Mackey JR. Effect of exercise training on C-reactive protein in postmenopausal breast cancer survivors: A randomized controlled trial. Brain, Behavior, and Immunity 19: 381-388, 2005.
46. Faustino-Rocha AI, Gama A, Oliveira PA, Alvarado A, Neuparth MJ, Ferreira R, and Ginja M. Effects of lifelong exercise training on mammary tumorigenesis induced by MNU in female Sprague–Dawley rats. Clinical and Experimental Medicine 1-10, 2016.
47. Fink T, Ebbesen P, Koppelhus U, and Zachar V. Natural Killer Cell-Mediated Basal and Interferon-Enhanced Cytotoxicity against Liver Cancer Cells is Significantly Impaired Under In Vivo Oxygen Conditions. Scandinavian Journal of Immunology 58: 607-612, 2003.
48. Foley JM, Stark KD, Zajchowski S, and Meckling KA. Fatty Acids and Exercise Affect Glucose Transport But Not Tumour Growth in F-344 Rats. Canadian Journal of Applied Physiology 29: 604-622, 2004.
49. Fong DYT, Ho JWC, Hui BPH, Lee AM, Macfarlane DJ, Leung SSK, Cerin E, Chan WYY, Leung IPF, Lam SHS, Taylor AJ, and Cheng K-k. Physical activity for cancer survivors: meta-analysis of randomised controlled trials. The BMJ 344: e70, 2012.

50. Gannon NP, Vaughan RA, Garcia-Smith R, Bisoffi M, and Trujillo KA. Effects of the exercise-inducible myokine irisin on malignant and non-malignant breast epithelial cell behavior in vitro. International Journal of Cancer 136: E197-E202, 2015.
51. Garcia E, Becker VGC, McCullough DJ, Stabley JN, Gittemeier EM, Opoku-Acheampong AB, Siemann DW, and Behnke BJ. Blood flow responses to mild-intensity exercise in ectopic versus orthotopic prostate tumors; dependence upon host-tissue hemodynamics and vascular reactivity. Journal of Applied Physiology 121. 15-24, 2016.
52. Gatti R, De Palo EF, Antonelli G, and Spinella P. IGF-I/IGFBP system: Metabolism outline and physical exercise. Journal of Endocrinological Investigation 35: 699-707, 2012.
53. George SM, Neuhouser ML, Mayne ST, Irwin ML, Albanes D, Gail MH, Alfano CM, Bernstein L, McTiernan A, Reedy J, Smith AW, Ulrich CM, and Ballard-Barbash R. Postdiagnosis Diet Quality Is Inversely Related to a Biomarker of Inflammation among Breast Cancer Survivors. Cancer Epidemiology Biomarkers & Prevention 19: 2220-2228, 2010.
54. Goh J, Kirk EA, Lee SX, and Ladiges WC. Exercise, Physical Activity and Breast Cancer: The Role of Tumor-Associated Macrophages. Exerc Immunol Rev 18: 158-176, 2012.
55. Goh J, Tsai J, Bammler TK, Farin FM, Endicott E, and Ladiges WC. Exercise training in transgenic mice is associated with attenuation of early breast cancer growth in a dose-dependent manner. PloS One 8: e80123, 2013.
56. Guinan E, Hussey J, Broderick JM, Lithander FE, O'Donnell D, Kennedy MJ, and Connolly EM. The effect of aerobic exercise on metabolic and inflammatory markers in breast cancer survivors—a pilot study. Supportive Care in Cancer 21: 1983-1992, 2013.
57. Gwak JM, Jang MH, Kim DI, Seo AN, and Park SY. Prognostic Value of Tumor-Associated Macrophages According to Histologic Locations and Hormone Receptor Status in Breast Cancer. PloS One 10: e0125728, 2015.
58. Ha M, Sung J, and Song Y-M. Serum total cholesterol and the risk of breast cancer in postmenopausal Korean women. Cancer Causes & Control 20: 1055-1060, 2009.
59. Hagstrom AD, Marshall PWM, Lonsdale C, Papalia S, Cheema BS, Toben C, Baune BT, Fiatarone Singh MA, and Green S. The effect of resistance training on markers of immune function and inflammation in previously sedentary women recovering from breast cancer: a randomized controlled trial. Breast Cancer Research and Treatment 155: 471-482, 2016.
60. Hartog H, Boezen HM, de Jong MM, Schaapveld M, Wesseling J, and van der Graaf WTA. Prognostic value of insulin-like growth factor 1 and insulin-like growth factor binding protein 3 blood levels in breast cancer. The Breast 22: 1155-1160, 2013.
61. Hasmim M, Noman MZ, Lauriol J, Benlalam H, Mallavialle A, Rosselli F, Mami-Chouaib F, Alcaide-Loridan C, and Chouaib S. Hypoxia-Dependent Inhibition of Tumor Cell Susceptibility to CTL-Mediated Lysis Involves NANOG Induction in Target Cells. The Journal of Immunology 187: 4031-4039, 2011.
62. Hawsawi Y, El-Gendy R, Twelves C, Speirs V, and Beattie J. Insulin-like growth factor — Oestradiol crosstalk and mammary gland tumourigenesis. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 1836: 345-353, 2013.
63. Henriksson P, Ericsson S, Stege R, Eriksson M, Rudling M, Berglund L, and Angelin B. Hypocholesterolaemia and increased elimination of low-density lipoproteins in metastatic cancer of the prostate. The Lancet 334: 1178-1180, 1989.
64. Higgins KA, Park D, Lee GY, Curran WJ, and Deng X. Exercise-induced lung cancer regression: mechanistic findings from a mouse model. Cancer 120: 3302-3310, 2014.
65. His M, Dartois L, Fagherazzi G, Boutten A, Dupré T, Mesrine S, Boutron-Ruault M-C, Clavel-Chapelon F, and Dossus L. Associations between serum lipids and breast cancer incidence and survival in the E3N prospective cohort study. Cancer Causes & Control 28: 77-88, 2017.
66. Hochberg Z, Hertz P, Maor G, Oiknine J, and Aviram M. Growth hormone and insulin-like growth factor-I increase macrophage uptake and degradation of low density lipoprotein. Endocrinology 131: 430-435, 1992.
67. Hoffman-Goetz L, Arumugam Y, and Sweeny L. Lymphokine activated killer cell activity following voluntary physical activity in mice. J Sports Med Phys Fitness 34: 83-90, 1994.
68. Hoffman-Goetz L, MacNeil B, Arumugam Y, and Simpson JR. Differential Effects of Exercise and Housing Condition on Murine Natural Killer Cell Activity and Tumor Growth. International Journal of Sports Medicine 13: 167-171, 1992.
69. Hoffman-Goetz L, May KM, and Arumugam Y. Exercise training and mouse mammary tumour metastasis. Anticancer Research 14: 2627-2631, 1994.
70. Hoffman SA, Paschkis KE, DeBias DA, Cantarow A, and Williams TL. The Influence of Exercise on the Growth of Transplanted Rat Tumors. Cancer Research 22: 597-599, 1962.
71. Hojman P, Dethlefsen C, Brandt C, Hansen J, Pedersen L, and Pedersen BK. Exercise-induced muscle-derived cytokines inhibit mammary cancer cell growth. American Journal of Physiology - Endocrinology and Metabolism 301: E504-E510, 2011.
72. Holick CN, Newcomb PA, Trentham-Dietz A, Titus-Ernstoff L, Bersch AJ, Stampfer MJ, Baron JA, Egan KM, and Willett WC. Physical Activity and Survival after Diagnosis of Invasive Breast Cancer. Cancer Epidemiology Biomarkers & Prevention 17: 379-386, 2008.
73. Holmes MD, Chen WY, Feskanich D, Kroenke CH, and Colditz GA. Physical activity and survival after breast cancer diagnosis. JAMA 293: 2479-2486, 2005.
74. Hovland A, Jonasson L, Garred P, Yndestad A, Aukrust P, Lappegård KT, Espevik T, and Mollnes TE. The complement system and toll-like receptors as integrated players in the pathophysiology of atherosclerosis. Atherosclerosis 241: 480-494, 2015.
75. Idorn M, and Hojman P. Exercise-Dependent Regulation of NK Cells in Cancer Protection. Trends in Molecular Medicine 22: 565-77, 2016.
76. Irwin ML, McTiernan A, Bernstein L, Gilliland FD, Baumgartner R, Baumgartner K, and Ballard-Barbash R. Relationship of Obesity and Physical Activity with C-Peptide, Leptin, and Insulin-Like Growth Factors in Breast Cancer Survivors. Cancer Epidemiology Biomarkers & Prevention 14: 2881-2888, 2005.
77. Irwin ML, McTiernan A, Manson JE, Thomson CA, Sternfeld B, Stefanick ML, Wactawski-Wende J, Craft L, Lane D, Martin LW, and Chlebowski R. Physical Activity and Survival in Postmenopausal Women with Breast Cancer: Results from the Women's Health Initiative. Cancer Prevention Research 4: 522-529, 2011.

78. Irwin ML, Smith AW, McTiernan A, Ballard-Barbash R, Cronin K, Gilliland FD, Baumgartner RN, Baumgartner KB, and Bernstein L. Influence of Pre- and Postdiagnosis Physical Activity on Mortality in Breast Cancer Survivors: The Health, Eating, Activity, and Lifestyle Study. Journal of Clinical Oncology 26: 3958-3964, 2008.
79. Irwin ML, Varma K, Alvarez-Reeves M, Cadmus L, Wiley A, Chung GG, DiPietro L, Mayne ST, and Yu H. Randomized Controlled Trial of Aerobic Exercise on Insulin and Insulin-like Growth Factors in Breast Cancer Survivors: The Yale Exercise and Survivorship Study. Cancer Epidemiology Biomarkers & Prevention 18: 306-313, 2009.
80. Isanejad A, Alizadeh AM, Amani Shalamzari S, Khodayari H, Khodayari S, Khori V, and Khojastehnjad N. MicroRNA-206, let-7a and microRNA-21 pathways involved in the anti-angiogenesis effects of the interval exercise training and hormone therapy in breast cancer. Life Sciences 151: 30-40, 2016.
81. Italiani P, and Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Frontiers in Immunology 5: 514, 2014.
82. Jacobs B, and Ullrich E. The Interaction of NK Cells and Dendritic Cells in the Tumor Environment: How to Enforce NK Cell & DC Action Under Immunosuppressive Conditions? Current Medicinal Chemistry 19: 1771-1779, 2012.
83. Jacobs EJ, and Gapstur SM. Cholesterol and Cancer: Answers and New Questions. Cancer Epidemiology Biomarkers & Prevention 18: 2805-2806, 2009.
84. Jadeski L, and Hoffman-Goetz L. Exercise and in vivo natural cytotoxicity against tumour cells of varying metastatic capacity. Clinical & Experimental Metastasis 14: 138-144, 1996.
85. Janelsins MC, Davis PG, Wideman L, Katula JA, Sprod LK, Peppone LJ, Palesh OG, Heckler CE, Williams JP, Morrow GR, and Mustian KM. Effects of Tai Chi Chuan on Insulin and Cytokine Levels in a Randomized Controlled Pilot Study on Breast Cancer Survivors. Clinical Breast Cancer 11: 161-170, 2011.
86. Jen KLC, Djuric Z, DiLaura NM, Buison A, Redd JN, Maranci V, and Hryniuk WM. Improvement of Metabolism among Obese Breast Cancer Survivors in Differing Weight Loss Regimens. Obesity Research 12: 306-312, 2004.
87. Jensen M, Tawadros S, Sedlacek H-H, Schultze JL, and Berthold F. NK cell depletion diminish tumour-specific B cell responses. Immunology Letters 93: 205-210, 2004.
88. Jones LW, Antonelli J, Masko EM, Broadwater G, Lascola CD, Fels D, Dewhirst MW, Dyck JR, Nagendran J, Flores CT, Betof AS, Nelson ER, Pollak M, Dash RC, Young ME, and Freedland SJ. Exercise modulation of the host-tumor interaction in an orthotopic model of murine prostate cancer. Journal of Applied Physiology 113: 263-272, 2012.
89. Jones LW, Courneya KS, Mackey JR, Muss HB, Pituskin EN, Scott JM, Hornsby WE, Coan AD, Herndon JE, Douglas PS, and Haykowsky M. Cardiopulmonary Function and Age-Related Decline Across the Breast Cancer Survivorship Continuum. Journal of Clinical Oncology 30: 2530-2537, 2012.
90. Jones LW, Eves ND, Courneya KS, Chiu BK, Baracos VE, Hanson J, Johnson L, and Mackey JR. Effects of Exercise Training on Antitumor Efficacy of Doxorubicin in MDA-MB-231 Breast Cancer Xenografts. Clinical Cancer Research 11: 6695-6698, 2005.
91. Jones LW, Hornsby WE, Goetzinger A, Forbes LM, Sherrard EL, Quist M, Lane AT, West M, Eves ND, Gradison M, Coan A, Herndon JE, and Abernethy AP. Prognostic significance of functional capacity and exercise behavior in patients with metastatic non-small cell lung cancer. Lung Cancer 76: 248-252, 2012.
92. Jones LW, Viglianti BL, Tashjian JA, Kothadia SM, Keir ST, Freedland SJ, Potter MQ, Moon EJ, Schroeder T, Herndon JE, 2nd, and Dewhirst MW. Effect of aerobic exercise on tumor physiology in an animal model of human breast cancer. Journal of Applied Physiology 108: 343-348, 2010.
93. Jones LW, Watson D, Herndon JE, Eves ND, Haithcock BE, Loewen G, and Kohman L. Peak oxygen consumption and long-term all-cause mortality in nonsmall cell lung cancer. Cancer 116: 4825-4832, 2010.
94. Jones SB, Thomas GA, Hesselsweet SD, Alvarez-Reeves M, Yu H, and Irwin ML. Effect of Exercise on Markers of Inflammation in Breast Cancer Survivors: The Yale Exercise and Survivorship Study. Cancer Prevention Research 6: 109-118, 2013.
95. Kang D-W, Lee J, Suh S-H, Ligibel JA, Courneya KS, and Jeon JY. Effects of Exercise on Insulin, IGF-axis, Adipocytokines, and Inflammatory Markers in Breast Cancer Survivors: A Systematic Review and Meta-Analysis. Cancer Epidemiology Biomarkers & Prevention 26: 355-365, 2016.
96. Kasymjanova G, Correa JA, Kreisman H, Dajczman E, Pepe C, Dobson S, Lajeunesse L, Sharma R, and Small D. Prognostic Value of the Six-Minute Walk in Advanced Non-small Cell Lung Cancer. Journal of Thoracic Oncology 4: 602-607, 2009.
97. Kaye JA, Meier CR, Walker AM, and Jick H. Statin use, hyperlipidaemia, and the risk of breast cancer. British Journal of Cancer 86: 1436-1439, 2002.
98. Ke Z, Yip SP, Li L, Zheng X-X, and Tong K-Y. The Effects of Voluntary, Involuntary, and Forced Exercises on Brain-Derived Neurotrophic Factor and Motor Function Recovery: A Rat Brain Ischemia Model. PloS One 6: e16643, 2011.
99. Kenfield SA, Stampfer MJ, Giovannucci E, and Chan JM. Physical Activity and Survival After Prostate Cancer Diagnosis in the Health Professionals Follow-Up Study. Journal of Clinical Oncology 29: 726-732, 2011.
100. Kim EJ, Choi M-R, Park H, Kim M, Hong JE, Lee J-Y, Chun HS, Lee KW, and Yoon Park JH. Dietary fat increases solid tumor growth and metastasis of 4T1 murine mammary carcinoma cells and mortality in obesity-resistant BALB/c mice. Breast Cancer Research 13: R78-R78, 2011.
101. Kitahara CM, Berrington de González A, Freedman ND, Huxley R, Mok Y, Jee SH, and Samet JM. Total Cholesterol and Cancer Risk in a Large Prospective Study in Korea. Journal of Clinical Oncology 29: 1592-1598, 2011.
102. Kos WL, Loria RM, Snodgrass MJ, Cohen D, Thorpe TG, and Kaplan AM. Inhibition of host resistance by nutritional hypercholesteremia. Infection and Immunity 26: 658-667, 1979.
103. Kruijsen-Jaarsma M, Revesz D, Bierings MB, Buffart LM, and Takken T. Effects of Exercise on Immune Function in Patients with Cancer: a Systematic Review. Exercise Immunology Review 19: 120-143, 2013.
104. Kubatka P, Kruzliak P, Rotrekl V, Jelinkova S, and Mladosievicova B. Statins in oncological research: From experimental studies to clinical practice. Critical Reviews in Oncology/Hematology 92: 296-311, 2014.

105. Lappano R, Recchia AG, De Francesco EM, Angelone T, Cerra MC, Picard D, and Maggiolini M. The Cholesterol Metabolite 25-Hydroxycholesterol Activates Estrogen Receptor α-Mediated Signaling in Cancer Cells and in Cardiomyocytes. PloS One 6: e16631, 2011.
106. Li T, Wei S, Shi Y, Pang S, Qin Q, Yin J, Deng Y, Chen Q, Wei S, Nie S, and Liu L. The dose–response effect of physical activity on cancer mortality: findings from 71 prospective cohort studies. British Journal of Sports Medicine 50: 339-345, 2016.
107. Li X, Tang H, Wang J, Xie X, Liu P, Kong Y, Ye F, Shuang Z, Xie Z, and Xie X. The effect of preoperative serum triglycerides and high-density lipoprotein-cholesterol levels on the prognosis of breast cancer. The Breast 32: 1-6, 2017.
108. Ligibel JA, Giobbie-Hurder A, Olenczuk D, Campbell N, Salinardi T, Winer EP, and Mantzoros CS. Impact of a mixed strength and endurance exercise intervention on levels of adiponectin, high molecular weight adiponectin and leptin in breast cancer survivors. Cancer Causes & Control 20: 1523-1528, 2009.
109. Lin S, Gan Z, Han K, Yao Y, and Min D. Interleukin-6 as a prognostic marker for breast cancer: a meta-analysis. Tumori 101: 535-41, 2015.
110. Lira FS, Neto JCR, Antunes BMM, and Fernandes RA. The Relationship Between Inflammation, Dyslipidemia and Physical Exercise: From the Epidemiological to Molecular Approach. Current Diabetes Reviews 10: 391-396, 2014.
111. Liu JUN, Zhang H, Jia L, and Sun H. Effects of Treg cells and IDO on human epithelial ovarian cancer cells under hypoxic conditions. Molecular Medicine Reports 11: 1708-1714, 2015.
112. Llaverias G, Danilo C, Mercier I, Daumer K, Capozza F, Williams TM, Sotgia F, Lisanti MP, and Frank PG. Role of Cholesterol in the Development and Progression of Breast Cancer. The American Journal of Pathology 178: 402-412, 2011.
113. Lu Y, John EM, Sullivan-Halley J, Vigen C, Gomez SL, Kwan ML, Caan BJ, Lee VS, Roh JM, Shariff-Marco S, Keegan THM, Kurian AW, Monroe KR, Cheng I, Sposto R, Wu AH, and Bernstein L. History of Recreational Physical Activity and Survival After Breast Cancer: The California Breast Cancer Survivorship Consortium. American Journal of Epidemiology 181: 944-955, 2015.
114. Ludewig B, Jäggi M, Dumrese T, Brduscha-Riem K, Odermatt B, Hengartner H, and Zinkernagel RM. Hypercholesterolemia Exacerbates Virus-Induced Immunopathologic Liver Disease Via Suppression of Antiviral Cytotoxic T Cell Responses. The Journal of Immunology 166: 3369-3376, 2001.
115. MacKinnon LT. Overtraining effects on immunity and performance in athletes. Immunol Cell Biol 78: 502-509, 2000.
116. MacNeil B, and Hoffman-Goetz L. Chronic exercise enhances in vivo and in vitro cytotoxic mechanisms of natural immunity in mice. Journal of Applied Physiology 74: 388-395, 1993.
117. MacNeil B, and Hoffman-Goetz L. Effect of Exercise on Natural Cytotoxicity and Pulmonary Tumor Metastases in Mice. Medicine and Science in Sports and Exercise 25: 922-928, 1993.
118. Malicka I, Siewierska K, Pula B, Kobierzycki C, Haus D, Paslawska U, Cegielski M, Dziegiel P, Podhorska-Okolow M, and Wozniewski M. The effect of physical training on the N-methyl-N-nitrosourea-induced mammary carcinogenesis of Sprague-Dawley rats. Experimental Biology and Medicine 240: 1408-1415, 2015.
119. Malik J, Stulc T, Wichterle D, Melenovsky V, Chytilova E, Lacinova Z, Marek J, and Ceska R. Hyperlipidemia is associated with altered levels of insulin-like growth factor-I. Physiological Research 57: 919-925, 2008.
120. Mann S, Beedie C, and Jimenez A. Differential Effects of Aerobic Exercise, Resistance Training and Combined Exercise Modalities on Cholesterol and the Lipid Profile: Review, Synthesis and Recommendations. Sports Medicine (Auckland, NZ) 44: 211-221, 2014.
121. Mansourian M, Haghjooy-Javanmard S, Eshraghi A, Vaseghi G, Hayatshahi A, and Thomas J. Statins Use and Risk of Breast Cancer Recurrence and Death: A Systematic Review and Meta-Analysis of Observational Studies. J Pharm Pharm Sci 19: 72-81, 2016.
122. McClellan JL, Davis JM, Steiner JL, Enos RT, Jung SH, Carson JA, Pena MM, Carnevale KA, Berger FG, and Murphy EA. Linking tumor-associated macrophages, inflammation, and intestinal tumorigenesis: role of MCP-1. American Journal of Physiology - Gastrointestinal and Liver Physiology 303: G1087-G1095, 2012.
123. McClellan JL, Steiner JL, Day SD, Enos RT, Davis MJ, Singh UP, and Murphy EA. Exercise effects on polyp burden and immune markers in the Apc(Min)(/+) mouse model of intestinal tumorigenesis. International Journal of Oncology 45: 861-868, 2014.
124. McCullough DJ, Nguyen LM-D, Siemann DW, and Behnke BJ. Effects of exercise training on tumor hypoxia and vascular function in the rodent preclinical orthotopic prostate cancer model. Journal of Applied Physiology 115: 1846-1854, 2013.
125. McCullough DJ, Nguyen LM, Siemann DW, and Behnke BJ. Effects of exercise training on tumor hypoxia and vascular function in the rodent preclinical orthotopic prostate cancer model. Journal of Applied Physiology 115: 1846-1854, 2013.
126. McCullough DJ, Stabley JN, Siemann DW, and Behnke BJ. Modulation of blood flow, hypoxia, and vascular function in orthotopic prostate tumors during exercise. Journal of the National Cancer Institute 106: dju036, 2014.
127. Meneses-Echavez JF, Correa-Bautista JE, González-Jiménez E, Schmidt Río-Valle J, Elkins M, Lobelo F, and Ramírez-Vélez R. The effect of exercise training on mediators of inflammation in breast cancer survivors: a systematic review with meta-analysis. Cancer Epidemiology Biomarkers & Prevention 25: 1009-17, 2016.
128. Meyerhardt JA, Giovannucci EL, Holmes MD, Chan AT, Chan JA, Colditz GA, and Fuchs CS. Physical Activity and Survival After Colorectal Cancer Diagnosis. Journal of Clinical Oncology 24: 3527-3534, 2006.
129. Meyerhardt JA, Giovannucci EL, Ogino S, Kirkner GJ, Chan AT, Willett W, and Fuchs CS. Physical activity and survival in male colorectal cancer survivors. Archives of Internal Medicine 169: 2102-2108, 2009.
130. Meyerhardt JA, Heseltine D, Niedzwiecki D, Hollis D, Saltz LB, Mayer RJ, Thomas J, Nelson H, Whittom R, Hantel A, Schilsky RL, and Fuchs CS. Impact of Physical Activity on Cancer Recurrence and Survival in Patients With Stage III Colon Cancer: Findings From CALGB 89803. Journal of Clinical Oncology 24: 3535-3541, 2006.
131. Meyerhardt JA, Ogino S, Kirkner GJ, Chan AT, Wolpin B, Ng K, Nosho K, Shima K, Giovannucci EL, Loda M, and Fuchs CS. Interaction of Molecular Markers and Physical Activity on Mortality in Patients with Colon Cancer. Clinical Cancer Research 15: 5931-5936, 2009.

132. Michigan A, Johnson TV, and Master VA. Review of the Relationship between C-Reactive Protein and Exercise. Molecular Diagnosis & Therapy 15: 265-275, 2012.
133. Milne HM, Wallman KE, Gordon S, and Courneya KS. Effects of a combined aerobic and resistance exercise program in breast cancer survivors: a randomized controlled trial. Breast Cancer Research and Treatment 108: 279-288, 2008.
134. Monninkhof EM, Elias SG, Vlems FA, van der Tweel I, Schuit AJ, Voskuil DW, van Leeuwen FE, and TFPAC obo. Physical Activity and Breast Cancer: A Systematic Review. Epidemiology 18: 137-157, 2007.
135. Moore SC, Lee IM, Weiderpass E, Campbell PT, Sampson JN, Kitahara CM, Keadle SK, Arem H, Berrington de Gonzalez A, Hartge P, Adami HO, Blair CK, Borch KB, Boyd E, Check DP, Fournier A, Freedman ND, Gunter M, Johannson M, Khaw KT, Linet MS, Orsini N, Park Y, Riboli E, Robien K, Schairer C, Sesso H, Spriggs M, Van Dusen R, Wolk A, Matthews CE, and Patel AV. Association of Leisure-Time Physical Activity With Risk of 26 Types of Cancer in 1.44 Million Adults. JAMA Intern Med 176: 816-825, 2016.
136. Moorman PG, Jones LW, Akushevich L, and Schildkraut JM. Recreational Physical Activity and Ovarian Cancer Risk and Survival. Annals of Epidemiology 21: 178-187, 2011.
137. Morikawa T, Kuchiba A, Yamauchi M, Meyerhardt JA, Shima K, Nosho K, Chan AT, Giovannucci E, Fuchs CS, and Ogino S. Association of CTNNB1 (β-Catenin) Alterations, Body Mass Index, and Physical Activity with Survival in Patients with Colorectal Cancer. JAMA 305: 1685-1694, 2011.
138. Murai T. Cholesterol lowering: role in cancer prevention and treatment. Biological Chemistry 369: 1-11, 2015.
139. Murphy EA, Davis JM, Barrilleaux TL, McClellan JL, Steiner JL, Carmichael MD, Pena MM, Hebert JR, and Green JE. Benefits of exercise training on breast cancer progression and inflammation in C3(1)SV40Tag mice. Cytokine 55: 274-279, 2011.
140. Myers J. Exercise and Cardiovascular Health. Circulation 107: e2-e5, 2003.
141. Nagaiah G, Hazard HW, and Abraham J. Role of obesity and exercise in breast cancer survivors. Oncology (Williston Park, NY) 24: 342-346, 2010.
142. Nelson ER, Chang C-y, and McDonnell DP. Cholesterol and Breast Cancer Pathophysiology. Trends in Endocrinology and Metabolism 25: 649-655, 2014.
143. Nelson ER, Wardell SE, Jasper JS, Park S, Suchindran S, Howe MK, Carver NJ, Pillai RV, Sullivan PM, Sondhi V, Umetani M, Geradts J, and McDonnell DP. 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. Science (New York, NY) 342: 1094-1098, 2013.
144. Nelson RH. Hyperlipidemia as a Risk Factor for Cardiovascular Disease. Primary Care 40: 195-211, 2013.
145. Nieman KM, Romero IL, Van Houten B, and Lengyel E. Adipose tissue and adipocytes support tumorigenesis and metastasis. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1831: 1533-1541, 2013.
146. Niu J, Jiang L, Guo W, Shao L, Liu Y, and Wang L. The Association between Leptin Level and Breast Cancer: A Meta-Analysis. PloS One 8: e67349, 2013.
147. Noman MZ, Buart S, Romero P, Ketari S, Janji B, Mari B, Mami-Chouaib F, and Chouaib S. Hypoxia-Inducible miR-210 Regulates the Susceptibility of Tumor Cells to Lysis by Cytotoxic T Cells. Cancer Research 72: 4629-4641, 2012.
148. Noman MZ, Hasmim M, Messai Y, Terry S, Kieda C, Janji B, and Chouaib S. Hypoxia: a key player in antitumor immune response. A Review in the Theme: Cellular Responses to Hypoxia. American Journal of Physiology - Cell Physiology 309: C569-C579, 2015.
149. Ohira T, Schmitz KH, Ahmed RL, and Yee D. Effects of weight training on quality of life in recent breast cancer survivors. Cancer 106: 2076-2083, 2006.
150. Pedersen BK. Exercise-induced myokines and their role in chronic diseases. Brain, Behavior, and Immunity 25: 811-816, 2011.
151. Pedersen L, Idorn M, Olofsson GH, Lauenborg B, Nookaew I, Hansen RH, Johannesen HH, Becker JC, Pedersen KS, Dethlefsen C, Nielsen J, Gehl J, Pedersen BK, Thor Straten P, and Hojman P. Voluntary Running Suppresses Tumor Growth through Epinephrine- and IL-6-Dependent NK Cell Mobilization and Redistribution. Cell Metabolism 23: 554-62, 2016.
152. Peel AB, Thomas SM, Dittus K, Jones LW, and Lakoski SG. Cardiorespiratory Fitness in Breast Cancer Patients: A Call for Normative Values. Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease 3: e000432, 2014.
153. Pelton K, Coticchia CM, Curatolo AS, Schaffner CP, Zurakowski D, Solomon KR, and Moses MA. Hypercholesterolemia induces angiogenesis and accelerates growth of breast tumors in vivo. The American Journal of Pathology 184: 2099-2110, 2014.
154. Pierce BL, Ballard-Barbash R, Bernstein L, Baumgartner RN, Neuhouser ML, Wener MH, Baumgartner KB, Gilliland FD, Sorensen BE, McTiernan A, and Ulrich CM. Elevated Biomarkers of Inflammation Are Associated With Reduced Survival Among Breast Cancer Patients. Journal of Clinical Oncology 27: 3437-3444, 2009.
155. Pierce BL, Neuhouser ML, Wener MH, Bernstein L, Baumgartner RN, Ballard-Barbash R, Gilliland FD, Baumgartner KB, Sorensen B, McTiernan A, and Ulrich CM. Correlates of circulating C-reactive protein and serum amyloid A concentrations in breast cancer survivors. Breast Cancer Research and Treatment 114: 155-167, 2008.
156. Pierce JP, Stefanick ML, Flatt SW, Natarajan L, Sternfeld B, Madlensky L, Al-Delaimy WK, Thomson CA, Kealey S, Hajek R, Parker BA, Newman VA, Caan B, and Rock CL. Greater Survival After Breast Cancer in Physically Active Women With High Vegetable-Fruit Intake Regardless of Obesity. Journal of Clinical Oncology 25: 2345-2351, 2007.
157. Printz C. Researchers find link between high cholesterol and breast cancer. Cancer 120: 3429-3429, 2014.
158. Protani M, Coory M, and Martin JH. Effect of obesity on survival of women with breast cancer: systematic review and meta-analysis. Breast Cancer Research and Treatment 123: 627-635, 2010.
159. Ravnskov U, McCully KS, and Rosch PJ. The statin-low cholesterol-cancer conundrum. QJM 105: 383-388, 2012.
160. Ren L, Yu Y, Wang L, Zhu Z, Lu R, and Yao Z. Hypoxia-induced CCL28 promotes recruitment of regulatory T cells and tumor growth in liver cancer. Oncotarget 7: 75763-75773, 2016.
161. Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, and Kzhyshkowska J. Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. Frontiers in Physiology 5: 75, 2014.

162. Richman EL, Kenfield SA, Stampfer MJ, Paciorek A, Carroll PR, and Chan JM. Physical Activity after Diagnosis and Risk of Prostate Cancer Progression: Data from the Cancer of the Prostate Strategic Urologic Research Endeavor. Cancer Research 71: 3889-3895, 2011.
163. Rodrigues dos Santos C, Domingues G, Matias I, Matos J, Fonseca I, de Almeida JM, and Dias S. LDL-cholesterol signaling induces breast cancer proliferation and invasion. Lipids in Health and Disease 13: 16-16, 2014.
164. Rodrigues dos Santos C, Fonseca I, Dias S, and Mendes de Almeida JC. Plasma level of LDL-cholesterol at diagnosis is a predictor factor of breast tumor progression. BMC Cancer 14: 132-132, 2014.
165. Rogers LQ, Fogleman A, Trammell R, Hopkins-Price P, Vicari S, Rao K, Edson B, Verhulst S, Courneya KS, and Hoelzer K. Effects of a physical activity behavior change intervention on inflammation and related health outcomes in breast cancer survivors: pilot randomized trial. Integrative Cancer Therapies 12: 323-335, 2013.
166. Ruden E, Reardon DA, Coan AD, Herndon JE, Hornsby WE, West M, Fels DR, Desjardins A, Vredenburgh JJ, Waner E, Friedman AH, Friedman HS, Peters KB, and Jones LW. Exercise Behavior, Functional Capacity, and Survival in Adults With Malignant Recurrent Glioma. Journal of Clinical Oncology 29: 2918-2923, 2011.
167. Sáez MdC, Barriga C, García JJxE, Rodríguez AB, and Ortega E. Exercise-induced stress enhances mammary tumor growth in rats: Beneficial effect of the hormone melatonin. Molecular and Cellular Biochemistry 294: 19-24, 2006.
168. Salomon F, Cuneo RC, Hesp R, and Sonksen PH. The effects of treatment with recombinant human growth hormone on body composition and metabolism in adults with growth hormone deficiency. New England Journal of Medicine 321: 1797-1803, 1989.
169. Sarkar S, Germeraad WTV, Rouschop KMA, Steeghs EMP, van Gelder M, Bos GMJ, and Wieten L. Hypoxia Induced Impairment of NK Cell Cytotoxicity against Multiple Myeloma Can Be Overcome by IL-2 Activation of the NK Cells. PloS One 8: e64835, 2013.
170. Schmidt S, Monk JM, Robinson LE, and Mourtzakis M. The integrative role of leptin, oestrogen and the insulin family in obesity-associated breast cancer: potential effects of exercise. Obesity Reviews 16: 473-487, 2015.
171. Schmitz KH, Ahmed RL, Hannan PJ, and Yee D. Safety and Efficacy of Weight Training in Recent Breast Cancer Survivors to Alter Body Composition, Insulin, and Insulin-Like Growth Factor Axis Proteins. Cancer Epidemiology Biomarkers & Prevention 14: 1672-1680, 2005.
172. Scott E, Daley AJ, Doll H, Woodroofe N, Coleman RE, Mutrie N, Crank H, Powers HJ, and Saxton JM. Effects of an exercise and hypocaloric healthy eating program on biomarkers associated with long-term prognosis after early-stage breast cancer: a randomized controlled trial. Cancer Causes & Control 24: 181-191, 2012.
173. Sheng ZJ, Qin CJ, Wei CW, Miao LC, Hua ZG, Rui C, Lin L, and Cai WM. The effect of aerobic exercise and Macrothele raven venom on tumor-bearing mice. International Journal of Sports Medicine 36: 93-100, 2015.
174. Silveira EMS, Rodrigues MF, Krause MS, Vianna DR, Almeida BS, Rossato JS, Oliveira LP, Curi R, and de Bittencourt PIH. Acute exercise stimulates macrophage function: possible role of NF-κB pathways. Cell Biochemistry and Function 25: 63-73, 2007.
175. Steiner JL, and Murphy EA. Importance of chemokine (CC-motif) ligand 2 in breast cancer. The International Journal of Biological Markers 27: e179-185, 2012.
176. Sternfeld B, Weltzien E, Quesenberry CP, Castillo AL, Kwan M, Slattery ML, and Caan BJ. Physical Activity and Risk of Recurrence and Mortality in Breast Cancer Survivors: Findings from the LACE Study. Cancer Epidemiology Biomarkers & Prevention 18: 87-95, 2009.
177. Strohmaier S, Edlinger M, Manjer J, Stocks T, Bjørge T, Borena W, Häggström C, Engeland A, Nagel G, Almquist M, Selmer R, Tretli S, Concin H, Hallmans G, Jonsson H, Stattin P, and Ulmer H. Total Serum Cholesterol and Cancer Incidence in the Metabolic Syndrome and Cancer Project (Me-Can). PloS One 8: e54242, 2013.
178. Sturgeon KM, Digiovanni L, Good J, Salvatore D, Fenderson D, Domchek SM, Stopfer JE, Galantino ML, Bryan C, Hwang W-T, and Schmitz K. Exercise Induced Dose Response Alterations in Adiponectin and Leptin Levels are Dependent on Body Fat Changes in Women at Risk for Breast Cancer. Cancer Epidemiology Biomarkers & Prevention 25(8): 1195-200, 2016.
179. Swisher AK, Abraham J, Bonner D, Gilleland D, Hobbs G, Kurian S, Yanosik MA, and Vona-Davis L. Exercise and dietary advice intervention for survivors of triple-negative breast cancer: effects on body fat, physical function, quality of life, and adipokine profile. Supportive Care in Cancer 23: 2995-3003, 2015.
180. Tas F, Karabulut S, Bilgin E, Tastekin D, and Duranyildiz D. Clinical significance of serum insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein-3 (IGFBP-3) in patients with breast cancer. Tumor Biology 35: 9303-9309, 2014.
181. The Endogenous H, and Breast Cancer Collaborative G. Insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and breast cancer risk: pooled individual data analysis of 17 prospective studies. The Lancet Oncology 11: 530-542, 2010.
182. Tian L, Goldstein A, Wang H, Ching Lo H, Sun Kim I, Welte T, Sheng K, Dobrolecki LE, Zhang X, Putluri N, Phung TL, Mani SA, Stossi F, Sreekumar A, Mancini MA, Decker WK, Zong C, Lewis MT, and Zhang XHF. Mutual regulation of tumour vessel normalization and immunostimulatory reprogramming. Nature 544: 250-254, 2017.
183. Touvier M, Fassier P, His M, Norat T, Chan DSM, Blacher J, Hercberg S, Galan P, Druesne-Pecollo N, and Latino-Martel P. Cholesterol and breast cancer risk: a systematic review and meta-analysis of prospective studies. British Journal of Nutrition 114: 347-357, 2015.
184. Traversari C, Sozzani S, Steffensen KR, and Russo V. LXR-dependent and -independent effects of oxysterols on immunity and tumor growth. European Journal of Immunology 44: 1896-1903, 2014.
185. Uhlenbruck G, and Order U. Can Endurance Sports Stimulate Immune Mechanisms against Cancer and Metastasis? International Journal of Sports Medicine 12: S63-S68, 1991.
186. Undela K, Srikanth V, and Bansal D. Statin use and risk of breast cancer: a meta-analysis of observational studies. Breast Cancer Research and Treatment 135: 261-269, 2012.

187. van Gemert WA, May AM, Schuit AJ, Oosterhof BYM, Peeters PH, and Monninkhof EM. Effect of weight loss with or without exercise on inflammatory markers and adipokines in postmenopausal women: the SHAPE-2 trial A Randomized Controlled Trial. Cancer Epidemiology Biomarkers & Prevention 25: 799-806, 2016.
188. Vandersluis AD, Venier NA, Colquhoun AJ, Sugar L, Pollak M, Kiss A, Fleshner NE, Klotz LH, and Venkateswaran V. Exercise does not counteract the effects of a "westernized" diet on prostate cancer xenografts. The Prostate 73: 1223-1232, 2013.
189. Vaupel P. Hypoxia and Aggressive Tumor Phenotype: Implications for Therapy and Prognosis. The Oncologist 13: 21-26, 2008.
190. Vignali DAA, Collison LW, and Workman CJ. How regulatory T cells work. Nature reviews Immunology 8: 523-532, 2008.
191. Williams CB, Yeh ES, and Soloff AC. Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy. NPJ Breast Cancer 2: 15025, 2016.
192. Wood IS, de Heredia FP, Wang B, and Trayhurn P. Cellular hypoxia and adipose tissue dysfunction in obesity. The Proceedings of the Nutrition Society 68: 370-377, 2009.
193. World Health Organisation. Breast Cancer: Prevention and Control. http://www.who.int/cancer/detection/breastcancer/en/; accessed 8/12/2017
194. Wu Q, Ishikawa T, Sirianni R, Tang H, McDonald JG, Yuhanna IS, Thompson B, Girard L, Mineo C, Brekken RA, Umetani M, Euhus DM, Xie Y, and Shaul PW. 27-Hydroxycholesterol Promotes Cell-autonomous ER-positive Breast Cancer Growth. Cell Reports 5: 637-645, 2013.
195. Yan L, and Demars LC. Effects of Non-motorized Voluntary Running on Experimental and Spontaneous Metastasis in Mice. Anticancer Research 31: 3337-3344, 2011.
196. Yanagita S, Amemiya S, Suzuki S, and Kita I. Effects of spontaneous and forced running on activation of hypothalamic corticotropin-releasing hormone neurons in rats. Life Sciences 80: 356-363, 2007.
197. You T, Arsenis NC, Disanzo BL, and LaMonte MJ. Effects of Exercise Training on Chronic Inflammation in Obesity. Sports Medicine 43: 243-256, 2013.
198. Yuan Z-Y, Luo R-Z, Peng R-J, Wang S-S, and Xue C. High infiltration of tumor-associated macrophages in triple-negative breast cancer is associated with a higher risk of distant metastasis. OncoTargets and Therapy 7: 1475-1480, 2014.
199. Zhang Q-B, Meng X-T, Jia Q-A, Bu Y, Ren Z-G, Zhang B-H, and Tang Z-Y. Herbal Compound Songyou Yin and Moderate Swimming Suppress Growth and Metastasis of Liver Cancer by Enhancing Immune Function. Integrative Cancer Therapies 15: 368-375, 2016.
200. Zhang QB, Zhang BH, Zhang KZ, Meng XT, Jia QA, Zhang QB, Bu Y, Zhu XD, Ma DN, Ye BG, Zhang N, Ren ZG, Sun HC, and Tang ZY. Moderate swimming suppressed the growth and metastasis of the transplanted liver cancer in mice model: with reference to nervous system. Oncogene 35: 4122-4131, 2016.
201. Zhang Y, Cheng S, Zhang M, Zhen L, Pang D, Zhang Q, and Li Z. High-Infiltration of Tumor-Associated Macrophages Predicts Unfavorable Clinical Outcome for Node-Negative Breast Cancer. PloS One 8: e76147, 2013.
202. Zheng Q, Dunlap SM, Zhu J, Downs-Kelly E, Rich JN, Hursting SD, Berger NA, and Reizes O. Leptin deficiency suppresses MMTV-Wnt-1 mammary tumor growth in obese mice and abrogates tumor initiating cell survival. Endocrine-Related Cancer 18: 491-503, 2011.
203. Zheng XI, Cui X-X, Gao ZHI, Zhao Y, Shi YI, Huang M-T, Liu YUE, Wagner GC, Lin Y, Shih WJ, Rao CV, Yang CS, and Conney AH. Inhibitory effect of dietary atorvastatin and celecoxib together with voluntary running wheel exercise on the progression of androgen-dependent LNCaP prostate tumors to androgen independence. Experimental and Therapeutic Medicine 2: 221-228, 2011.
204. Zhong S, Zhang X, Chen L, Ma T, Tang J, and Zhao J. Statin use and mortality in cancer patients: Systematic review and meta-analysis of observational studies. Cancer Treatment Reviews 41: 554-567, 2015.
205. Zhu Z, Jiang W, Zacher JH, Neil ES, McGinley JN, and Thompson HJ. Effects of Energy Restriction and Wheel Running on Mammary Carcinogenesis and Host Systemic Factors in a Rat Model. Cancer Prevention Research 5: 414-422, 2012.
206. Zielinski MR, Muenchow M, Wallig MA, Horn PL, and Woods JA. Exercise delays allogeneic tumor growth and reduces intratumoral inflammation and vascularization. Journal of Applied Physiology 96: 2249-2256, 2004.

Anxiety and perceived psychological stress play an important role in the immune response after exercise

Jason P. Edwards[1], Neil P. Walsh[1], Bethany C. Diment[1,2] and Ross Roberts[3]

[1] College of Health and Behavioural Sciences, Bangor University, Bangor, Gwynedd, UK.
[2] Norwich Medical School, University of East Anglia, Norwich, Norfolk, UK.
[3] Institute for the Psychology of Elite Performance, Bangor University, Bangor, Gwynedd, UK.

ABSTRACT

There are common pathways by which psychological stress and exercise stress alter immunity. However, it remains unknown whether psychological stress plays a role in the in vivo immune response to exercise. We examined the relationship between anxiety and perceived psychological stress reported before exercise and in vivo immunity after exercise using skin sensitisation with Diphenylcyclopropenone (DPCP). In a randomised design, sixty four, thoroughly familiarised, males completed widely used psychological instruments to assess state-anxiety and perceived psychological stress before exercise, and ran either 30 minutes at 60% (30MI) or 80% (30HI) $\dot{V}O_{2peak}$, 120 minutes at 60% (120MI) $\dot{V}O_{2peak}$ or rested (CON) before DPCP sensitisation. Cutaneous recall to DPCP was measured as the dermal thickening response to a low-dose series DPCP challenge 4-weeks after sensitisation. After accounting for exercise (R^2 = 0.20; P < 0.01), multiple-regression showed that pre-exercise state-anxiety (STAI-S; ΔR^2 = 0.19; P < 0.01) and perceived psychological stress (ΔR^2 = 0.13; P < 0.05) were moderately associated with the DPCP response after exercise. The STAI-S scores before exercise were considered low-to-moderate in these familiarised individuals (median split; mean STAI-S of low 25 and moderate 34). Further examination showed that the DPCP response after exercise (30MI, 30HI or 120MI) was 62% lower in those reporting low vs. moderate state-anxiety before exercise (mean difference in dermal thickening: -2.6 mm; 95% CI: -0.8 to -4.4 mm; P < 0.01). As such, the results indicate a beneficial effect of moderate (vs. low) state-anxiety and perceived psychological stress on in vivo immunity after exercise. Moreover, correlations were of comparable strength for the relationship between physiological stress (heart rate training impulse) and the summed dermal response to DPCP (r = -0.37; 95% CI: -0.05 to -0.62; P = 0.01), and state-anxiety and the summed dermal response to DPCP (r = 0.39; 95% CI: 0.08 to 0.63; P < 0.01). In conclusion, state-anxiety and perceived psychological stress levels before exercise play an important role in determining the strength of the in vivo immune response after exercise. These findings indicate a similar strength relationship for the level of state-anxiety prior to exercise and the level of physiological stress during exercise with the in vivo immune response after exercise. Future research is required to investigate exercise-immune responses in athletes, military personnel and others in physically demanding occupations experiencing higher levels of psychological stress than those reported in this study e.g. related to important competition, military operations and major life events. Nevertheless, the present findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise.

Keywords: Running, Immunity, In vivo, Diphencyprone, STAI

INTRODUCTION

Numerous studies report an increase in upper respiratory tract infection (URTI) symptoms following a bout of strenuous exercise and during periods of heavy training in athletes (25, 33, 37), and there is widespread agreement that a transient suppression of immune function is at least partly responsible (48). A multitude of training and lifestyle stressors are thought to be involved in the observed decrease in immune function in athletes and military personnel; including, prolonged training sessions, exposure to environmental extremes (e.g. heat, cold and high altitude), poor nutrition and poor sleep (41-43, 47, 48). For example, prolonged heavy exercise (≥ 2 h) transiently decreases *in vitro* measures of immunity in isolated blood samples (48) and more clinically meaningful *in vivo* measures of immunity instigated at the skin, including delayed type hypersensitivity (DTH) and contact hypersensitivity (CHS) (6, 16, 24). Indeed, recent work highlights the immunosuppressive effect of prolonged exercise (2 h) on the induction of CHS using the novel antigen Diphenylcyclopropenone (DPCP) (16, 24). Besides the immunosuppressive effects of prolonged heavy training sessions, the training environment and lifestyle stressors such as nutritional deficits (e.g. energy, macro- and micro- nutrients) and poor sleep (e.g. total deprivation and disruption) have long been implicated in the decrease in immune function in athletes and military personnel (41-43). Somewhat surprisingly, field studies (multi-stres-

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

sor environment) and laboratory studies mimicking real-world athletic and military scenarios by exposing participants to these stressors, either separately or combined, demonstrate only subtle and short-lived modulation of immunity at rest and in response to exercise (5, 28, 31, 40). Rather than decrease immunity, some studies actually show a beneficial 'priming' effect of stressors such as short-term sleep disruption (1 night) (28), intermittent cold exposure (29) and intermittent hypoxic exposure on immunity (50). As such, there is a pressing need for research investigating other likely behavioural, environmental and lifestyle candidates involved in the observed decrease in immune function in athletes and military personnel.

Given the well-known and marked influence of psychological stress on immunity and infection resistance (10, 13), and the likely shared mechanisms by which psychological stress and exercise stress alter immunity (36); i.e. principally through activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic-adrenal-medullary (SAM) axis and subsequent immunomodulatory hormones, it has been hypothesised that psychological stress can play a role in the decrease in immunity with prolonged heavy exercise and heavy training (8, 36, 49). Unfortunately, exercise immunologists rarely report measures of psychological stress in their studies and so there is little by way of empirical evidence to support this hypothesis (38). That there are striking similarities in the way acute and chronic psychological stress and acute and chronic exercise stress influence immunity provides indirect support for this hypothesis. For example, although chronic psychological stress is widely accepted to decrease immunity and increase infection risk (10, 13), short-lasting, moderate-intensity psychological stress can enhance *in vivo* immunity (21) and is considered a fundamental adaptive response to help us survive (13). Similarly, prolonged heavy exercise and heavy training are widely accepted to decrease immunity and increase infection risk (48), but short-lasting, moderate-intensity exercise stress can enhance *in vivo* immunity (34).

With this information in mind, using a multiple linear regression model, we tested, and provide evidence supporting our hypothesis that the level of anxiety and perceived psychological stress reported by an individual prior to exercise play an important role in determining the strength of the *in vivo* immune response to DPCP after exercise.

METHODS

Using the CHS responses to exercise from a previous study (16), here we present previously unpublished and novel insights regarding the influence of anxiety and perceived psychological stress on *in vivo* immunity after exercise.

Participants

Sixty four healthy, non-smoking, recreationally active males (age 22 ± 3 years; height 180 ± 6 cm; body mass 76.7 ± 11.5 kg; $\dot{V}O_{2peak}$ 57 ± 6 mL/kg/min) gave written informed consent to participate in the study. Participants had no previous history of exposure to DPCP and were excluded if they were taking any medication or dietary supplements, or had a history of atopy or any other immune-related or inflammatory dermatological condition. Participants were required to abstain from alcohol and exercise for 24 h before and 48 h after the experimental trials. The study received local ethics committee approval, and all protocols were conducted in accordance with the Declaration of Helsinki (2013).

Participants were matched for age and aerobic fitness (gas exchange threshold and $\dot{V}O_{2peak}$) before being randomly assigned to one of four groups. Groups were 1) 120 min of seated rest (CON); 2) 30 min of moderate-intensity (60% $\dot{V}O_{2peak}$) exercise (30MI); 3) 30 min of high-intensity (80% $\dot{V}O_{2peak}$) exercise (30HI); or 4) 120 min of moderate-intensity (60% $\dot{V}O_{2peak}$) exercise (120MI).

Preliminary measures and familiarisation

$\dot{V}O_{2peak}$ was estimated by means of a ramped exercise test on a treadmill (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany) as described (16). At least 24 h after the preliminary measures and approximately 7 days before the experimental trial, participants were informed of their group allocation and attended the laboratory for familiarisation. For exercising participants, the calculated exercise intensity was verified, and the participant was familiarised by running for 50% of their allocated exercise duration. During this visit, all participants were familiarised with blood sampling and other relevant procedures.

Experimental procedures

On the day of the experimental trial, participants were transported to the laboratory at 0730 h and provided with a standard breakfast (0.03 MJ/kg) before completing widely used, validated psychological instruments. The level of anxiety was assessed using the state aspect of the State Trait Anxiety Inventory (STAI-S): the STAI-S is one of the most commonly used scales to measure anxiety, which has been defined as an unpleasant emotional state that exists at a given moment in time and at a particular level of intensity, and is characterised by subjective feelings of tension, apprehension, nervousness, and worry (45). The STAI-S consists of 20-items, with responses being measured on a four-point Likert scale (from 1 'not at all' to 4 'very much so') and a range of scores from 20−80 (composite reliability = 0.94). Perceived psychological stress was assessed using the Perceived Stress Scale (PSS): the PSS is a widely used psychological instrument for measuring the perception of stress, and measures the degree to which life situations are considered stressful by the individual during the previous month (11). The PSS is a 14-item inventory, with responses measured on a five-point Likert scale (from 0 'never' to 4 'very often') and a range of scores from 0−56 (composite reliability = 0.73). Average PSS score for young adults has been reported as 21 ± 7 and high PSS score in post-traumatic stress disorder patients as 34 ± 8 (11, 26). Participants assigned to 120MI began running on a treadmill at 1100 h, and those assigned to 30HI and 30MI began at 1230 h, so that all participants completed the exercise at the same time of day (1300 h). Heart rate was monitored continuously during the experimental trials (Polar FT1, Polar Electro, Kempele, Finland). Immediately after the exercise, participants showered and returned to the laboratory within 15 min of completion before being sensitised to DPCP at 1320 h, exactly 20

min after exercise cessation. This short standardised delay in sensitisation allowed cutaneous blood flow to return to baseline (16).

Blood collection and analysis

Blood samples were collected before, immediately after, and 1 h after exercise or seated rest by venepuncture into two separate vacutainer tubes (Becton Dickinson, Oxford, UK), one containing K_3EDTA, and one containing lithium heparin. The samples were spun at 1500 g for 10 minutes in a refrigerated centrifuge. Plasma was aliquoted into Eppendorf tubes, and immediately frozen at -80 °C for later analysis. Plasma epinephrine and norepinephrine were determined on K_3EDTA plasma, and plasma cortisol was determined on lithium heparin plasma using commercially available ELISA kits (CatCombi, IBL International, Hamburg, Germany and DRG Instruments, Marburg, Germany, respectively). The intra-assay coefficient of variation for plasma epinephrine, norepinephrine and cortisol was 4.1%, 4.1% and 4.4%, respectively.

Induction of CHS

The sensitising exposure to the novel antigen DPCP involved application of an occluded patch, constituting a 12-mm aluminium Finn chamber (Epitest Oy, Tuusula, Finland) on scanpor hypoallergenic tape containing an 11-mm filter paper disc (16). The paper disc was soaked in 22.8 μL of 0.125% DPCP in acetone (patch = 30 μg/cm^2 DPCP) and allowed to dry for 5 min before being applied to the skin on the lower back for exactly 48 h.

Elicitation

The magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to DPCP. Twenty eight days after the initial sensitisation to DPCP, all participants received a challenge with a low-concentration dose-series of DPCP on individual patches, each comprising an 8-mm aluminium Finn chamber on scanpor hypoallergenic tape containing a 7-mm filter paper disc. Patches were applied to the volar aspect of the upper arm in the following concentrations: 10 μL of DPCP: 0.0048%, 1.24 μg/cm^2; 0.0076%, 1.98 μg/cm^2; 0.0122%, 3.17 μg/cm^2; 0.0195%, 5.08 μg/cm^2; 0.0313%, 8.12 μg/cm^2; and, 10 μL of 100% acetone served as a control patch for background subtraction. Patches were applied in randomly allocated order at the local site to minimise any anatomical variability in responses. Elicitation patches were removed after 6 h, and the strength of immune reactivity was assessed as the cutaneous responses 48 h after application (16).

Assessment of CHS responses

Dermal thickness was determined at each elicitation site using a high-frequency ultrasound scanner (Episcan, Longport Inc, Reading, UK). The ultrasound probe was placed over the centre of each patch site together with ultrasound gel. The mean of three measurements was taken from each 12-mm scan image assessed at a later time by a blinded investigator. Mean skinfold thickness was determined from triplicate measurements at each elicitation site using modified spring-loaded skin callipers (Harpenden Skinfold Calliper, British Indicators, England, UK). As previously described (24), this method provides an objective measure of skin oedema (inflammatory swelling). Skinfold thickness was recorded to the nearest 0.1 mm by placing the jaws of the calliper at the outer diameter of the response site and measuring skin thickness only (no subcutaneous fat). Skinfold thickness assessed using skinfold callipers has previously been shown to be strongly related (r = 0.93) with high-frequency ultrasound readings of dermal thickness (16). Mean skin erythema was determined from triplicate measurements at each elicitation site using an erythema meter (ColorMeter DSM11, Cortex Technology, Hadsund, Denmark) which provides an objective measure of skin redness (24). Mean background values were determined from triplicate measurements at the acetone patch site for both thickness and redness. To determine the increase in thickness and redness, the value from the acetone-only site was subtracted from each elicitation site value. The values for increase in dermal thickness, skinfold thickness and erythema over all the doses were summed to give an approximation of the area under the dose–response curve, representative of the overall reactivity of each participant to DPCP (24).

Statistical analyses

Hierarchical linear regression analysis was used to examine the relationship between STAI-S and PSS (in 2 separate models) and *in vivo* immunity after exercise. In step 1 of each model, the influence of exercise on the summed dermal thickening response to DPCP was accounted for by calculating the training impulse (TRIMP) to reflect the level of physiological stress, as described (2). In step 2, the influence of each psychological measure on the summed dermal thickening response to DPCP was assessed. Sample size was deemed appropriate for the multiple linear regression analysis with 2 steps, in line with recommendations (46). To further illustrate the influence of anxiety on *in vivo* immunity after exercise, we performed additional analyses by categorising the population based on STAI-S scores using a median split; whereby, the levels before exercise were defined as low anxiety (LOW: STAI-S ≤ 29; mean 25) and moderate anxiety (MOD: STAI-S ≥ 30; mean 34): the STAI-S ranges for LOW and MOD are in line with those reported in the literature (30, 45). Independent t-tests were used to compare the summed dermal responses to DPCP in LOW and MOD in each group (30MI, 30HI, 120MI and CON). Comparisons of psychological measures between groups (30MI, 30HI, 120MI and CON) were made using one-way ANOVA. A two-way, mixed-model ANOVA was used to analyse DPCP responses across the full dose-series challenge (anxiety level x dose) and circulating stress hormones (anxiety level x time) with significant differences identified using *post hoc* Tukey HSD, where appropriate. Pearson correlation coefficients were also calculated between physiological stress (TRIMP) and the DPCP response, and anxiety and the DPCP response. To determine the influence of anxiety on the threshold DPCP dose that elicits a response, logarithmic transformation was performed on the DPCP data (LOW *vs.* MOD). This enabled the calculation of the x-intercept when $y = 0$, using linear regression on the linear portion of the dose-response curve. A threshold dose for a response to DPCP was then calculated by back transformation (antilog). Data are presented as mean ± SD, unless otherwise stated and statistical significance was accepted at $P < 0.05$. Data were checked for normality and where appropriate natural log transformation was performed before analysis. Statistical analyses were performed using common statistical

software packages (SPSS 22; IBM, Chicago, IL, and GraphPad Prism 5.0, San Diego, CA). Cohen's *d* effect sizes (*d*) are presented to indicate the meaningfulness of group differences for DPCP responses; whereby, values greater than 0.2, 0.5, and 0.8 represent small, medium, and large effects, respectively (9).

RESULTS

STAI-S Anxiety

Prior to exercise, there were no differences in psychological measures between groups (e.g. STAI-S scores for 30MI, 30HI, 120MI and CON) and participants reported low-to-moderate STAI-S scores (Fig. 1A). In step 1 of the regression model (Table 1), exercise (TRIMP; 78 ± 60 AU) was a significant predictor accounting for 20% of the variance in the summed dermal thickening response to DPCP ($P < 0.01$); whereby, greater physiological stress was associated with a lower DPCP response following exercise. In step 2, STAI-S score was a significant predictor over and above exercise, accounting for an additional 19% of the variance in DPCP response ($P < 0.01$); together, exercise and anxiety accounted for 39% of the variance in the dermal thickening response to DPCP (Table 1). Pearson correlation coefficients were of comparable, moderate strength for the relationship between physiological stress and the summed dermal response to DPCP (TRIMP; $r = -0.37$, $R^2 = 0.13$, $P = 0.01$), and anxiety and the summed dermal response to DPCP (STAI-S score; $r = 0.39$, $R^2 = 0.15$, $P < 0.01$). This association between anxiety before exercise and *in vivo* immunity after an exercise challenge indicates that LOW were more likely to have a lower DPCP response following exercise stress than MOD (Fig. 1B). When reported as the summed response to the five DPCP challenge doses, dermal thickening response was 62% lower in LOW than MOD (LOW 1.6 ± 2.3 and MOD 4.2 ± 3.1 mm; $P < 0.01$; $d = 1.0$).

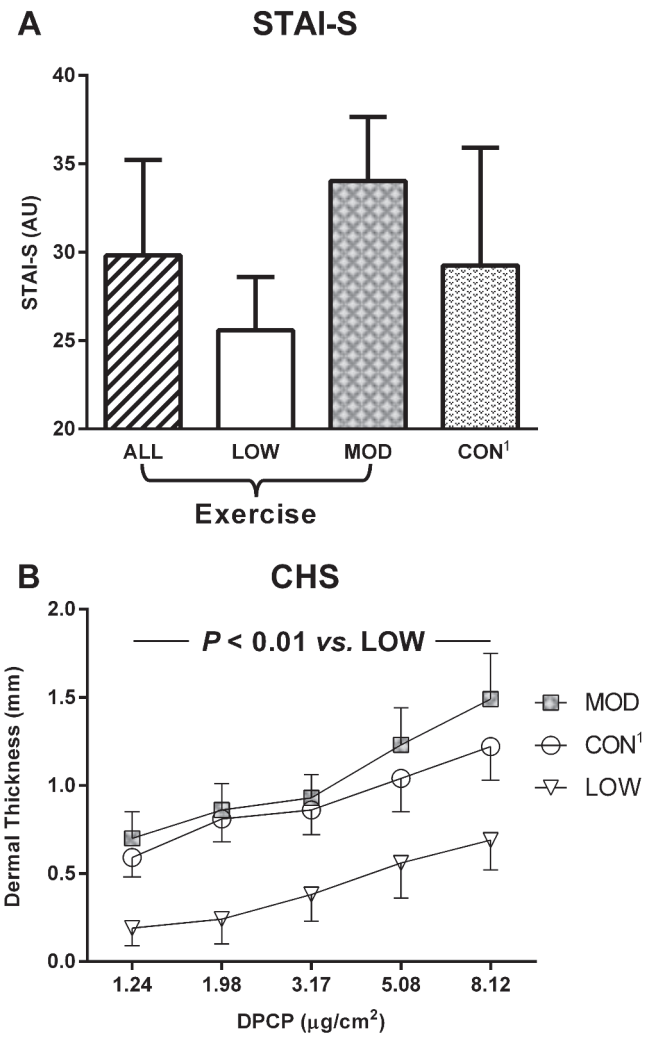


FIGURE 1. Effect of state-anxiety (STAI-S) prior to exercise on the *in vivo* immune response after exercise. (A) Low (LOW) and moderate (MOD) levels of state-anxiety. Data are Mean ± SD. (B) Contact hypersensitivity (CHS) assessed as elicitation challenge 28 d after DPCP induction. Dermal thickening response to the full dose-series challenge with DPCP is shown (30MI and 30HI). Data are Mean ± SEM for clarity. [1]Shown for comparison.

The ubiquitous influence of anxiety on *in vivo* immunity after exercise challenge (but not rested CON) is further illustrated in the comparisons between LOW and MOD in each group (30MI, 30HI, 120MI and CON; Fig. 2A-D). Responses to DPCP assessed as skinfold thickness and erythema (data not shown for brevity), were smaller in LOW *vs.* MOD for 30MI ($P < 0.01$) and 30HI ($P < 0.05$; Fig. 2A-B), but not CON. The suppressive effect of LOW *vs*. MOD was also apparent in 120MI ($P = 0.05$; $d = 0.9$; Fig. 2C) which is particularly striking given that the suppressive effect of prolonged exercise on the induction of DPCP immune memory has been reported (16). The lower CHS response to exercise in LOW *vs.* MOD is also illustrated in the smaller dermal thickening response across the full dose-series of DPCP in LOW *vs.* MOD ($F(1, 35) = 11.1$, $P < 0.01$; Fig. 1B for 30MI and 30HI). Furthermore, the threshold dose for a positive response to DPCP was calculated using the linear part of the dose-response curves. Compared with MOD, LOW required a 4-times greater DPCP dose (1.5 µg/cm^2) to elicit a positive response.

Perceived Stress Scale

Participants reported low-to-moderate PSS scores (16.5 ± 5.3). After accounting for the influence of exercise in step 1 of the regression model (Table 1), PSS score was a significant, moderate predictor (in step 2), accounting for an additional 13% of the variance in DPCP response ($P < 0.05$); together, exercise and PSS score accounted for 33% of the variance in the dermal thickening response to DPCP (Table 1). This association between the perception of psychological stress in the last month (i.e. the degree to which life situations are considered stressful) and *in vivo* immunity after exercise challenge indicates that participants reporting lower life stress were more likely to have a lower DPCP response following an exercise challenge than participants reporting moderate life stress.

Circulating stress hormones

When comparing LOW and MOD, a significant anxiety level x time interaction was observed for circulating epinephrine concentration ($F(2, 88) = 5.9$; $P < 0.01$); whereby, epinephrine was lower in LOW than MOD at pre-exercise (LOW 0.25 ± 0.17 *vs*. MOD 0.58 ± 0.46 nmol/L; $P < 0.01$), but not different at post or 1 h post-exercise. Similarly, an independent *t*-test

TABLE 1. Multiple linear regression analysis examining the influence of state-anxiety and perceived psychological stress level prior to exercise on the subsequent *in vivo* immune response after exercise. Contact hypersensitivity (CHS) assessed as the summed dermal thickening response to the full dose-series elicitation challenge with DPCP 28 d after DPCP induction. After accounting for the negative influence of exercise in step 1, separate models show the positive influence of anxiety (from low to moderate levels), assessed using STAI-S in step 2 (A) and perceived psychological stress (from low to moderate levels) over the last month, assessed using PSS in step 2 (B), respectively.

Dependent variable: CHS	B	SE	*β*	*t*	Δ*F*	R^2	ΔR^2
A. Step 1							
Exercise (TRIMP)[1]	-0.005	0.002	-0.44	-2.93	8.56	0.20**	0.20**
Step 2							
STAI-S	0.06	0.02	0.44	3.24	10.50	0.39**	0.19**
B. Step 1							
Exercise (TRIMP)[1]	-0.005	0.002	-0.44	-2.93	8.56	0.20**	0.20**
Step 2							
PSS	0.06	0.02	0.36	2.54	6.45	0.33**	0.13*

[1]TRIMP = training impulse; STAI-S = State Trait Anxiety Inventory; PSS = Perceived Stress Scale;* $P < 0.05$; ** $P < 0.01$.

showed that circulating cortisol concentration was also lower pre-exercise in LOW than MOD (LOW 545 ± 190 *vs*. MOD 699 ± 289 nmol/L; $P < 0.05$); albeit, there was no significant interaction. Nevertheless, the lower circulating epinephrine and cortisol concentration in LOW than MOD before exercise represent large ($d = 0.94$) and medium ($d = 0.63$) effects, respectively. Circulating norepinephrine was not different between LOW and MOD.

DISCUSSION

The aim of this work was to investigate the influence of anxiety and perceived psychological stress on the *in vivo* immune response after exercise. The findings support our hypothesis that the level of anxiety and perceived psychological stress reported by the individual prior to exercise play an important role in determining the strength of the subsequent *in vivo* immune response after exercise (Table 1 and Fig. 1): *in vivo* immunity was assessed by DPCP sensitisation after exercise and recall responses measured 28 d later. Moreover, the findings indicate a similar, moderate strength relationship for the level of anxiety prior to exercise (STAI-S; $r = 0.39$) and the level of physiological stress during exercise (TRIMP; $r = -0.37$) with the *in vivo* immune response after exercise challenge. The ubiquitous influence of anxiety on the immune response after exercise is further evidenced by a lower *in vivo* immune response to DPCP in individuals reporting low compared with moderate anxiety, regardless of the intensity and duration of the exercise challenge (30MI, 30HI and 120MI, Fig. 2A−C). These findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise.

The findings of the present study demonstrate an important interaction between the a priori level of anxiety and perceived psychological stress and the subsequent immune response after an exercise challenge. We previously showed no significant influence of 30MI or 30HI on *in vivo* immunity (16), but these new insights show a lower *in vivo* immune response in individuals reporting low compared with moderate anxiety in 30MI and 30HI (Fig. 2A−B). Moreover, although we have previously shown a suppressive effect of 120MI compared with rested control on *in vivo* immunity (16), particularly striking is the 50% lower *in vivo* immune response in individuals reporting low compared with moderate anxiety on 120MI (Fig. 2C). Given that DPCP is benign, determining the clinical significance of these findings, with specific regard to infection (skin and other) is an important avenue for future research. Preferably, the strength of the cutaneous recall response to DPCP could be generalised beyond skin immunity to indicate the immune system's general ability to respond to an infectious challenge. The available evidence in this regard is supportive as cutaneous immune measures are impaired in individuals with acute infectious illness (3, 22), diabetes and psoriasis (1) and predict mortality in critically ill HIV-infected patients (17). That we show lower pre-exercise circulating cortisol and epinephrine in the low compared with moderate anxiety group raises the possibility that stress hormones may modulate the immune response to subsequent exercise; indeed, stress hormones are considered to play important roles in preparing the immune system for challenge (13, 15). For example, administration of physiological doses of corticosterone and epinephrine increased T-cell drainage away from the site of DTH challenge to lymph nodes, which in-turn enhanced the DTH response in rats (15). In addition, adrenalectomy has been shown to eliminate stress-induced immune-enhancement in rats, likely by reducing the glucocorticoid and epinephrine response (15). Nevertheless, post-exercise circulating cortisol and epinephrine were not different between individuals reporting low and moderate anxiety in the present study; as such, further research is required into the underlying mechanisms.

Regarding the timing of the psychological measurements, the findings were unlikely due to an acute anticipatory effect prior to exercise as our participants underwent thorough familiarisation to all procedures, including running 50% of their allocated exercise duration; indeed, the success of familiarisation is shown as similar STAI-S scores prior to exercise and rested CON (Fig. 1A). In addition, our findings for the relationship between STAI-S score and the *in vivo* immune response after exercise are further supported by the relation-

ship between PSS score and the *in vivo* immune response after exercise: PSS assesses the perception of stress, and measures the degree to which life situations spanning the last month are considered stressful (whereas STAI-S provides an acute measure of anxiety) (11). As such, the PSS findings provide added confidence regarding the observed association between psychological stress and the *in vivo* immune response after exercise challenge. It remains to be shown whether individuals are predisposed to respond to stressful situations, such as competitive sport or military scenarios, in a predictable manner with regards to neuro-endocrine-immune responses. In support of this notion, there is some evidence that personality traits predict endocrine stress-reactivity (4, 19); nevertheless, further research is required to investigate this novel concept in exercise immunology, and to establish whether the findings of the present study extend to other immune measures e.g. vaccination responses (7) and mucosal immunity (23). Further research is also required to disentangle the influence of psychological and physiological strain during prolonged exercise (e.g. during endurance and ultra-endurance events) on *in vivo* immunity. Psychological stress measurements were made before exercise in the present study and it is reasonable to assume that psychological stress during more prolonged exercise (e.g. 120MI) might also play a role in the observed decrease in the *in vivo* immune response (Fig. 2C).

Bridging the gap between exercise immunology and psycho-neuro-immunology

Research investigators have long since acknowledged a role for psychological stress in the decrease in immunity associated with heavy exercise and training but there is little empirical research to support this hypothesis (8, 36). Since Clow and Hucklebridge's Exercise Immunology Review article highlighting this working hypothesis in 2001 (8) there have been > 3,000 peer-reviewed publications in exercise immunology (using the search terms 'exercise' and 'immune', Web of Science™) yet < 5% of these publications include the search terms 'psychological stress' or 'anxiety'. Closer inspection of this small subset of exercise immunology publications reveals that the large majority mention a putative role for psychological stress or anxiety in exercise-immune modulation; however, only a small handful of original investigations either attempt to manipulate psychological stress or include objective measures of psychological stress (27, 32, 38, 39). The present study answers the recent calls to physiologists (51) and exercise immunologists (49) to incorporate objective psychological measurements in their human studies.

The findings herein support the recommendation that exercise immunologists should include aspects of mental health (e.g. psychological stress and others), in a broader conceptual framework of exercise-immune interactions alongside other

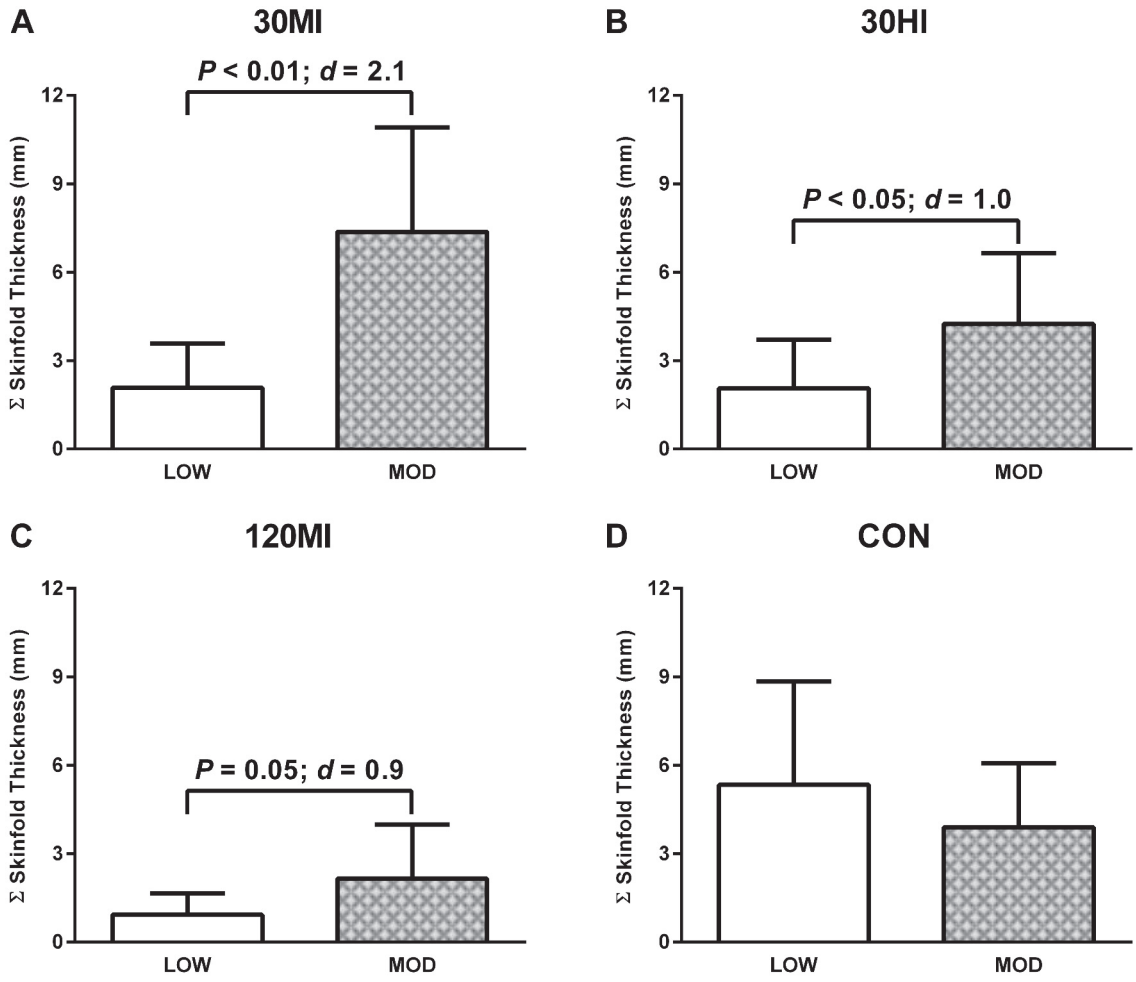


FIGURE 2. Effect of state-anxiety prior to exercise on the *in vivo* immune response after exercise of varying intensity and duration. (A–D) Summed increase in skinfold thickening response to DPCP challenge for each exercise group (30MI, 30HI and 120MI) and rested CON. Data are Mean ± SD.

factors thought to decrease immunity in athletes and military personnel (e.g. prolonged training sessions, poor nutrition etc.). This will inform and direct research questions and experimental designs with the aim of improving our understanding of the complicated exercise-immune interactions and with the potential to provide effective countermeasures to immune impairment in those concerned. To this end, the exercise immunologist's toolkit will be enhanced by joining forces with experts in the ever expanding field of psycho-neuro-immunology to begin to disentangle the psychosocial and physiological underpinning of decreased immunity and increased infection risk in high level athletes, military personnel and others in physically demanding occupations. Our finding that pre-exercise anxiety and perceived psychological stress accounted for additional variance in post-exercise *in vivo* immunity after accounting for exercise (using TRIMP) emphasises the importance of incorporating psychological measurements in studies investigating the immune response to exercise. As do the similar strength correlations for pre-exercise anxiety (STAI-S; $r = 0.39$) and physiological stress during exercise (TRIMP; $r = -0.37$) with *in vivo* immunity after exercise. These findings indicate a beneficial effect of moderate (*vs*. low) anxiety and perceived psychological stress on *in vivo* immunity after exercise (Fig. 2A and D); as such, the findings accord with the immune-enhancement theory of moderate stress (13, 20, 21). Further research is required to investigate exercise-immune responses in athletes, military personnel and others in physically demanding occupations (e.g. firefighters and mountain rescue workers) experiencing higher levels of psychological stress than those reported in this study e.g. as might occur in relation to important competition, major life events etc. The immuno-suppressive effects of chronic high stress in rats (3 weeks of restraint and shaking stress) (14) and humans (examination period) (44) are widely acknowledged (13). As such, research is required to test the hypothesis that chronic high levels of psychological stress exacerbate the decrease in *in vivo* immunity after exercise. Irrespective, the present findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise, and for coaches and support staff to monitor anxiety and psychological stress alongside more traditional physiological measures of training stress. Accordingly, recent evidence highlights that aspects of mental health such as psychological stress and depression are important risk factors for illness in Olympic athletes (18). In time, studies may demonstrate the utility of interventions to alter psychological stress in order to optimise immunity and host defence in athletes, military personnel and those in physically demanding occupations. There is good reason for optimism as an 8-week mindfulness meditation programme increased the antibody response to influenza vaccine in employees working in a highly stressful environment (*vs.* waiting-list controls) (12). Also, although somewhat limited methodologically, preliminary work in competitive athletes showed that a 3-week stress management intervention reduced the number of days out due to illness and injury (35).

CONCLUSIONS

In conclusion, these findings show that anxiety and perceived psychological stress levels prior to exercise play an important role in determining the strength of the *in vivo* immune response after exercise. Moreover, these findings indicate a similar, moderate strength relationship for the level of state-anxiety prior to exercise and the level of physiological stress during exercise with the *in vivo* immune response after exercise. Future research is required to investigate exercise-immune responses in athletes and others in physically demanding occupations experiencing higher levels of psychological stress than those reported in this study e.g. related to important competition and major life events. Nevertheless, these findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise.

ACKNOWLEDGEMENTS

Bethany Diment received a 125th anniversary research scholarship from Bangor University. This study received no external funding. None of the authors had a conflict of interest.

REFERENCES

1. Bangsgaard N, Engkilde K, Menne T, Lovendorf M, Jacobsen GK, Olsen J, and Skov L. Impaired hapten sensitization in patients with autoimmune disease. Clin Exp Immunol 165: 310-317, 2011.
2. Banister EW. Modelling elite athletic performance. In: Physiological testing of elite athletes, edited by Green H, McDougal J, and Wenger H. Champaign, IL: Human Kinetics, 1991, p. 403-424.
3. Bennett BK, Hickie IB, Vollmer-Conna US, Quigley B, Brennan CM, Wakefield D, Douglas MP, Hansen GR, Tahmindjis AJ, and Lloyd AR. The relationship between fatigue, psychological and immunological variables in acute infectious illness. Aust N Z J Psychiatry 32: 180-186, 1998.
4. Bibbey A, Carroll D, Roseboom TJ, Phillips AC, and de Rooij SR. Personality and physiological reactions to acute psychological stress. Int J Psychophysiol 90: 28-36, 2013.
5. Booth CK, Coad RA, Forbes-Ewan CH, Thomson GF, and Niro PJ. The physiological and psychological effects of combat ration feeding during a 12-day training exercise in the tropics. Mil Med 168: 63-70, 2003.
6. Bruunsgaard H, Hartkopp A, Mohr T, Konradsen H, Heron I, Mordhorst CH, and Pedersen BK. In vivo cell-mediated immunity and vaccination response following prolonged, intense exercise. Med Sci Sports Exerc 29: 1176-1181, 1997.
7. Burns VE. Using vaccinations to assess in vivo immune function in psychoneuroimmunology. Methods Mol Biol 934: 371-381, 2012.
8. Clow A, and Hucklebridge F. The impact of psychological stress on immune function in the athletic population. Exerc Immunol Rev 7: 5-17, 2001.
9. Cohen J. Statistical power analysis for the behavioral sciences. Hillsdale, NJ: Erlbaum, 1988, p. 79-80.
10. Cohen S, Tyrrell DA, and Smith AP. Psychological stress and susceptibility to the common cold. N Engl J Med 325: 606-612, 1991.
11. Cohen S, and Williamson G. Psychological stress in a probability sample of the United States. In: The social psychology of health, edited by Spacapan S, and Oskamp S. Newbury Park, CA: Sage, 1988, p. 31-67.

12. Davidson RJ, Kabat-Zinn J, Schumacher J, Rosenkranz M, Muller D, Santorelli SF, Urbanowski F, Harrington A, Bonus K, and Sheridan JF. Alterations in brain and immune function produced by mindfulness meditation. Psychosom Med 65: 564-570, 2003.
13. Dhabhar FS. Effects of stress on immune function: the good, the bad, and the beautiful. Immunol Res 58: 193-210, 2014.
14. Dhabhar FS, and McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav Immun 11: 286-306, 1997.
15. Dhabhar FS, and McEwen BS. Enhancing versus suppressive effects of stress hormones on skin immune function. Proc Natl Acad Sci U S A 96: 1059-1064, 1999.
16. Diment BC, Fortes MB, Edwards JP, Hanstock HG, Ward MD, Dunstall HM, Friedmann PS, and Walsh NP. Exercise intensity and duration effects on in vivo immunity. Med Sci Sports Exerc 47: 1390-1398, 2015.
17. Dolan MJ, Clerici M, Blatt SP, Hendrix CW, Melcher GP, Boswell RN, Freeman TM, Ward W, Hensley R, and Shearer GM. In vitro T cell function, delayed-type hypersensitivity skin testing, and CD4+ T cell subset phenotyping independently predict survival time in patients infected with human immunodeficiency virus. J Infect Dis 172: 79-87, 1995.
18. Drew MK, Vlahovich N, Hughes D, Appaneal R, Peterson K, Burke L, Lundy B, Toomey M, Watts D, Lovell G, Praet S, Halson S, Colbey C, Manzanero S, Welvaert M, West N, Pyne DB, and Waddington G. A multifactorial evaluation of illness risk factors in athletes preparing for the Summer Olympic Games. J Sci Med Sport 20: 745-750, 2017.
19. Edelstein RS, Yim IS, and Quas JA. Narcissism predicts heightened cortisol reactivity to a psychosocial stressor in men. J Res Pers 44: 565-572, 2010.
20. Edwards KM, Burns VE, Carroll D, Drayson M, and Ring C. The acute stress-induced immunoenhancement hypothesis. Exerc Sport Sci Rev 35: 150-155, 2007.
21. Edwards KM, Burns VE, Reynolds T, Carroll D, Drayson M, and Ring C. Acute stress exposure prior to influenza vaccination enhances antibody response in women. Brain Behav Immun 20: 159-168, 2006.
22. Haider S, Coutinho Mde L, Emond RT, and Sutton RN. Tuberculin anergy and infectious mononucleosis. Lancet 2: 74, 1973.
23. Hanstock HG, Walsh NP, Edwards JP, Fortes MB, Cosby SL, Nugent A, Curran T, Coyle PV, Ward MD, and Yong XH. Tear fluid SIgA as a noninvasive biomarker of mucosal immunity and common cold risk. Med Sci Sport Exer 48: 569-577, 2016.
24. Harper Smith AD, Coakley SL, Ward MD, Macfarlane AW, Friedmann PS, and Walsh NP. Exercise-induced stress inhibits both the induction and elicitation phases of in vivo T-cell-mediated immune responses in humans. Brain Behav Immun 25: 1136-1142, 2011.
25. 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.
26. Hu E, Koucky EM, Brown WJ, Bruce SE, and Sheline YI. The role of rumination in elevating perceived stress in posttraumatic stress disorder. J Interpers Violence 29: 1953-1962, 2014.
27. Huang CJ, Webb HE, Garten RS, Kamimori GH, and Acevedo EO. Psychological stress during exercise: lymphocyte subset redistribution in firefighters. Physiol Behav 101: 320-326, 2010.
28. Ingram LA, Simpson RJ, Malone E, and Florida-James GD. Sleep disruption and its effect on lymphocyte redeployment following an acute bout of exercise. Brain Behav Immun 47: 100-108, 2015.
29. Jansky L, Pospisilova D, Honzova S, Ulicny B, Sramek P, Zeman V, and Kaminkova J. Immune system of cold-exposed and cold-adapted humans. Eur J Appl Physiol Occup Physiol 72: 445-450, 1996.
30. Julian LJ. Measures of anxiety: State-Trait Anxiety Inventory (STAI), Beck Anxiety Inventory (BAI), and Hospital Anxiety and Depression Scale-Anxiety (HADS-A). Arthritis Care Res (Hoboken) 63 Suppl 11: S467-472, 2011.
31. Laing SJ, Oliver SJ, Wilson S, Walters R, Bilzon JL, and Walsh NP. Neutrophil-degranulation and lymphocyte-subset response after 48 hr of fluid and/or energy restriction. Int J Sport Nutr Exerc Metab 18: 443-456, 2008.
32. Moreira A, Arsati F, Lima-Arsati YB, Simoes AC, and De Araujo VC. Monitoring stress tolerance and occurrences of upper respiratory illness in basketball players by means of psychometric tools and salivary biomarkers. Stress Health 27: e166-e172, 2011.
33. Nieman DC, Johanssen LM, Lee JW, and Arabatzis K. Infectious episodes in runners before and after the Los Angeles Marathon. J Sports Med Phys Fitness 30: 316-328, 1990.
34. Pascoe AR, Singh MAF, 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.
35. Perna FM, Antoni MH, Baum A, Gordon P, and Schneiderman N. Cognitive behavioral stress management effects on injury and illness among competitive athletes: a randomized clinical trial. Ann Behav Med 25: 66-73, 2003.
36. Perna FM, Schneiderman N, and LaPerriere A. Psychological stress, exercise and immunity. Int J Sports Med 18 Suppl 1: S78-83, 1997.
37. Peters EM, and Bateman ED. Ultramarathon running and upper respiratory tract infections. An epidemiological survey. S Afr Med J 64: 582-584, 1983.
38. Rehm KE, Elci OU, Hahn K, and Marshall GD. The impact of self-reported psychological stress levels on changes to peripheral blood immune biomarkers in recreational marathon runners during training and recovery. Neuroimmunomodulation 20: 164-176, 2013.
39. Rehm KE, Sunesara I, Tull MT, and Marshall GD. Psychological stress moderates the relationship between running volume and cd4+ T cell subpopulations. J Biol Reg Homeos Ag 30: 449-457, 2016.
40. Severs Y, Brenner I, Shek PN, and Shephard RJ. Effects of heat and intermittent exercise on leukocyte and sub-population cell counts. Eur J Appl Physiol Occup Physiol 74: 234-245, 1996.
41. Shephard RJ. Immune changes induced by exercise in an adverse environment. Can J Physiol Pharmacol 76: 539-546, 1998.
42. Shephard RJ, and Shek PN. Heavy exercise, nutrition and immune function: is there a connection? Int J Sports Med 16: 491-497, 1995.
43. Shephard RJ, and Shek PN. Interactions between sleep, other body rhythms, immune responses, and exercise. Can J Appl Physiol 22: 95-116, 1997.

44. Smith A, Vollmer-Conna U, Bennett B, Wakefield D, Hickie I, and Lloyd A. The relationship between distress and the development of a primary immune response to a novel antigen. Brain Behav Immun 18: 65-75, 2004.
45. Spielberger CD. Manual for the State-Trait Anxiety Inventory. Palo Alto, CA: Consulting Psychologists Press, 1983, p. 12-14.
46. Tabachnick BG, and Fidell LS. Using multivariate statistics. Essex, UK: Pearson Education, 2013, p. 159-160.
47. 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.
48. 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.
49. Walsh NP, and Oliver SJ. Exercise, immune function and respiratory infection: an update on the influence of training and environmental stress. Immunol Cell Biol 94: 132-139, 2016.
50. Wang JS, Chen WL, and Weng TP. Hypoxic exercise training reduces senescent T-lymphocyte subsets in blood. Brain Behav Immun 25: 270-278, 2011.
51. Wehrwein EA, and Carter JR. The mind matters: psychology as an overlooked variable within physiology studies. Physiology (Bethesda) 31: 74-75, 2016.

Effects of lifelong training on senescence and mobilization of T lymphocytes in response to acute exercise.

Luciele G. Minuzzi[1], Luís Rama[1], Matheus Uba Chupel[1], Fátima Rosado[1], João Valente dos Santos[1,2], Richard Simpson[3], António Martinho[4], Artur Paiva[5], Ana M. Teixeira[1]

[1] Research Center for Sport and Physical Activity (IUD/DTP/04213/2016) Faculty of Sports Science and Physical Education, University of Coimbra, Portugal
[2] Faculty of Physical Education and Sport, Lusófona University of Humanities and Technologies, Lisbon, Portugal
[3] Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, Texas, USA.
[4] Portuguese Institute for Blood and Transplantation, São Martinho do Bispo, Coimbra, Portugal
[5] Flow Cytometry Unit-Clinical Pathology Service, University Hospital Centre of Coimbra, Portugal

ABSTRACT

Background/Purpose: *Ageing has profound impact on the immune system, mainly on T-cells. However, it has been suggested that chronic exercise may delay immunosenescence. Master athletes represent an interesting sub-demographic group to test this theory since they maintain a high training frequency and load throughout life. The purpose of this study was to evaluate the effects of lifelong training on the senescence and mobilization of T lymphocytes in response to acute exercise.*

Material and Methods: *Nineteen athletes who regularly participated in training and competitions for more than 20 years throughout their lives and a control group of 10 healthy individuals participated in this study. All subjects performed a progressive test to exhaustion on a cycle ergometer. Blood samples were obtained before (Pre), 10 min after the test (Post) and 1 h after the test (1h). Phenotypic study of peripheral blood T-cells was performed by flow cytometry. Genes of interest expression was done on T-cells purified by cell sorting.*

Results: *Master athletes had a lower percentage of senescent naïve, central memory and effector memory $CD8^+$ T-cells and senescent naïve and effector memory $CD4^+$ T-cells. Age had a positive effect on SLEC $CD8^+$ T-cells and a negative effect on naïve $CD8^+$ T-cells. VO_{2max} positively correlated with the proportion of naïve $CD4^+$ T-cells and negatively correlated with the percentage of total lymphocytes. No differences were founded for $CD4^+$ and $CD8^+$ T-cells and their subsets between master athletes and the control group at all times of measurement. No differences were observed in the CD45RA expressing effector memory cells (EMRA) for the various study conditions. The mRNA expression of the CCR7 gene for naïve $CD8^+$ T-cells and the Fas-L gene for effector-terminal $CD8^+$ T-cells was not different between masters and controls and did not change in response to the maximal protocol test.*

Conclusion: *In conclusion, maintaining high levels of aerobic fitness during the natural course of aging may help prevent the accumulation of senescent T-cells.*

Keywords: Immunosenescence; KLRG1; Cytomegalovirus; CCR7; Fas-L; Immune space.

INTRODUCTION

The age-associated decline in immune function, referred to as immunosenescence, is well characterized in the adaptive immune system and, in particular, within T-cells. The characteristics of immunosenescence in the T cell pool, include low numbers and proportions of naïve T-cells (especially $CD8^+$ T-cells) and a large number of memory T-cells ($CD8^+$ T-cells especially in late stage of differentiation) (1, 9, 21, 22); poor vaccination responses (18, 41); and a CD4:CD8 ratio <1.0 (17). These changes are largely driven by infection with cytomegalovirus (455). Depletion of the naïve T cell pool may result in fewer naïve cells capable of responding to new antigens; in fact, the extended pool of memory cells may actually prevent populations of naïve cells from being able to proliferate and expand in sufficient numbers (26).

The onset of senescent T cells occurs due to excess clonal expansions occurring as part of a normal immune response to pathogens that invade or reactivate infections in the body throughout life (33). Repeated exposure to antigenic stimuli throughout life (like reactivation of latent viral infections) leads to further rounds of cell division and premature senescence. As these senescent T cells still preserve effector cell functions (like death of virus-infected cells) and are highly pro-inflammatory, their accumulation in blood and tissues

Corresponding author:
Ana Maria Teixeira, Pavilhão III, Estádio Universitário de Coimbra, Santa-Clara, 3040-156 Coimbra, Portugal
E-mail: ateixeira@fcdef.uc.pt
Tel.: +351 239 802 770, Fax: +351 239 802 779

may also contribute to a number of pathologies associated with inflammation (33).

Spielmann et al. (2011) demonstrated that the proportions of senescent $CD4^+$ and $CD8^+$ T-cells increased with advanced age, at a corresponding rate of 10% and 10.2% per decade, respectively. This was accompanied by a reduction in the proportions of naive $CD4^+$ and $CD8^+$ T-cells (10% and 9.9% per decade, respectively). Interestingly, the authors found that subjects who had above-average VO_{2max} values had fewer senescent $CD4^+$ and $CD8^+$ T-cells and increased numbers of naive $CD8^+$ T-cells than those with lower VO_{2max} values, even after adjustment for age, body mass index, and percentage of body fat. Surprisingly, the authors found that the well-accepted association between age and senescent T-cells no longer existed when age was adjusted for VO_{2max}, indicating that aerobic fitness may be a strong determinant of T-cell phenotypic changes with greater impact than chronological age. This effect was limited to senescent cells, and VO_{2max} was not associated with increased memory cells after adjustment for age. The study was the first to show that aerobic fitness is associated with a moderation of natural age-related accumulation of senescent T cells in peripheral blood, highlighting the beneficial effects of maintaining a physically active lifestyle on immunosenescence (38).

Many aspects of immune function change with age, and some of these changes can be restored temporarily by exercise by delaying the onset of immune ageing or by rejuvenating aged immune profiles (20, 28, 34, 36, 40). This theory has been developed based on the evidence that exercise "stimulates" the immune function (43, 44). For example, moderate or intense training throughout life leads to strong response to vaccines against influenza, resulting in higher percentages of protected subjects (2). Furthermore, the immunosurveillance of lymphocytes, the cells which seek tissue antigens derived from viruses, bacteria or malignant transformation is thought to be facilitated by lymphocytosis and subsequent transient exercise-induced lymphopenia (10, 40). Also, some forms of exercise are anti-inflammatory, and if regularly repeated throughout life, there is a lower morbidity and mortality from diseases with an immunological and inflammatory etiology (11).

Many aspects of immune function change with age, and some of these changes can be restored temporarily by exercise. A recent theory proposed that exercise can trigger preventive and / or restorative mechanisms of T-cell immunosenescence (28, 34) involving a three steps process. Firstly, as shown by many studies (4, 32, 33), cells in a late differentiated stage are mobilized to the peripheral blood during exercise. Secondly, these cells extravasate from the blood to peripheral and / or inflamed tissues 1-2 h after exercising (6, 43, 44). At these sites, it is thought that T-cells are exposed to a variety of pro-apoptotic stimuli (e.g., reactive oxygen species, glucocorticoids, cytokines) that may cause apoptosis of these cells (15). The third and final phase of this hypothesis, proposes that the naïve T-cell repertoire is then able to expand in response to the "immune space" that has been created, initiated by a hypothetical negative feedback loop that governs the number of naïve and memory cells (12, 28).

If exercise can reverse the immunosenescent phenotype by causing selective apoptosis (28) is still a topic of debate (30, 40). For example, exercise could also prevent accumulation of terminally differentiated/exhausted T-cells with age by preventing CMV reactivation (31). Moreover, there is evidence for and against this model, including the idea suggesting that not all cells bearing a so-called "senescent" phenotype are actually senescent (40). More recent advances showed that many CMV-specific $CD8^+$ T cells, identified using MHC-class I tetramers, are multifunctional, producing IFN-γ, IL-2 and TNF-α and exhibiting potent cytotoxic activity (25). In addition, these cells have telomeres of intermediate length, despite ''senescent'' cell-surface characteristics (e.g., $CD45RA^+CD27^-$) (25).

Although, many studies indicate that exercise can slow human biological aging, the effects of long-term exercise on T cell function are not well known (5, 19, 23, 39). Therefore, the aim of this study was to evaluate the effects of training throughout life on the senescence of T cell and on the mobilization of senescent T cell subsets in response to a maximal acute exercise test.

The hypothesis of increasing the frequency of terminally differentiated T-cells in the blood after acute exercise suggests that the selective mobilization and death of these cells by acute and regular exercise could eventually allow naïve T-cells to occupy vacant immune space and increase the T cell repertoire (32). The analysis of this hypothesis in master athletes is original and was tested in this study.

MATERIALS AND METHODS

Participants

Nineteen master athletes volunteered to participate in this study. The subjects (n=15 men and n=4 women) were swimmers, judo and track and field athletes over 40 years old who had participated in training and competitions for more than 20 years throughout their life. The study also included a healthy, non-smokers, body mass and age-matched control group of 10 subjects (n=7 men and n=3 women) who performed no regular physical training in the last 20 years (Table 1). The inclusion criteria for masters were a minimum of twenty years of regular training and competition participation and being currently participating in regular training and competition. The inclusion criteria for the control group, was to not have performed regular physical training in the last 20 years. Exclusion criteria for both groups included smokers, any known cardiovascular, musculoskeletal or neurological disease and the use of any medication or supplementation. The master athlete sports history was accessed through a self-reported regarding the beginning of athletic life, the modalities practiced and the duration dedicated to each category, including the starting and finishing year, the hours per week and the months per year in the mentioned sport and the episodes of injury. A second questionnaire regarding the planning of the training period, including the development of the activities, namely type of activity, category, hours / week, classification of effort intensity (easy, moderate, intense or very intense), competitions in which the athlete participated and the results obtained, was also applied.

All subjects completed a medical and health questionnaire and gave their written informed consent to participate in this study. The experimental procedure was approved by the Ethics and Human Subjects Review Board of the Faculty of

Sports Science and Physical Education, University of Coimbra.

Table 1. Participants characteristics

	Masters	Control
Age (years)	53.5 ± 8.94	53.7 ± 6.04
Height (cm)	171.5 ± 5.86	169.7 ± 8.23
Body mass (kg)	74.7 ± 15.17	70.45 ± 13.71
BMI ($kg.m^{-2}$)	25.7 ± 4.65	24.6 ± 3.18
VO_{2max} (L.min-1)	2859.5 ± 697.22	2094.55 ± 4.14 **a**
VO_{2max} (mL.kg-1.min-1)	40.36 ± 11.55	29.29 ± 697.22 **b**

Values are Mean ± Standard Deviation (SD). N= Masters (19), Control (10). BMI= Body Mass Index; VO_{2max} = Maximal Oxygen Consumption. **a**= P<0.01 compared to masters; **b**= P<0.001 compared to masters.

Experimental design

All participants agreed to refrain from caffeine and alcohol in the previous 24 h and avoided strenuous exercise 72 h prior to the laboratory procedures. Participants arrived at the laboratory at 09:00am. First, they completed a comprehensive health-screening questionnaire. All subjects completed a maximal oxygen uptake (VO_{2max}) test on an electro-magnetically braked cycle ergometer (Lode Excalibur Sport V4.67, Groningen, The Netherlands). Participants began cycling at 75W and the power output was increased by 25W every 3 min until volitional fatigue. Oxygen uptake (breath-by-breath) was measured using an automated gas-analysis system (Quark CPET COSMED, COSMED, Rome, Italy). Heart-rate, using short range telemetry (COSMED, Rome, Italy), and ratings of perceived exertion Borg Cr-10 scale (RPE) were recorded at each 3-min stage and at the end of the test. Intravenous blood samples were collected in 6ml vacuum tubes containing EDTA as an anticoagulant (Becton-Dickinson, Oxford, UK) before (Pre), immediately after (Post), and 1 h after exercise.

Peripheral Blood Cell Counts

Total leukocyte and lymphocyte counts were determined using an automated cell counter (Coulter ACT Diff, Beckham Coulter, USA).

Flow cytometry

Specific lymphocyte populations were identified by immunofluorescent antibody staining of whole blood by eight-colour flow cytometry (FACSCanto II, BD Bioscience, San Jose, CA, USA). The following monoclonal antibodies (mAbs) were used: anti-human CD3-PB (pacific blue, clone UCHT1, Pharmingen, San Diego, C.A. USA), anti-human CD4-APC-H7 (allophycocyanin-hilite 7, Clone 13B8.2, Beckman Coulter, Miami, FL, USA), anti-human CD8-KO (Krome Orange, Clone 5MZ.332, Beckman Coulter, Miami, FL, USA), anti-human CD25-PE (phycoerythrin, Clone 2A3, BD Bioscience, San Jose, CA, USA), anti-human CD127-FITC (fluorescein isothiocyanate, Clone R.34.34, Beckman Coulter), anti-human CD27-PECy5 (phycoerythrin-cyanine 5, clone R.8.01, Beckman Coulter), anti-human CD45RA-PECy7 (phycoerythrin-cyanine 7, Clone L48, BD Bioscience) and anti-human/mouse KLRG1-APC (allophycocyanin, Clone 2F1/KLRG1, Biolegend, San Diego, CA).

Briefly, peripheral blood cells (PBCs) were labelled and incubated for 10 min at room temperature in the dark. After this, 2 ml of FACS lysing Solution (BD Biosciences) was added; cells were incubated at room temperature for a further 10 min and then washed with 2ml of PBS. The tubes were then centrifuged at 540 g for 5 min and the supernatant was discarded. The cells were resuspended in 0.5 ml PBS, and immediately acquired in a flow cytometer.

For acquisition, initially, 1.000.000 events, corresponding to all nucleated cells present in the sample, were collected and information stored. Blood lymphocytes were identified and electronically gated using the forward and side light-scatter mode. T lymphocytes were identified according to their positivity for CD3 and typical light scatter. Two parameter dot plots were generated from the gated lymphocyte cell population to identify $CD3^+/CD4^+$ or $CD3^+/CD8^+$ T-cell subset populations. CD4+ and CD8+ T-cells were subsequently further differentiated into naïve, central memory (CM), effector memory (EM), and CD45RA expressing effector memory cells (EMRA) subsets by CD45RA expression in combination with CD27. Senescent T-cells were detected based on the expression of $KLRG1^+$. The flow cytometer was routinely calibrated using Calibrite beads (BD Biosciences) and single labelled antibody tubes were used for further compensation adjustments. Data analysis was performed in the Infinicyt version 1.7 software (Cytognos, Salamanca, Spain). Absolute counts were calculated using a dual platform methodology (flow cytometry and haematological cell analyser – Beckman Coulter LH 750, Miami, USA) (Figure 1).

Cell purification by fluorescence-activated cell sorting

$CD8^+$ naïve and EMRA T cell populations were purified by FACS (using a FACSAria II flow cytometer; BD) according to their typical phenotype. The purified cell populations were subsequently used for mRNA expression studies.

Analysis of mRNA expression in purified CD8 T-cells:

The content of sorted purified cells were transferred to a 1.5-mL *Eppendorf* tube and centrifuged for 5 min at 300 g, and the pellet ressuspended in 350 µL of RLT Lysis Buffer (Qiagen, Hilden, Germany). Total RNA was extracted with the RNeasy Micro kit (Qiagen) in accordance with the manufacturer instructions. Total RNA was eluted in a 20-µL volume of RNase-free water and was reverse-transcribed with Tetra cDNA Synthesis (Bioline, London, UK) in accordance with the manufacturer instructions. Relative quantification of gene expression by real-time polymerase chain reaction (PCR) was performed in the LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCRs were carried out by using 1x QuantiTect SYBR Green PCR Master Mix (Qiagen) and 1x QuantiTect Primer Assay (CCR7: QT00025718; FAS-L: QT00041685, Qiagen) in a final volume of 10 µL. The reactions were performed by using the following thermal profile: one cycle of 10 min at 95°C, 50

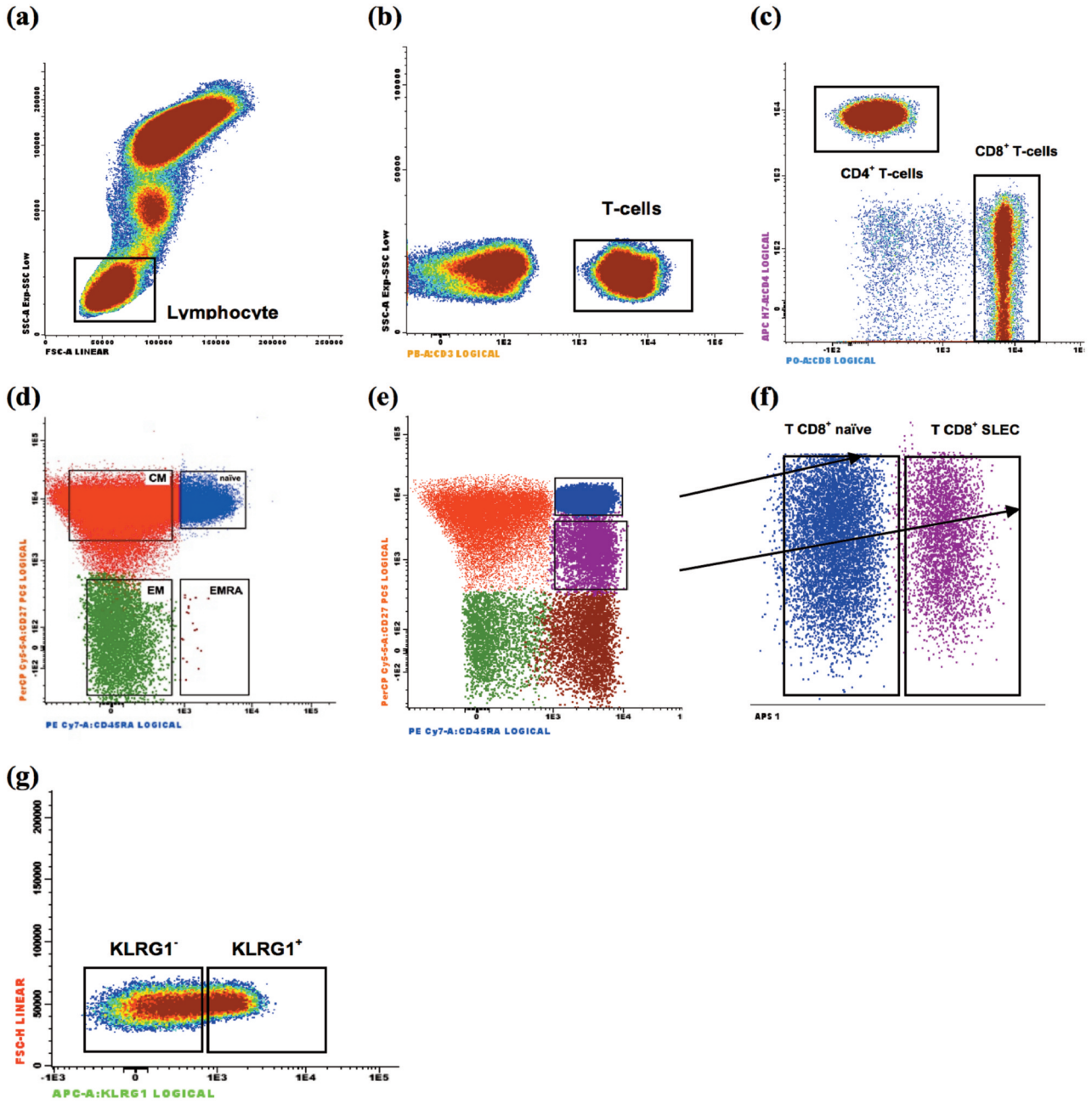


Figure 1. Representative analysis strategy used to define the different CD4+ and CD8+ T-cells populations and the % of these cells expressing the marker KLRG1.

The lymphocyte population was defined based on their low scatter *(side scatter)* and their small size *(forward scatter)* (Figure 1a). T lymphocytes were identified according to their CD3 positivity (Figure 1b). CD4+ and CD8+ T-cells were defined based on their positivity for CD4 and CD8, respectively (Figure 1c). Each subpopulation of $CD4^+$ and $CD8^+$ T-cells was divided into naïve ($CD27^+CD45RA^+$), central memory (CM; $CD27^+CD45RA^-$), effector memory (EM; $CD27^-CD45RA^-$) and CD45RA expressing effector memory cells (EMRA; $CD27^-CD45RA^+$) (Figure 1d, e). $CD8^+$ T-cells were further subdivided into another population, short-lived effector cells (SLEC, $CD27^{-/low}CD45RA^{++}$) (Figure 1f). All populations were analysed for their positivity for the KLRG1 marker (Figure 1g).

cycles of 10 s at 95°C, 20 s at 55°C and 30 s at 72°C, one cycle of 5 s at 95°C, 1 min at 65°C and continuous at 97°C, and one cycle of 10 s at 21°C. Real-time PCR results were analysed with the LightCycler software (Roche Diagnostics). GeNorm software (Primer Design Ltd., Southampton, UK) was used to select the reference genes to normalize data. The reference genes used for gene expression analysis were cytochrome c1 (CYC1; QT00209454) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; QT012504271). The normalized expression levels of the genes of interest were calculated by using the delta Ct (change in threshold cycle) method.

Statistical analysis

Descriptive results are presented as means ± standard deviation (SD). The Gaussian distribution for each parameter was assessed by a Shapiro-Wilk test. Alpha level was set at 0.05. All these routine analyses were assessed using SPSS software v19.0 (IBM Company, NY, USA).

For the analyses of change, accounting for the multilevel design of the study [level 1 units (intra-individual) within each level 2 unit (individuals of different groups)], hierarchical random effects models (REM) were constructed using a multilevel modelling approach (MLwiN v2.26, Center for Multilevel Modelling, University of Bristol, Bristol, UK) (3).

Analysis models that contained variables measured at different levels of a hierarchy are known as multilevel regression models. The following additive polynomial multilevel model was adopted to describe the changes in response to a maximum effort protocol:

$$yij = (\alpha + \mu j) + (\beta + \nu j)\, xij + (z1ij + z2ij + \cdots + znij) + \varepsilon ij$$

This equation is an example of REM in which level 1 regression coefficients are treated as random variables at level 2. In this example, the minutes from start (x) is in both the fixed and random parts of the model. This is seen clearly when equation 1 is rearranged into fixed and random parts:

$$yij = (\alpha + \beta j\, xij) + (z1ij + z2ij + \cdots + znij) + (\mu j + \nu jxij + \varepsilon ij)$$

where y is the %Total Ly, %Total LyT, %LyT, %Total CD4$^+$, %CD4$^+$, %Total CD8$^+$ and %CD8$^+$; naïve, central memory (CM), effector memory (EM) and CD45RA expressing effector memory cells (EMRA) of CD4$^+$ T-cells (%CD4); naïve, central memory (CM), effector memory (EM) and CD45RA expressing effector memory cells (EMRA) of CD8$^+$ T-cells (%CD8); LyT/KLRG1$^+$, %CD4$^+$/KLRG1$^+$, %CD4$^+$/naïve/KLRG1$^+$, %CD4$^+$/CM/KLRG1$^+$ and %CD4$^+$/EM/KLRG1$^+$, %CD4$^+$/EMRA/KLRG1$^+$ on measurement occasion i in the j^{th} individual, α is the constant for each j^{th} individual, $\beta_j\, x_{ij}$ is the slope for the dependent parameters over time (i.e., minute of measurement) for the j^{th} individual; and z_1 to z_n were the coefficients of explanatory variables (e.g., age, sex, VO_{2max} etc.) at assessment occasion i in the j^{th} individual. These were the fixed parameters in the model.

Both μj, $v_j x_{ij}$ and ε_{ij} formed the random parameters in the model. They were assumed to be independent and follow a normal distribution, with means equal to zero and variance σ^2. $\varepsilon_{ij} \sim N[0,\mathrm{var}(\varepsilon_{ij})]$ was the level 1 residual (within-individual variance) for the i^{th} assessment of %Total Ly, %Total LyT, %LyT, %Total CD4$^+$, %CD4$^+$, %Total CD8$^+$ or %CD8$^+$; naïve, central memory (CM), effector memory (EM) or CD45RA expressing effector memory cells (EMRA) of CD4$^+$ T-cells (%CD4); naïve, central memory (CM), effector memory (EM) or CD45RA expressing effector memory cells (EMRA) of CD8$^+$ T-cells (%CD8); LyT/KLRG1$^+$, %CD4$^+$/KLRG1$^+$, %CD4$^+$/naïve/KLRG1$^+$, %CD4$^+$/CM/KLRG1$^+$ or %CD4$^+$/EM/KLRG1$^+$, %CD4$^+$/EMRA/KLRG1$^+$ in the j^{th} individual. Also, $\mu_j \sim N[0,\mathrm{var}(\mu)]$ was the between individuals intercept variance and $v_{ij}x_{ij} \sim N[0,\mathrm{var}(v_{ij}x_{ij})]$ was the between individuals slope variance; thus, being used as the level 2 residuals (between subjects) variances for the j^{th} individual. The equation $\mu_j \times v_{ij}x_{ij} \sim N[0,\mathrm{var}(\mu_j \times v_{ij}x_{ij})]$ explained the intercept-slope covariance relationships among the intercepts and slopes in the model (3, 13).

Models were built using a stepwise procedure, i.e. predictor variables (z fixed effects) were added one at a time, and likelihood ratio statistics were used to judge the statistical fit of the model (3). Predictor variables (z) were accepted as significant if the estimated mean coefficient was greater than twice the standard error of the estimate. If the retention criterion was not met, the predictor variable was discarded. Minute of measurement power functions were introduced into the linear models to allow for the nonlinearity of changes in the dependent parameters. The following variables were introduced as predictors in the multilevel models: minute of measurement, minute of measurment^2, age, height, body mass and VO_{2max}. Dummy variables were created for sex and sample groups with female participants and controls, respectively, as reference categories. A total of twenty-eight independent multilevel REMs was constructed.

RESULTS

Participants anthropometric and physiologic characteristics are shown in table 1. Data from the questionnaires on training history and training habits showed that on average, master athletes had a practice of history of 24.6 ± 1.8 years and trained 10.3 ± 0.2 months per year with approximately 5 hours per week (5.5 ± 0.4 hours) per month trained.

The results obtained for the different cell subpopulations after analysis using multilevel models are summarized in tables 2, 3, 4, 5 and 6.

Table 2 shows the results from multilevel models for the proportion of total Ly, total LyT, total CD4 and CD4 T cells, total CD8 and CD8 T-cells. For the six models, the random effects are significant within individuals, indicating that %Total Ly, %Total LyT, %Total CD4, %CD4, %Total CD8 and %CD8 T-cells are increasing significantly at each minute of measurement within individuals (P<0.05). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts [constant/constant ($\mu_j \times \mu_j$), P<0.05] but not in terms of the slopes of their lines [minute of measurement/minute of measurement ($v_j x_{ij} \times v_j x_{ij}$), P>0.05], except for proportion of total CD4$^+$ cells (P<0.05). Also, the variance of these intercepts and slopes are not significantly correlated [constant/age ($\mu_j \times v_j x_{ij}$), P>0.05], except proportion of total CD8$^+$cells. The variance between individuals is not, therefore, different at different minutes of measurement, except for %Total CD4 and %Total CD8. The fixed effects that significantly predicted %Total Ly, %Total LyT, %Total CD4, %CD4, %Total CD8 and %CD8 indicates that once the time of measurement is controlled (1 minute predicts 0.4%, 0.1, -0.5, -0.05, -0.6, 0.1 and 0.4, respectively). Once time of measurement is accounted for no significant effect of sports participation (controls *vs* masters) was noted for all variables. VO2max had an independent, significant and negative association with proportion of total Ly.

Table 3 summarizes the results from multilevel models for the proportion of CD4 naïve, CM, EM and EMRA. For the four models, the random effects are significant within individuals. The proportion of CD4 naïve, CM, EM and EMRA are increasing significantly at each minute of measurement within individuals (P<0.05). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts (P<0.05) but not in terms of the slopes of their lines (P>0.05). Also, the variance of these intercepts and slopes are not significantly correlated (P>0.05). The variance between individuals is not, therefore, different at different minutes of measurement. The fixed effects that significantly predicted %CD4 naïve, %CD4 CM, %CD4 EM and %CD4 EMRA indicate that once the time of measurement is controlled (1 minute predicts -0.01%,

Table 2. Multilevel regression models for the proportion of Total lymphocytes, Total T-lymphocytes, T-lymphocytes, Total $CD4^+$, $CD4^+$, Total $CD8^+$ and $CD8^+$ T-cells.

	%Total Ly	%Total LyT	%LyT	%Total CD4	%CD4	%Total CD8	%CD8
Fixed effects							
Constant	31.3845±4.0097	18.1119±1.0056	75.4511±2.9632	13.1916±1.0026	59.0956±1.6833	6.0599±0.5640	32.5100±1.5327
Minute of measurement	0.3709±0.0756	0.0930±0.0339	-0.5072±0.0838	-0.0470±0.0190	-0.6316±0.0675	0.1038±0.0238	0.3606±0.0578
Minute of measurement ^{2}	-0.0075±0.0012	-0.0023±0.0008	0.0092±0.0013	0.0005±0.0002	0.0112±0.0011	-0.0021±0.0004	-0.0066±0.0009
Age	NS	NS	NS	NS	NS	NS	NS
Females *vs* males	NS	NS	-6.4202±3.1680	-3.4520±1.1493	NS	NS	NS
Controls *vs* masters	NS	NS	NS	NS	NS	NS	NS
Height	NS	NS	NS	NS	NS	NS	NS
Body mass	NS	NS	NS	NS	NS	NS	NS
VO_{2max}	-0.023±0.0010	NS	NS	NS	NS	NS	NS
Random effects							
Level 1							
Constant (ε_{ij})	10.8641±2.0350	5.5379±1.0374	13.3507±2.5010	0.6474±0.1702	8.6572±1.6217	1.0756±0.2820	6.3568±1.1908
Level 2							
Constant (μ_j)	26.6720±7.9999	24.0956±6.8292	55.4478±15.7697	6.6531±1.8609	73.9934±20.2087	8.2070±2.3434	62.1141±16.8800
CA centered ($v_j x_{ij}$)	NS	NS	NS	0.0005±0.0002	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	NS	NS	NS	NS	-0.0344±0.0169	NS

Abbreviations: %Total Ly, percentage of lymphocytes to the total number of white blood cells; %Total LyT, percentage of T-lymphocytes to the total lymphocytes; %LyT, T-lymphocytes percentage; %Total $CD4^+$, percentage of $CD4^+$ T-lymphocytes to the total lymphocytes; %CD4, $CD4^+$ T-lymphocytes percentage; %Total $CD8^+$, percentage of $CD8^+$ T-lymphocytes to the total lymphocytes; %CD8, $CD8^+$ T-lymphocytes percentage; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of Total Ly, Total LyT, LyT, Total CD4, CD4, Total CD8 and CD8 in %. Random effects values are presented as estimated mean variance ± SEE (Total Ly, Total LyT, LyT, Total CD4, CD4, Total CD8 and CD8 in $\%^2$).

Table 3. Multilevel regression models for naïve, central memory (CM), effector memory (EM) and CD45RA expressing effector memory cells (EMRA) $CD4^+$ T-cells.

	%CD4			
	Naïve	CM	EM	EMRA
Fixed effects				
Constant	116.9764±48.9146	50.9481±2.9232	-78.6447±38.3960	1.2080±0.5510
Minute of measurement	-0.0061±0.0140	-0.4028±0.0789	0.2614±0.0515	-0.0097±0.0073
Minute of measurement ^{2}	NS	0.0071±0.0012	-0.0046±0.0008	NS
Age	NS	NS	0.2820±0.1317	NS
Females *vs* males	NS	9.1919±3.2184	-11.8384±3.5127	NS
Controls *vs* masters	NS	NS	NS	NS
Height	-0.6979±0.3126	NS	NS	NS
Body mass	NS	NS	NS	NS
VO_{2max}	0.0599±0.0024	NS	NS	NS
Random effects				
Level 1				
Constant (ε_{ij})	10.8009±2.0115	11.6590±3.0609	5.0347±0.9431	6.8678±2.0783
Level 2				
Constant (μ_j)	78.9011±21.7285	65.4753±19.2889	34.9337±9.6265	2.9954±0.5611
CA centered ($v_j x_{ij}$)	NS	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	NS	NS	NS

Abbreviations: %CD4, $CD4^+$ T-lymphocytes percentage; CM, central memory; EM, effector memory; EMRA, CD45RA expressing effector memory cells; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of naïve, CM, EM and EMRA in %. Random effects values are presented as estimated mean variance ± SEE (naïve, CM, EM and EMRA in $\%^2$).

Table 4. Multilevel regression models for naïve, central memory (CM), effector memory (EM) CD45RA expressing effector memory cells (EMRA) $CD8^+$ T-cells.

	%CD8				
	Naïve	SLEC	CM	EM	EMRA
Fixed effects					
Constant	59.3528±12.6252	-52.4484±17.0325	36.0371±2.1766	14.2907±1.4504	23.8093±2.9579
Minute of measurement	-0.4232±0.06554	0.1254±0.0259	-0.2643±0.0707	NS	0.4659±0.0876
Minute of measurement ^{2}	0.0074±0.0010	-0.0023±0.0004	0.0052±0.0011	NS	-0.0085±0.0014
Age	-0.5964±0.2192	0.1840±0.0774	NS	NS	NS
Females *vs* males	-8.9289±4.0912	NS	NS	NS	NS
Controls *vs* masters	NS	NS	NS	NS	NS
Height	NS	0.2856±0.0969	NS	NS	NS
Body mass	NS	NS	NS	NS	NS
VO_{2max}	NS	NS	NS	NS	NS
Random effects					
Level 1					
Constant (ε_{ij})	7.6713±1.4370	1.2250±0.3217	9.5113±1.7816	8.3741±1.5687	14.4887±3.8030
Level 2					
Constant (μ_j)	85.4878±23.1379	22.7971±6.1739	128.3961±34.5698	55.5906±15.3509	240.0121±65.5292
CA centered ($v_j x_{ij}$)	NS	0.0007±0.0003	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	-0.1136±0.0410	NS	NS	NS

Abbreviations: %CD8, $CD8^+$ T-lymphocytes percentage; SLEC: short-lived effector cells; CM, central memory; EM, effector memory; EMRA, CD45RA expressing effector memory cells; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of naïve, CM, EM and EMRA in %. Random effects values are presented as estimated mean variance ± SEE (naïve, CM, EM and EMRA in $\%^2$).

-0.4, 0.3 and -0.01, respectively) no significant effect of sports participation (controls vs masters) was noted for all variables. VO_{2max} had an independent, significant and positive association with the proportion of CD4 naïve T cells.

Table 4 summarizes the results from multilevel models for the proportion of naïve, SLEC, CM, EM and EMRA CD8+ T-cells. For the five models, the random effects are significant within individuals. The proportion of naïve, SLEC, CM, EM and EMRA $CD8^+$ T-cells are increasing significantly at each minute of measurement within individuals ($P<0.05$). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts ($P<0.05$) but not in terms of the slopes of their lines ($P>0.05$), except for the proportion of $CD8^+$ SLEC T-cells ($P>0.05$). Also, the variance of these intercepts and slopes are not significantly correlated ($P<0.05$). The variance between individuals is not, therefore, different at different minutes of measurement, except for the proportion of $CD8^+$ SLEC T-cells. The fixed effects that significantly predicted naïve, SLEC, CM and EMRA $CD4^+$ SLEC T-cells indicate that once the time of measurement is controlled (1 minute predicts -0.4%, 0.1, -0.3 and 0.5, respectively) no significant effect of sports participation (controls *vs* masters) was noted for all variables.

The effects of exercise and age on total T-lymphocytes, $CD4^+$ and $CD8^+$ T-cells and the KLRG1 expression are shown in Table 5. Total $CD3^+$, $CD4^+$ and $CD8^+$ T-cells percentages did not increase with exercise. Senescent $CD4^+$ and $CD8^+$ T-cells were elevated in control compared to masters ($p < 0.05$) before and 1 h after exercise (Table 5).

Table 5. Values (%) of cells $CD3^+$, $CD3^+$ T-cells and $CD3^+CD4^+/CD8^+$ T-cells and KLRG1 expression in response to a maximum effort protocol.

	Pre		Post		1h	
%	**Masters**	**Control**	**Masters**	**Control**	**Masters**	**Control**
$CD3^+$	66.28±10.45	70.99±6.94	62.46±9.06	66.38±7.66	67.34±12.10	72.16±10.13
$CD3^+KLRG1^+$	6.58±4.08	9.19±4.92	9.70±5.77	12.23±6.09	8.68±5.33	13.69±7.57
$CD3^+CD4^+$	58.66±8.30	58.73±9.68	51.95±10.70	53.15±10.20	60.44±8.53	60.67±9.58
$CD3^+CD4^+KLRG1^+$	8.05±5.06*	13.49±8.17	11.35±6.79	17.07±9.80	7.31±4.97*	12.78±6.17
$CD3^+CD8^+$	30.64±8.20	31.93±8.18	35.63±10.24	35.26±8.49	28.37±9.77	30.73±7.14
	25.22±12.94	36.54±14.9			22.14±12.83	
$CD3^+CD8^+KLRG1^+$	*	1	32.32±15.90	42.63±12.58	*	37.90±11.80

Values are Mean ± SD. N= 9 (Control); N= 19 (Masters). * $P<0.05$ compared to control.

Table 6 summarizes the results from multilevel models for the proportion of LyT, $CD4^+$, $CD4^+$ naïve, central memory, effector-memory and EMRA expressing KLRG1. The random effects describe the two levels of variance [within individuals (Level 1 of the hierarchy) and between individuals (Level 2 of the hierarchy)]. For the six models, the random effects are significant within individuals, indicating that proportion of senescent LyT, $CD4^+$, naïve, CM, EM and EMRA $CD4^+$ T-cells are increasing significantly at each minute of measurement within individuals

Table 6. Multilevel regression models for the proportion of T-lymphocytes, $CD4^+$ and naïve, central memory, effector memory, CD45RA expressing effector memory $CD4^+$ T-cells expressing KLRG1.

			%$CD4^+$			
	%LyT/$KLRG1^+$	%$CD4^+$/$KLRG1^+$	naïve/$KLRG1^+$	CM/$KLRG1^+$	EM/$KLRG1^+$	EMRA/$KLRG1^+$
Fixed effects						
Constant	23.7922±2.9145	13.8351±2.0220	10.9632±1.9997	8.8069±1.5148	NS	NS
Minute of measurement	0.4936±0.0856	0.3158±0.0583	0.1216±0.0496	0.1227±0.0507	0.4557±0.1047	0.3514±0.1577
Minute of measurement ^{2}	-0.0090±0.0013	-0.0055±0.0009	-0.0019±0.0008	-0.0022±0.0008	-0.0087±0.0016	-0.0076±0.0028
Age	NS	NS	NS	NS	NS	NS
Controls *vs* masters	-6.7829±3.0608	-5.8145±2.4227	-5.3182±2.5761	-4.3505±1.9356	NS	NS
VO_{2max}	NS	NS	NS	NS	NS	NS
Random effects						
Level 1						
Constant (ε_{ij})	14.0597±2.6338	6.0097±1.1357	3.5199±0.7511	3.6377±0.7756	18.3809±3.6048	34.3770±12.1479
Level 2						
Constant (μ_j)	69.5709±19.5392	33.8227±9.5824	34.0991±10.6367	18.6913±6.0067	183.2635±52.5409	349.0878±131.5839
Minute of measurement ($v_j x_{ij}$)	NS	NS	NS	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	NS	NS	NS	NS	NS

Abbreviations: %LyT, T-Lymphocytes percentage; %CD4, $CD4^+$ T-cell percentage; CM, central memory; EM, effector memory; EMRA, CD45RA expressing effector memory cells; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of LyT/$KLRG1^+$, $CD4^+$/$KLRG1^+$, $CD4^+$/naïve/$KLRG1^+$, $CD4^+$/CM/$KLRG1^+$, $CD4^+$/EM/$KLRG1^+$, $CD4^+$/EMRA/$KLRG1^+$ in %. Random effects values are presented as estimated mean variance ± SEE (LyT/$KLRG1^+$, $CD4^+$/$KLRG1^+$, $CD4^+$/naïve/$KLRG1^+$, $CD4^+$/CM/$KLRG1^+$, $CD4^+$/EM/$KLRG1^+$, $CD4^+$/EMRA/$KLRG1^+$ in $\%^2$).

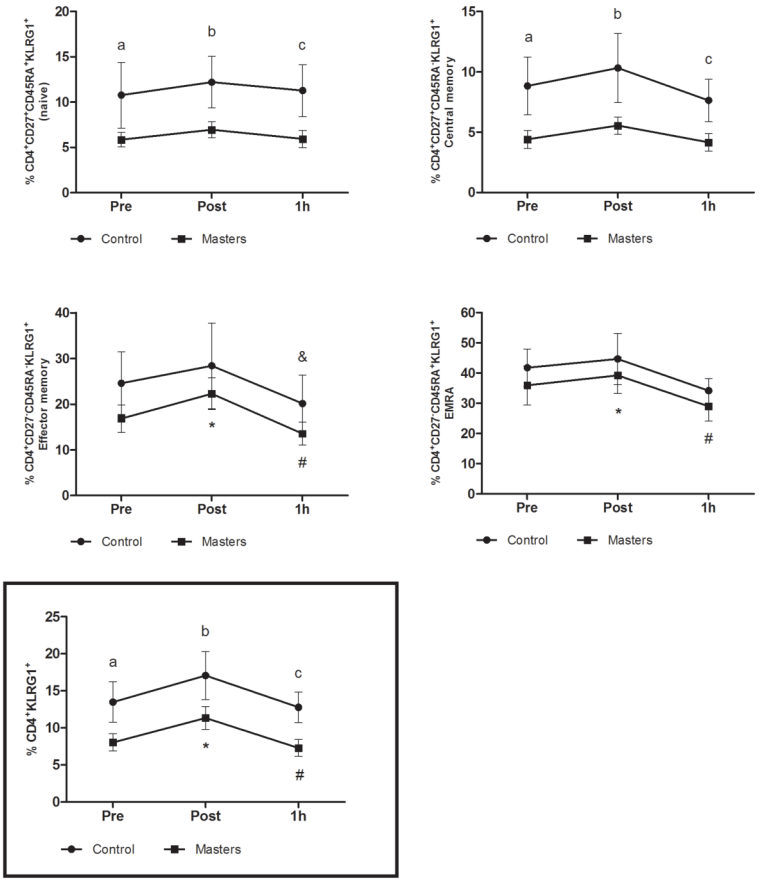


Figure 2: Proportion of naïve, central memory, effector memory and CD45RA expressing effector memory $CD4^+$ T cells expressing KLRG1 before and in response to a maximum effort protocol for control and master athlete groups. The values are presented as the mean ± standard deviation. a P<0.05 compared to master athletes in Pre. b P <0.05 compared to master athletes in Post. c P<0.05 compared to the master athletes in 1h. * P <0.05 Post compared to Pre. # P<0.05 1h compared to Post. & P<0.05 1h compared to Pre. Abbreviations: %$CD4^+KLRG1^+$ = percentage of $CD4^+$ T-cells expressing KLRG1. Pre (before), Post (10 min after test), 1 h (1 h after test). Control (N = 9); Masters (N = 19).

(P<0.05). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts [constant/constant ($\mu_j \times \mu_j$), P<0.05] but not in terms of the slopes of their lines [minute of measurement/minute of measurement ($v_j x_{ij} \times v_j x_{ij}$), P>0.05]. Also, the variance of these intercepts and slopes are not significantly correlated [constant/age ($\mu_j \times v_j x_{ij}$), P>0.05]. The variance between individuals is not, therefore, different at different minutes of measurement. The fixed effects that significantly predicted %LyT/$KLRG1^+$, %$CD4^+$/$KLRG1^+$, %$CD4^+$/naïve/$KLRG1^+$, %$CD4^+$/CM/$KLRG1^+$ indicate that once the time of measurement is controlled (1 minute predicts 0.5%, 0.3, 0.1 and 0.1, respectively), *masters* had significant less proportion of senescent LyT (-6.7829±3.0608%), $CD4^+$ T-cells (-5.8145±2.4227), naïve (-5.3182±2.5761) and effector memory (-4.3505±1.9356%) $CD4^+$ senescent T-cells than controls, while no significant effect of sports participation (controls vs masters) was noted for the proportion of effector memory and EMRA senescent $CD4^+$ T-cells.

Considering that the proportion of senescent $CD4^+$ T-cells and subsets of naïve, CM, EM and EMRA $CD4^+$ T-cells increases with each minute of measurement, we compared the mean values of the proportion of senescent $CD4^+$ T-cells and subsets at Pre, Post and 1h using ANOVA for repeated measures. The results of this analysis are presented in Figure 2. Senescent $CD4^+$ T-cells are mobilized by exercise only in the master athletes group, as shown by the significant increase in senescent $CD4^+$ T-cells at Post (Figure 2), corroborating the results of the multilevel analysis (Table 6). The proportion of

Table 7. Multilevel regression models for the proportion of $CD8^+$, naïve, short-lived effector cell, central memory, effector memory and CD45RA expressing effector memory $CD8^+$ T-cells expressing KLRG1.

		%$CD8^+$				
	%$CD8^+$/$KLRG1^+$	naïve/$KLRG1^+$	SLEC/$KLRG1^+$	CM/$KLRG1^+$	EM/$KLRG1^+$	EMRA/$KLRG1^+$
Fixed effects						
Constant	NS	15.8408±7.023	53.0178±3.6679	25.4383±3.3874	40.3917±5.7790	42.8580±4.2705
Minute of measurement	0.6729±0.1108	0.1058±0.0538	0.6824±0.1846	0.6063±0.0877	0.4867±0.1173	0.2627±0.1247
Minute of measurement ^{2}	-0.0117±0.0017	-0.0019±0.0008	-0.0120±0.0029	-0.0106±0.0014	-0.0091±0.0018	-0.0056±0.0019
Age	NS	NS	NS	NS	NS	NS
Controls *vs* masters	-13.0661±4.9568	-9.3624±2.0992	NS	-7.0282±3.0654	-12.0034±5.4968	NS
VO_{2max}	NS	NS	NS	NS	NS	NS
Random effects						
Level 1						
Constant (ε_{ij})	23.3417±4.3484	5.3239±1.0110	64.9409±12.1082	14.3256±2.7075	26.0568±6.8358	27.2463±7.4122
Level 2						
Constant (μ_j)	114.6349±40.0272	25.0755±7.1753	328.8600±92.0476	85.0265±24.0171	344.0468±94.8657	466.6415±131.9003
Minute of measurement						
($v_j x_{ij}$)	NS	NS	NS	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	NS	NS	NS	NS	NS

Abbreviations: %LyT, T-Lymphocytes percentage; %CD8, $CD8^+$ T-Lymphocytes percentage; SLEC, short-lived effector cells; CM, central memory; EM, effector memory; EMRA, CD45RA expressing effector memory cells; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of LyT/$KLRG1^+$, $CD8^+$/$KLRG1^+$, $CD8^+$/naïve/$KLRG1^+$, $CD8^+$/CM/$KLRG1^+$, $CD8^+$/EM/$KLRG1^+$, $CD8^+$/EMRA/$KLRG1^+$ in %.
Random effects values are presented as estimated mean variance ± SEE (LyT/$KLRG1^+$, $CD8^+$/$KLRG1^+$, $CD8^+$/naïve/$KLRG1^+$, $CD8^+$/CM/$KLRG1^+$, $CD8^+$/EM/$KLRG1^+$, $CD8^+$/EMRA/$KLRG1^+$ in $\%^2$).

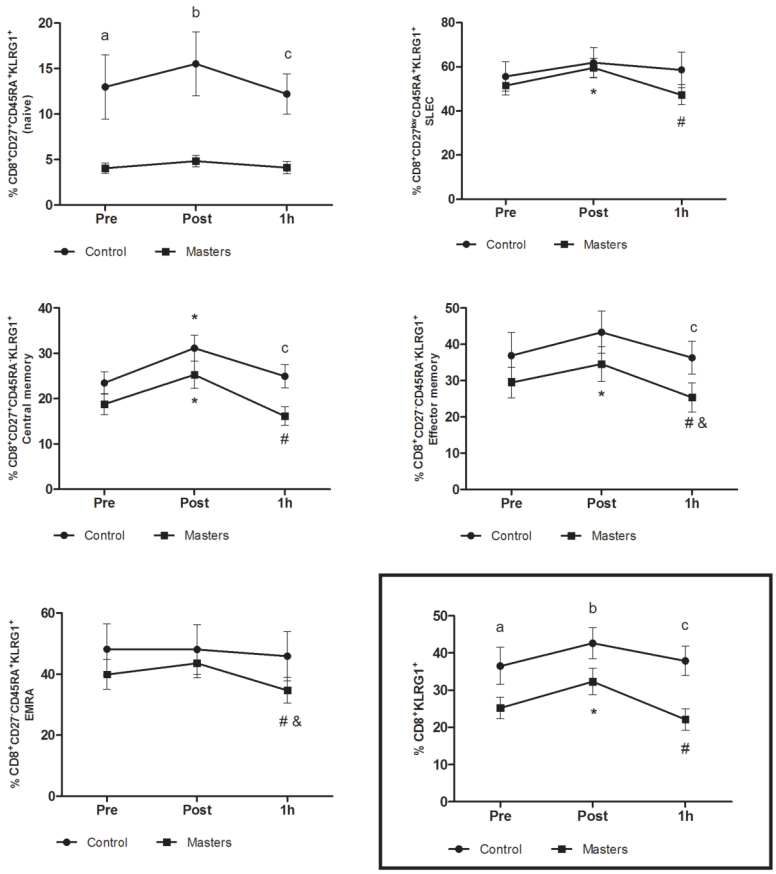


Figure 3: Proportion of senescent naïve, central memory, effector memory and CD45RA expressing effector memory $CD8^+$ T-cells before and in response to a maximum effort protocol for control and master athletes groups. The values are presented as the mean ± standard deviation. a P<0.05 compared to master athletes in Pre. b P <0.05 compared to master athletes in Post. c P<0.05 compared to the master athletes in 1h. * P <0.05 Post compared to Pre. # P<0.05 1h compared to Post. & P<0.05 1h compared to Pre. Abbreviations: %$CD8^+$/$KLRG1^+$ = percentage of $CD8^+$ T-cells expressing KLRG1. Pre (before), Post (10 min after test), 1 h (1 h after test). Control (N = 9); Masters (N = 19).

senescent $CD4^+$ T-cells return to Pre-values in 1h (Figure 2). The results indicated that the proportions of senescent naïve and CM $CD4^+$ T-cells are not different between the two groups. The senescent EM and EMRA $CD4^+$ T-cells increased in Post for master athletes and return to the baseline values in 1h. In the control group, the only difference observed was the proportion of the senescent $CD4^+$ EM T-cells, with lower values at 1h when compared to Pre.

Table 7 summarizes the results from multilevel models for the proportion of senescent $CD8^+$, naïve, SLEC, CM, EM e EMRA $CD8^+$ T-cells. The random effects are significant within individuals, indicating that proportion of $CD8^+$, naïve, SLEC, CM, EM e EMRA $CD8^+$ T-cells expressing $KLRG1^+$ are increasing significantly at each minute of measurement within individuals (P<0.05). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts [constant/constant ($\mu_j \times \mu_j$), P<0.05] but not in terms of the slopes of their lines [minute of measurement/minute of measurement ($v_j x_{ij} \times v_j x_{ij}$), P>0.05]. Also, the variance of these intercepts and slopes are not significantly correlated [constant/age ($\mu_j \times v_j x_{ij}$), P>0.05]. The variance between individuals is not, therefore, different at different minutes of measurement. The fixed effects that significantly predicted the proportion of senescent $CD8^+$, naïve, SLEC, CM, EM e EMRA $CD8^+$ T-cells indicates that once the time of measurement is controlled

(1 minute predicts 0.7, 0.1, 0.7, 0.6 and 0.5 and 0.3 respectively), master athletes had significant less senescent $CD8^+$ T-cells (-13.0661±4.9568), $CD8^+$ naïve (-9.3624±2.0992), central memory (-7.0282±3.0654) and effector memory (-12.0034±5.4968) senescent T-cells than controls.

Senescent $CD8^+$ T-lymphocytes and subsets increased in response to each minute of measurement of the test protocol. The senescent $CD8^+$ T-cells are mobilized by the exercise and return to the pre-exercise values at 1h in the master athletes group (Figure 3). The senescent $CD8^+$ T-cells and the senescent SLEC, CM, EM $CD8^+$ T-cells are also mobilized by exercise in the master athletes group. The values returned to Pre-values 1h after the end of the protocol. In the control group, only the senescent $CD8^+$ CM T-cells increased at Post and the values remained elevated after 1h. The senescent $CD8^+$ EMRA T-cells did not increase at Post but decreased in 1h to values below those observed at Post and Pre in the master athletes group ($P < 0.05$).

The mRNA expression of the CCR7 for $CD8^+$ naïve T-cells and the Fas-L for $CD8^+$ EMRA T-cells was not different between masters and controls. The mRNA expression for both genes did not change in response to the maximal effort test (Figure 4).

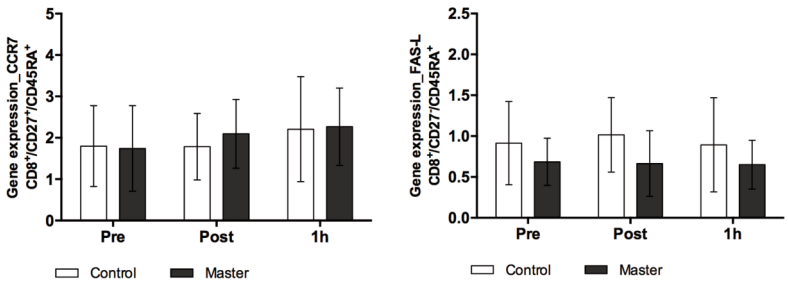


Figure 4. Effect of acute exercise on expression of CCR7 genes in $CD8^+$ naïve and Fas-L in $CD8^+$ EMRA T-cells.
Data are presented as mean ± SD. Pre (before), Post (10 min after test), 1 h (1 h after test). Control (N = 9); Masters (N = 19).

DISCUSSION

Differences between masters and controls, suggesting lifelong training effects, were observed for senescent $CD4^+$ T cells and naïve and CM $CD4^+$ T-cells subsets, and for senescent $CD8^+$ T-cells and naïve, CM, EM $CD8^+$ T-cells subsets. The proportion of senescent $CD4^+$ and $CD8^+$ EMRA cells did not differ between conditions. The results also indicate that the proportion of $CD4^+$ and $CD8^+$ T-cells and their subsets are not different between masters and controls at all times of measurement (Tables 2, 3 and 4). Age had a negative effect on the proportion of $CD8^+$ naïve T-cells and a positive effect on the proportion of $CD8^+$ SLEC T-cells. VO_{2max} negatively influenced the proportion of total lymphocytes and showed a positive effect on the proportion of $CD4^+$ naïve T-cells.

In general, studies in both mice and humans, have showed a gradual reduction of naïve $CD4^+$ and $CD8^+$ T-cell numbers with aging, a consequence of the decreased production of these cells by the thymus. Despite this decrease in thymic production from puberty to old age, it is known that humans have a remarkable ability to maintain relatively constant numbers of lymphocytes over many decades through the homeostatic proliferation of peripheral T-cells in healthy adults (1). However, there are important differences between the $CD4^+$ and $CD8^+$ T-cells populations. It has been demonstrated, in the elderly, that the low thymus functionality shows a thymocyte production tendentious for $CD4^+$ T-cells (7). In fact, all subjects with a very low thymic function, defined as the percentage of double positive thymocytes below 10%, had CD4/CD8 ratios higher than 2 (7). This suggests a dysregulation of homeostasis, mainly affecting the subgroup of $CD8^+$ naïve T-cells. $CD8^+$ T-cells undergo major expansion after activation and can establish a stable set of highly differentiated memory cells at rest. In contrast, the ability of $CD4^+$ T-cells to expand and survive appears to be lower, so that the vast majority of activated $CD4^+$ T-cells can undergo apoptosis quickly. Furthermore, the total $CD4^+$ T-cell repertoire appears to be less likely to have late-differentiated compared to $CD8^+$ T lymphocytes (1). We observed a negative influence of age on $CD8^+$ naïve T-cells, and a positive effect of VO_{2max} on $CD4^+$ naïve T-cells. This suggests that the effect of age is greater than training on $CD8^+$ naïve T-cells, whereas individuals with a better physical condition (higher VO_{2max} values), tend to have higher numbers of $CD4^+$ naïve T-cells (Table 2).

On the other hand, the results showed a positive effect of age on $CD8^+$ SLEC T-cells. $CD8^+$ SLEC T-cells are cells that still express CD45RA but lose expression of CD27 (Figure 1e) and have high expression of KLRG1 (Figure 3). When visualized by APS (Automatic Population Separator) these were shown to be a distinct population of the $CD8^+$ naïve T-cells (Figure 1f). We classified these cells based on the definition found in the literature for short-lived effector cells (SLEC) cells (46). Yuzefpolskiy and colleagues found that cells with high expression of KLRG1 ($KLRG1^{hi}$) represent the SLEC population. In addition, it appears that SLEC cells have a preferential localization at non-lymphoid sites, suggesting that they are continuously encountering antigens at peripheral sites, thus undergoing a terminal differentiation process (46). We suggest that future studies should develop new methodologic approaches for a better characterization of this population.

Changes with age have been observed in gene expression of several genes in the naïve compartment of $CD4^+$ T and $CD8^+$ T-cells (Mirza et al., 2011). Taking this into account, we quantified the expression of CCR7 mRNA in $CD8^+$ naïve T-cells. CCR7 participates in both regulation of trafficking and homing of leukocytes to lymph nodes (8). T-cells become progressively differentiated, suffering loss of the surface molecules, initially CD45RA undergoes downregulation, followed by loss of CCR7 expression and ultimately a decrease in CD28 and CD27 expression (24). The CCR7 gene expression was not modified by the maximal exercise test and was the same for masters and controls (Figure 4). This suggests that neither exercise nor age seem to affect the homing capacity of these cells.

Exercise induces leukocytosis, but the response of major leukocyte subtypes is not uniform. According to Simpson and colleagues (2015) there are 3 main characteristics that are shared by all leukocyte subsets that are preferentially redistributed after a single exercise session, namely: 1) the cells mobilized by the exercise tend to have an increased cytotoxic/effector function and have a mature/differentiated phenotype; 2) these cells redistributed with exercise tend to present phenotypes associated with tissue migration; 3) leukocyte subtypes which are preferentially redistributed with exercise show high expression of adrenergic receptors (β2-ARS) and glucocorticoid receptors, and are therefore highly sensitive to catecholamines and cortisol (35). This is an important factor to consider since aging is associated with profound declines in immunity and older individuals (> 50 years) having a diminished ability to redistribute certain leukocyte subtypes in response to a single exercise bout (37). Spielmann and colleagues (2014) showed that this was due to a lower mobilization of naïve T-cells, possibly due to the reduction in the thymic output. Thus, senescent T-cells accumulate with age, reducing the repertoire of naïve T-cells and increasing the risk of infection in the host. Senescent T-lymphocytes are cells that have already encountered an antigen, and express the KLRG1 and/or CD57 receptor, do not expand clonally after antigenic restimulation and prevail in the blood of older adults. Because this response is likely to be influenced by certain lifestyle factors, we examined the association between aerobic fitness (VO_{2max}) and age-related senescent T-cell accumulation. We found no interactions between VO_{2max} or age in any of the subsets of KLRG1$^+$ cells, contrary to previous studies (38). However, master athletes, which had higher aerobic capacity values than controls, showed lower values of senescent CD4$^+$, CD8$^+$ T-cells and their subsets when compared to controls. The exception was observed for senescent CD4$^+$ and CD8$^+$ EMRA T-cells. This suggests that repetitions of lifelong exercise avoid accumulating of senescent T-cells, corroborating the theory of immune space proposed by Simpson (28). Therefore, we postulate that maintaining high levels of aerobic fitness during the natural course of aging may help prevent the accumulation of senescent T-cells, enhancing the beneficial effects of maintaining a physically active lifestyle in the immune system of adult subjects. If it is possible to maintain an adequate number of naïve T-cells, the immune system will be better able to recognize and respond to new pathogens (38).

The theory of the immune space indicates that exercise can make space by the mobilization of senescent or terminally differentiated T-cells and death of this cells by apoptosis as a result of the apoptotic signals produced by exercise (28). We are able to confirm the mobilization of senescent CD4$^+$ and CD8$^+$ T-cells by exercise (Fig. 2 and 3). Regarding cells of a late-stage differentiated phenotype (EMRA), the results showed that only EMRA CD4$^+$ T-cells (not CD8$^+$ T-cells) were mobilized during exercise. Because the 'post' sample was collected 10-minutes after exercise, is it possible that we have missed the peak cell mobilization (29). A recent study showed that the mobilization and apoptosis of low and highly differentiated T-cell subsets immediately after exercise was dependent on the kind of exercise performed with continuous exercise deleting mainly low differentiated T-cells (14). We assessed an apoptotic marker on the sorted CD8$^+$ EMRA T-cells. The Fas-L gene expression in these cells was not different between the groups and between the measurement points. Fas and Fas-L are two molecules involved in the regulation of cell death, and their interaction leads to apoptosis of thymocytes that fail to properly rearrange TCR genes and those that recognize autoantigens, a process called negative selection. In addition, the Fas and Fas-L interaction leads to activation-induced cell death, a form of apoptosis by repeated TCR stimulation, responsible for the peripheral deletion of activated T-cells (42). Since the gene expression of Fas-L was not different between the groups, it is possible that cellular homing markers are involved (16). We suggest that future research should include the determination of cellular homing markers and other markers of apoptosis such as caspase-3 and anti-apoptotic marker like Bcl-2 to refute or corroborate this hypothesis. The results of a study similar to ours showed that in athletes (65-85 years old) who underwent intense training, there was less apoptosis of CD45RO$^+$ and CD45RA$^+$ T-cells than in athletes with moderate training routine or untrained people, as demonstrated by a higher expression of Bcl-2 and a lower expression of caspase-3 (27). However, these were not measured after acute exercise. Therefore, it is necessary to determine which mechanisms are responsible for the lower number of senescent T-cells in master adult athletes and if this response is maintained in elderly master athletes. The secretion of IL-7, that is produced by skeletal muscle and regulates Bcl-2 expression, could explain why athletes would be protected from spontaneous T cell apoptosis (27). Not all cells bearing the classic 'senescent' phenotype are senescent and there are new markers of senescence (i.e. P16ink4a) and markers of exhaustion (i.e. PD-1) that should be looked at on these cell types.

CONCLUSION

CD8$^+$ naïve T-cells decreased and CD8$^+$ SLEC T-cell increased with age while VO_{2max} positively influenced the proportion of CD4$^+$ naïve T-cell, suggesting that individuals with better physical condition are better at preserving their CD4$^+$ naïve T-cell population. However similar values of total naïve CD4$^+$ and CD8$^+$ T-cells and their memory subsets were found for the master and control groups. The main differences between groups were found in the KLRG1 senescence marker expression. Lifelong training decreased the percentage of the senescent naïve, CM and EM CD8$^+$ T-cells and senescent naïve and CM CD4$^+$ T-cells. In both CD4$^+$ and CD8$^+$ T-cell subsets, the percentage of senescent EMRA T cells was also lower in the master athletes. This suggests that lifelong training induces the death of these cells and corroborates the immune space hypothesis proposed by Simpson and colleagues (27). In addition, we postulate that maintaining high levels of aerobic fitness during the natural course of aging may help preserve the proportion of CD4$^+$ naïve cells and prevent the accumulation of senescent T-cells, hallmarks of a "younger" immune system.

ACKNOWLEDGMENTS

Firstly, the authors would like to thank all the master athletes that volunteered to participate in this study. The research

Center for Sport and Physical Activity (IUD/DTP/04213/2016) and the Faculty of Sport Sciences and Physical Education, University of Coimbra funded the study. Also, the present manuscript was accomplished with support from CAPES, Coordination for the Improvement of Higher Education Personnel, Brazil (grants numbers 1417/13-4) and from FCT, the Portuguese Foundation for Science and Technology (SFRH/BPD/100470/2014), Lisbon, Portugal.

REFERENCES

1. Appay V, Sauce D. Naive T cells: The crux of cellular immune aging? Exp Gerontol 54: 90–3, 2014.
2. De Araújo AL, Silva LCR, Fernandes JR, Matias M de ST, Boas LS, Machado CM, Garcez-Leme LE, Benard G. Elderly men with moderate and intense training lifestyle present sustained higher antibody responses to influenza vaccine. Age (Dordr) 37: 105, 2015.
3. Baxter-Jones ADG, Mirwald RL, mckay HA, Bailey DA. A longitudinal analysis of sex differences in bone mineral accrual in healthy 8-19-year-old boys and girls. Ann Hum Biol 30: 160–175, 2003.
4. Campbell JP, Riddell NE, Burns VE, Turner M, van Zanten JJCSV, Drayson MT, Bosch JA. Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. Brain Behav Immun 23: 767–75, 2009.
5. Cosgrove C, Galloway SDR, Neal C, Hunter AM, mcfarlin BK, Spielmann G, Simpson RJ. The impact of 6-month training preparation for an Ironman triathlon on the proportions of naïve, memory and senescent T cells in resting blood. Eur J Appl Physiol 112: 2989–98, 2012.
6. Dhabhar FS. Effects of stress on immune function: the good, the bad, and the beautiful. Immunol Res 58: 193–210, 2014.
7. Ferrando-Martínez S, Ruiz-Mateos E, Hernández A, Gutiérrez E, Rodríguez-Méndez MDM, Ordoñez A, Leal M. Age-related deregulation of naive T cell homeostasis in elderly humans. Age (Omaha) 33: 197–207, 2011.
8. Förster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol 8: 362–71, 2008.
9. Fülöp T, Larbi A, Pawelec G. Human T cell aging and the impact of persistent viral infections. Front Immunol 4: 271, 2013.
10. Gleeson M, Bishop NC. The T cell and NK cell immune response to exercise. [Online]. Ann Transplant 10: 43–8, 2005. Http://www.ncbi.nlm.nih.gov/pubmed/17037088.
11. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol 11: 607–15, 2011.
12. Haugen F, Norheim F, Lian H, Wensaas AJ, Dueland S, Berg O, Funderud A, Skålhegg BS, Raastad T, Drevon C a. IL-7 is expressed and secreted by human skeletal muscle cells. Am J Physiol Cell Physiol 298: C807–C816, 2010.
13. Jackowski SA, Baxter-Jones ADG, Gruodyte-Raciene R, Kontulainen SA, Erlandson MC. A longitudinal study of bone area, content, density, and strength development at the radius and tibia in children 4???12??Years of age exposed to recreational gymnastics. Osteoporos Int 26: 1677–1690, 2015.
14. Krüger K, Alack K, Ringseis R, Mink L, Pfeifer E, Schinle M, Gindler K, Kimmelmann L, Walscheid R, Muders K, Frech T, Eder K, Mooren FC. Apoptosis of T-Cell Subsets after Acute High-Intensity Interval Exercise. Med Sci Sports Exerc. 48(10):2021-9, 2016.
15. Krüger K, Frost S, Most E, Völker K, Pallauf J, Mooren FC. Exercise affects tissue lymphocyte apoptosis via redox-sensitive and Fas-dependent signaling pathways. Am J Physiol Regul Integr Comp Physiol 296: R1518-27, 2009.
16. Krüger K, Mooren FC. T cell homing and exercise. [Online]. Exerc Immunol Rev 13: 37–54, 2007. Http://www.ncbi.nlm.nih.gov/pubmed/18198659.
17. Luz Correa B, Ornaghi AP, Cerutti Muller G, Engroff P, Pestana Lopes R, Gomes Da Silva Filho I, Bosch J a., Bonorino C, Bauer ME. The inverted CD4:CD8 ratio is associated with cytomegalovirus, poor cognitive and functional states in older adults. Neuroimmunomodulation 21: 206–212, 2014.
18. Moro-García MA, Alonso-Arias R, López-Vázquez A, Suárez-García FM, Solano-Jaurrieta JJ, Baltar J, López-Larrea C. Relationship between functional ability in older people, immune system status, and intensity of response to CMV. Age (Dordr) 34: 479–95, 2012.
19. Moro-García MA, Fernández-García B, Echeverría A, Rodríguez-Alonso M, Suárez-García FM, Solano-Jaurrieta JJ, López-Larrea C, 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.
20. Müller L, Pawelec G. Aging and immunity - Impact of behavioral intervention. Brain Behav Immun 39: 8–22, 2013.
21. Pawelec G. Hallmarks of human {"}immunosenescence{"}: adaptation or dysregulation? Immun Ageing 9: 15, 2012.
22. Pawelec G. Immunosenenescence: role of cytomegalovirus. Exp Gerontol 54: 1–5, 2014.
23. Prieto-Hinojosa A, Knight A, Compton C, Gleeson M, Travers PJ. Reduced thymic output in elite athletes. Brain Behav Immun 39: 75–9, 2014.
24. Reinke S, Geissler S, Taylor WR, Schmidt-Bleek K, Juelke K, Schwachmeyer V, Dahne M, Hartwig T, Akyuz L, Meisel C, Unterwalder N, Singh NB, Reinke P, Haas NP, Volk H-D, Duda GN. Terminally Differentiated CD8+ T Cells Negatively Affect Bone Regeneration in Humans. Sci Transl Med 5: 177ra36-177ra36, 2013.
25. Riddell NE, Griffiths SJ, Rivino L, King DCB, Teo GH, Henson SM, Cantisan S, Solana R, Kemeny DM, MacAry PA, Larbi A, 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.
26. Senchina DS, Kohut ML. Immunological outcomes of exercise in older adults. [Online]. Clin Interv Aging 2: 3–16, 2007. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2684080&tool=pmcentrez&rendertype=abstract [10 Apr. 2014].
27. Silva LCR, de Araújo AL, Fernandes JR, Matias M de ST, Silva PR, Duarte AJS, Garcez Leme LE, Benard G. Moderate and intense exercise lifestyles attenuate the effects of aging on telomere length and the survival and composition of T cell subpopulations. Age (Dordr) 38: 24, 2016.
28. Simpson RJ. Aging, persistent viral infections, and immunosenescence: can exercise "make space"? Exerc Sport Sci Rev 39: 23–33, 2011.

29. Simpson RJ, Bigley AB, Agha N, Hanley PJ, Bollard CM. Mobilizing Immune Cells With Exercise for Cancer Immunotherapy. Exerc Sport Sci Rev 45: 163–172, 2017.
30. Simpson RJ, Bigley AB, Spielmann G, LaVoy ECP, Kunz H, Bollard CM. Human cytomegalovirus infection and the immune response to exercise [Online]. Exerc Immunol Rev 22: 8–26, 2016. http://www.ncbi.nlm.nih.gov/pubmed/26853134 [1 Sep. 2016].
31. Simpson RJ, Bosch J a. Special issue on Exercise Immunology: current perspectives on aging, health and extreme performance. Brain Behav Immun , 2014.
32. Simpson RJ, Cosgrove C, Chee MM, McFarlin BK, Bartlett DB, Spielmann G, O'Connor DP, Pircher H, Shiels PG, Brian M, Bartlett DB, Spielmann G, Daniel O, Pircher H, Shiels PG, McFarlin BK, O'Connor DP. Senescent phenotypes and telomere lengths of peripheral blood T-cells mobilized by acute exercise in humans. Exerc Immunol Rev 16: 40–55, 2010.
33. Simpson RJ, Florida-James GD, Cosgrove C, Whyte GP, Macrae S, Pircher H, 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.
34. Simpson RJ, Guy K. Coupling aging immunity with a sedentary lifestyle: has the damage already been done?--a minireview. Gerontology 56: 449–458, 2010.
35. Simpson RJ, Kunz H, Agha N, Graff R. Exercise and the Regulation of Immune Functions. Prog Mol Biol Transl Sci 135: 355–80, 2015.
36. Simpson RJ, Lowder TW, Spielmann G, Bigley AB, LaVoy EC, Kunz H. Exercise and the aging immune system. Ageing Res Rev 11: 404–20, 2012.
37. Spielmann G, Bollard CM, Bigley AB, Hanley PJ, Blaney JW, LaVoy ECP, Pircher H, 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: 142–51, 2014.
38. Spielmann G, McFarlin BK, O'Connor DP, Smith PJW, Pircher H, Simpson RJ. Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. Brain Behav Immun 25: 1521–9, 2011.
39. Teixeira AM, Rama L, Carvalho HM, Borges G, Carvalheiro T, Gleeson M, Alves F, Trindade H, Paiva A. Changes in naïve and memory T-cells in elite swimmers during a winter training season. Brain Behav Immun 39: 186–93, 2014.
40. Turner JE. Is immunosenescence influenced by our lifetime "dose" of exercise? Biogerontology 17: 581–602, 2016.
41. Turner JE, Campbell JP, Edwards KM, Howarth LJ, Pawelec G, Aldred S, Moss P, Drayson MT, Burns VE, Bosch JA. Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults. Age (Dordr) 36: 287–97, 2014.
42. Volpe E, Sambucci M, Battistini L, Borsellino G. Fas–Fas Ligand: Checkpoint of T Cell Functions in Multiple Sclerosis. Front Immunol 7: 382, 2016.
43. Walsh N, Gleeson MM, Shephard R, Woods J, Bishop N, Fleshner M, Green C, Pedersen B, Laurie H-G, Rogers C, Northoff H, Abbasi A, Simon P. Position statement. Part one: Immune function and exercise. Exerc Immunol Rev 17: 6–63, 2011.
44. Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, Shephard RJ, Oliver SJ, Bermon S, Kajeniene A. Position statement. Part two: Maintaining immune health. [Online]. Exerc Immunol Rev 17: 64–6103, 2011. http://www.ncbi.nlm.nih.gov/pubmed/21446353 [24 Nov. 2017].
45. Wills M, Akbar A, Beswick M, Bosch J, Caruso C, Giuseppina C-R, Dutta A, Franceschi C, Fulop T, Effrossyni G-K, Goronzy J, Griffiths S, Henson S, Dietmar H-B, Hill A, Kern F, Klenerman P, Macallan D, Macualay R, Maier A, Mason G, Melzer D, Morgan M, Moss P, Janko N-Z, Pachnio A, Riddell N, Roberts R, Sansoni P, Sauce D, Sinclair J, Solana R, Strindhall J, Trzonkowski P, van Lier R, Vescovini R, Wang G, Westendorp R, Pawelec G. Report from the second cytomegalovirus and immunosenescence. Immun Ageing 8, 2011.
46. Yuzefpolskiy Y, Baumann FM, Kalia V, Sarkar S. Early CD8 T-cell memory precursors and terminal effectors exhibit equipotent in vivo degranulation. Cell. Mol. Immunol. (July 28, 2014). doi: 10.1038/cmi.2014.48.

Aerobic exercise inhibits acute lung injury: from mouse to human evidence Exercise reduced lung injury markers in mouse and in cells

Nicole Cristine Rigonato-Oliveira[1], BreAnne MacKenzie[2], Andre Luis Lacerda Bachi[2], Manoel Carneiro Oliveira-Junior[2], Alana Santos-Dias[2], Maysa Alves Rodrigues Brandao-Rangel[2,3], Humberto Delle[1], Tamara Costa-Guimaraes[2,4], Nilsa Regina Damaceno-Rodrigues[5], Larissa Hilário Dulley[2], Marcela Anhesini Benetti[2], Christiane Malfitano[6], Kátia de Angelis[7], Regiane Albertini[3], Ana Paula Ligeiro Oliveira[1], Asghar Abbasi[8], Hinnak Northoff[9*], Rodolfo Paula Vieira[2,3,4*]

[1] Nove de Julho University, São Paulo - SP, Brazil.
[2] Brazilian Institute of Teaching and Research in Pulmonary and Exercise Immunology (IBEPIPE), São José dos Campos - SP, Brazil.
[3] Federal University of São Paulo (UNIFESP), Post-graduation Program in Sciences of Human Movement and Rehabilitation, Santos - SP, Brazil.
[4] Universidade Brasil, São Paulo - SP, Brazil.
[5] University of Sao Paulo, School of Medicine, Department of Pathology (LIM 59), Sao Paulo - SP, Brazil.
[6] Science Department of Health, Universidade Federal de Lavras (UFLA), Lavras - MG, Brazil.
[7] Federal University of São Paulo (UNIFESP), Campus São Paulo, São Paulo - SP, Brazil.
[8] Division of Respiratory & Critical Care Physiology & Medicine, Los Angeles Biomedical Research Institute at Harbor - UCLA Medical Center, California - CA, USA.
[9] Institute of Clinical and Experimental Transfusion Medicine (IKET), University of Tübingen, Tübingen, Germany.

[*] HN and RPV have equally contributed to this work.

ABSTRACT

Acute respiratory distress syndrome (ARDS) is defined as hypoxemic respiratory failure with intense pulmonary inflammation, involving hyperactivation of endothelial cells and neutrophils. Given the anti-inflammatory effects of aerobic exercise (AE), this study investigated whether AE performed daily for 5 weeks would inhibit extra-pulmonary LPS-induced ARDS. C57Bl/6 mice were distributed into Control, Exercise, LPS and Exercise+LPS groups. AE was performed on a treadmill for 5x/week for four weeks before LPS administration. 24hours after the final AE physical test, animals received 100ug of LPS intra-peritoneally. In addition, whole blood cell culture, neutrophils and human endothelial cells were pre-incubated with IL-10, an anti-inflammatory cytokine induced by exercise. AE reduced total protein levels ($p<0.01$) and neutrophil accumulation in bronchoalveolar lavage (BAL) ($p<0.01$) and lung parenchyma ($p<0.01$). AE reduced BAL inflammatory cytokines IL-1β, IL-6 and GM-CSF ($p<0.001$), CXCL1/KC, IL-17, TNF-alpha and IGF-1 ($p<0.01$). Systemically, AE reduced IL-1β, IL-6 and IFN-gamma ($p<0.001$), CXCL1/KC ($p<0.01$) and TNF-alpha ($p<0.05$). AE increased IL-10 levels in serum ($p<0.001$) and BAL ($p<0.001$). Furthermore, AE increased superoxide dismutase SOD ($p<0.01$) and decreased superoxide anion accumulation in the lungs ($p<0.01$). Lastly, pre-incubation with IL-10 significantly reduced LPS-induced activation of whole blood cells, neutrophils and HUVECs, as observed by reduced production of IL-1β, IL-6, IL-8 and TNF-alpha. Our data suggest that AE inhibited LPS-induced lung inflammation by attenuating inflammatory cytokines and oxidative stress markers in mice and human cell culture via enhanced IL-10 production.

Key words: exercise immunology, lung inflammation, immune response, acute lung injury, cytokines.

INTRODUCTION

Acute and chronic exercise alters both the innate and adaptive immune response (38, 39). Anti-inflammatory effects are observed in individuals who perform low to moderate intensity exercise, as well as endurance exercise with marginal effects on innate immune cell numbers. In contrast, acute high intensity and/or long duration exercise induce profound increases in the number of neutrophils and lymphocytes (13, 38). The innate immune response is the first line of defense against infectious pathogens, while adaptive immune response corresponds to a pathogen-specific immune response (5).

In addition to exercise's ability to modulate the systemic immune response in healthy subjects (38, 39), a growing number of studies have shown that exercise also positively modu-

Corresponding author:
Prof. Dr. Rodolfo de Paula Vieira, rodrelena@yahoo.com.br
Universidade Brasil, Instituto de Pesquisa, Rua Carolina Fonseca 285, Itaquera, São Paulo - SP, Brazil, 08230-030

lates the innate and adaptive pulmonary immune response in the context of respiratory diseases, such as asthma (33-37), chronic obstructive pulmonary diseases (COPD) (37), lung fibrosis (26, 27), as well as acute respiratory distress syndrome (ARDS) (8, 28, 30). In general, these studies have shown that low and moderate intensity exercise when performed regularly, results in NF-kB deactivation, anti-inflammatory cytokine release, and reduced oxidative stress.

ARDS is characterized by hypoxemic respiratory failure, intense bilateral pulmonary inflammation, accumulation of proteinaceous exudate in the alveoli, and disruption of alveolar membrane barrier; processes which are mediated by pro-inflammatory cytokines (29). The mortality rate increases progressively with age, ranging from 24 percent among patients 15 to 19 years of age to 60 percent among patients 85 years of age older (overall 41%) (31). Survivors need special attention to non-pulmonary comorbidities, such as skeletal muscle wasting and weakness, in which physical and exercise therapy could present beneficial effects, as recently demonstrated in an experimental study (10). At the time of this submission no correlation studies have been performed linking physical fitness to ARDS outcome.

Given the ability of chronic, moderate AE to suppress systemic and pulmonary inflammation (13) as well as oxidative stress (7), the aim of the present study was to investigate whether chronic, moderate intensity aerobic exercise (AE) prior to injury attenuates extra-pulmonary LPS-induced ARDS.

METHODS

Animals and experimental design

This study was approved by the research ethics committee at the Nove de Julho University (UNINOVE), protocol: AN0035.2013.

Forty male C57Bl/6 mice, 8 weeks of age,weighing between 20 and 25g were maintained in the animal center of the UNINOVE in standard conditions. Mice (n=10/group) were distributed into Control (Control), LPS (LPS), Exercise (Exe) and Exercise+LPS (Exe+LPS).

Treadmill aerobic exercise test and training

Exercise physical test and training was performed as previously described (34). Briefly, the animals from all groups were adapted to the treadmill training during three days (15 min, 25% grade, 0.3Km/h) before the first physical test. Then, maximum exercise capacity test was performed as follow; 5 minutes warm-up (25% grade, 0.3Km/h) followed by an increase in the treadmill speed (0.1Km/h each 2.5 min) until the animals' exhaustion, until they were not able to run even after 10 mechanical stimuli. Maximal exercise capacity (100%) was set as the maximum speed reached by each mouse. Mice were then trained using moderate intensity exercise (60% of average maximum speed) for 60 min/session, 5 days/week for 4 weeks. The maximum exercise capacity test was repeated for all animals after four weeks (34).

Extra-pulmonary model of LPS-induced ARDS

Twenty-four hours after the last maximum exercise capacity test, the animals were anesthetized (ketamine 100 mg/kg and xylazine 10 mg/kg) and received intra-peritoneal administration of 100 μg of Escherichia coli LPS (026:B6; L3755, Sigma Aldrich, St. Louis, MO, USA) suspended in phosphate buffered solution (PBS). Control and Exercise groups were submitted to intra-peritoneal injection of PBS. Treadmill training was performed for 4 weeks followed by the final physical test. Then, twenty-four hours after final physical test, animals received LPS administration, as stated above. Then, twenty-four hours later, animals were anesthetized and the following analysis were performed.

Blood collection and processing

One milliliter of blood was collected via cava vein and immediately centrifuged at 950g, 4ºC, during 7 min. The serum was collected and stored at -70ºC for cytokines measurement.

Bronchoalveolar lavage (BAL) fluid

To assess lung inflammation total and differential cells counts of BAL fluid was performed. BAL was obtained through a gentle washing of 3 x 0.5 ml PBS. The volume recovered was centrifuged at 900g, at 4ºC, for 7 min. The supernatant was stored at -70ºC for cytokines analysis and the cell pellet was resuspended in 1mL PBS. The total number of cells was counted using a hemocytometer and the differential cells count was performed through a cytospin preparation, stained with Diff Quick and 300 cells were counted according to the hematological criteria (28, 30).

Total proteins measurement in BAL

Total proteins in BAL fluid was quantified using the bicinchoninic acid (BCA) method to evaluate lung vascular permeability as previously described (30).

Cytokines measurements

The levels of IL-1β, IL-6, CXCL1/KC, IL-10, IL-17, TNF-α, GM-CSF, IFN-γ and IGF-1 were evaluated using ELISA kits from R&D Systems (Minneapolis, MN, USA) and BioLegend (San Diego, CA, USA) according to the manufacturer's instructions.

Histomorphometric study

To evaluate the effects of exercise on parenchymal inflammation, a hallmark of ARDS, lungs were collected, fixed in 10% formalin and submitted to histological routine. Briefly, 5μm thickness lung slices were stained with hematoxylin and eosin. 15 random fields of the lung parenchyma of each mouse were photographed. Image Pro Plus 4.0 software was used to measure the air and tissue area of all photomicrographs. Neutrophils were counted according to morphological criteria by an experienced researcher; groups were blinded. The number of neutrophils per square millimeter of lung tissue was presented (30, 35).

Oxidative stress and superoxide dismutase evaluation

The cranial lobe was washed with PBS and frozen at -70°C for analysis of oxidative stress. Approximately 50mg was used for the evaluation of superoxide dismutase (SOD) and QL (superoxide anion). After homogenization for 30 seconds in 1.15% phenyl methyl sulfonyl fluoride (PMSF) (100mmol/L) and in isopropanol 10uL/mL, the samples were centrifuged for 10min, 4ºC, at 950g and the supernatant was

frozen at -80°C. Protein content was measured by the Lowry's' method (21).

Chemiluminescence (QL): Lipid peroxidation was assessed by chemiluminescence. The chemiluminescence assay was performed with a liquid scintillation LKB Rack Beta 1215 spectrophotometer (LKB Producer AB, USA) in order to room temperature (25°C to 27°C) to out-of-coincidence. Supernatants were diluted in 140 mM KCl and sodium phosphate buffer 20 mM pH 7.4 and added to glass tubes, which were placed in scintillation vials; 3 mM tert-butyl hydroperoxide was added, and chemiluminescence was determined at the emission maximum (14, 17).

Superoxide Dismutase (SOD): SOD activity was measured by spectrophotometry in lung homogenates by inhibition of pyrogallol autoxidation rate at 420 nm. The enzymatic activity was reported as U/mg protein. The technique used was based on inhibition of superoxide radical reaction with piragalol. Since it is unable to determine the concentration of the enzyme or its activity in terms of substrate consumed per unit time, quantification was expressed in relative units. One unit of SOD is defined as the amount of enzyme that inhibits the reaction by 50% of the oxidation rate detector. The oxidation of pyrogallol leads to the formation of a colored product, which was detected at 420nm for 2min. The SOD activity was determined by measuring the rate of formation of the oxidized pyrogallol. In the reaction medium, 20uL of lung homogenate was used, 973uL Tris-Phosphate buffer 50 mmol/L (pH 8.2), 8uL pyrogallol (to 24 mmol/L) and 4uL of CAT (30umol/L). This curve obtained was used as a blank. A standard curve was also performed using three different concentrations of SOD (0,25U, and 0.5U 1U), and values were calculated based on the slope-intercept of the standard curve.

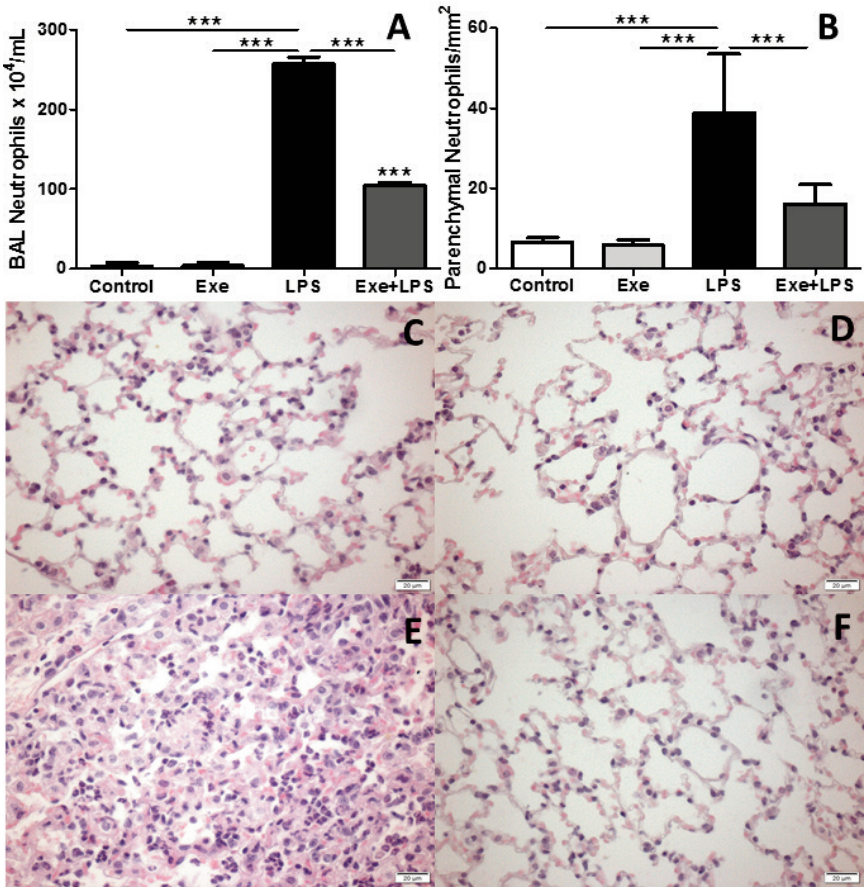


Figure 1. Neutrophils count in BAL and parenchyma. Results expressed as the number of neutrophils x 10^5 in BAL per mL (Figure 1A) and as number of neutrophils per mm2 of lung parenchyma (Figure 1B). *** p<0.001. Figures 1C-F are representative photomicrographs of lung parenchyma stained with hematoxylin and eosin, respectively from Control (Figure 1C), Exercise (Figure 1D), LPS (Figure 1E) and Exercise+LPS (Figure 1F) groups. *** p<0.001.

Human Cell Cultures Studies

For all *in vitro* studies, IL-10 was used in order to mimic the effects observed in the *in vivo* exercise protocol, which was performed prior to LPS inoculation and resulted in a strong release of anti-inflammatory cytokine IL-10. In summary, we cell cultures (whole blood cells, neutrophils and endothelial cells) were pre-incubated with human recombinant IL-10 (10 ng/mL) for one hour and followed by stimulation with lipopolysaccharide (LPS) (Escherichia coli 026:B6; L3755, Sigma Aldrich, St. Louis, MO, USA; final concentration 10 ng/mL) for 4 hours, at 37ºC, 5% CO2 at humid atmosphere. Therefore, we had four final conditions in our cell culture performed in triplicate: Control (medium stimulated only), IL-10 (stimulated with IL-10), LPS (only LPS-stimulated) and IL-10+LPS (incubated with IL-10 for 1 hour + LPS stimulation and recovery after 4 hours). After 4 hours of LPS stimulation, the cells were centrifuged for 7 minutes, at 4ºC. IL-1β, IL-6, IL-8 and TNF-alpha; indicators of cellular activation were measured in both the serum and supernatant.

Whole blood cell culture

Blood samples were drawn from each mouse and were cultured using whole blood culture system as described previously (1). Briefly, venous blood samples were drawn from the antecubital vein in a sitting position and collected into endotoxin-free K3-EDTA tubes (Vacuette, Greiner bio-one-Frickenhausen, Germany). 3 × 4-ml whole blood were stimulated with or without IL-10 and lipopolysaccharide (LPS) in 6 well plate in humid atmosphere as described above.

Human umbilical vein endothelial cells (HUVEC) culture

HUVEC (3 x 10^4 cells/300µL medium) were cultivated in vascular cell basal media (ATCC PCS100030) supplemented with endothelial cell growth kit (ATCC PCS100040) components. The stimulation protocol was applied as described above.

Human neutrophils

Five milliliter of venous blood were collected and the neutrophils were isolated by using the EasySep™ Human Neutrophil Enrichment Kit (Stemcell, Cod 19257, Cambridge, MA, USA), according to the manufacturer's instructions. 5 x 10^4/mL neutrophils per well per mL were seeded in 24 well plate and stimulated as described above.

Statistical analysis

Graph pad prism 5.0 software was used to perform two-way ANOVA, followed by Student–Newman–Keuls post hoc test. Differences were considered significant for $p< 0.05$.

RESULTS

Aerobic exercise inhibits pulmonary inflammation

During lung injury, cytokines hone neutrophils to the lung to clear and kill invading pathogens. Neutrophil influx was measured in both the bronchial alveolar lavage fluid (BAL) and lung parenchyma. AE reduced neutrophil influx in the BAL of Exercise+LPS mice (Figure 1A). Likewise, the number of neutrophils was reduced in the lung parenchyma (Figure 1B). Representative photomicrographs of lung parenchyma stained with hematoxylin and eosin (H+E) reveal reduced neutrophil number in the alveolar spaces and in the parenchyma of Exercise+LPS animals (Figure 1F) compared to LPS alone (Figure 1E). Neutrophil numbers were low in sedentary (Figure 1C) and exercised animals (Figure 1D).

Aerobic exercise modulates pulmonary cytokine and growth factor release

A pro-inflammatory cytokine "storm" is implicated in death due to ARDS. Therefore, pro and anti-inflammatory cytokine levels were assessed in the BALF using ELISA technique. AE reduced BAL levels of inflammatory cytokines including interleukin (IL) IL-1β (Figure 2A), IL-6 (Figure 2B), chemokine (C-X-C motif) ligand 1 (CXCL1)/KC (Figure 2C), IL-17 (Figure 2E), tumor necrosis factor (TNF)-alpha (Figure 2F), Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Figure 2G), and IGF-1 ($p<0.01$) (Figure 2I). In contrast, anti-inflammatory cytokine IL-10 was increased in both exercise only and Exe+LPS mice (Figure 2D). Interferon-gamma was not significantly regulated in this model (Figure 2H). The results are expressed as picograms of cytokines per mL of BAL. *** $p<0.001$; ** $p<0.01$; * $p<0.05$. In summary, pro-inflammatory cytokines were reduced in exercised mice and anti-inflammatory cytokine IL-10 was increased.

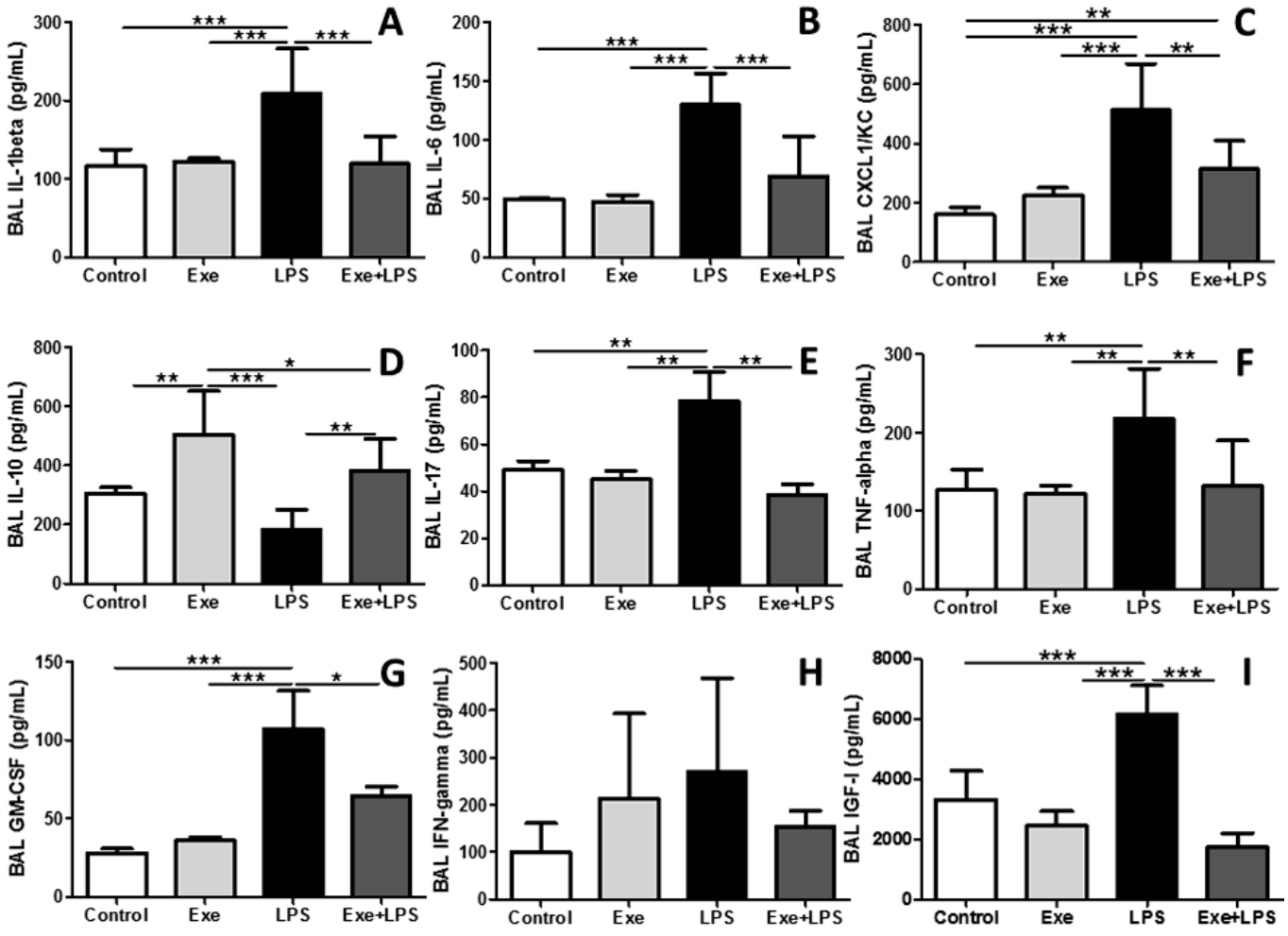


Figure 2. Pro and anti-inflammatory cytokines in BAL. The results are expressed as picograms of cytokines per mL of BAL. *** $p<0.001$; ** $p<0.01$; * $p<0.05$.

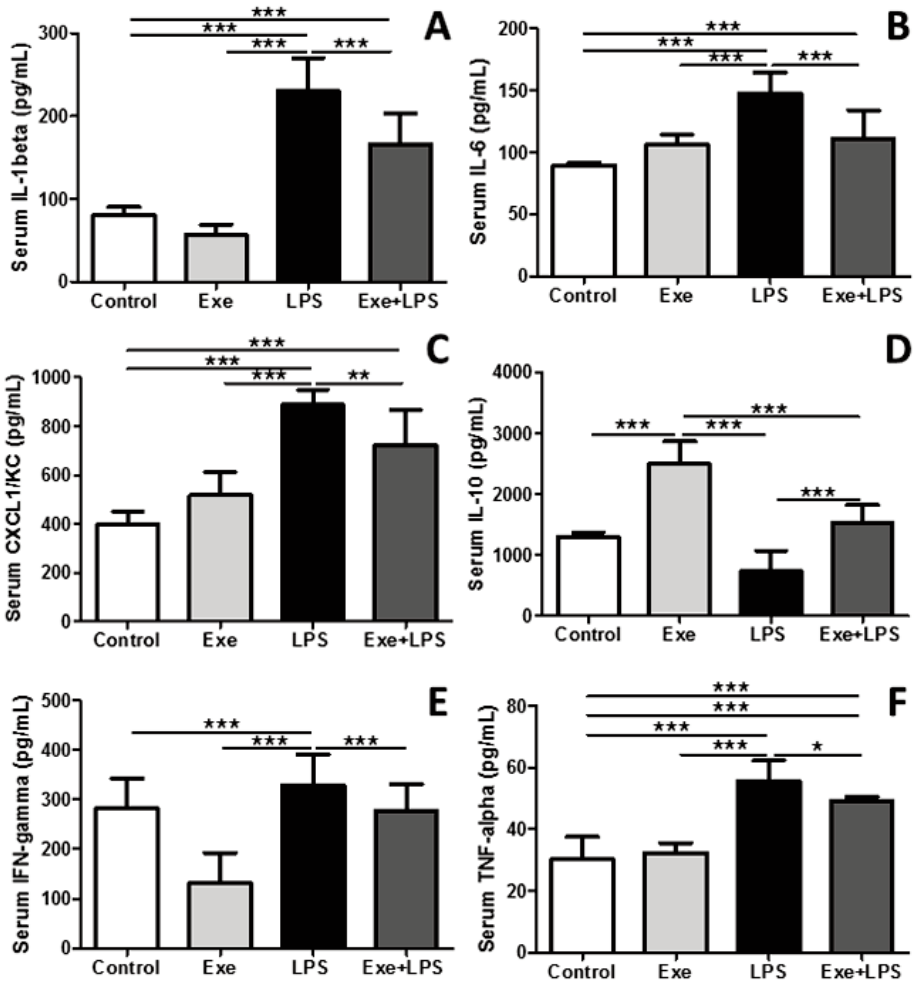


Figure 3. Pro and anti-inflammatory cytokines in serum. The results are expressed as picograms of cytokines per mL or serum. *** p<0.001; ** p<0.01; * p<0.05.

Aerobic exercise modulates systemic cytokine release

Serum cytokine levels can also indicate extent of severity of ARDS. Therefore, serum was collected and cytokines were measured by ELISA (Figure 3). IL-β (Figure 3A), IL-6 (Figure 3B), CXCL1 (Figure 3C), and TNF-alpha (Figure 3F) were reduced in Exercise+LPS animals. Anti-inflammatory cytokine IL-10 was increased in serum of exercised animals (Figure 3D). IFN-gamma was slightly reduced in Exe+LPS mice versus LPS mice (Figure 3E). In summary, exercising before injury resulted in reduced levels of pro-inflammatory cytokines in the serum.

Aerobic exercise inhibits oxidative stress and increases antioxidant enzyme (SOD)

Increased protein levels in BAL fluid indicates alveolar damage and leakage. Animals that performed AE before LPS injury, had less total protein in BAL fluid (Figure 4A). The reactive oxygen species, superoxide anion, expressed as anion units per mg of lung protein was also reduced in BAL fluid of Exe+LPS mice (Figure 4B). Exercise increased the antioxidant superoxide dismutase (SOD) (Figure 4C). In summary, AE decreased oxidative stress and alveolar damage.

IL-10 pre-incubation inhibits whole blood cells, endothelial cells and neutrophils activation

To mimic the observed exercised-induced production of IL-10 post-exercise in vivo, human whole blood cells, endothelial cells and neutrophils were incubated with IL-10 prior to LPS administration. Pre-incubation with IL-10 resulted in reduced production of IL-1β ($p<0.001$), IL-6 ($p<0.001$), IL-8 ($p<0.001$) and TNF-α ($p<0.001$) by human whole blood cell culture (Figure 5A to D, respectively). Similarly, pre-incubation with IL-10 also reduced the production of IL-1β ($p<0.001$), IL-6 ($p<0.01$), IL-8 ($p<0.001$) and TNF-α ($p<0.001$) by human endothelial cells (Figure 5 E through H, respectively). In addition, pre-incubation with IL-10 also inhibited the production of IL-1β ($p<0.001$), IL-6 ($p<0.01$), IL-8 ($p<0.001$) and TNF-α ($p<0.01$) by human neutrophils (Figure 5 I through M, respectively). Taken together, increased IL-10 significantly inhibited the activation of human structural and inflammatory cells that play a pivotal role in the pathophysiology of ARDS.

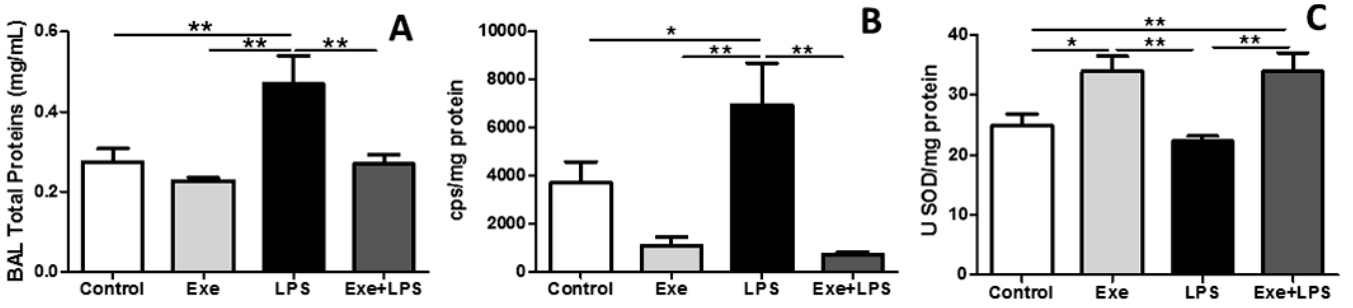


Figure 4. Total proteins and reactive oxygen specie and antioxidant enzyme in the lungs. Total protein (Figure 4A). Reactive oxygen species (ROS), mainly superoxide anion, expressed as anion units per mg of lung protein (Figure 4B). Total superoxide dismutase (SOD) per mg of lung protein (Figure 4C). ** p<0.01 and * p<0.05.

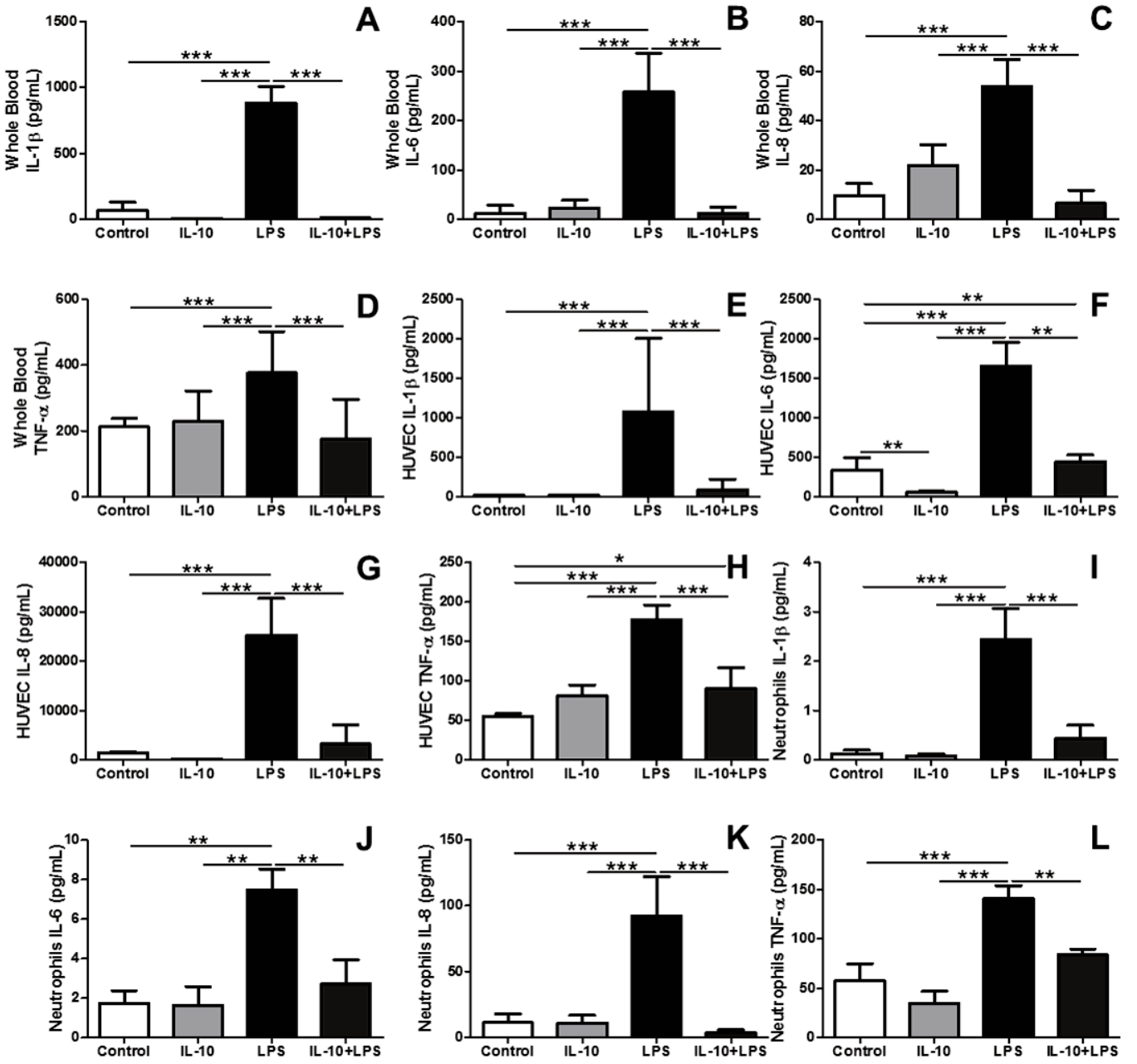


Figure 5. Pro-inflammatory cytokines produced by human whole blood cells (Figure 5 A, B, C and D), HUVEC cells (Figure 5 E, F, G and H) and neutrophils (Figure 5 I, J, K and L). The results are expressed as picograms of cytokines per mL of BAL. *** p<0.001; ** p<0.01; * p<0.05.

DISCUSSION

This study demonstrated for the first time that chronic, moderate intensity aerobic exercise (AE) may have prophylactic effects against ARDS from extra pulmonary origin, a disease that often occurs unexpectedly and for which there is no cure. While pro-inflammatory cytokines were reduced, as well as oxidative stress levels, anti-inflammatory cytokine IL-10, was increased in LPS+Exe mice compared to LPS (sedentary) animals providing additional evidence that chronic, moderate, AE is an effective, cost-efficient, preventative, medicine against pulmonary disease.

In contrast to sedentary mice receiving LPS, Exe+LPS mice showed decreased neutrophil accumulation both in BAL fluid and in the parenchyma as well as less damage to the alveolar epithelium as measured by protein content in the BAL. Reduced injury in Exe+LPS mice was likely due to the reduction of neutrophils and neutrophil chemoattractants including reduced: IL-1β, IL-6, (CXCL1)/KC, IL-17, TNF-α, GM-CSF, and IGF-1.

While exercise alone does not regulate CXCL1/KC, increased CXCL1/KC in BAL is related to endothelial damage and contributes to leukocyte recruitment (22). Thus decreased CXCL1/KC in Exe+LPS animals compared to LPS animals was likely due to decreased neutrophil damage in the endothelial compartment. Likewise, increased IGF-1 is associated with ARDS and has been demonstrated to activate fibroblasts (repair) and permit epithelial cell death (32). Like

CXCL1/KC, IGF-1 was not regulated by exercise alone, however, levels were reduced in Exe+LPS animals indicating decreased injury compared to LPS animals.

Recent studies have implicated that IL-17 may play an important role in ARDS. In addition to forming a positive feedback loop involving IL-1β and IL-6 (24), IL-17 promotes IL-6, IL-8, TNF-α, IL-1β, G-CSF, and monocyte chemo-attractant protein (MCP)-1 (11, 23). IL-17 is increased in ARDS patient BAL fluid and has been shown to increase permeability across alveolar epithelial monolayers (19). Furthermore, IL-17 knockout mice exposed to acute lung injury showed reduced inflammation (20). While exercise alone did not regulate IL-17, IL-1β, or IL-6, these cytokines were reduced in Exe+LPS groups compared to LPS alone, confirming the old notion that exercise can suppress endotoxin-stimulated inflammatory cytokines in blood culture (40). However, exercise alone increased IL-10 which has been demonstrated to repress the release of cytokines by macrophages and lymphocytes, including IL-17 (15). In addition, although it was not evaluated in the present study, exercise-induced IL-10 release have been attributed to increased mobilization of T-regulatory cells (21) and also by IL-10 derived from bronchial epithelial cells (38). Furthermore, low concentrations of IL-10 are associated with poor prognosis for ARDS. (3, 9).

GM-CSF has been shown to prime the neutrophilic response to LPS by up-regulating toll-like receptors (TLR2 and TLR4), the main receptors for LPS. In addition, the over-production of GM-CSF in ARDS contributes to pulmonary destruction by neutrophils, as GM-CSF is also a neutrophil chemo-attractant. In horses that underwent moderate, chronic AE, TLR expression was reduced in pulmonary alveolar macrophages compared to unconditioned animals (10). In this model, moderate AE blunted GM-CSF production, which likely repressed LPS-induced TLR expression and contributed to reduced inflammation in the Exe+LPS group. While GM-CSF was not significantly regulated by exercise alone, increases in the anti-inflammatory cytokine IL-10 (observed in both the Exe and Exe+LPS groups) may have had a repressive effect on GM-CSF and other cytokine production.

Recently, SNPs in genes involved in oxidative stress have been identified in ARDS patients and may contribute to patient prognosis (2). Levels of oxidative stress and anti-oxidant activity were maintained at "reduced" exercise-only levels (compared to uninjured mice) in Exe+LPS mice. Importantly, these results indicated that at least for rodents, chronic, moderate AE significantly represses oxidative stress levels and enhances anti-oxidant activity after 24 hours of basal, sedentary activity. Further studies exploring the length of "sedentary" time for which AE modulates both oxidative stress and cytokine activity would help to further define a general, prophylactic exercise program against a variety of pulmonary diseases.

While chronic, moderate AE in rodents has been repeatedly demonstrated to improve outcomes in a variety of lung disease models, in order to assess the extent by which physical fitness at the time of injury effects ARDS prognosis, longitudinal studies correlating with previous physical fitness, not just body mass index, should be performed.

REFERENCES

1. Abbasi A, Hauth M, Walter M, Hudemann J, Wank V, Niess AM, Northoff H. Exhaustive exercise modifies different gene expression profiles and pathways in LPS-stimulated and un-stimulated whole blood cultures. Brain Behav Immun: 39:130–41, 2014.
2. Acosta-Herrera M, Pino-Yanes M, Blanco J, Ballesteros JC, Ambrós A, Corrales A, Gandía F, Subirá C, Domínguez D, Baluja A, Añón JM, Adalia R, Pérez-Méndez L, Flores C, Villar J Common variants of NFE2L2 gene predisposes to acute respiratory distress syndrome in patients with severe sepsis. Crit Care: 19:256, 2015.
3. Armstrong L, Millar AB. Relative production of tumour necrosis factor alpha and interleukin 10 in adult respiratory distress syndrome. Thorax 1997; 52:442–6, 1997.
4. Brüggemann TR, Ávila LC, Fortkamp B, Greiffo FR, Bobinski F, Mazzardo-Martins L, Martins DF, Duarte MM, Dafre A, Santos AR, Silva MD, Souza LF, Vieira RP, Hizume-Kunzler DC. Effects of Swimming on the Inflammatory and Redox Response in a Model of Allergic Asthma. Int J Sports Med, 2015.
5. Cayrol C, Girard J-P. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. Curr Opin Immunol: 31:31–7, 2014.
6. Dal-Pizzol F, Ritter C. Functional disability 5 years after ARDS. N Engl J Med: 365:274-5-6, 2011.
7. Debevec T, Pialoux V, Mekjavic IB, Eikein O, Mury P, Millet GP. Moderate exercise blunts oxidative stress induced by normobaric hypoxic confinement. Med Sci Sports Exerc:46:33–41, 2014.
8. de Araújo CC, Silva JD, Samary CS, Guimarães IH, Marques PS, Oliveira GP, do Carmo LG, Goldenberg RC, Bakker-Abreu I, Diaz BL, Rocha NN, Capelozzi VL, Pelosi P, Rocco PR. Regular and moderate exercise before experimental sepsis reduces the risk of lung and distal organ injury. J Appl Physiol: 112:1206–14, 2012.
9. Donnelly SC, Strieter RM, Reid PT, Kunkel SL, Burdick MD, Armstrong I, Mackenzie A, Haslett C. The association between mortality rates and decreased concentrations of interleukin-10 and interleukin-1 receptor antagonist in the lung fluids of patients with the adult respiratory distress syndrome. Ann Intern Med: 125:191–6, 1996.
10. Files DC, Liu C, Pereyra A, Wang ZM, Aggarwal NR, D'Alessio FR, Garibaldi BT, Mock JR, Singer BD, Feng X, Yammani RR, Zhang T, Lee AL, Philpott S, Lussier S, Purcell L, Chou J, Seeds M, King LS, Morris PE, Delbono O. Therapeutic exercise attenuates neutrophilic lung injury and skeletal muscle wasting. Sci Transl Med. 2015 Mar 11;7(278):278ra32.
11. Frellstedt L, Waldschmidt I, Gosset P, Desmet C, Pirottin D, Bureau F, Farnir F, Franck T, Dupuis-Tricaud MC, Lekeux P, Art T. Training modifies innate immune responses in blood monocytes and in pulmonary alveolar macrophages. Am J Respir Cell Mol Biol: 51:135–42, 2014.
12. Gaffen SL. Recent advances in the IL-17 cytokine family. Curr Opin Immunol: 23:613–9, 2011.
13. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol:11:607–15, 2011.

14. Gleeson M, Walsh NP. The BASES expert statement on exercise, immunity, and infection. J Sports Sci: 30:321–4, 2012.
15. Gonzalez-Rothi RJ, Straub L, Cacace JL, Schreier H. Liposomes and pulmonary alveolar macrophages: functional and morphologic interactions. Exp Lung Res: 17:687–705, 1991.
16. Gu Y, Yang J, Ouyang X, Liu W, Li H, Yang J, Bromberg J, Chen SH, Mayer L, Unkeless JC, Xiong H. Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. Eur J Immunol: 38:1807–13, 2008.
17. Herridge MS, Tansey CM, Matté A, Tomlinson G, Diaz-Granados N, Cooper A, Guest CB, Mazer CD, Mehta S, Stewart TE, Kudlow P, Cook D, Slutsky AS, Cheung AM; Canadian Critical Care Trials Group. Functional disability 5 years after acute respiratory distress syndrome. N Engl J Med: 364:1293–304, 2011.
18. Irigoyen M-C, Paulini J, Flores LJF, Flues K, Bertagnolli M, Moreira ED, Consolim-Colombo F, Belló-Klein A, De Angelis K. Exercise training improves baroreflex sensitivity associated with oxidative stress reduction in ovariectomized rats. Hypertension: 46:998–1003, 2005.
19. Li JT, Melton AC, Su G, Hamm DE, LaFemina M, Howard J, Fang X, Bhat S, Huynh KM, O'Kane CM, Ingram RJ, Muir RR, McAuley DF, Matthay MA, Sheppard D. Unexpected Role for Adaptive αβTh17 Cells in Acute Respiratory Distress Syndrome. J Immunol: 195:87–95, 2015.
20. Li Q, Gu Y, Tu Q, Wang K, Gu X, Ren T. Blockade of Interlukin-17 restrains the development of acute lung injury. Scand J Immunol 2015.
21. Lowder T, Dugger K, Deshane J, Estell K, Schwiebert LM. Repeated bouts of aerobic exercise enhance regulatory T cell responses in a murine asthma model. Brain Behav Immun. 2010 Jan;24(1):153-9.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem: 193:265–75, 1951.
23. Maniatis NA, Orfanos SE. The endothelium in acute lung injury/acute respiratory distress syndrome. Curr Opin Crit Care: 14:22–30, 2008.
24. Memtsoudis SG, Bombardieri AM, Ma Y, Walz JM, Chiu YL, Mazumdar M. Mortality of patients with respiratory insufficiency and adult respiratory distress syndrome after surgery: the obesity paradox. J Intensive Care Med: 27:306–11, 2012.
25. Moon H-G, Kang CS, Choi J-P, Choi DS, Choi HI, Choi YW, Jeon SG, Yoo JY, Jang MH, Gho YS, Kim YK. Acetyl salicylic acid inhibits Th17 airway inflammation via blockade of IL-6 and IL-17 positive feedback. Exp Mol Med: 45:e6, 2013.
26. Orme J, Romney JS, Hopkins RO, Pope D, Chan KJ, Thomsen G, Crapo RO, Weaver LK. Pulmonary function and health-related quality of life in survivors of acute respiratory distress syndrome. Am J Respir Crit Care Med: 167:690–4, 2003.
27. Pereira PR, Oliveira-Junior MC, MacKenzie B, Chiovatto JE, Matos Y, Greiffo FR, Rigonato-Oliveira NC, Brugemman TR, Delle H, Idzko M, Albertini R, Ligeiro Oliveira AP, Damaceno-Rodrigues NR, Caldini EG, Fernandez IE, Castro-Faria-Neto HC, Dolhnikoff M, Eickelberg O, Vieira RP. Exercise Reduces Lung Fibrosis Involving Serotonin/Akt Signaling. Med Sci Sports Exerc 2016.
28. Prata LO, Oliveira FMS, Ribeiro TMS, Almeida PW, Cardoso JA, Rodrigues-Machado Mda G, Caliari MV. Exercise attenuates pulmonary injury in mice with bleomycin-induced pulmonary fibrosis. Exp Biol Med (Maywood): 237:873–83, 2012.
29. Ramos DS, Olivo CR, Quirino Santos Lopes FDT, Toledo AC, Martins MA, Lazo Osório RA, Dolhnikoff M, Ribeiro W, Vieira RP. Low-intensity swimming training partially inhibits lipopolysaccharide-induced acute lung injury. Med Sci Sports Exerc:42:113–9, 2010.
30. ARDS Definition Task Force, Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS Acute respiratory distress syndrome: the Berlin Definition. JAMA: 307:2526–33, 2012.
31. Reis Gonçalves CT, Reis Gonçalves CG, de Almeida FM, Lopes FD, dos Santos Durão AC, dos Santos FA, da Silva LF, Marcourakis T, Castro-Faria-Neto HC, Vieira RP, Dolhnikoff M. Protective effects of aerobic exercise on acute lung injury induced by LPS in mice. Crit Care: 16:R199, 2012.
32. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD Incidence and outcomes of acute lung injury. N Engl J Med: 353:1685–93, 2005.
33. Schnapp LM, Donohoe S, Chen J, Sunde DA, Kelly PM, Ruzinski J, Martin T, Goodlett DR. Mining the acute respiratory distress syndrome proteome: identification of the insulin-like growth factor (IGF)/IGF-binding protein-3 pathway in acute lung injury. Am J Pathol: 169:86–95, 2006.
34. Silva RA, Vieira RP, Duarte ACS, Lopes FD, Perini A, Mauad T, Martins MA, Carvalho CR. Aerobic training reverses airway inflammation and remodelling in an asthma murine model. Eur Respir J : 35:994–1002, 2010.
35. Vieira RP, Claudino RC, Duarte ACS, Santos AB, Perini A, Faria Neto HC, Mauad T, Martins MA, Dolhnikoff M, Carvalho CR. Aerobic exercise decreases chronic allergic lung inflammation and airway remodeling in mice. Am J Respir Crit Care Med: 176:871–7, 2007.
36. Vieira RP, de Andrade VF, Duarte ACS, Dos Santos AB, Mauad T, Martins MA, Dolhnikoff M, Carvalho CR. Aerobic conditioning and allergic pulmonary inflammation in mice. II. Effects on lung vascular and parenchymal inflammation and remodeling. Am J Physiol Lung Cell Mol Physiol: 295:L670-9, 2008.
37. Vieira RP, Duarte AC, Santos ABG, Medeiros MC, Mauad T, Martins MA, Carvalho CR, Dolhnikoff M. Exercise reduces effects of creatine on lung. Int J Sports Med: 30:684–90, 2009.
38. Vieira RP, Toledo AC, Ferreira SC, Santos AB, Medeiros MC, Hage M, Mauad T, Martins Mde A, Dolhnikoff M, Carvalho CR. Airway epithelium mediates the anti-inflammatory effects of exercise on asthma. Respir Physiol Neurobiol. 2011 Mar 15;175(3):383-9.
39. Vieira R de P, Toledo AC, Silva LB, Almeida FM, Damaceno-Rodrigues NR, Caldini EG, Santos AB, Rivero DH, Hizume DC, Lopes FD, Olivo CR, Castro-Faria-Neto HC, Martins MA, Saldiva PH, Dolhnikoff M. Anti-inflammatory effects of aerobic exercise in mice exposed to air pollution. Med Sci Sports Exerc: 44:1227–34, 2012.

40. 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, Simon P Position statement. Part one: Immune function and exercise. Exerc Immunol Rev: 17:6–63, 2011.
41. Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, Shephard RJ, Oliver SJ, Bermon S, Kajeniene A. Position statement. Part two: Maintaining immune health. Exerc Immunol Rev: 17:64–103, 2011.
42. Weinstock C, König D, Harnischmacher R, Keul J, Berg A, Northoff H. Effect of exhaustive exercise stress on the cytokine response. Med Sci Sports Exerc: 29:345–54, 1997.

Acknowledgements: Sao Paulo Research Foundation (FAPESP) grant 2012/15165-2. Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) grant 311335-2015-2. Comissão de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES) grants 12804/13-4 and 1303/13-9. NCRO holds a MSc fellowship from FAPESP 2013/24076-6. BM holds a postdoctoral fellowship from FAPESP 2014/23196-0. MCOJ holds a PhD fellowship from FAPESP 2012/14604-8. ASAS holds a MSc fellowship from CAPES. LHD holds a scientific initiation fellowship from FAPESP 2012/25435-7. MAB holds a scientific initiation fellowship from FAPESP 2012/24880-7.

Microparticles and Exercise in Clinical Populations

Patrick J Highton[1,2], Naomi Martin[2,3], Alice C Smith[1,2], James O Burton[2], Nicolette C Bishop[1,2].

[1] School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, United Kingdom
[2] Leicester Kidney Exercise Team, John Walls Renal Unit, University Hospitals of Leicester and Department of Infection, Immunity and Inflammation, University of Leicester, Leicestershire, United Kingdom
[3] School of Allied Health Sciences, Faculty of Health and Life Sciences, De Montfort University, Leicester, United Kingdom

ABSTRACT

Microparticles (MPs) are shed membrane vesicles released from a variety of cell types in response to cellular activation or apoptosis. They are elevated in a wide variety of disease states and have been previously measured to assess both disease activity and severity. However, recent research suggests that they also possess bioeffector functions, including but not limited to promoting coagulation and thrombosis, inducing endothelial dysfunction, increasing pro-inflammatory cytokine release and driving angiogenesis, thereby increasing cardiovascular risk. Current evidence suggests that exercise may reduce both the number and pathophysiological potential of circulating MPs, making them an attractive therapeutic target. However, the existing body of literature is largely comprised of in vitro or animal studies and thus drawing meaningful conclusions with regards to health and disease remains difficult. In this review, we highlight the role of microparticles in disease, comment on the use of exercise and dietary manipulation as a therapeutic strategy, and suggest future research directions that would serve to address some of the limitations present in the research to date.

Keywords: Exercise, microparticles, immunology,

INTRODUCTION

Microparticles (MPs) are shed membrane vesicles, usually ranging in size from 0.1 to 1 μm. They are distinct from exosomes, which tend to be smaller (<0.1 μm) and have a different method of formation (1) – this review will focus solely on MPs, their pathophysiology within clinical populations and the potential of exercise as a therapeutic strategy.

Causes of formation

MPs are released from the cell membrane during apoptosis or activation, elicited by a variety of stimuli. For example, the activation stimuli could be inflammation, oxidative stress or mechanical/haemodynamic fluctuations depending on the parent cell in question. After their formation, MPs express surface proteins and antigens that are suggestive of their cellular origin, through which they can be identified by laboratory techniques (the most common MP cellular sources and their corresponding surface antigens are listed in Table 1). The MP membrane might also include negatively charged phospholipids, the majority of which are phosphatidylserine (PS) which is exposed on the outer layer (2). For a list of possible detection methods and a comparison of their minimum detectable vesicle sizes, see Van Der Pol et al (3).

Table 1. The most common cellular sources of MPs, along with the corresponding antigens exposed on their outer surface. As many cellular sources can be represented by several cell surface markers, differences may occur in the literature when studies have used different markers for the same MP derivation, which could lead to inaccuracies. CD = Cluster of Differentiation, PECAM = platelet-endothelial cell adhesion molecule, ICAM = intercellular adhesion molecule. *The value of measuring PS (by assessing the degree of ligation with its detector reagent Annexin-V) to quantify 'all MPs' has been questioned; as many as 80% of MPs do not bind with Annexin-V *in vitro* and therefore do not express PS on their outer surface (4). PS-negative MPs which do not bind with Annexin-V demonstrate reduced procoagulant activity compared to their PS-positive counterparts (4) however their functional significance remains unclear and warrants further investigation.

MP Cellular Source	Surface antigen/s used for determination
All cells	Phosphatidylserine*
Leukocyte	CD11a, CD45
Granulocyte	CD11b, CDF66
Platelet	CD31 (PECAM-1), CD40L, CD41/a, CD42b, CD61
Monocyte	CD11b, CD14, CD16
Endothelial cell	CD31, CD51, CD54 (ICAM-1), CD62E (E-Selectin), CD62P (P-Selectin), CD105 (endoglin), CD144 (VE-Cadherin), CD146 (S-Endo 1)
Neutrophil	CD66b
Erythrocyte	CD235a
Lymphocyte	CD3, CD4, CD36

Mechanisms of formation

A resting, inactivated phospholipid membrane will display phospholipid asymmetry, i.e. different phospholipids displayed on the outer and inner layer (PS is displayed on the inner layer in a healthy membrane(5)). This asymmetry is maintained by the enzymes gelsolin, aminophospholipid

Corresponding author:
Nicolette C Bishop, School of Sport, Exercise and Health Sciences Loughborough University, LE11 3TU, N.C.Bishop@lboro.ac.uk

translocase, floppase, scramblase and calpain (for a full review of these enzymes' kinetics and how they pertain to MP formation, see Piccin, Murphy, & Smith, 2007)(6). Cellular activation or apoptosis causes the endoplasmic reticulum to release calcium into the cytosol, which alters the actions of these enzymes, resulting in a restructuring of the cytoskeleton and a reversal of the phospholipid asymmetry and therefore externalisation of PS. This causes outward 'blebbing' of the cell membrane and ultimately fissure, resulting in a released vesicle that might express both PS and surface proteins related to its parental cell on its outer membrane. This process is displayed in Figure 1.

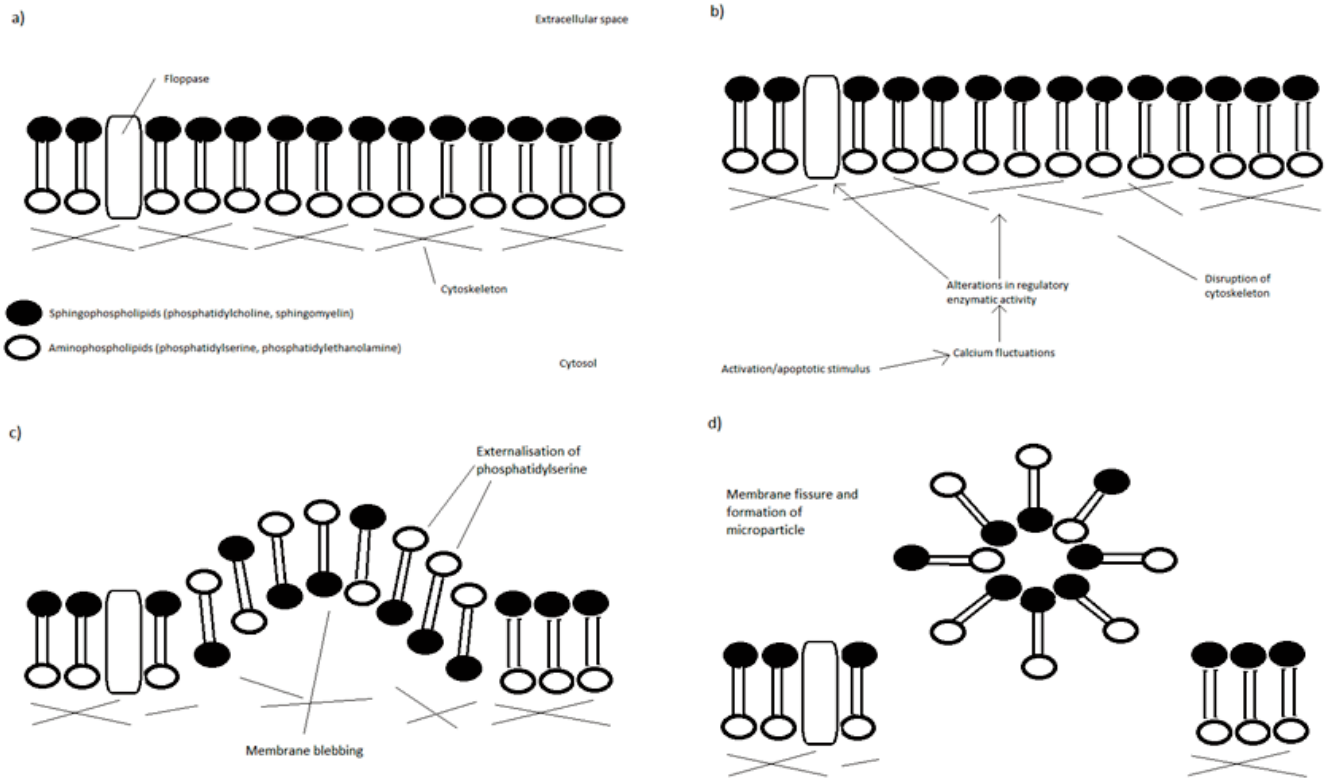


Figure 1. The steps involved in the process of MP formation. a) Demonstrating a healthy membrane, with phospholipid asymmetry and the presence of the regulatory enzyme floppase (interchangeable in this diagram with the other regulatory enzymes mentioned). b) Activation or apoptotic stimuli causes fluctuations of cytosolic calcium, altering the activity of the regulatory enzymes and causing cytoskeletal disruption. c) Membrane blebbing, and loss of phospholipid asymmetry resulting in externalisation of phosphatidylserine. d) Fissure of the membrane, resulting in the formation of an MP which is now a distinct vesicle from its original membrane. This MP will express surface antigens representative of its parent cell, which can be assessed to identify the origin of the MP. The MP size and number of phospholipids present in the membrane in Figure d) is not truly representative; the purpose of this diagram is to illustrate the formation process. In reality, the MP is of far greater size relative to the phospholipids, which are also present in far greater abundance in the MP membrane.

Less is known about how MPs are acutely cleared from the circulation. Firstly, clearance must exceed production, meaning that the activation/apoptotic stimulus must either be removed or decreased to a large enough extent to allow the return of cell quiescence, thus reducing MP formation. Beyond reduced production, proposed mechanisms for the removal of MPs from the circulation include direct binding of phagocytes to either PS or opsonisation proteins (e.g. complement) on the MP surface (7), IgM-mediated phagocytosis by macrophages (8), and destruction by circulating phospholipases (9). Particle size may influence the method employed to clear MPs (8), however this requires further investigation. Conversely, MPs may also adhere to the endothelium or form thrombi due to their reported expression of adhesion molecules (e.g. P-Selectin) (10) or Tissue Factor (TF) (11), respectively, meaning they are not removed from the circulation but are not detectable using standard techniques, creating the illusion of their absence. Further investigation is necessary to elucidate how MPs are acutely removed from the circulation.

Sources

MPs can be derived from many different cellular sources as shown in Table 1, including leukocytes, platelets, erythrocytes and endothelial cells (12). The stimuli for the release of MPs from these cells differ depending on the cell, as various conditions will initiate activation and/or apoptosis of each cell type. Whilst MPs are present in healthy populations (13), the primary aim of this review is to explain the pathophysiological role of MPs in clinical populations and the potential impact of exercise.

Methods of Detection

There are several different laboratory techniques that are regularly used for the identification of circulating MPs in the literature. Broadly, these include: flow cytometry; transmission electron microscopy; nanoparticle tracking analysis, and resistive pulse sensing. Whilst flow cytometry generally has a higher minimum detectable threshold than other techniques

and can be time and labour intensive, it provides the most information with regards to MP size, complexity and cellular surface marker expression, and therefore remains the 'gold standard' technique most applicable to clinical research (3,14). Flow cytometry also provides high throughput whilst remaining relatively cheap, making it desirable when compared to other techniques (15). More novel techniques for detection of MPs include Raman microspectroscopy, micro nuclear magnetic resonance, and small-angle X-ray scattering. However, whilst these techniques may offer new insights in MP research, they are very specialised and not yet commercially available (16). Lastly, it must also be noted that sample collection and preparation techniques, including needle gauge and anticoagulant used for sample collection, tourniquet use, centrifugation protocol, freezing and thawing protocol, and buffer filtration may influence the detection of total (17,18) and phenotype-specific MPs (19) and thus should be considered when interpreting results. The lack of uniformity in MP collection and analysis protocols used in the literature makes the results difficult to interpret.

Microparticles in Disease

Elevated MP levels have been found in a variety of disease states (20), leading to the investigation into their use as prognostic markers to both comment on the current pathophysiological condition and predict future outcomes. There now exists a steadily growing body of literature that suggests that MPs can also display biological effector functions, i.e. they are able to influence other cells or systems (21), primarily in a pathophysiological manner.

Biomarker functions

As MPs are released upon cell stress, they are elevated in a variety of disease states and might be used as biomarkers of disease severity. MPs are elevated in a number of chronic systemic inflammatory conditions (22) including rheumatoid arthritis (23) and systemic lupus erythematosus (24), cardiovascular diseases (25) including stroke and acute coronary syndrome patients (26,27), various forms of cancer including colon, prostate, breast, ovarian and gastric cancer (28,29), HIV (30), and various forms of renal disease including predialysis chronic kidney disease, patients receiving varying dialysis modalities and renal transplant recipients (31,32). Many other conditions have been associated with increased MP levels – their rather unspecific nature of release (i.e. upon an activation or stress stimuli) means that a wide variety of stimuli can elicit MP shedding from a large number of cell types. For this reason, elevations in total or phenotype-specific MP counts may not be unique for each disease (33). Therefore, it may be more pertinent to 'profile' trends in the changes of many MP surface markers in different disease states to identify a panel of a combination of markers, the changes of which can be much more sensitive to disease severity or risk than the measurement of one MP phenotype or marker alone (34). This profiling method has been successful in strengthening the use of MPs as biomarkers in conditions such as various liver diseases (35), malaria (36) and atherosclerosis (37). However, this approach is not always successful in delineating different diseases, for instance in various forms of cancer (34). In this case, combining the identification of surface makers with measures of micro RNA content can increase the biomarker sensitivity of MPs (38,39) and improve diagnostic power.

Bioeffector functions

More recently MPs have also been considered as biologically active with effector functions rather than simply biomarkers of disease (21). The majority of this research has occurred either *in vitro* or *ex vivo*, owing to the difficulty of isolating the effects of MPs in an *in vivo* setting and the ethical issues involving MP infusion in human participants due to their potential pathophysiological impact. Several studies have used *in vivo* study designs to investigate MP infusion in animals, for instance to explore the mechanism behind MP-associated coagulation (40) and thrombus formation (41) in mice, however the primary purpose of this review is to comment on the current state of the literature concerning MPs in diseased human populations.

Endothelial MPs (EMPs) can induce endothelial activation and dysfunction (42) by reducing endothelium-dependent vasodilation in response to acetylcholine (43,44) and decreasing the release of the vasodilation-inducing nitric oxide (NO) (44,45) when incubated *in vitro* with rat aortic rings. This can reduce the ability of the vasculature to respond to fluctuations in haemodynamic pressure, inducing cardiac stress and left ventricular hypertrophy (46), and increasing cardiovascular mortality (47). Similarly, angiotensin II, which promotes vasoconstriction via activation of the renin angiotensin system and thus increases cardiovascular risk (48) can increase EMP release when incubated *in vitro* with murine endothelial cells (49), indicating endothelial damage. Increased circulating count of MPs of all cellular derivations has been positively associated with the circulating concentration of several reactive oxygen species (ROS), including plasma glutathione peroxidase and superoxide (50,51). When EMPs are incubated *in vitro* with human umbilical vein endothelial cells (HUVECs), the detrimental changes seen in angiogenesis (e.g. a reduction in total capillary length) were alleviated the presence of superoxide dismutase (52), implicating ROS production as a potential mechanism by which MPs can impair vascular function.

EMPs released from HUVECs in response to the pro-inflammatory cytokine TNF-α have a high calcium content and can induce osteogenesis and calcification when incubated with vascular smooth muscle cells (53). Similarly, platelet MPs (PMPs) incubated with rat aortic rings can promote angiogenesis via increased vascular endothelial growth factor (VEGF) activity (54) whilst EMPs incubated with HUVECs can increase PI3K activity, which plays a critical role in angiogenesis (55). Whilst angiogenesis is important for maintaining vascular health and homeostasis (56), excessive or dysregulated angiogenesis has been implicated in many conditions, including cancer (via loss of tumour growth suppression), some autoimmune disorders, atherosclerosis, pulmonary hypertension and inflammatory bowel disease, among others (57). These effects on the vasculature may increase cardiovascular risk and thus risk of mortality (58,59). Lastly, PS externalised on MPs can bind with the pro-thrombotic and procoagulant TF to initiate and promote thrombosis and coagulation (60–62) increasing the risk of embolism and driving ath-

erosclerosis (63). Elevated MP counts might therefore be predictive of mortality in a variety of conditions (64–66).

Whilst the 'quantity of MPs (i.e. concentration in the circulation) is important, their 'quality' (i.e. sourced from healthy or dysfunctional parent cells, their protein and RNA contents and composition) also impacts their transfer of information and thus bioeffector functions (67). EMPs released from a healthy endothelium help to maintain a protective low-grade procoagulant activity by increasing platelet clot stability (68), whilst EMPs released from damaged endothelial cells (e.g. due to atherosclerosis) can further induce endothelial dysfunction in a 'vicious cycle' manner by promoting atherogenesis (21,25). MPs isolated from human atherosclerotic plaque contain the metalloprotease TNF-α converting enzyme which increases TNF-α shedding from HUVECs, whilst this enzyme is not found in MPs isolated from healthy human internal arteries (37). Similarly, EMPs isolated from acute myocardial infarction, when incubated with rat aortic rings, caused a significant reduction in acetylcholine-induce endothelium dependent relaxation which was not seen when EMPs isolated from non-ischaemic patients were incubated at the same concentration (43) suggesting a difference in the quality of these MPs. Similarly, MPs were found at similar levels in the circulation of cardiac surgery patients and healthy controls, however the MPs from the patient group expressed significantly more TF, and thus promoted thrombogenesis to a greater extent in an *in vivo* model (11). Furthermore, the mRNA and micro RNA composition of EMPs as well as the ability of EMPs to transfer these RNAs to their target cells differs in certain disease conditions, for instance in coronary heart disease (69). Similarly, activation status may alter the micro RNA composition of MPs (70,71).

Consequently, any intervention which reduces the level of circulating MPs and positively alters their composition in clinical populations might be a therapeutic strategy, which could ultimately reduce morbidity and mortality. However, caution must be exercised when interpreting findings from *in vitro* studies. Whilst *in vitro* studies provide useful and direct information regarding how a particular variable impacts MP kinetics, they cannot account for the plethora of other factors that may influence these parameters in an *in vivo* setting. This is particularly pertinent in patients that suffer from systemic conditions that may alter a wide array of factors that could be expected to alter MP kinetics.

It should be noted that beneficial effects of MPS are also reported in the literature. MPs deliver RNAs, growth factors and cell surface receptors to target cells and as such are necessary for cellular communication (72). MP functions that are detrimental when aberrantly regulated (e.g. accelerated thrombosis) are a necessary response to vascular injury and important in wound healing. Additionally, platelet-derived MPs can inhibit apoptosis of polymorphonuclear leukocytes, potentially mediated by the influence of TGF-β1 (73). Similarly, shedding of endothelial cell MPs prevents an accumulation of caspase 3 and thus promotes cell survival via prevention of premature endothelial cell detachment and apoptosis (74). Some leukocyte derived MPs stimulate NO production (75) and can release anti-inflammatory effectors such as Annexin 1 (76) which can prevent endothelial leukocyte adhe-

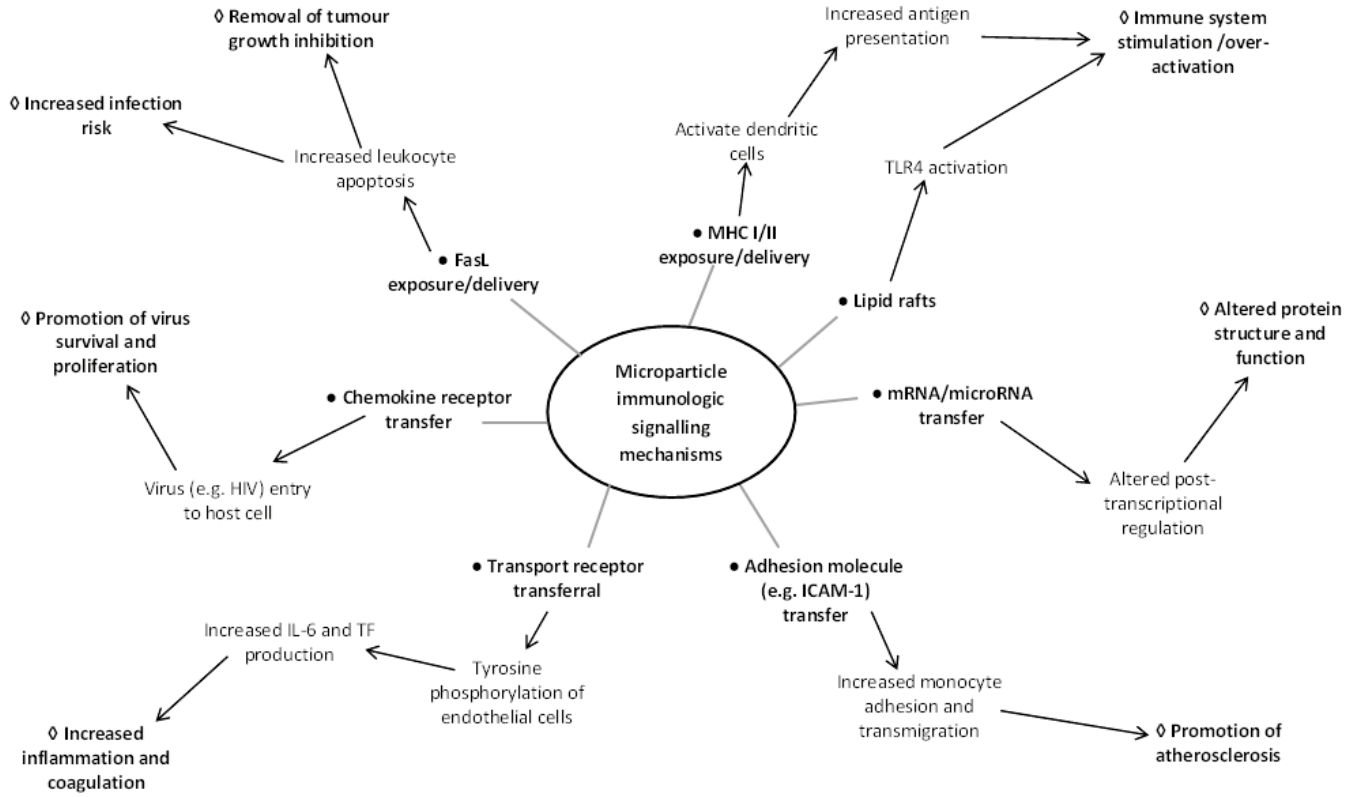


Figure 2. Examples of the immunologic signalling mechanisms MPs utilise to facilitate their bioeffector functions. Included are the typical pathways that these mechanisms initiate, and the typical end result. • = signalling mechanism, ◊ = typical physiological consequence.

sion and thus endothelial dysfunction (77). However, the vast majority of the research to date focusses on the deleterious effects of MPs (67) and thus they are considered to be largely pathophysiological in nature.

Signalling Mechanisms

MPs exert their bioeffector functions by implementing a variety of immunologic signalling mechanisms. MPs may bring about activated T-cell apoptosis by exposing Fas ligand (FasL – a death receptor ligand) (78) which can contribute to immune suppression and has been implicated in indirectly promoting tumour growth (79). MPs also mediate antigen presentation via the exposure of MHC class I and II molecules (80) which they can present to dendritic cells to facilitate immune surveillance (81). Similarly, the lipid component of MPs can activate Toll Like Receptor 4 on macrophages, stimulating antigen presentation (82). Additionally, MPs promote inflammation by transferring transport receptors to target cells (83) – for instance MPs from activated leukocytes increase tyrosine phosphorylation of endothelial cells, thus inducing their activation and increasing TF and IL-6 production (84). The pro-atherosclerotic role of MPs is mediated in part by the ability of MPs to transfer Intercellular Adhesion Molecule-1 (ICAM-1) to endothelial cells, thus increasing monocyte adhesion to the endothelium and promoting atherosclerotic plaque progression (85). MPs can also alter protein structure and function by transferring genetic information to their target cells, for instance mRNA and microRNA, which subsequently alter post-transcriptional regulation (83,86). Lastly, MPs may promote virus survival and growth via transfer of chemokine receptors, For instance, MPs released from HIV-infected cells can transfer CCR5 and CXCR4 to cells lacking these receptors, therefore making them susceptible to HIV (87). The immunologic signalling mechanisms of extracellular vesicles are discussed in greater depth by van der Pol et al (83) and are summarised in Figure 2.

Exercise and Microparticles

The beneficial effects of regular, moderate intensity aerobic and resistance exercise are well documented in the general population, and include improved body composition (88), increased physical capacity (89), reduced cardiovascular disease risk (90), reduced systemic inflammation (91), enhanced immune function (92) and reduced mortality (93). Exercise may also influence MP release via haemodynamic mechanisms. Aerobic exercise elicits increased blood flow to meet the extra oxygen demands of the working muscles, which can modify haemodynamic activation of both freely circulating cells and cells adhered to the endothelium via alterations in shear stress. Shear stress is a product of blood viscosity and flow rate; therefore aerobic exercise-induced increased blood flow can increase shear stress (94), which has been implicated in MP formation and release via modulation of cell membrane quiescence (95–97) due to mechanic and haemodynamic cellular activation. When considering platelets, increased shear stress may increase GPIb-dependent binding to endothelial Von Willebrand Factor, which can initiate PMP formation and thrombosis (96). The mechanism by which increased shear stress elicits increased MP shedding from other cell types is less clear and warrants further investigation. Additionally, both reduced physical activity and enforced physical inactivity can cause endothelial dysfunction (impaired flow-mediated dilation) accompanied by increased circulating resting EMP levels (13,98). Whilst acute aerobic exercise can also transiently increase MP formation as explained above, regular aerobic exercise training has been shown to improve endothelial function in cardiovascular disease populations (99,100) and therefore may be expected to reduce resting MP levels. Acute aerobic exercise may also increase cellular activation by transiently increasing catecholamine (e.g. norepinephrine) levels (101), thus increasing MP shedding by lowering membrane quiescence. Lastly, acute aerobic exercise can increase leukocyte apoptosis (102), potentially triggered by increases in cellular oxidative stress caused by increased reactive oxygen species production (103). As MPs are released by apoptotic cells (104), this exercise-induced apoptosis also increases MP production.

Healthy Population

There has been a great deal of recent research investigating the effects of acute and chronic exercise on MP kinetics in healthy participants undertaking aerobic exercise. However, the findings seem to be conflicting; some studies report increased post-exercise MP counts of platelet origin, particularly after strenuous exercise (105–109), which suggests a pro-thrombotic effect due to the high TF expression usually found on platelet-derived MPs (110,111). Mechanical activation of platelets and thus accelerated MP shedding is cited as the cause of this. Conversely, other studies have found no change in EMP or PMP levels following high-intensity (100% peak power output) cycling (112) or even shown a reduction in circulating EMPs following cycling of various intensities (55-100% peak power output) (113). This disparity may be caused by training status; the studies mentioned above which found increased MPs used healthy but sedentary (i.e. exercise frequency of $\leq$ 1/week) participants, whilst those that found decreased MPs used trained participants (either author-defined as 'fit' (112) or trained triathletes and cyclists (113)). This hypothesis is supported by studies investigating chronic regular aerobic exercise training, which display both an attenuation in the acute exercise-induced increase in MPs (neutrophil and platelet derived) (108,109) and a reduction in resting EMP counts (97,114) following prolonged training (e.g. 3 times/week for 6 months). Therefore, in the general population, it seems that whilst acute aerobic exercise may increase circulating MP counts, regular aerobic exercise training can either attenuate or abolish this effect and reduce resting circulating MP levels. This may be due to an adaptation effect caused by the repeated exposure of the endothelium to high SS elicited by aerobic exercise, which would prevent endothelial leukocyte adhesion and endothelial cell activation and/or apoptosis. Regular exercise training also improves endothelial function and increases resting NO availability (115), which may partially mitigate the increased SS caused by increased blood flow and thus prevent MP formation as explained above (52).

Clinical Populations

There is less research concerning exercise and MPs in clinical populations, especially considering the importance of health improvements when compared to the already 'healthy population'. When compared to healthy controls, patients with vas-

cular disease referred for stress echocardiography (incremental intravenous Dobutamine infusion until 85% of age-predicted maximum heart rate was reached) displayed a diminished post-test increase in leukocyte, granulocyte and monocyte MPs, whilst platelet, erythrocyte and endothelial MPs increased as normal (116). Similarly, a single bout of high intensity interval cycling (intervals completed at 100% peak power output) did not affect platelet or endothelial MP counts in coronary heart disease patients (112). It is unclear why patients with cardiovascular deficiencies would exhibit reduced MP release, as they would be expected to display reduced exercise-induced vasodilation due to arterial stiffening (117) and potentially increased shear stress-mediated MP release. A possible explanation is that CVD patients display reduced cardiac contractile power due to a reduction in stroke volume mediated by left ventricular hypertrophy and a reduced ejection fraction. Therefore, the haemodynamic response to aerobic exercise may be blunted in CVD patients (118), blunting the subsequent MP response. However, following 12 weeks of either continuous or interval aerobic exercise training, coronary artery disease patients displayed no change in resting EMP levels, despite showing improvements in endothelial function as measured by flow mediated dilation (119). In the same study, baseline EMP levels were inversely correlated with increases in peak VO_2 consumption, suggesting that pre-existing elevated EMP levels (perhaps suggesting the presence of endothelial dysfunction) may prevent subsequent aerobic training adaptation. A possible explanation, similar to above, is that cardiovascular disease-associated contractile and endothelial impairments create a vascular 'dormancy' which reduces MP increases in response to exercise. This increase is seen as a normal physiological response (116), the absence of which may prevent the chronic training-associated improvements in MP levels seen in the general population. However, renal transplant recipients displayed reduced circulating EMP levels following 6 months of aerobic exercise training compared to non-exercise controls (120). Renal transplant recipients are considered at heightened risk of cardiovascular events (121) and display impaired flow mediated dilation (122) and increased prevalence of left ventricular hypertrophy (123), demonstrating that exercise can improve MP levels in a population displaying cardiovascular decrements. This was accompanied by a reduced endothelial progenitor cell concentration, which may signify either reduced vascular repair capacity or reduced vascular damage (which is more likely considering the reduced EMP levels), therefore reducing the repair stimulus and subsequent progenitor cell response.

There is a clear lack of uniformity concerning the effects of exercise on MP levels and composition in clinical populations in the current body of literature. This may be in part due to the different methods of isolation used. Flow cytometry was the most commonly used technique to measure MPs in the studies mentioned above, however the processing and isolation protocols used were not uniform which may have impacted the MP counts. Whilst it is evident that regular exercise training is effective in reducing cardiovascular risk in both healthy and diseased populations, the disparity seen in the MP literature may suggest that one size does not fit all. For instance, it is unclear why acute aerobic exercise, particularly of a very high intensity, would elicit an increase in circulating MP counts in sedentary healthy individuals (105,106) but not in the cardiovascular disease population (112). It is possible that MP release is increased following exercise in cardiovascular disease patients but they are not measurable in the circulation, for instance because they have formed clots or adhered to endothelial cells, thereby promoting their pathophysiological influence. As previously mentioned, little is known about the clearance of MPs from the circulation (124) and therefore this may require further investigation. Similarly, patients from different disease populations display differing MP responses to comparable exercise interventions. More research is necessary to investigate the effects of different exercise regimens on MP kinetics in various patient populations in order to tailor rehabilitation programmes more effectively to patients depending on their comorbid conditions. For instance, cardiac rehabilitation programmes in coronary heart disease patients typically consist of regular moderate intensity aerobic exercise (125) however this type of training does not seem to influence MP levels in this population (119). Similarly, the lack of changes in MP kinetics seen after high intensity training requires further investigation. As the primary cause of MP formation during exercise is suggested to be cellular activation caused by haemodynamic activation, it is unclear why this type of training would not elicit increased MP release in certain populations (112,119). Lastly, resistance training is an effective training modality for reducing cardiovascular risk in diseased populations (126) however the impact on MP kinetics is under-researched. Research investigating the role of resistance training in modulating MP levels and reducing cardiovascular risk will allow more well-rounded exercise programmes to be designed for specific patient populations.

Interestingly, MPs may also play a role in the adaption to exercise training. MPs and exosomes released during aerobic exercise have been proposed to contain proteins and nucleic acids (for instance heat shock protein 70) (127) that are hypothesised to mediate organ crosstalk and promote systemic adaption to aerobic exercise (128). As such, it is been hypothesised that small extracellular vesicles released from the muscle during aerobic exercise may mediate many of the systemic adaptations to endurance exercise that prevent or lessen the severity of health conditions such as obesity and Type 2 Diabetes Mellitus (129). However, this concept requires further investigation.

Diet and Body Composition

Diet, body composition and gender also influence MP levels, and these relationships may be modulated by exercise. Inactive, obese males displayed reduced circulating EMP levels following moderate-to-high intensity cycling compared to a non-exercise control trial completed in a randomised counterbalanced manner, whilst overweight females displayed increased EMP levels compared to their non-exercise control trial (130). The cause of this gender disparity is unknown; the authors suggest a possible modulating effect of oestrogen with regards to cardiovascular disease risk. Indeed, in healthy individuals, increased EMP and PMP counts have been observed in females, the levels of which may be altered by the menstrual cycle stage (i.e. luteal versus follicular phase) and the associated fluctuations in oestrogen and progesterone (131). Addi-

tionally, other cardiovascular risk factors such as central obesity, elevated total cholesterol and reduced high-density lipoprotein cholesterol may be more prevalent in females (132). MP kinetics may therefore be another mechanism by which cardiovascular risk differs based on gender. Furthermore, females display elevated endothelial progenitor cell counts compared to males (133), suggesting stimulated repair mechanisms in response to greater activation or damage, which would explain the increased EMP levels. Regardless of gender, excessive adipose tissue has been associated with elevated circulating PMP levels compared to age-matched non-obese controls (134). This elevation was partially reduced by a 12 week calorie-restricted diet (1200 kcal/day for women, 1700 kcal/day for men) which reduced BMI by roughly 10%, and was reduced to a greater degree by a 12 week programme of calorie restriction and regular aerobic exercise (3 times/week, 60 minutes, 12-14 RPE) which reduced BMI by roughly 12%. Diet coupled with exercise was also more efficacious in reducing fat tissue mass, visceral and subcutaneous fat area, and total and LDL cholesterol, offering other possible explanations for the reduced PMP count beyond simply reducing BMI. The increased MP levels seen in obesity may be partially caused by the MP response to a high fat and/or carbohydrate diet. Postprandial hypertriglyceridaemia and hyperglycaemia caused by high dietary fat and carbohydrate intake can induce vascular dysfunction (i.e. impaired vasodilatation), possibly mediated by increased oxidative stress and NO inactivation (135), or increased adhesion molecule (VCAM-1, ICAM-1) expression, increasing leukocyte infiltration (136). As such, EMP and total MP are elevated in response to high fat and carbohydrate meals (137,138), offering a possible explanation for the increased coagulation and thrombotic activity of the TF pathway seen during hypertriglyceridaemia and hyperglycaemia (139). However, this response may be ameliorated by exercise. Moderate intensity cycling (60-75% VO_2 to elicit an energy expenditure of 4-6 kcal/kg) completed 1 hour before ingestion of a high-fat meal blunted the postprandial increase in EMP levels that was seen in the non-exercise control trial (140). However, 100 mins of cycling at 70% VO_2 peak completed the previous evening did not affect the increase in EMPs seen in response to a high fat meal ingested the following morning (141). This suggests a more direct effect on MPs rather than indirect via lipid alterations, as moderate exercise completed the day before the consumption of a high fat meal can attenuate postprandial lipaemia (142). MPs would also be affected if they were dependent on blood lipid levels.

It is unclear whether or not the increase in MP counts often seen after consumption of a high fat meal represents a clinically significant effect that could elicit pathophysiological consequences. In some disease states, such as diabetes or coronary artery disease, the disparity between the MP counts of the disease population and the healthy population is comparable with the magnitude of the increase seen between pre- and post-prandial conditions (141,143). Whilst this suggests the potential to exert pathophysiological effects, the transient nature of the post-prandial MP increase may prevent the development of any clinically significant health decrements. Further research is required to investigate the impact of regular high fat meal consumption on MP kinetics and the possible downstream pathophysiological consequences.

Clinical Implications and Further Research

In summary, elevated MP levels and altered composition are seen in a number of disease states, having a number of pathophysiological effector functions. Exercise may help to reduce MP levels and thus diminish their pathophysiological potential but more research is needed to elucidate these effects, particularly in clinical populations that display elevated cardiovascular risk. Studies investigating chronic exercise training in clinical populations are needed to investigate MP levels and composition, and how they relate to measures of systemic inflammation, thrombotic potential, vascular damage and various cardiovascular risk factors. Additionally, the effects of resistance exercise with regards to MPs are under-researched, as resistance exercise can be a powerful therapeutic tool for reducing morbidity and maintaining physical function in clinical populations (144). Increases in blood pressure and associated reductions in arterial compliance (145) caused by skeletal muscle contraction during resistance exercise could create an environment that promotes MP shedding, an interesting topic for future research. The evidence to date is encouraging, and suggests that, whilst acute exercise can increase circulating MP counts, regular exercise training can diminish this effect and eventually reduce overall resting MP counts, partially preventing their pathophysiological effects. This effect has been demonstrated within as little as 5 weeks of regular aerobic exercise training (109). However, given the widespread systemic effects of exercise and the numerous pathways eliciting MP release from various cell types, more research must be done to better understand how exercise affects the number and bioeffector function of microparticles.

REFERENCES

1. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. Trends Cell Biol [Internet]. 2009;19(2):43–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19144520
2. Shantsila E, Kamphuisen PW, Lip GYH. Circulating microparticles in cardiovascular disease: implications for atherogenesis and atherothrombosis. J Thromb Haemost [Internet]. 2010;8(11):2358–68. Available from: http://doi.wiley.com/10.1111/j.1538-7836.2010.04007.x
3. van der Pol E, Coumans F a W, Grootemaat a. E, Gardiner C, Sargent IL, Harrison P, et al. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. J Thromb Haemost. 2014;12(7):1182–92.
4. Connor DE, Exner T, Ma DDF, Joseph JE. The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. Thromb Haemost. 2010;103(5):1044–52.
5. Bevers EM, Comfurius P, Dekkers DWC, Zwaal RF a. Lipid translocation across the plasma membrane of mammalian cells. Biochim Biophys Acta - Mol Cell Biol Lipids. 1999;1439:317–30.
6. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev [Internet]. 2007;21(3):157–71. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0268960X0600052X

7. Flaumenhaft R. Formation and fate of platelet microparticles. Blood Cells, Mol Dis. 2006;36(2):182–7.
8. Litvack ML, Post M, Palaniyar N. IgM promotes the clearance of small particles and apoptotic microparticles by macrophages. PLoS One [Internet]. 2011;6(3):e17223. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3063157&tool=pmcentrez&rendertype=abstract
9. Fourcade O, Simon MF, Viodé C, Rugani N, Leballe F, Ragab A, et al. Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. Cell. 1995;80(6):919–27.
10. Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. Atherosclerosis. 2001;158(2):277–87.
11. Biró É, Sturk-Maquelin KN, Vogel GMT, Meuleman DG, Smit MJ, Hack CE, et al. Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner. J Thromb Haemost. 2003;1(12):2561–8.
12. Burger D, Schock S, Thompson CS, Montezano AC, Hakim AM, Touyz RM. Microparticles: biomarkers and beyond. Clin Sci [Internet]. 2013;124(7):423–41. Available from: http://clinsci.org/lookup/doi/10.1042/CS20120309
13. Navasiolava NM, Dignat-George F, Sabatier F, Larina IM, Demiot C, Fortrat J-O, et al. Enforced physical inactivity increases endothelial microparticle levels in healthy volunteers. Am J Physiol Heart Circ Physiol [Internet]. 2010;299(2):H248-56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20472757\nhttp://ajpheart.physiology.org/content/299/2/H248.full-text.pdf+html
14. Strasser EF, Happ S, Weiss DR, Pfeiffer A, Zimmermann R, Eckstein R. Microparticle detection in platelet products by three different methods. Transfusion. 2013;53(1):156–66.
15. Szatanek R, Baj-Krzyworzeka M, Zimoch J, Lekka M, Siedlar M, Baran J. The methods of choice for extracellular vesicles (EVs) characterization. Int J Mol Sci. 2017;18(6).
16. van der Pol E, Coumans F, Varga Z, Krumrey M, Nieuwland R. Innovation in detection of microparticles and exosomes. J Thromb Haemost. 2013;11(SUPPL.1):36–45.
17. Dey-Hazra E, Hertel B, Kirsch T, Woywodt A, Lovric S, Haller H, et al. Detection of circulating microparticles by flow cytometry: Influence of centrifugation, filtration of buffer, and freezing. Vasc Health Risk Manag. 2010;6(1):1125–33.
18. van der Heyde HC, Gramaglia I, Combes V, George TC, Grau GE. Flow Cytometric Analysis of Microparticles. Methods Mol Biol. 2011;699:337–54.
19. van Ierssel SH, Van Craenenbroeck EM, Conraads VM, Van Tendeloo VF, Vrints CJ, Jorens PG, et al. Flow cytometric detection of endothelial microparticles (EMP): Effects of centrifugation and storage alter with the phenotype studied. Thromb Res [Internet]. Elsevier Ltd; 2010;125(4):332–9. Available from: http://dx.doi.org/10.1016/j.thromres.2009.12.019
20. Roseblade A, Luk F, Rawling T, Ung A, Grau GER, Bebawy M. Cell-derived microparticles: New targets in the therapeutic management of disease. J Pharm Pharm Sci. 2013;16(2):238–53.
21. Chironi GN, Boulanger CM, Simon A, Dignat-George F, Freyssinet J-M, Tedgui A. Endothelial microparticles in diseases. Cell Tissue Res [Internet]. 2009;335(1):143–51. Available from: http://link.springer.com/10.1007/s00441-008-0710-9
22. Distler JHW, Huber LC, Gay S, Distler O, Pisetsky DS. Microparticles as mediators of cellular cross-talk in inflammatory disease. Autoimmunity [Internet]. 2006;39(8):683–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17178565
23. Knijff-Dutmer E a J, Koerts J, Nieuwland R, Kalsbeek-Batenburg EM, Van De Laar M a FJ. Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis. Arthritis Rheum. 2002;46(6):1498–503.
24. Sellam J, Proulle V, Jüngel A, Ittah M, Miceli Richard C, Gottenberg J-E, et al. Increased levels of circulating microparticles in primary Sjögren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity. Arthritis Res Ther [Internet]. 2009;11(5):R156. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2787287&tool=pmcentrez&rendertype=abstract
25. Vanwijk MJ, Vanbavel E, Sturk a, Nieuwland R. M icroparticles in cardiovascular diseases. Cardiovasc Res. 2003;59:277–87.
26. Simak J, Gelderman MP, Yu H, Wright V, Baird a. E. Circulating endothelial microparticles in acute ischemic stroke: A link to severity, lesion volume and outcome. J Thromb Haemost. 2006;4(6):1296–302.
27. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, et al. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation. 2000;101(8):841–3.
28. Kanazawa S, Nomura S, Kuwana M, Muramatsu M, Yamaguchi K, Fukuhara S. Monocyte-derived microparticles may be a sign of vascular complication in patients with lung cancer. Lung Cancer [Internet]. 2003;39(2):145–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12581566
29. Goon PKY, Lip GYH, Boos CJ, Stonelake PS, Blann a. D. Circulating Endothelial Cells, Endothelial Progenitor Cells, and Endothelial Microparticles in Cancer. Neoplasia [Internet]. Neoplasia Press, Inc.; 2006;8(2):79–88. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1476558606801156
30. Baker J, Hullsiek K, Bradford R, Prosser R, Tracy R, Key N. Circulating levels of tissue factor microparticle procoagulant activity are reduced with antiretroviral therapy and are associated with persisten inflammation and coagulation in HIV, Baker.pdf. Journal of Aquired Immune Deficiency Syndrome; 2013. p. 367–71.
31. Burton JO, Hamali H a., Singh R, Abbasian N, Parsons R, Patel AK, et al. Elevated Levels of Procoagulant Plasma Microvesicles in Dialysis Patients. PLoS One [Internet]. 2013;8(8):e72663. Available from: http://dx.plos.org/10.1371/journal.pone.0072663
32. Daniel L, Dou L, Berland Y, Lesavre P, Mecarelli-Halbwachs L, Dignat-George F. Circulating microparticles in renal diseases. Nephrol Dial Transplant [Internet]. 2008;23(7):2129–32. Available from: http://ndt.oxfordjournals.org/cgi/doi/10.1093/ndt/gfn029
33. Pisetsky DS, Ullal AJ, Gauley J, Ning TC. Microparticles as mediators and biomarkers of rheumatic disease. Rheumatology. 2012;51(10):1737–46.
34. Julich H, Willms A, Lukacs-Kornek V, Kornek M. Extracellular vesicle profiling and their use as potential disease specific biomarker. Front Immunol. 2014;5(AUG):1–6.
35. Kornek M, Lynch M, Mehta SH, Lai M, Exley M, Afdhal NH, et al. Circulating microparticles as disease-specific biomarkers of severity of inflammation in patients with hepatitis C or nonalcoholic steatohepatitis. Gastroenterology. 2012;143(2):448–58.

36. Bertrand J, Mfonkeu P, Gouado I, Fotso Kuaté H, Zambou O, Amvam Zollo PH, et al. Elevated Cell-Specific Microparticles Are a Biological Marker for Cerebral Dysfunctions in Human Severe Malaria. PLoS One. 2010;5(10).
37. Canault M, Leroyer AS, Peiretti F, Lesèche G, Tedgui A, Bonardo B, et al. Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/ADAM17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1. Am J Pathol [Internet]. 2007;171(5):1713–23. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2043531&tool=pmcentrez&rendertype=abstract
38. Sun L, Hu J, Xiong W, Chen X, Li H, Jie S. MicroRNA expression profiles of circulating microvesicles in hepatocellular carcinoma. Acta Gastroenterol Belg. 2013;76(4):386–92.
39. Li L, Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, et al. Human bile contains MicroRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. Hepatology. 2014;60(3):896–907.
40. Falati S, Liu Q, Gross P, Merrill-Skoloff G, Chou J, Vandendries E, et al. Accumulation of Tissue Factor into Developing Thrombi In Vivo Is Dependent upon Microparticle P-Selectin Glycoprotein Ligand 1 and Platelet P-Selectin. J Exp Med. 2003;197(11):1585–98.
41. Chou J, Mackman N, Merrill-Skoloff G, Pedersen B, Furie BC, Furie B. Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. Blood. 2004;104(10):3190–7.
42. Amabile N, Guerin AP, Leroyer AP, Mallat Z, Nguyen C, Boddaert J, et al. Circulating Endothelial Microparticles Are Associated with Vascular Dysfunction in Patients with End-Stage Renal Failure. J Am Soc Nephrol [Internet]. 2005;16(11):3381–8. Available from: http://www.jasn.org/cgi/doi/10.1681/ASN.2005050535
43. Boulanger CM, Scoazec a, Ebrahimian T, Henry P, Mathieu E, Tedgui a, et al. Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. Circulation. 2001;104(22):2649–52.
44. Martin S, Tesse A, Hugel B, Martinez C, Morel O, Freyssinet J-M, et al. Shed Membrane Particles From T Lymphocytes Impair Endothelial Function and Regulate Endothelial Protein Expression. Circulation [Internet]. 2004;109(13):1653–9. Available from: http://circ.ahajournals.org/cgi/doi/10.1161/01.CIR.0000124065.31211.6E
45. Brodsky S V, Zhang F, Nasjletti A, Goligorsky MS. Endothelium-derived microparticles impair endothelial function in vitro. Am J Physiol Heart Circ Physiol. 2004;286:H1910–5.
46. Treasure CB, Klein JL, Vita J a, Manoukian S V, Renwick GH, Selwyn a P, et al. Hypertension and left ventricular hypertrophy are associated with impaired endothelium-mediated relaxation in human coronary resistance vessels. Circulation [Internet]. 1993;87(1):86–93. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8419028
47. Bauml MA, Underwood DA. Left ventricular hypertrophy: An overlooked cardiovascular risk factor. Cleve Clin J Med. 2010;77(6):381–7.
48. Schmieder RE, Hilgers KF, Schlaich MP, Schmidt BM. Renin-angiotensin system and cardiovascular risk. Lancet. 2007;369(9568):1208–19.
49. Burger D, Montezano AC, Nishigaki N, He Y, Carter A, Touyz RM. Endothelial microparticle formation by angiotensin II is mediated via ang II receptor type I/NADPH Oxidase/rho kinase pathways targeted to lipid rafts. Arterioscler Thromb Vasc Biol. 2011;31(8):1898–907.
50. Mastronardi ML, Mostefai HA, Meziani F, Martínez MC, Asfar P, Andriantsitohaina R. Circulating microparticles from septic shock patients exert differential tissue expression of enzymes related to inflammation and oxidative stress*. Crit Care Med [Internet]. 2011;39(7):1739–48. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00003246-201107000-00017
51. Helal O, Defoort C, Robert S, Marin C, Lesavre N, Lopez-Miranda J, et al. Increased levels of microparticles originating from endothelial cells, platelets and erythrocytes in subjects with metabolic syndrome: Relationship with oxidative stress. Nutr Metab Cardiovasc Dis. 2011;21(9):665–71.
52. Mezentsev A, Merks RMH, Riordan EO, Chen J, Mendelev N, Goligorsky MS, et al. Endothelial microparticles affect angiogenesis in vitro: role of oxidative stress. Am J Physiol Hear Circ Physiol. 2005;10595:1106–14.
53. Buendia P, Montes de Oca a., Madueno J a., Merino a., Martin-Malo a., Aljama P, et al. Endothelial microparticles mediate inflammation-induced vascular calcification. FASEB J [Internet]. 2015;29(1):173–81. Available from: http://www.fasebj.org/cgi/doi/10.1096/fj.14-249706
54. Brill A, Dashevsky O, Rivo J, Gozal Y, Varon D. Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization. Cardiovasc Res. 2005;67:30–8.
55. Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, et al. Endothelial progenitor cell - Derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. Blood. 2007;110(7):2440–8.
56. Moreno PR, Sanz J, Fuster V. Promoting Mechanisms of Vascular Health. Circulating Progenitor Cells, Angiogenesis, and Reverse Cholesterol Transport. J Am Coll Cardiol. American College of Cardiology Foundation; 2009;53(25):2315–23.
57. Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003;9(6):653–60.
58. Nevskaya T, Bykovskaia S, Lyssuk E, Shakhov I, Zaprjagaeva M, Mach E, et al. Circulating endothelial progenitor cells in systemic sclerosis: relation to impaired angiogenesis and cardiovascular manifestations. Clin Exp Rheumatol [Internet]. 2008;26(3):421–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18578963
59. Siervo M, Ruggiero D, Sorice R, Nutile T, Aversano M, Stephan BCM, et al. Angiogenesis and biomarkers of cardiovascular risk in adults with metabolic syndrome. J Intern Med. 2010;268(4):338–47.
60. Sinauridze EI, Kireev DA, Popenko NY, Pichugin A V., Panteleev MA, Krymskaya O V., et al. Platelet microparticle membranes have 50- to 100fold higher specific procoagulant activity than activated platelets. Thromb Haemost [Internet]. 2007;97:425–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17139370
61. Polgar J, Matuskova J, Wagner DD. The P-selectin, tissue factor, coagulation triad. J Thromb Haemost. 2005;3(8):1590–6.
62. Ando M, Iwata A, Ozeki Y, Tsuchiya K, Akiba T, Nihei H. Circulating platelet-derived microparticles with procoagulant activity may be a potential cause of thrombosis in uremic patients. Kidney Int [Internet]. 2002;62(5):1757–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12371977

63. Kleinegris MC, Ten Cate-Hoek AJ, Ten Cate H. Coagulation and the vessel wall in thrombosis and atherosclerosis. Pol Arch Med Wewn. 2012;122(11):557–66.
64. Amabile N, Guerin a. P, Tedgui a., Boulanger CM, London GM. Predictive value of circulating endothelial microparticles for cardiovascular mortality in end-stage renal failure: a pilot study. Nephrol Dial Transplant [Internet]. 2012;27(5):1873–80. Available from: http://ndt.oxfordjournals.org/cgi/doi/10.1093/ndt/gfr573
65. Bharthuar A, Khorana A a., Hutson A, Wang JG, Key NS, Mackman N, et al. Circulating microparticle tissue factor, thromboembolism and survival in pancreaticobiliary cancers. Thromb Res [Internet]. Elsevier B.V.; 2013;132(2):180–4. Available from: http://dx.doi.org/10.1016/j.thromres.2013.06.026
66. Wang CC, Tseng CC, Hsiao CC, Chang HC, Chang LT, Fang WF, et al. Circulating endothelial-derived activated microparticle: A useful biomarker for predicting one-year mortality in patients with advanced non-small cell lung Cancer. Biomed Res Int. 2014;2014.
67. Herring JM, Mcmichael MA, Smith SA. Microparticles in health and disease. J Vet Intern Med. 2013;27(5):1020–33.
68. Jy W, Jimenez JJ, Mauro LM, Horstman LL, Cheng P, Ahn ER, et al. Endothelial microparticles induce formation of platelet aggregates via a von Willebrand factor/ristocetin dependent pathway, rendering them resistant to dissociation. J Thromb Haemost. 2005;3(6):1301–8.
69. Finn NA, Eapen D, Manocha P, Al Kassem H, Lassegue B, Ghasemzadeh N, et al. Coronary heart disease alters intercellular communication by modifying microparticle-mediated microRNA transport. FEBS Lett [Internet]. Federation of European Biochemical Societies; 2013;587(21):3456–63. Available from: http://dx.doi.org/10.1016/j.febslet.2013.08.034
70. Laffont B, Corduan A, PÍe H, Duchez AC, Cloutier N, Boilard E, et al. Activated platelets can deliver mRNA regulatory Ago2•microRNA complexes to endothelial cells via microparticles. Blood. 2013;122(2):253–61.
71. Schiro A, Wilkinson FL, Weston R, Smyth J V., Serracino-Inglott F, Alexander MY. Endothelial microparticles as conveyors of information in atherosclerotic disease. Atherosclerosis [Internet]. Elsevier Ltd; 2014;234(2):295–302. Available from: http://dx.doi.org/10.1016/j.atherosclerosis.2014.03.019
72. Ferraris V. Microparticles: The good, the bad, and the ugly. J Thorac Cardiovasc Surg [Internet]. Elsevier Ltd; 2015;149:312–3. Available from: http://dx.doi.org/10.1016/j.jtcvs.2014.08.051
73. Brunetti M, Martelli N, Manarini S, Mascetra N, Musiani P, Cerletti C, et al. Polymorphonuclear leukocyte apoptosis is inhibited by platelet-released mediators, role of TGF-β1. Thromb Haemost. 2000;84(1):478–83.
74. Hussein M, Boing A, Sturk A, Hau C, Nieuwland R. Inhibition of microparticle release triggers endothelial cell apoptosis and detachment. Thromb Haemost. 2007;98:1096–107.
75. Agouni A, Mostefai HA, Porro C, Carusio N, Favre J, Richard V, et al. Sonic hedgehog carried by microparticles corrects endothelial injury through nitric oxide release. FASEB J [Internet]. 2007;21(11):2735–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17428963
76. Gasser O, Schifferli JA. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. Blood. 2004;104(8):2543–8.
77. Dalli J, Norling L V., Renshaw D, Cooper D, Leung KY, Perretti M. Annexin 1 mediates the rapid anti-inflammatory effects of neutrophil-derived microparticles. Blood. 2008;112(6):2512–9.
78. Martínez-Lorenzo MJ, Anel A, Gamen S, Monleón I, Lasierra P, Larrad L, et al. Activated Human T Cells Release Bioactive Fas Ligand and APO2 Ligand in Microvesicles. J Immology. 1999;163(3):1274–81.
79. Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL. Fas Ligand − Positive Membranous Vesicles Isolated from Sera of Patients with Oral Cancer Induce Apoptosis of Activated T Lymphocytes Fas Ligand – Positive Membranous Vesicles Isolated from Sera of Patients with Oral Cancer Induce Apoptosis of Activated T. Clin Cancer Res. 2005;11:1010–20.
80. Van Niel G, Mallegol J, Bevilacqua C, Candalh C, Brugiere S, Tomaskovic-Crook E, et al. Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. Gut [Internet]. 2003;52(12):1690–7. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14633944
81. Mallegol J, Van Niel G, Lebreton C, Lepelletier Y, Candalh C, Dugave C, et al. T84-Intestinal Epithelial Exosomes Bear MHC Class II/Peptide Complexes Potentiating Antigen Presentation by Dendritic Cells. Gastroenterology. 2007;132(5):1866–76.
82. Thomas LM, Salter RD. Activation of macrophages by P2X7-induced microvesicles from myeloid cells is mediated by phospholipids and is partially dependent on TLR4. J Immunol [Internet]. 2010;185(6):3740–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2933301&tool=pmcentrez&rendertype=abstract
83. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol Rev [Internet]. 2012;64(3):676–705. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22722893
84. Mesri M, Altieri DC. Leukocyte Microparticles Stimulate Endothelial Cell Cytokine Release and Tissue Factor Induction in a JNK1 Signaling Pathway. J Biol Chem. 1999;274(33):23111–8.
85. Rautou PE, Leroyer AS, Ramkhelawon B, Devue C, Duflaut D, Vion AC, et al. Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. Circ Res. 2011;108(3):335–43.
86. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan MLG, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood. 2012;119(3):756–66.
87. Mack M, Kleinschmidt A, Bruhl H, Klier C, Nelson PJ, Cihak J, et al. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. Nat Med [Internet]. 2000;6(7):769–75. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10888925
88. Peterson MD, Sen A, Gordon PM. Influence of Resistance Exercise on Lean Body Mass in Aging Adults: A Meta-Analysis. Med Sci Sport Exerc. 2011;43(2):249–58.

89. Chou C-H, Hwang C-L, Wu Y-T. Effect of Exercise on Physical Function, Daily Living Activities, and Quality of Life in the Frail Older Adults: A Meta-Analysis. Arch Phys Med Rehabil. 2012;93(2):237–44.
90. Li J, Siegrist J. Physical activity and risk of cardiovascular disease-a meta-analysis of prospective cohort studies. Int J Environ Res Public Health. 2012;9(2):391–407.
91. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo M a. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol [Internet]. Nature Publishing Group; 2011;11(9):607–15. Available from: http://www.nature.com/doifinder/10.1038/nri3041
92. Nieman DC, Pedersen BK. Exercise and Immune Function. Sport Med. 1999;27(2):73–80.
93. Kokkinos P, Myers J, Faselis C, Panagiotakos DB, Doumas M, Pittaras A, et al. Exercise capacity and mortality in older men: A 20-year follow-up study. Circulation. 2010;122(8):790–7.
94. Long DS, Smith ML, Pries AR, Ley K, Damiano ER. Microviscometry reveals reduced blood viscosity and altered shear rate and shear stress profiles in microvessels after hemodilution. Proc Natl Acad Sci U S A [Internet]. 2004;101(27):10060–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15220478
95. Miyazaki Y, Nomura S, Miyake T, Kagawa H, Kitada C, Taniguchi H, et al. High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles. Blood. 1996;88(9):3456–64.
96. Reininger AJ, Heijnen HFG, Schumann H, Specht HM, Schramm W, Ruggeri ZM. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. Blood. 2006;107(9):3537–45.
97. Kim J-S, Kim B, Lee H, Thakkar S, Babbitt DM, Eguchi S, et al. Shear Stress-Induced Mitochondrial Biogenesis Decreases the Release of Microparticles from Endothelial Cells 4 5. Am J Physiol Hear Circ Physiol. 2015;19122:425–33.
98. Boyle LJ, Credeur DP, Jenkins NT, Padilla J, Leidy HJ, Thyfault JP, et al. Impact of reduced daily physical activity on conduit artery flow-mediated dilation and circulating endothelial microparticles. J Appl Physiol [Internet]. 2013;115(10):1519–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24072406\nhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3841822
99. Hambrecht R, Fiehn E, Weigl C, Gielen S, Hamann C, Kaiser R, et al. Regular physical exercise corrects endothelial dysfunction and improves exercise capacity in patients with chronic heart failure. Circulation [Internet]. 1998;98(24):2709–15. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9851957
100. Gokce N, Vita JA, Bader DS, Sherman DL, Hunter LM, Holbrook M, et al. Effect of exercise on upper and lower extremity endothelial function in patients with coronary artery disease. Am J Cardiol [Internet]. 2002;90(2):124–7. Available from: http://www.sciencedirect.com/science/article/pii/S0002914902024335
101. Perini R, Orizio C, Comande A, Castellano M, Beschi M, Veicsteinas A. Plasma norepinephrine and heart rate dynamics during recovery from submaximal exercise in man. Eur J Appl Physiol. 1989;58(8):879–83.
102. Mooren F, Lechtermann A, Völker K. Exercise-induced apoptosis of lymphocytes depends on training status. Med Sci Sports Exerc. 2004;36(9):1476–83.
103. Phaneuf S, Leeuwenburgh C. Apoptosis and exercise. Med Sci Sports Exerc [Internet]. 2001;33(3):393–6. Available from: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00005768-200103000-00010
104. Distler JHW, Huber LC, Hueber AJ, Reich CF, Gay S, Distler O, et al. The release of microparticles by apoptotic cells and their effects on macrophages. Apoptosis. 2005;10(4):731–41.
105. Chen Y-W, Chen J-K, Wang J-S. Strenuous exercise promotes shear-induced thrombin generation by increasing the shedding of procoagulant microparticles from platelets. Thromb Haemost [Internet]. 2010;104(2):293–301. Available from: http://www.schattauer.de/index.php?id=1214&doi=10.1160/TH09-09-0633
106. Chaar V, Romana M, Tripette J, Broquere C, Huisse MG, Hue O, et al. Effect of strenuous physical exercise on circulating cell-derived microparticles. Clin Hemorheol Microcirc. 2011;47(1):15–25.
107. Sossdorf M, Otto GP, Claus R a., Gabriel HH, Lösche W. Release of pro-coagulant microparticles after moderate endurance exercise. Platelets [Internet]. 2010;21(5):389–91. Available from: http://www.tandfonline.com/doi/full/10.3109/09537101003698564
108. Chen Y-W, Chen Y-C, Wang J-S. Absolute hypoxic exercise training enhances in vitro thrombin generation by increasing procoagulant platelet-derived microparticles under high shear stress in sedentary men. Clin Sci [Internet]. 2013;124(10):639–49. Available from: http://clinsci.org/lookup/doi/10.1042/CS20120540
109. Chen Y-C, Ho C-W, Tsai H-H, Wang J-S. Interval and continuous exercise regimens suppress neutrophil-derived microparticle formation and neutrophil-promoted thrombin generation under hypoxic stress. Clin Sci (Lond) [Internet]. 2015;128(7):425–36. Available from: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed13&NEWS=N&AN=25371035
110. Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JWA, Radder JK. Elevated Numbers of Tissue-Factor Exposing Microparticles Correlate With Components of the Metabolic Syndrome in Uncomplicated Type 2 Diabetes Mellitus. Circulation [Internet]. 2002;106(19):2442–7. Available from: http://circ.ahajournals.org/cgi/doi/10.1161/01.CIR.0000036596.59665.C6
111. Del Conde I, Shrimpton CN, Thiagarajan P, López J a. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood. 2005;106(5):1604–11.
112. Guiraud T, Gayda M, Juneau M, Bosquet L, Meyer P, Théberge-Julien G, et al. A Single Bout of High-Intensity Interval Exercise Does Not Increase Endothelial or Platelet Microparticles in Stable, Physically Fit Men With Coronary Heart Disease. Can J Cardiol. 2013;29(10):1285–91.
113. Wahl P, Jansen F, Achtzehn S, Schmitz T, Bloch W, Mester J, et al. Effects of High Intensity Training and High Volume Training on Endothelial Microparticles and Angiogenic Growth Factors. PLoS One [Internet]. 2014;9(4):e96024. Available from: http://dx.plos.org/10.1371/journal.pone.0096024
114. Babbitt DM, Diaz KM, Feairheller DL, Sturgeon KM, Perkins AM, Veerabhadrappa P, et al. Endothelial activation microparticles and inflammation status improve with exercise training in african americans. Int J Hypertens [Internet]. 2013;2013:538017. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3652180&tool=pmcentrez&rendertype=abstract

115. Green DJ, Maiorana A, O'Driscoll G, Taylor R. Effect of exercise training on endothelium-derived nitric oxide function in humans. J Physiol [Internet]. 2004;561(Pt 1):1–25. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1665322&tool=pmcentrez&rendertype=abstract
116. Augustine D, Ayers L V., Lima E, Newton L, Lewandowski AJ, Davis EF, et al. Dynamic release and clearance of circulating microparticles during cardiac stress. Circ Res. 2014;114(1):109–13.
117. Safar ME, Levy BI, Struijker-Boudier H. Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular diseases. Circulation. 2003;107(22):2864–9.
118. Sullivan MJ, Knight JD, Higginbotham MB, Cobb FR. Relation between central and peripheral hemodynamics during exercise in patients with chronic heart failure. Muscle blood flow is reduced with maintenance of arterial perfusion pressure. Circulation. 1989;80(4):769–81.
119. Van Craenenbroeck EM, Frederix G, Pattyn N, Beckers P, Van Craenenbroeck AH, Gevaert A, et al. Effects of aerobic interval training and continuous training on cellular markers of endothelial integrity in coronary artery disease: A SAINTEX-CAD substudy. Am J Physiol Hear Circ Physiol [Internet]. 2015;309(11):H1876–82. Available from: http://ajpheart.physiology.org/content/309/11/H1876
120. Pitha J, Králová Lesná I, Stávek P, Mahrová A, Racek J, Sekerková A, et al. Effect of exercise on markers of vascular health in renal transplant recipients. Physiol Res. 2015;64(6):945–9.
121. Stoumpos S, Jardine a G, Mark PB. Cardiovascular morbidity and mortality after kidney transplantation. Transpl Int. 2015;28(1):10–21.
122. Hausberg M, Kisters K, Kosch M, Rahn KH, Barenbrock M. Flow-mediated vasodilation and distensibility of the brachial artery in renal allograft recipients. Kidney Int. 1999;55(3):1104–10.
123. Rigatto C, Foley R, Jeffery J, Negrijn C, Tribula C, Parfrey P. Electrocardiographic Left Ventricular Hypertrophy in Renal Transplant Recipients: Prognostic Value and Impact of Blood Pressure and Anemia. J Am Soc Nephrol [Internet]. 2003;14(2):462–8. Available from: http://www.jasn.org/cgi/doi/10.1097/01.ASN.0000043141.67989.39
124. Rand ML, Wang H, Bang KW a, Packham M a, Freedman J. Rapid clearance of procoagulant platelet-derived microparticles from the circulation of rabbits. J Thromb Haemost [Internet]. 2006;4(7):1621–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16839364
125. Heran BS, Chen JMH, Ebrahim S, Moxham T, Oldridge N, Thompson DR, et al. Exercise-based cardiac rehabilitation for coronary heart disease. Cochrane Database Syst Rev. 2011;(7):1–73.
126. Pollock ML, Franklin BA, Balady GJ, Chaitman BL, Fleg JL, Fletcher B, et al. Resistance Exercise in Individuals With and Without Cardiovascular Disease. Benefits, Rationale, Safety and Prescription. Blood Press. 2000;8721(71):828–33.
127. Frühbeis C, Helmig S, Tug S, Simon P, Krämer-Albers EM. Physical exercise induces rapid release of small extracellular vesicles into the circulation. J Extracell Vesicles. 2015;4(1).
128. Safdar A, Tarnopolsky MA. Exosomes as Mediators of the Systemic Adaptations to Endurance Exercise. Cold Spring Harb Perspect Med. 2017;7(5).
129. Safdar A, Saleem A, Tarnopolsky MA. The potential of endurance exercise-derived exosomes to treat metabolic diseases. Nat Rev Endocrinol [Internet]. Nature Publishing Group; 2016;12(9):504–17. Available from: http://dx.doi.org/10.1038/nrendo.2016.76
130. Durrer C, Robinson E, Wan Z, Martinez N, Hummel ML, Jenkins NT, et al. Differential Impact of Acute High-Intensity Exercise on Circulating Endothelial Microparticles and Insulin Resistance between Overweight/Obese Males and Females. PLoS One [Internet]. 2015;10(2):e0115860. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4339732&tool=pmcentrez&rendertype=abstract
131. Toth B, Nikolajek K, Rank A, Nieuwland R, Lohse P, Pihusch V, et al. Gender-specific and menstrual cycle dependent differences in circulating microparticles. Platelets. 2007;18(7):515–21.
132. Ong KL, Tso AWK, Lam KSL, Cheung BMY. Gender difference in blood pressure control and cardiovascular risk factors in Americans with diagnosed hypertension. Hypertension. 2008;51(4 PART 2 SUPPL.):1142–8.
133. Fadini GP, De Kreutzenberg S, Albiero M, Coracina A, Pagnin E, Baesso I, et al. Gender differences in endothelial progenitor cells and cardiovascular risk profile: The role of female estrogens. Arterioscler Thromb Vasc Biol. 2008;28(5):997–1004.
134. Murakami T, Horigome H, Tanaka K, Nakata Y, Ohkawara K, Katayama Y, et al. Impact of weight reduction on production of platelet-derived microparticles and fibrinolytic parameters in obesity. Thromb Res. 2007;119(1):45–53.
135. Ceriello A, Taboga C, Tonutti L, Quagliaro L, Piconi L, Bais B, et al. Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: Effects of short- and long-term simvastatin treatment. Circulation. 2002;106(10):1211–8.
136. Lupattelli G, Lombardini R, Schillaci G, Ciuffetti G, Marchesi S, Siepi D, et al. Flow-mediated vasoactivity and circulating adhesion molecules in hypertriglyceridemia: Association with small, dense LDL cholesterol particles. Am Heart J. 2000;140(3):521–6.
137. Tushuizen ME, Nieuwland R, Scheffer PG, Sturk a., Heine RJ, Diamant M. Two consecutive high-fat meals affect endothelial-dependent vasodilation, oxidative stress and cellular microparticles in healthy men. J Thromb Haemost. 2006;4(5):1003–10.
138. Ferreira AC, Peter A a., Mendez AJ, Jimenez JJ, Mauro LM, Chirinos J a., et al. Postprandial hypertriglyceridemia increases circulating levels of endothelial cell microparticles. Circulation. 2004;110(23):3599–603.
139. Boden G, Rao a. K. Effects of hyperglycemia and hyperinsulinemia on the tissue factor pathway of blood coagulation. Curr Diab Rep. 2007;7(3):223–7.
140. Strohacker K, Breslin W, Carpenter K, Davidson T, Agha N, McFarlin B. Moderate-intensity, premeal cycling blunts postprandial increases in monocyte cell surface CD18 and CD11a and endothelial microparticles following a high-fat meal in young adults. Appl Physiol Nutr Metab. 2012;37(3):530–9.
141. Harrison M, Murphy RP, O'Connor PL, O'Gorman DJ, McCaffrey N, Cummins PM, et al. The endothelial microparticle response to a high fat meal is not attenuated by prior exercise. Eur J Appl Physiol. 2009;106(4):555–62.

142. Herd SL, Kiens B, Boobis LH, Hardman a. E. Moderate exercise, postprandial lipemia, and skeletal muscle lipoprotein lipase activity. Metabolism. 2001;50(7):756–62.
143. Koga H, Sugiyama S, Kugiyama K, Watanabe K, Fukushima H, Tanaka T, et al. Elevated Levels of VE-Cadherin-Positive Endothelial Microparticles in Patients With Type 2 Diabetes Mellitus and Coronary Artery Disease. J Am Coll Cardiol [Internet]. 2005;45(10):1622–30. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0735109705004821
144. Levinger I, Goodman C, Hare D. The effect of resistance training on functional capacity and quality of life in individuals with high and low numbers of metabolic risk factors. Diabetes [Internet]. 2007;30(9):2205–2210. Available from: http://care.diabetesjournals.org/content/30/9/2205.short
145. DeVan AE, Anton MM, Cook JN, Neidre DB, Cortez-Cooper MY, Tanaka H. Acute effects of resistance exercise on arterial compliance. J Appl Physiol. 2005;98(6):2287–91.

Acknowledgments
The research was supported by the National Institute for Health Research (NIHR) Leicester Biomedical Research Centre and the Stoneygate Trust. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Toll like receptor expression induced by exercise in obesity and metabolic syndrome: A systematic review

Isabel Rada[1], Louise Deldicque[2], Marc Francaux[2], Hermann Zbinden-Foncea[1*]

[1] Exercise Science Laboratory, School of Kinesiology, Faculty of Medicine, Universidad Finis Terrae, Santiago, Chile
[2] Institute of Neuroscience, Université catholique de Louvain, Louvain-la-Neuve, Belgium

ABSTRACT

Background: *Obesity and metabolic syndrome are disorders that correlate with the activation of pro-inflammatory pathways and cytokine production, to which Toll like receptors (TLR) contribute. Exercise may act as an anti-inflammatory modulator, but there is no consensus about the role of the TLR in this tuning.*
The present styudy aims to systematically review the current evidence on exercise-induced TLR regulation in animals and humans suffering from obesity and metabolic syndrome.
Methods: *Pubmed and Scopus databases were searched for publications from 1990 to September 2015. Search terms included: "Toll like Receptor", "TLR", "exercise", "obesity", "diabetes", and "metabolic syndrome". Elegibility criteria comprised: randomized control trials, cross-sectional and cohort studies; human or animal models with metabolic syndrome; any type of exercise; TLR expression measurement in any tissue by a clearly reported technique. The quality of selected studies was assessed using a modified version of the Downs and Black Quality Assessment Checklist. Data of study design; population; exercise type, timing and training elements; measurement technique, tissue analyzed and main outcome were extracted and categorized to facilitate data synthesis.*
Results: *17 studies were included, of which 11 publications obtained a high, 5 a moderate and 1 a low score for quality assessment. A total of 8 human studies were analyzed: 6 studies used endurance continuous or interval training protocols, 1 study resistance training and the remaining study was performed following a marathon race. Blood cells were analyzed in seven studies, of which four studies sampled peripheral blood mononuclear cells (PBMC), three analyzed whole blood and one study sampled skeletal muscle. Nine animal studies were included: 8 used endurance training and 1 acute aerobic exercise. A variety of tissues samples were explored such as PBMC, skeletal muscle, adipose, vascular and nervous tissue. Globally, the animal studies showed a marked tendency towards a down-regulation of TLR2 and 4 expression accompagnied with, a reduced activation of nuclear factor-kappaB (NF-κB) signaling and cytokine production, and an improvement in insulin sensitivity and body composition.*
Conclusion: *While animal studies showed a marked tendency towards TLR2 and 4 down-regulation after chronic endurance exercise, the current evidence in human is not sufficiently robust to conclude any role of TLR in the anti-inflammatory properties of exercise.*

Keywords: TLR2, TLR4, nuclear factor-kappaB, chronic inflammation, immunomodulation, cytokines, diabetes, metabolic disorders

INTRODUCTION

Overweight and obesity have become increasingly prevalent. According to the World Health Organization (WHO) approximately two billion adults are overweight and over half a billion are obese (37). Over half the adult population in developed countries is either overweight or obese (14). Obesity prevalence is directly associated to the country income level (37). As it is a main risk factor for metabolic co-morbidities related to premature death (32), obesity is a key public health concern. Associated endocrine and metabolic alterations lead to the development of the metabolic syndrome (MetS), a collection of medical conditions including insulin resistance, type 2 diabetes mellitus, dyslipidemia, hypertension, obstructive sleep apnea and non-alcoholic fatty liver disease (14).

Obesity is often associated with a chronic low-grade inflammation state caused by a pathological expansion of the adipose tissue, leading to elevated levels of free fatty acid (FFA). Activation of innate immune receptors, such as Toll like receptors (TLR), in obesity and MetS is possibly part of the chronic pro-inflammatory process (11). TLR are pattern-recognition receptors (PRR) expressed in different cell compartments. In mammals, there are 12 known members involved in detection and immune response to diverse exogenous signals such as pathogen-associated molecular patterns (PAMP), as well as endogenous ligands like damage-associated molecular pattern molecules (DAMP) involved in sterile inflammation induced by tissue damage and cellular stress (15). Within the recognized ligands, FFAs and endotoxin like lipopolysaccharides (LPS), activate I kappa B kinase (IKK), nuclear factor-kappa B (NF-κB) signaling (1) and also the mitogen-activated protein kinase (MAPK) pathway (12).

**Correspondence to:*
Hermann Zbinden-Foncea, PhD, School of Kinesiology
Universidad Finis Terrae, 1509 Pedro de Valdivia Av.,
Providencia, Santiago, CHILE
Phone: (+56) 2 2420 7226, Fax: (+56) 2 2420 7692
E-mail address: hzbinden@uft.cl

TLR activation may have an important protective role against viral infections when detecting gram- negative and gram-positive bacteria (1). However, in obesity conditions the majority of animal studies have reported increased TLR expression associated to a pro-inflammatory state (11). Inversely, suppression of TLR2 and TLR4 expression or their deficit reduces chronic inflammation, suggesting a pathogenic role for TLR in obese animal model (11). In humans with obesity and type 2 diabetes, elevated TLR activity has been correlated with the severity of insulin resistance (15). Exercise modulates TLR expression in monocytes, adipose tissue and skeletal muscle, which could be a molecular mechanism by which exercise exerts its anti-inflammatory effect (8). Reduced TLR4 expression at the cell surface of leukocytes has been found in young and old physically active individuals, in whom cytokine production following LPS stimulation was lower compared with sedentary participants (21). Moreover, exercise reduces plasma FFA levels, thereby decreasing TLR activity (32).

Exercise is a well-known tool to counteract the chronic pro-inflammatory state and the consequences of metabolic syndrome but the mechanisms are not totally elucidated, certainly when focusing on TLR signaling. Exercise is able to modify TLR expression in some conditions in both human and animal models (9). However, currently no consensus exists regarding exercise-induced TLR regulation. This mechanism possibly reduces the low-grade inflammation that often accompanied obesity and MetS. Hence, the present study aims to systematically review the current evidence on TLR expression in any tissue of either obese/MetS animals or humans undergoing an exercise program. We aim to better understand how exercise affects immune signaling and to set a precedent that could contribute to the development of guidelines related to physical activity that could be useful for clinical practitioners.

METHODS

This systematic review was conducted using the PRISMA statement for reporting systematic reviews (19).

Search strategy and study selection

In September 2015, the electronic databases PubMed and Scopus were queried. The following search terms were used as keywords: “Toll Like Receptor”, “TLR”, “exercise”, “obesity”, “obese”, “diabetes”, and “ metabolic syndrome” using the Boolean connectors AND/OR respectively. The query was limited to articles published between 1990 - September 2015 in English, Spanish, Portuguese and French.

The following criteria were applied: 1) Population type: human or animal with metabolic syndrome, e.g., obesity, diabetes, hyperglycemia, hypertension, dyslipidemia, and atherosclerosis (11). No age restriction was applied and all activity levels (active or sedentary) were admitted. 2) Study type: randomized control trials, cross-sectional studies and cohort studies. 3) Exercise type: any type of exercise, e.g., endurance and/or resistance training. 4) Outcomes type: all types of Toll like receptor expression, e.g., mRNA or protein in any tissue, measured by a clearly reported technique.

After removing duplicates, IR began the study selection process by screening titles and abstracts. Subsequently, the full text versions of the selected publications were reviewed in detail by IR and HZ to ensure the existence of metabolic syndrome in the studied subjects. Populations with pathologies unassociated to metabolic conditions were excluded. Alternative interventions in addition to exercise were admitted, such as pharmacological administration, TLR agonist, insulin clamps, and pathogen injection. The papers were discussed by IR and HZ and discrepancies were resolved in consensus.

Data collection and synthesis

IR extracted the data from the selected studies and HZ checked these data. The template used was classified by population type (human or animal) and sorted by the study characteristics (author, year and study type). The following information was provided: participants’ characteristics (sample number, sex-age distribution and metabolic disorder); exercise intervention type (e.g., endurance, or resistance); timing (e.g., acute or chronic); training elements (duration, frequency, intensity, volume, and mode); measurement technique; tissue analyzed; and main outcome. A qualitative synthesis was performed with the extracted data; the quantitative analysis was not feasible given the diverse analytical techniques to report the main outcome.

Quality assessment

The Downs et al. checklist (6) adapted by Munn et al. (23) was selected to measure the quality of the included (non)-randomized studies. The Munn’s list (2010) was complemented with items from the original list and some descriptions were adapted to be applicable to both human and animal studies based on the Gold Standard Publication Checklist to improve the Quality of Animal studies (10). In animals experiments, the characterization was evaluated by specific criteria (species, genetic background, origin and source) added in the third item of the checklist. The item 4 of the original Downs et al. checklist (1998) was re-included to obtain the duration, frequency, intensity, volume and mode of exercise; for animal studies, the time schedule, housing, nutrition and drug administration were also obtained (10). The confounding variables that had to be reported to score the item 5 in human studies were sex and age. In case of animal models, the weight at the beginning of the experiment had to be stated as well. To consider the number and characteristics of patients lost during follow-up, the original Item 9 of Downs et al. (1998) was reincorporated. The Item 11 was modified to include the assessesment of source, recruitment, and selection procedure. The items 12, 21 and 22 of Munn et al. (2010), for which a representative sample from the entire population is required, were discarded because the participants have to be screened to ensure the presence of metabolic disorder. For animal models, the sample size calculation had to be reported and had to include the minimum of animals required by the calculation (10).

The original Item 13 of the Downs et al. (1998) checklist was adapted and its description complemented to verify intervention supervision and the proper performance of the exercise. Item 15 of the Downs et al. (1998) checklist was incorporated to ensure that the data examiner was blinded from the charac-

teristics of the subjects tested (name, sex, age, and activity level). Item 16 of the Munn et al. (2010) list was omitted since the data dredging was relevant for the present study. Item 20 demands a standardized technique to be clearly described to ensure the validity and reliability of the main outcome. The original Item 24 of the Downs et al. (1998) checklist was re-included to ensure the randomization of the intervention groups. Item 25 demands separate analysis to ensure adequate adjustment for confounding analyses in the presence of significant differences in sex, age, or weight at the beginning of the trial. Supplementary 1 contains the checklist template adapted for this systematic review and the description of the items.

The Downs et al. (1998) scale was used to score each item. Questions were answered "YES" if the description requested was provided (score = 1); and "NO" (score = 0) when no report was given or was unclear. IR checked every item, filling a template with data extracted from the paper to support the assigned rate. The results were discussed with HZ to assign the final scores. Study quality was determined using Munn's scoring scale (23): positive answers were summed and converted to a percentage: high quality studies scored over 70%, moderate between 60-74%, and low quality scored under 60%.

Results

Literature Search

Querying both databases generated 477 records, of which 214 duplicates were removed. The remaining 263 titles and abstracts were screened. One hundred ninety-four did not meet the eligibility criteria: 1 by language, 1 by year of publication, 42 by study design, 134 by intervention (no exercise training), 16 by outcomes (no TLR expression report in analyzed tissue). Sixty-nine full texts were reviewed to confirm eligibility and the presence of metabolic syndrome in the studied populations, upon which 52 studies were excluded: 41 for being healthy, 2 due to chronic fatigue, 2 due to brain ischemia, 1 due to Alzheimer's disease, 1 due to lower back pain, 1 due to induced hypoxia, 1 due to severe liver injury, 1 due to myopathy, 1 due to myositis, 1 due to stroke. Seventeen records met the eligibility criteria and were included for qualitative synthesis (Figure 1).

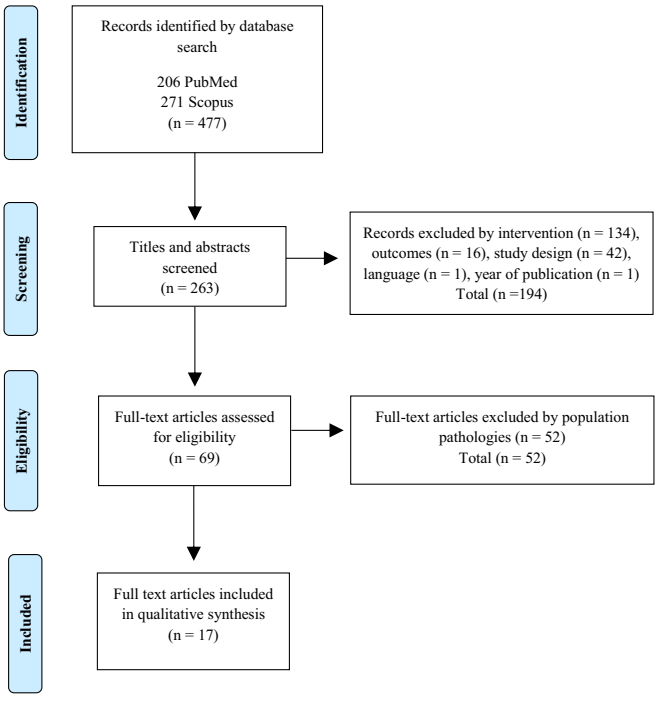


Figure 1. Search results through the review process

Quality assessment of studies included

Among the 17 studies, the quality scores ranged from 56 - 94% (Supplementary 2). Eleven publications obtained a high score, five a moderate and one a low score. All publications had clear descriptions of the hypothesis/objective. The main outcome to be measured, related to Toll like receptors and exercise was mentioned within the introduction or methods in 16 papers. The participant source and inclusion/exclusion criteria were reported in 5 of the 8 human studies. In animal studies, the origin, source, species and genetic background was mentioned in 8 of 9 studies. Exercise intervention type and its protocol was described in 7 of 8 human publications; while in animal studies this information was detailed with time schedule, housing and nutrition in 7 of 9 studies. The distribution of principal confounding variables such as sex and age were stated in 7 of 8 human studies; besides the distribution of these variables in animals, the initial weight was provided only in 3 of 9 studies.

All 17 studies described simple outcome data from findings associated with Toll like receptors and exercise interventions. The 17 papers reported estimates of random variability (standard error/standard deviation), probability values, and number/characteristics of patients lost during follow up. Of the 8 human studies, 4 papers did not report the participants´ source or selection procedure while the remaining four clearly described the selection process by screening. Four of the nine studies in animal calculated the sample size and included the minimum sample amount. The exercise interventions were supervised by an examiner or monitored in all 17 studies. Internal validity by blinding the examiner from participants' characteristics was reported in 3 of the 17 publications. Appropriate statistical analyses were performed in 9 of 17 studies. Toll like receptors expression measurement technique and the tissue analyzed was stated in all 17 studies. Randomization of the intervention groups was specified in 13 of 17 papers, and an adequate adjustment for confounding variables within the groups was performed in 11 of 17 publications.

Characteristics of included studies

Methods

The main characteristics of the 17 selected studies are presented in Table 1 and Table 2. All 17 studies were intervention studies, of which 12 were randomized control trials (RCTs), 4 non-randomized control trials (NRS) and one quasi randomized trial (qRCT). All publications were in English and published between 2010 and 2015. Eleven studies were from the Americas: 7 from the United States, 3 from Brazil, and one from Canada. The remaining 7 studies were European or Asian: 3 from Germany, 3 from China and 1 from Japan.

Table 1: Summary of the 8 human studies included in the systematic review

Human studies							
Reference	**Study type**	**Population types and conditions**	**# Subjects, gender and age**	**Exercise type**	**Timing/duration of intervention and intensity**	**Analytical technique/ blood cell type or tissue**	**Primary outcome**
Liu et al, 2015(20)	RCT	• Type 2 diabetic (AR/DCT) • Healthy control subjects (CTL)	Total: n=62 • AR+DCT: n=42, 30F + 12M, 52.6y • CTL: n=20; 13F + 7M, 51.2y	Chronic endurance and resistance training with elastic band	• AR: aerobic ex 12 weeks, 3d/wk, 10-30 min at 40-60% VO_2max + resistance ex 2-3d/wk, 2 sets of 8-10 repetitions at 50-60% 1RM • DCT: drug therapy and diet control • CTL: No intervention	• Real-time quantitative PCR • Western blot • PBMC (Mononuclear blood cells)	• Before treatment: higher TLR4 mRNA and protein expression levels in AR and DCT than in CTL. • After exercise intervention: TLR4 mRNA and protein expressions were significantly lower in AR group than DCT group.
Coen et al, 2010(5)	RCT	• Hypercholesterolemic physically inactive subjects (R/RE) • Hypercholesterolemic physically active subjects (ACTL)	Total: n=49 • R: n=17, 9M + 8F, 52.1y • RE: n=16, 7M + 9F, 52.2y • ACTL: n=16, 7M + 9F, 51.6y	Chronic endurance (treadmill and walking) and resistance training (leg press, leg extension, leg curl, chest press, lat pull-down, seated row, leg adduction, and leg abduction).	• R: 20 weeks of rosuvastatin calcium (10mg/d) treatment • RE: same as R with the last 10 weeks including ex training (3d/wk). A session consisted in 20min at 60-70% of HR reserve + 2 sets of 8 resistance exercises at 80% 1RM • ACTL: No intervention	• Color flow cytometry • Whole blood	• Higher TLR4 expression after treatment in R. • Percentage of $CD14^+$ monocytes expressing TLR4 was not different between and within R, RE and ACTL throughout the study. • More TLR4 expressed in pro-inflammatory than classic monocytes.
Lambert et al, 2008(16)	RCT	• Sedentary obese elderly subjects • AR/DCT	Total: n=16 • DCT: n=8, 4F + 4M, 69.6y • AR: n=8, 4F + 4M, 68.5y	Chronic endurance (treadmill, step-ups, climbing, stationary cycling, stair-master exercise) and resistance training (squats, leg press, knee extension, knee flexion, row, seated chest press, biceps curl, and triceps extensions)	• AR: 12 weeks (3d/wk), endurance ex for 20min at 75-90% HRpeak + 30-40min resistance ex at 65-80% 1RM (6-12 repetitions) • DCT: 12 weeks of weight loss therapy (energy deficit diet and behavior therapy)	• Real-time quantitative PCR • Muscle vastus lateralis	• AR reduced TLR4 mRNA. • Expression remained unchanged in DCT.
Nickel et al, 2011(25)	NRS	• Obese amateur marathon runners (ONE) • Lean non elite (LNE) • Lean elite (LE)	Total: n=47, M, 40y • ONE: n=15 • LNE: n=16 • LE: n=16	Chronic endurance training	• 10 weeks (4d/wk) of continuous endurance and interval training • Intensity given by individual target HR • ONE and LNE: 40km/wk; LE: 55km/wk	• Real-time quantitative PCR (all groups) and western blot (only in LNE) • PBMC	• Before training: TLR2, 4 and 7 mRNA: no differences between the groups. • After training: higher TLR2 mRNA in LNE, up-regulation of TLR4 and 7 mRNA in all groups. • No exercise-induced changes in MyD88mRNA gene-expression between the groups. • In LNE, increase in TLR4 and 7 protein expression after training, no change in TLR2 and MyD88. No protein analysis in ONE and LE.
Robinson et al, 2015(33)	RCT	• Sedentary pre-diabetic subjects • HIIT/MICT	Total: n=38 • HIIT: n=20, 17F + 3M, 52y • MICT: n=18, 14F + 4M, 52y	Chronic endurance training (cycle ergometer, treadmill, outdoor walking, elliptical training)	• HIIT: 10 sessions spread over 2 weeks consisting in 4-10 repetitions of 1-min intervals at 85-90% Wpeak and 1-min rest at 20% Wpeak • MICT: 10 sessions spread over 2 weeks consisting in 20-50 min of continuous training at 32.5% Wpeak	• Flow cytometry • Whole blood	• Decreased TLR4 expression in lymphocytes and $CD14^+$ monocytes in both MICT and HIIT. • Decreased TLR4 expression in $CD15^+$ neutrophils only in MICT, no change in HIIT. • TLR2 expression was reduced in both MICT and HIIT in lymphocytes but not in monocytes and neutrophils.

Reyna et al, 2013(31)	NRS	• Sedentary and lean subjects (LE) • Obese (OB) • Type 2 diabetic (T2DM)	Total: n=36 (no gender distribution reported) • LE: n=17, 39y • OB: n = 8, 40y • T2DM: n=11, 50y	Chronic endurance training (cycle ergometer)	• 40min/d of endurance ex for 15 consecutive days • Each session consisted in 4 x 8min at 70% VO_2peak followed by 2min of rest • First session within the week after insulin clamp, which was repeated 36h after last session	• Western blot • PBMC	• Before intervention higher TLR4 protein content in T2DM and OB. • After ex training and insulin infusion: no change in TLR2 and 4 expressions in any group.
Nickel et al, 2012(24)	NRS	• Obese amateur marathon runners (ONE) • Lean non elite (LNE) • Lean elite (LE)	Total: n=47, M, 40y • ONE: n=15 • LNE: n=16 • LE: n=16	Acute endurance bout (marathon)	• Marathon race	• Real-time quantitative PCR (all groups) and western blot (only in LNE) • PBMC	• Immediately after race: no change in TLR2 mRNA levels in any group, down-regulation in TLR4 in only LNE group, TLR7 mRNA levels decreased in all groups. No change in TLR2, 4 and 7 protein in LNE, no protein analysis in LE and ONE. • 24 hours post-race: no change in TLR2 mRNA levels in any group, increase in TLR4 and 7 mRNA levels in all groups. Only TLR7 protein expression decreased in LNE, no protein analysis in LE and ONE.
Philips et al, 2012(28)	qRCT	• Obese post-menopausal women intervention (RE) • Obese post-menopausal women control (OCTL)	Total: n=23, F • RE: n=11, 64.8y • OCTL: n=12, 66.4y	Chronic resistance training (chest press, shoulder press, and seated row or cable press, abdominal crunches, and back extensions)	• RE: 12 weeks (3d/wk), 3 sets of 10 repetitions at 100% of 8RM • OCTL: stretching, health education and safety talks • Test: 1 week after last session: a single resistance bout with 3 sets of 10 exercises at 8 RM with 1.5min recovery	• Real-time quantitative PCR • Whole blood • Subcutaneus adipose tissue (SCAT)	• TLR4 mRNA expression in whole blood was not different between the groups after interventions. • After RE intervention: TLR4 mRNA expression remained unchanged in SCAT. • Total volume load was negatively correlated to TLR4 mRNA in SCAT. • RE improved body strength and reduced circulating CRP, leptin and TNF levels

RCT: Randomized Control Trial; NRS: Non-Randomized Control Study; qRCT: quasi-Randomized Control Trials; ex: exercise; min: minutes ; wk: week; d: day; h: hour; HR: heart rate; Wpeak: peak power output; VO2max: maximum oxygen consumption; VO_2peak: peak oxygen uptake; 1RM: One Repetition Maximun; m/min: meters per minute; PBMC: peripheral blood mononuclear cells; TLR: Toll Like Receptor; mRNA: Messenger RNA; CRP: C-Reactive Protein; TNF: Tumor Necrosis Factor; MyD88: Myeloid differentiation primary response gene 88.

Table 2: Summary of the 9 Animal studies included in the systematic review

Animal studies							
Reference	**Study type**	**Population types and conditions**	**# Animals, sex and age**	**Exercise intervention type**	**Timing/duration of intervention and intensity**	**Analytical technique/ blood cell type or tissue**	**Primary outcomes**
Li et al, 2015(18)	RCT	• Sprague Dawley rats • Diet induced obesity • ND/ND-EX/DIO-S/DIO-EX	Total: n=122, male no age reported • RD-S: n=31 • RD-EX: n=29 • DIO-S: n=30 • DIO-EX: n=32	Chronic endurance (treadmill running)	• ND: regular diet • S: sedentary • DIO: high fat diet for 12 weeks • EX: 8 weeks, 5d/wk, 60min/session, 18m/min	• Western blot • Muscle soleus • Muscle ventricle	• Higher protein and mRNA levels of TLR2 and 4 in soleus and ventricule of DIO-S versus ND-S and DIO-EX. • TLR2 and 4 protein and mRNA levels were lower in DIO-EX compared with ND-EX and DIO-S.

Wu et al, 2014(38)	RCT	• ApoE null C57BL/6J mice • Diet induced obesity • AS/AD/AEX	Total: n=36 • 10 week old male mice • no specific amount of mice per group	Chronic endurance training (treadmill)	• AS: atherosclerotic control mice • AD: 12 weeks of 10mg/kg simvastatin treatment • AEX: 12 weeks, 5d/wk, 60min/session, 13m/min (zero slope)	• Immunohistochemistry • Vascular tissue	• TLR4 protein expression was increased in AS. • TLR4 protein expression reduced in AD and AEX groups, with a larger reduction in AD compared with AEX. • Increased expression of miR-146a in both AD and AEX.
Carpenter et al, 2012(3)	RCT	• CD1 mice • Diet induced obesity • CTL-DIO/FEX/VEX and CTL non DIO	Total: n=24 • 6 male mice/group • 2-14 weeks at start of DIO (CTL-DIO/FEX/VEX but not CTL non DIO), 64-66 weeks at start of low-fat diet (LFD, 4 groups) + EX (FEX/VEX) for 8 weeks	Chronic endurance training (forced-treadmill running and voluntary wheel running)	• CTL-non DIO: normal diet, no exercise • CTL-DIO: induced obesity, no exercise • FEX: 8 weeks, 5d/wk (Monday to Friday), forced treadmill running, 60min at 12 to 20 m/min • VEX: 8 weeks, 5d/wk, (Monday to Friday) voluntary wheel running with 24h access	• Flow cytometry • PBMC (Monocytes blood cells)	• Before LFD, the expression of TLR4 in CTL non DIO was higher than in DIO. • After LFD, TLR2 expression was lower in VEX than in the other 3 groups. TLR4 expression was lower in VEX than in CTL non DIO and FEX.
Kawanishi et al, 2010(13)	RCT	• C57BL/6 mice • Diet induced obesity • non DIO/non DIO-EX/DIO/DIO-EX	Total: n=38, 4 wk-old • ND: n=7 • ND-EX: n=7 • DIO: n=12 • DIO-EX: n=12	Chronic endurance training (treadmill running)	• ND: regular diet with standard chow • DIO: high fat diet for 16 weeks • EX: 16 weeks, 5d/wk, 60min/session, 12-20m/min	• Real-time quantitative PCR • Adipose tissue	• TLR4 mRNA levels varied as an effect of diet and exercise training alone as well as diet combined with exercise. • Differences in TLR4 expression between ND vs DIO and DIO vs DIO-EX. • Down-regulation of TLR4 mRNA levels after DIO-EX compared with DIO.
Esposito et al, 2010(7)	RCT	• Outbred CD-1 mice • Diet induced obesity • DIO/DIO-EXE/ND	Total: n=36 • 12 male mice/group • 6-8 week-old	Chronic endurance training (treadmill running)	• DIO: high fat diet for 6 weeks • DIO-EXE: 6 weeks of DIO + ex as follows: 5d/week, 1h/session, 21-22 m/min, 1% grade • ND: normal diet with standard chow, sedentary	• Flow cytometry • intensity • PBMC (Monocytes blood cells)	• At the end of the intervention, DIO expressed more TLR4 than DIO-EXE and ND, with no difference between the latter two.
Zhou et al, 2011(39)	NRS	• C57BL/6J mice • Diet induced obesity • ND/DIO/OB-ND-EX	Total: n=20 • 4 mice/group • 6 week-old • no sex distribution	Chronic endurance training (treadmill)	• ND: standard chow • DIO: long-term high fat diet induced obesity • DIO-ND-EX: long-term high fat diet and switch to normal diet plus exercise for 4 weeks (5d/wk, 1h/session, 12m/min)	• Real-time quantitative PCR • Bone marrow-derived macrophages	• Lower TLR2 but higher TLR4 mRNA levels in DIO and DIO-ND-EX compared with ND, with no difference between DIO and DIO-ND-EX. • Exercise did not reverse the decrease in TLR2 mRNA levels in response to infection in DIO mice.

Oliveira et al, 2011(27)	RCT	• Wistar rats • Diet induced obesity • ND/DIO/DIO-EX/DIO-AEX	Total: n=40 • 8 week-old male rats • 10 rats/group	Chronic and acute endurance training (swimming)	• ND: normal diet with standard chow • DIO: high fat diet for 20 weeks • EX: 8 weeks, 5d/wk, 1h/session, progressive load increase up to 5% of body weight • AEX: 2 moderate bouts of 3 hours separated by one rest period of 45 min	• Real-time quantitative PCR and western blot • Skeletal muscle, liver and adipose tissue	• TLR4 protein expression was higher in DIO than in ND in all tissues. • After chronic training: DIO-EX group had decreased mRNA and protein TLR4 levels compared with DIO in all tissues. • After acute exercise: no changes in TLR4 protein expression in all tissues. Decrease in TLR4 mRNA levels only in skeletal muscle. • Reduction in TLR4/MyD88 interaction in both EX and AEX compared with DIO sedentary rats.
Ropelle et al, 2010(34)	RCT	• Wistar rats • Diet induced obesity • ND/DIO/DIO-EX	Total n=32-40 • 8-10 male rats/group • ND/DIO/DIO-EX: 4 week-old	Chronic and acute endurance training (swimming and treadmill)	• ND: normal diet with standard chow • DIO: high fat diet for 12 weeks • DIO-EX-A: acute single bout of 60min at 10-15m/min with 5% inclination. • DIO-EX-C: 4 weeks, 5d/wk, 1h/session, 2% body weight overload	• Western blot • Hypothalamus tissue	• DIO induced an increase in TLR4 expression. • Acute exercise did not reduce TLR4 expression in DIO. • No TLR expression report after chronic training, but reduced IKKb phosphorylation and increased IκBα expression in the hypothalamus of obese rats.
Oliveira et al, 2013(26)	RCT	• Wistar rats • Diet induced obesity • ND/DIO/DIO-EX	Total: n=24 • 8 male rats/group • 6 week-old	Acute endurance bout (swimming)	• ND: normal diet with standard chow D • DIO: high fat diet for 12 weeks • DIO-EX: 2 moderate bouts of 3h separated by one rest period of 45 min	• Western blot • White adipose tissue	• Higher interaction in TLR4/MyD88 in DIO compared with ND. • DIO-EXE reduced this interaction compared with DIO.

RCT: Randomized Control Trial; NRS: Non-Randomized Control Study; DIO: diet-induced obesity; ex: exercise;min: minutes; m: meter; wk: week; d: day; h: hour; HR: heart rate; m/min: meters per minute; ApoE: Apolipoprotein E; TLR: Toll Like Receptor; mRNA: Messenger RNA; TNF: Tumor Necrosis Factor; MyD88: Myeloid differentiation primary response gene 88; IKKb: I kappa B kinase b; IκBα: inhibitor of nuclear factor-kappa B

Participants

The included publications comprised 271 human subjects: 131 female and 104 male subjects. One study of 36 participants did not specify gender. Age ranged between 39 and 69.6 years. Five of the eight studies included obese subjects, two of which were based on an elderly population, two on obese amateur runners, and one on type 2 diabetic participants. One study was based on subjects solely diagnosed with type 2 diabetes. Another one set pre-diabetes as inclusion criteria, and the last one studied subjects with hypercholesterolemia. Two studies used the same sample of subjects and thus only one sample set was included (24). The inclusion of 348 animals was clearly reported, however one study did not specify the accurate total sample number, which approximately comprised 32 to 40 animals (4 groups comprising 8-10 animals per group). The animals' age ranged from 4 to 66 weeks at the start of the training intervention. All animal studies used high fat diet induced obesity as a metabolic condition. Moreover, one study induced atherosclerosis.

Human studies

Of the 8 selected human studies, 7 used an endurance exercise training protocol. Chronic long-term endurance protocols were applied in 4 studies, lasting 10-12 weeks, at a frequency of 3-5 days per week. Training sessions lasted initially 10 minutes and continued for another 20-50 minutes. The intensity was set between 60-70% of heart rate reserve (5), 40-60% of maximum oxygen consumption (VO_2max) (20) or 75-90% of peak heart rate (16). One study did not state the training

session duration, only that intensity was gradually increased up to an individual target heart rate (25).

Moreover there were two short-term intervention studies with 10 and 15 sessions of endurance exercise distributed over two weeks. One study was designed with two groups performing either 20 minutes of continuous exercise or intervals (33). The other short endurance protocol study included four cycles of 8:4 work ratio (31). These endurance training sessions were performed on a treadmill, elliptical trainer, step-ups, cycle ergometer, or by climbing stairs or walking outdoor. Furthermore, other exercise types such as marathon race were used to evaluate obese amateur runners (24). Resistance training combined with endurance exercise sessions was used in three studies (5, 16, 20). Muscle strengthening was performed using free weights or elastic bands, and the intensity was set between 50-80% of one repetition maximum (1RM). Finally, one remaining study exclusively used resistance training for 12 weeks with a frequency of 3 days per week at an intensity corresponding to 8RM. The participants completed two sets of 8 repetitions and the last set to fatigue on 10 different types of weight machines (28).

Animal intervention studies

Eight of the nine animal studies used chronic endurance training. Intervention duration ranged from 4 to 16 weeks, with a frequency of 5 days per week. The majority of the exercise sessions lasted 1 hour per day (3, 7, 13, 20, 27, 34, 38, 39). One study included a group performing voluntary wheel running (3). When exercise was imposed, the workload was set at 12 to 20 m/min on a treadmill (3, 7, 13, 18, 38, 39). Supervised chronic swimming exercise was performed with a load equal to 2% (34) and 5% of body weight and progressively increased (27). In those studies, measurements were also made after an acute bout of exercise: after 1 hour at 10–15 m/min with 5% of body weight (34) or following 2 swimming bouts of 3 hours separated by 45 minutes recovery (27). The latter protocol was also used in a study using acute aerobic exercise only (26).

Analytical technique/blood cell type or tissue

Different standardized techniques were used to analyze TLR expression. Of the human studies, five used real time quantitative polymerase chain reaction (qPCR) (16, 20, 24, 25, 28), two used color flow cytometry (5, 33) and three western blotting (24, 25, 31). Blood cells were analyzed in seven studies, of which four studies sampled peripheral blood mononuclear cells (PBMC) (20, 24, 25, 31), three studies sampled whole blood (5, 28, 33). Moreover, other tissues such as subcutaneous adipose tissue (28) and muscle vastus lateralis (16) were sampled in the remaining two human studies.

In the animal studies, four studies used western blot (18, 27, 34), three qPCR (13, 27, 39) and three color flow cytometry (3, 7), or immunohistochemistry (38). Blood was sampled in two studies analyzing PBMC (3, 7), whereas three assessed the adipose tissue (13) (26, 27) and two the skeletal muscles (18, 27). The remaining studies sampled the hypothalamus (34), liver, bone marrow-derived macrophages (39), and vascular tissue (38) and ventricle muscles.

Secondary outcomes

Besides determining TLR expression in control conditions and after exercise, additional measurements were taken in the 17 studies. The following assessments were made with regard to metabolic syndrome risk factors: body mass index (BMI) (5, 16, 24, 25, 28, 31, 33), waist circumference (24, 25), fat free mass (FFM) (16), fat mass (FM) (5, 13, 16, 24, 25, 27, 28, 34), epididymal white adipose tissue (18), blood pressure (18, 25, 33), fasting plasma glucose (20, 26, 27, 31, 33, 34, 39), insulin concentration (20, 26, 31, 33, 34), triglycerides (5, 20, 27, 34), cholesterol (5, 20, 34, 38), LDL (5, 20, 24, 25, 38), HDL (20, 38), serum glycosylated hemoglobin (20, 31, 33), NEFA (27, 31, 33, 34, 39), etc. Additionally, inflammatory markers as tumor necrosis factor (TNF-α) (3, 13, 16, 25, 26, 28, 31, 33, 38, 39), interleukin 1β (26, 33, 39), IL-6 (13, 16, 24, 25, 27, 28, 33, 38, 39), IL-8 (20), activation of mitogen-activted protein kinases (MAPK) (26, 27, 31, 34), C-reactive protein content (CRP) (5, 16, 24, 25, 31, 38), monocyte chemo attractant protein-1 (MCP-1) (3, 13, 26, 28, 38) and activation of NF-κB (20, 31, 34, 38, 39) were also assessed.

Synthesis of main results

Human models

Previous to exercise interventions, upregulation of TLR4 mRNA and protein expression in mononuclear cells of type 2 diabetic patients has been reported (20). Similarly, higher basal TLR4 protein content in PBMC has also been described in obese diabetic sedentary subjects (31). In trained subjects, the pre-intervention TLR2, 4 and 7 mRNA levels were the same for obese runners as lean elite and non-elite marathon athletes (25).

Regarding the effects of long term interventions, twelve weeks of combined endurance and resistance exercises elicited significant reductions in TLR4 mRNA levels (Lambert 2008) and both mRNA and protein expressions (Liu 2015). These combining interventions produced larger decrease in TLR4 expression in mononuclear cells (20) and vastus lateralis (16) than drug and diet control therapies. In a third study, 10mg/d of rosuvastatin, was administered for 20 weeks, alone or in combination with endurance and resistance exercises (5). The sedentary group showed a higher TLR4 expression on CD14+ monocyte surface after rosuvastatin chronic intake compared to pre- ingestion values. Moreover, there were no significant differences in TLR4 surface expression between physically active control subjects and those combining rosuvastatin treatment and exercise training over the course of the study (pre- vs. mid- vs. post-time measurements) (5). Endurance training in marathon runners significantly increased TLR2 mRNA levels in dendritic cells of non-elite runners in comparison to their basal pre-intervention values, whilst TLR4 and TLR7 mRNA levels were up-regulated in obese, non-elite and elite runners (25). Long-term resistance training did not change TLR4 mRNA level in whole blood samples in obese post-menopausal women, compared with sedentary controls (28).

Short-term endurance protocols such as 15 days of interval training did not induce changes in TLR4 or TLR2 protein con-

tent in peripheral blood mononuclear cells in obese, diabetic or lean participants, when compared with their baseline values (31). High interval training and continuous aerobic training protocols showed different results depending on the blood cell analyzed (33). TLR4 surface expression was reduced on lymphocytes and CD14+ monocytes vs. pre-exercise expression, with no significant differences between interventions; while the surface expression in CD15+ neutrophils was only reduced by continuous exercise. Surface TLR2 lymphocyte expression decreased with either interval or continuous training compared with pre-exercise values; although it was not the case for monocytes and neutrophils (33).

Immediately after a marathon, in peripheral blood mononuclear cells, TLR2 mRNA levels remained unchanged when compared to pre-exercise levels in the three included groups: elite, lean non-elite and obese runners (24). TLR4 mRNA was down-regulated in lean non-elite only and TLR7 mRNA was reduced in all groups. Twenty-four hours after the race, TLR2 mRNA level was still unchanged, whilst TLR4 and TLR7 mRNA levels increased in all groups compared to baseline. TLR2-4-7 protein expression was only analyzed in lean non-elite runners and the only difference found was a decrease in TLR7 24h after marathon vs. pre-exercise values (24).

Animal models

A high fat diet resulted in an increase in TLR4 and MyD88 interaction in white adipose tissue when compared with standard chow (26). Furthermore, an increased TLR4 expression was reported in monocytes, adipose tissue, soleus, ventricle muscle, vascular tissue and bone derived macrophages of obese animals compared to their respective controls (7, 13, 18, 27, 34, 38, 39).

An endurance training of 6 weeks consisting in running on a treadmill resulted in less TLR4 cell surface expression on monocytes of animals consuming a high fat diet vs. their sedentary controls (7). Twenty-week old mice were fed with a high fat diet for 50 weeks. When reaching 70 weeks, the mice started an eight-week intervention consisting in 24h access to voluntary wheel running, coupled to low fat diet. This intervention produced lower TLR2 cell surface expression on monocytes than mice receiving a normal diet, sedentary mice or mice subjected to 60 min of forced running (3). Similarly, at the end of the intervention, voluntary exercised mice had the lowest TLR4 expression at monocyte surface compared to the 3 other groups (3). Protocols of similar duration also induced decreased mRNA and protein levels of TLR 2-4 in the soleus and ventricle muscles (18) and reduced mRNA and protein levels of TLR4 in liver, skeletal muscle and adipose tissue (27) of obese exercising compared to obese non exercising animals.

Long-term endurance training reduced TLR4 mRNA levels in obese animals compared with sedentary obese controls. Similarly, active or sedentary animals fed with standard chow did not increase TLR4 mRNA levels, thus these effects seemed to be additive as assessed by the interaction exercise x diet (13). Moreover, the reduction of TLR4 surface expression in obese animals after long term exercise interventions has been also related to modification on vascular miRNA-146A levels, in which its increment reduces chronic inflammation (38).

Compared to lean animals, TLR2 mRNA was lower, whilst TLR4 mRNA levels were higher, in macrophages of mice subjected to high fat diet (39). A short duration endurance training was not able to reverse those changes (39). Similar results were obtained while TLR2 mRNA decrease was induced by Porphyromonas gingivalis infection in obese mice (39). TLR4 protein expression in liver, adipose tissue and skeletal muscle remained unchanged in comparison to pre-exercise values in obese animals submitted to two acute bouts of moderate intensity exercise, whilst TLR4 mRNA expression was decreased, but in skeletal muscle only (27). After the same protocol, a decreased TLR4/MyD88 interaction has been reported in adipose tissue (26). Finally, TLR4 expression in the hypothalamus of obese rats remained constant after a single bout of treadmill running compared to pre-exercise values (34).

DISCUSSION

The findings of the present systematic review revealed a variety of responses of TLR mRNA, protein and cell surface receptor expression following exercise in humans or animals with obesity/MetS. The animal studies showed a marked tendency towards TLR2 and 4 mRNA, protein down-regulation and a reduction in TLR4/MyD88 interaction after endurance training. In humans, evidence is not sufficiently robust to determine the regulation of TLR expression after chronic or acute exercise. Multiple interventions types were conducted including endurance, resistance or a combination of both under diverse durations, frequencies, intensities, volumes or modes. Long-term endurance training for a marathon increased TLR mRNA levels according to the activity level and body composition of the athletes (25), which was also reflected after the marathon event (24). Furthermore chronic interventions of combined endurance and resistance exercises resulted in reductions of TLR4 mRNA and protein levels (16, 20), whereas no differences in TLR4 surface expression were observed when chronic endurance and resistance training were also combined with rosuvastatin intake(5). Moreover, long-term resistance protocol did not induce different TL4 mRNA expression from sedentary conditions (28). Short-term interventions elicited conflicting results, while in one study TLR2 and 4 protein content remained constant after interval training (31), another study performing either interval or continuous exercise, resulted in a different TLR surface expression depending on the blood cell analyzed (33).

Nevertheless, the baseline TLR mRNA and surface protein expression on mononuclear cells was consistently high in the obese and type 2 diabetic compared to healthy sedentary subjects. Interestingly, in obese physically active subjects the expression of TLR2, 4 and 7 mRNA expressions did not differ from the lean athletes, which was related to a lower pro-inflammatory state compared to obese sedentary subjects. Those general outcomes may be influenced by important factors that will be discussed below.

Intervention heterogeneity

There is a considerable heterogeneity within the human interventions. Differences in endurance protocols either by longer

(5, 16, 20, 25) or shorter duration (Robins 2015, Reyna 2013), lead to variances in the amount of exercise performed, similarly with the variety given by the type of endurance exercise used as continuous or intermittent (31, 33). Meanwhile, some training protocols were also accompanied with resistance protocols (5, 16, 20). Short term protocols, completed in 2 weeks (31, 33), elicited conflicting results that could have been influenced by differences in intensity and training mode where continuous exercises could have greater impact than intermittent protocols (33). In addition to the previous variations, acute strenuous endurance exercise (24) and sole resistance interventions were also executed (28) and could have been influenced by the training status of the subjects. After a marathon, the responses of TLR expression in peripheral blood cells were influenced by the acute intervention itself (24) and probably also by the chronic long-term training needed to complete such as strenuous exercise (25). The same holds true for acute resistance exercise (28) and the influence of training background (5, 16, 20) if not controlled. Lastly, sole resistance training failed to induce changes of TLR expression in whole blood and sub-cutaneous adipose tissue (28).

The studies conducted in animals used endurance training protocols that globally resulted in TLR down-regulation (3, 7, 13, 18, 26, 34), whereas acute interventions (26, 34) tended not to modify TLR expression. In addition to duration, other aspects may influence the outcomes between the selected studies, such as: switches in the dietary regimen (39), statin intake (e.g. rosuvastatin) (5) or induced infection (39). These factors lead to increased heterogeneity in the intervention protocols that could impact the immune response to exercise and should therefore be considered in the interpretation of the results.

Sample heterogeneity

The first aspect of heterogeneity was related to the participants' characteristics. The human studies included adults (24, 25, 31) and elderly subjects (5, 16, 20, 28, 33) of both genders or solely female/male groups. Similarly, the animal studies included male mice (3, 7, 13, 38, 39) or rats (18, 26, 27, 34) with specific genetic backgrounds and with an age varying between 4 to 66 weeks at the beginning of the study. Hence, age could have influenced the response of TLR to exercise but the literature reports conflicting results from comparisons between young and older participants (35) and animals (2, 30). Therefore, at this stage, there is no conclusive dependent effect of age on TLR levels and further research is needed.

Another relevant issue is the tissue analyzed and the level of expression of TLR, i.e. mRNA and/or protein. In human studies, PBMC (20, 24, 25, 31) and whole blood (5, 28, 33) are the samples used in the majority of the studies whereas only one reported analysis in skeletal muscle (16). Differences in TLR expression has been reported in different PBMC, i.e. monocytes, neutrophils and lymphocytes, emphasizing that the sole measure of monocytes is not representative of the total circulating immune cells (33). The measure of TLR in human skeletal muscle has been less explored while its detection might be relevant to reveal direct effects of exercise on TLR expression (16). In addition, the report of the specific localization of TLR appears to be important since it can be located on the cell surface or be internalized into endosomes (22). Within the selected studies, only two used color flow cytometry and could clearly report the surface localization (5, 33). Although specific to the type of TLR tested (4), knowing the localization in more studies would improve the thoroughness of the methodology in the field and therefore contribute to a better understanding of the regulation of TLR and of the immune system in general.

Methodological quality

Regarding the quality of the selected studies, the majority was of high quality, a few had moderate and only one had a low score. The above indicates a proper reporting of methodological aspects that leads to a better understanding and reliability of the outcomes of the studies. However, there were some items that were not optimally described and should be considered for further research. Half of the human studies did not fully describe the participants' characteristics or the selection procedure, which renders difficult the precise characterization of the population and the comparison between studies. Another inconsistency was found in the statistics used in both human and animal studies, in which parametric methods were used without reporting normality. Following the arguments of Weissgerber et al (36), parametric methods are appropriate only if symmetrical distribution was reported. An additional aspect missed in the vast majority of the studies was the report of blinding the examiner from participants' characteristics, which is necessary to ensure a suitable methodology. Based on the Gold Standard Publication Checklist to improve the Quality of Animal studies (10), there was an overall adequate report in the animals studies. However, in a few studies, essential information like weight at the start of the experiment and/or description of sample size calculation was not available. It was, therefore not possible to determine whether the amount of animals used reached the minimum required for the main outcome.

Clinical perspective

Obesity and MetS are characterized by a low grade inflammation state, associated with endocrine and metabolic disorders (14). This low grade inflammation state increases the risk of death and co-morbidities which are a major public health concern. Chronic exercise has the capacity to counteract, at least partially, a pro-inflammatory state through reduced activation of the pro- inflammatory pathway and cytokine production (29). Studies in the present review reported reduced activation of IKK and NF-κB signaling after exercise training (16, 20, 26, 27, 34, 38, 39). This reduction in NF-κB signaling was also accompanied by lower levels of TNF-α (5, 13, 25-28, 38, 39), CRP (5, 28, 38), MCP-1 (26, 39), IL1β (39) and IL8-33 (20). In addition, IL6 was reduced after endurance training protocols (16, 27, 38, 39), but an acute increment was also reported as a result of muscle contraction following resistance training (28) and acute aerobic bouts (Ropelle et al., 2010, Nickel et al., 2013). Furthermore, in both trained human and animal studies, a reduction of MAPK activation, i.e. ERK and JNK phosphorylation, was described (26, 27, 31), which reflects the influence of exercise on the downstream signals of the TLR pathway.

Insulin resistance is one of the major complications provoked by metabolic disorder while exercise acts as an immunomodulator that can delay its aggravation and the appearance of type 2 diabetes mellitus (17). Within the selected studies, various markers of insulin resistance were reduced after the interventions: fasting blood glucose (20, 26, 27, 33), postprandial blood glucose, postprandial plasma insulin and glycated hemoglobin (20). In terms of insulin sensitivity, Reyna et al reported an improvement of about 10% in obese and about 30% in type 2 diabetic subjects (31). Decreased serum insulin levels (26, 27) and restored insulin signaling, reflected by an increase in insulin-induced tyrosine phosphorylation of the insulin receptors (34), were also described.

Concerning body composition, lower BMI and body weight (3, 25, 31, 33, 34), decrease in waist circumference (25) and fat-free mass (16) were found after endurance training. Modifications of lipid metabolism were also reported. Leptin concentrations were significantly reduced after resistance training (28). It was also reported that the level of oxidatively modified low-density lipoproteins decreased after strenuous endurance training and that serum LPS levels decreased after either acute exercise or training in animals (27).

Limitations

Considering the analytical techniques and the way the main outcomes were reported, a meta-analysis was not feasible. Hence, a quantifiable comparison of TLR expression was out of our possibilities. Another aspect to be mentioned is the presence of a large clinical and methodological heterogeneity, which makes difficult to do conclusive interpretations. Additional interventions in some studies such as drug administration, or induced infections, may influence immune environment and could induce bias when looking at the effect of exercise on TLR expression.

CONCLUSIONS

The findings of the present systematic review based on 17 human and animal studies show that exercise elicits a variety of responses in TLR mRNA, protein and cell surface receptor expression. These results may have been influenced by the sample hereogeneity given by the participant's characteristics and by the different tissues analyzed. Although not all studies reported changes in TLR level, reduction in cytokine production and NF-κB/MAPK activation indicate the amelioration of the chronic pro-inflammatory state, which was also related to increased insulin sensitivity. The effects of exercise on TLR expression and downstream anti-inflammatory responses seem to be related to exercise type and duration, suggesting that diverse signaling cascades may possibly be stimulated depending on the exercise protocols. To get a better insight, further human research is needed using interventions on a longer term, even up to years. Additionnally, TLR expression should be reported at both mRNA and protein levels, analyzing tissues sensitive to insulin (i.e. skeletal muscle, liver and adipose tissue).

ACKNOWLEDGEMENTS

This project was funded by the Chilean National Science and Technology Fund, FONDECYT Nº 11150576. There are no relevant conflicts of interest to declare.

REFERENCES

1. Beutler BA. TLRs and innate immunity. Blood 113: 1399-1407, 2009.
2. Boehmer ED, Goral J, Faunce DE, and Kovacs EJ. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. Journal of leukocyte biology 75: 342-349, 2004.
3. Carpenter KC, Strohacker K, Breslin WL, Lowder TW, Agha NH, and McFarlin BK. Effects of exercise on weight loss and monocytes in obese mice. Comp Med 62: 21-26, 2012.
4. Chaturvedi A, and Pierce SK. How location governs toll-like receptor signaling. Traffic (Copenhagen, Denmark) 10: 621-628, 2009.
5. Coen PM, Flynn MG, Markofski MM, Pence BD, and Hannemann RE. Adding exercise to rosuvastatin treatment: influence on C-reactive protein, monocyte toll-like receptor 4 expression, and inflammatory monocyte (CD14+ CD16+) population. Metabolism: clinical and experimental 59: 1775-1783, 2010.
6. Downs SH, and Black N. The feasibility of creating a checklist for the assessment of the methodological quality both of randomised and non-randomised studies of health care interventions. Journal of epidemiology and community health 52: 377-384, 1998.
7. Esposito LM, Simpson RJ, Strohacker K, Carpenter KC, and McFarlin BK. Defining a longitudinal survival model to examine forced treadmill running as a countermeasure for diet-induced weight gain. Lab Anim 44: 305-311, 2010.
8. Flynn MG, and McFarlin BK. Toll-like receptor 4: link to the anti-inflammatory effects of exercise? Exerc Sport Sci Rev 34: 176-181, 2006.
9. 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.
10. Hooijmans CR, Leenaars M, and Ritskes-Hoitinga M. A gold standard publication checklist to improve the quality of animal studies, to fully integrate the Three Rs, and to make systematic reviews more feasible. Altern Lab Anim 38: 167-182, 2010.
11. Jialal I, Kaur H, and Devaraj S. Toll-like receptor status in obesity and metabolic syndrome: a translational perspective. The Journal of clinical endocrinology and metabolism 99: 39-48, 2014.
12. Kawai T, and Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology 11: 373-384, 2010.
13. Kawanishi N, Yano H, Yokogawa Y, and Suzuki K. Exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages in high-fat-diet-induced obese mice. Exerc Immunol Rev 16: 105-118, 2010.
14. Klop B, Elte JWF, and Castro Cabezas M. Dyslipidemia in Obesity: Mechanisms and Potential Targets. Nutrients 5: 1218-1240, 2013.

15. Konner AC, and Bruning JC. Toll-like receptors: linking inflammation to metabolism. Trends in endocrinology and metabolism: TEM 22: 16-23, 2011.
16. Lambert CP, Wright NR, Finck BN, and Villareal DT. Exercise but not diet-induced weight loss decreases skeletal muscle inflammatory gene expression in frail obese elderly persons. Journal of applied physiology 105: 473-478, 2008.
17. Lancaster GI, and Febbraio MA. The immunomodulating role of exercise in metabolic disease. Trends in immunology 35: 262-269, 2014.
18. Li G, Liu JY, Zhang HX, Li Q, and Zhang SW. Exercise training attenuates sympathetic activation and oxidative stress in diet-induced obesity. Physiol Res 64: 355-367, 2015.
19. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JPA, Clarke M, Devereaux PJ, Kleijnen J, and Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. Bmj 339: 2009.
20. Liu Y, Liu SX, Cai Y, Xie KL, Zhang WL, and Zheng F. Effects of combined aerobic and resistance training on the glycolipid metabolism and inflammation levels in type 2 diabetes mellitus. J Phys Ther Sci 27: 2365-2371, 2015.
21. McFarlin BK, Flynn MG, Campbell WW, Craig BA, Robinson JP, Stewart LK, Timmerman KL, and Coen PM. Physical activity status, but not age, influences inflammatory biomarkers and toll-like receptor 4. The journals of gerontology Series A, Biological sciences and medical sciences 61: 388-393, 2006.
22. McGettrick AF, and O'Neill LA. Localisation and trafficking of Toll-like receptors: an important mode of regulation. Current opinion in immunology 22: 20-27, 2010.
23. Munn J, Sullivan SJ, and Schneiders AG. Evidence of sensorimotor deficits in functional ankle instability: a systematic review with meta-analysis. Journal of science and medicine in sport / Sports Medicine Australia 13: 2-12, 2010.
24. Nickel T, Emslander I, Sisic Z, David R, Schmaderer C, Marx N, Schmidt-Trucksass A, Hoster E, Halle M, Weis M, and Hanssen H. Modulation of dendritic cells and toll-like receptors by marathon running. European journal of applied physiology 112: 1699-1708, 2012.
25. Nickel T, Hanssen H, Emslander I, Drexel V, Hertel G, Schmidt-Trucksass A, Summo C, Sisic Z, Lambert M, Hoster E, Halle M, and Weis M. Immunomodulatory effects of aerobic training in obesity. Mediators of inflammation 2011: 308965, 2011.
26. Oliveira A, Araujo T, Carvalho B, Guadagnini D, Rocha G, Bagarolli R, Carvalheira J, and Saad MJ. Acute exercise induces a phenotypic switch in adipose tissue macrophage polarization in diet-induced obese rats. Obesity 21: 2545-2556, 2013.
27. Oliveira AG, Carvalho BM, Tobar N, Ropelle ER, Pauli JR, Bagarolli RA, Guadagnini D, Carvalheira JB, and Saad MJ. Physical exercise reduces circulating lipopolysaccharide and TLR4 activation and improves insulin signaling in tissues of DIO rats. Diabetes 60: 784-796, 2011.
28. Phillips MD, Patrizi RM, Cheek DJ, Wooten JS, Barbee JJ, and Mitchell JB. Resistance training reduces subclinical inflammation in obese, postmenopausal women. Medicine and science in sports and exercise 44: 2099-2110, 2012.
29. Rask-Madsen C, and Kahn CR. Tissue-specific insulin signaling, metabolic syndrome, and cardiovascular disease. Arteriosclerosis, thrombosis, and vascular biology 32: 2052-2059, 2012.
30. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, and Sambhara S. Cutting edge: impaired Toll-like receptor expression and function in aging. Journal of immunology 169: 4697-4701, 2002.
31. Reyna SM, Tantiwong P, Cersosimo E, Defronzo RA, Sriwijitkamol A, and Musi N. Short-term exercise training improves insulin sensitivity but does not inhibit inflammatory pathways in immune cells from insulin-resistant subjects. J Diabetes Res 2013: 107805, 2013.
32. Ringseis R, Eder K, Mooren FC, and Kruger K. Metabolic signals and innate immune activation in obesity and exercise. Exerc Immunol Rev 21: 58-68, 2015.
33. Robinson E, Durrer C, Simtchouk S, Jung ME, Bourne JE, Voth E, and Little JP. Short-term high-intensity interval and moderate-intensity continuous training reduce leukocyte TLR4 in inactive adults at elevated risk of type 2 diabetes. Journal of applied physiology 119: 508-516, 2015.
34. Ropelle ER, Flores MB, Cintra DE, Rocha GZ, Pauli JR, Morari J, de Souza CT, Moraes JC, Prada PO, and Guadagnini D. IL-6 and IL-10 anti-inflammatory activity links exercise to hypothalamic insulin and leptin sensitivity through IKKβ and ER stress inhibition. PLoS biology 8: e1000465, 2010.
35. Stewart LK, Flynn MG, Campbell WW, Craig BA, Robinson JP, McFarlin BK, Timmerman KL, Coen PM, Felker J, and Talbert E. Influence of exercise training and age on CD14+ cell-surface expression of toll-like receptor 2 and 4. Brain, behavior, and immunity 19: 389-397, 2005.
36. Weissgerber TL, Milic NM, Winham SJ, and Garovic VD. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS biology 13: e1002128, 2015.
37. WHO. Obesity World Health Organization. http://www.who.int/mediacentre/factsheets/fs311/en/.
38. Wu X, Zeng K, Liu W, Gao Y, Gong C, Zhang C, and Chen Y. Effect of aerobic exercise on miRNA-TLR4 signaling in atherosclerosis. International journal of sports medicine 35: 344-350, 2014.
39. Zhou Q, Leeman SE, and Amar S. Signaling mechanisms in the restoration of impaired immune function due to diet-induced obesity. Proceedings of the National Academy of Sciences 108: 2867-2872, 2011.

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:
Prof. Dr. Karsten Krüger
Institute of Sport Science, Dept. of Exercise and Health, University of Hannover
Am Moritzwinkel 6,
30167 Hannover, Germany.
karsten.krueger@sportwiss.uni-hannover.de

EDITOR

Karsten Krüger
Leibniz University of Hannover, Germany

ASSOCIATE EDITORS

Jonathan Peake
Queensland University of Technology, Australia

Richard Simpson
The University of Arizona, USA

Neil Walsh
Bangor University, UK

EDITORIAL BOARD

Stéphane Bermon
Monaco Institute of Sports Medicine and Surgery, Monaco

Katsuhiko Suzuki
Waseda University, Japan

David C. Nieman
Appalachian State University, USA

Maree Gleeson
University of Newcastle, Australia

Jeffrey A. Woods
University of Illinois, USA

Frank C. Mooren
University of Giessen, Germany

Lindy Castell
University of Oxford, UK