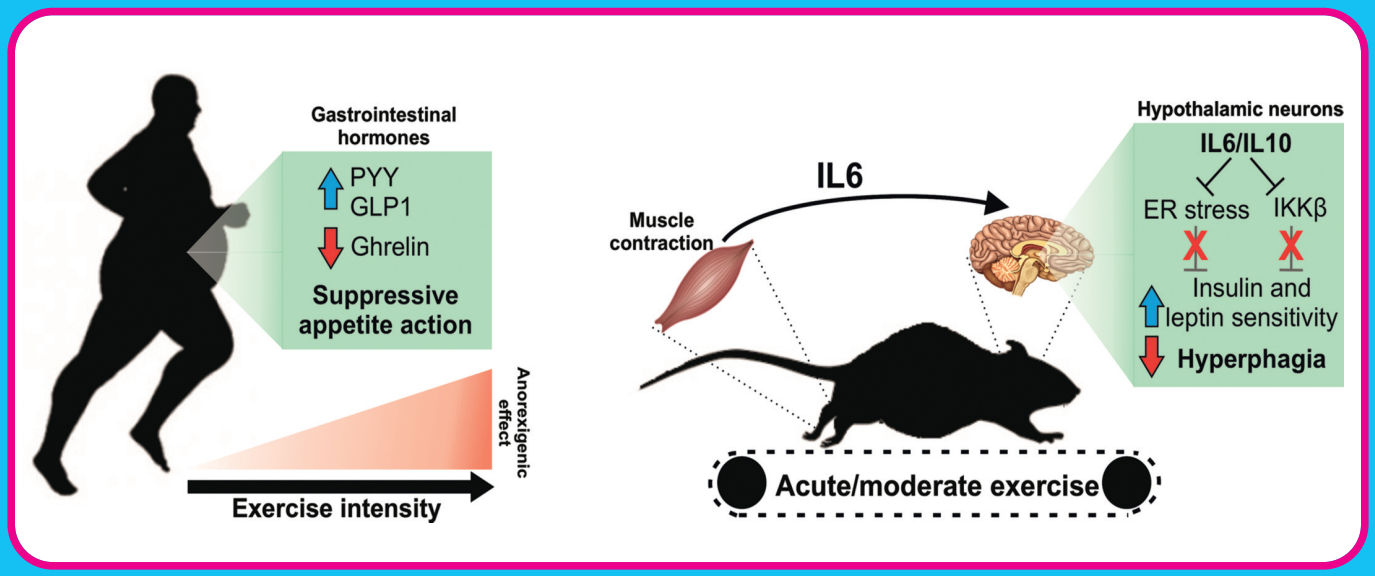
EXERCISE IMMUNOLOGY REVIEW



VOLUME 27 • 2021

The International Society of
Exercise and Immunology

EXERCISE IMMUNOLOGY REVIEW

An official Publication of the
International Society of Exercise and
Immunology (ISEI)

Upcoming Events

The 15th ISEI Symposium

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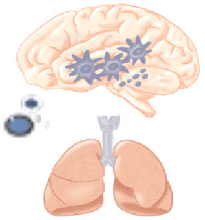

Application opens: **14th of December 21**
Application deadline: **31st of January 22**
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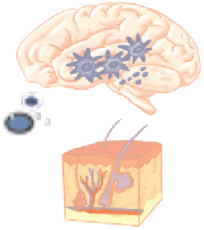
The skin-brain axis and the breaking of barriers: 02-10.09.2023



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EXERCISE IMMUNOLOGY REVIEW

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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)

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 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, International Society of Exercise and Immunology (ISEI).

From the Editors

I am pleased to welcome you as a reader of Issue 27 of the journal Exercise Immunology Review. In this issue, we publish 8 articles, all of which again provide exciting impulses for Exercise Immunology.

Rochete Ropelle et al. present in their review, that the anti-inflammatory effects of exercise involved in the control of hypothalamic satiety signaling. In a well-done systematic review article, Proschinger and colleagues provide an overview of the regulation of regulatory T cells by physical activity, including methodological guidance on their detection. Soares et al. analyzed in a systematic review and meta-analysis the effect of exercise-conditioned human serum on the viability of cancer cell cultures, discussing also immunological mechanisms on these processes. Favere et al. present a systematic review about exercise and Toll-like receptors. The article by do Brito Valente provide a focused overview about the immune response to regular exercise on the development of atherosclerosis.Lavero et al. describe the effects of exercise training on natural killer cell with a proteomics and systems biology approach. De Souza Teixeira et al. present a study about the effects of lifelong exerciseon the improvement in effector-memory CD4+ response and anti-inflammatory profile and their relation to clock gene expression in master athletes. Finally, Wadley et al. present findings that higher levels of physical activity are associated with reduced tethering and migration of pro-inflammatory monocytes in males with central obesity. I think the articles cover a broad field of Exercise Immunology and give impulses for our future research.

For EIR28 and beyond, our preference is to always solicit and publish topical review articles. While original research articles are also published in EIR, we encourage potential authors to combine their new data with an extended literature review and/or discussion of the topic. Please note that the submission deadline for EIR28 is 30. September 2021 Thank you, Rickie Simpson, Neil Walsh and Jonathan Peake, for the continuing close, trusting and friendly teamwork as we work towards keeping EIR as one of the top ranked journals in the Sports Science category. We thank all members of ISEI and the EIR Editorial Board for the confidence and trust you have placed in us. We greatly appreciate your ongoing support of EIR.

Finally, I would like to point out that the International Society of Exercise and Immunology (ISEI) now has a new homepage. On the page ***https://exerciseimmunology.com/*** you will now find an overview of the activities of the ISEI, its board, planned events and also this journal. Soon you will also be able to apply for a free membership again. Please note that the 15th ISEI Symposium will take place in Tucson, Arizona, USA, in October 2022. Further information will come soon on the website.

We hope you enjoy reading this new issue of EIR and we send out a special thanks to all contributors and reviewers of EIR27.

On behalf of the Editors,

Karsten Krüger

Physical Exercise: A Versatile Anti-Inflammatory Tool Involved in the Control of Hypothalamic Satiety Signaling

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ABSTRACT

The hypothalamus plays a critical role in the control of food consumption and energy expenditure. Fatty diets can elicit an inflammatory response in specific hypothalamic cells, including astrocytes, tanycytes, and microglia, disrupting anorexigenic signals in region-specific hypothalamic neurons, contributing to overeating and body weight gain. In this study, we present an update regarding the knowledge of the effects of physical exercise on inflammatory signaling and circuits to control hunger in the hypothalamus in obesity conditions. To try to understand changes in the hypothalamus, we review the use of magnetic resonance/anorexigenic hormone analysis in humans, as well as in animal models to explore the physiological and molecular mechanism by which exercise modulates satiety signals, such as the central anti-inflammatory response, myokine delivery from skeletal muscle, and others. The accumulation of scientific evidence in recent years allows us to understand that exercise contributes to weight control, and it is managed by mechanisms that go far beyond "burning calories."

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INTRODUCTION

The hypothalamus is recognized as the master regulator of energy homeostasis, directly controlling food intake and energy expenditure. Dysfunctions in hypothalamic neuronal regions or subpopulations are strongly associated with increased or reduced body weight. In the last decades, the hypothalamic inflammatory process has been linked to the signaling loss of anorexigenic hormones, leptin, and insulin (13, 19, 30, 88). Consequently, the functional disturbance of insulin and leptin in the hypothalamus favors a positive energy balance, culminating in body fat tissue enhancement. Body fat weight gain has been primarily associated with several comorbidities, and in this sense, obesity could be considered the century's juggernaut disease. Pharmacological treatments and invasive interventions such as bariatric surgery have been effective in weight loss; however, weight loss is not sustained with these alone. Thus, non-invasive, less costly, and side effects free interventions are needed to fight against this disease (21, 44, 79, 98).

Several researchers have sought to understand how nutritional and physical activity strategies can modulate energy homeostasis, with a direct impact on weight loss, mainly on preventing weight regain. The vital work published by King and coworkers was able to demonstrate that the appetite stimulus was transiently inhibited during and after high-intensity exercise in healthy lean men (62). Therefore, the term "exercise-induced anorexia" was adopted and opened up new exploratory avenues concerning the suppressive effects of exercise over appetite in humans (11, 12, 61). Furthermore, research using animal and human models has been performed to understand the molecular and hormonal mechanisms by which physical exercise can contribute to the control of food intake (11, 12, 61, 78, 108, 138). The knowledge that substances secreted by skeletal muscle (myokines) during and after contractile activity could reach and alter central nervous system (CNS) functioning changed the status quo of the predictable actions of exercise, transposing the benefits of physical exercise to another level of conceptualization. Some of these myokines orchestrate concatenated intracellular signaling, decreasing the proinflammatory molecules, and improving circulating

factors with suppressive effects on appetite, such as leptin and insulin. The understanding of endoplasmic reticulum stress over-response control and anti-apoptotic effects induced by these myokines, which protect the brain areas from neuronal damage caused by obesity, has projected sport sciences into this new field of investigation, called "immunometabolism and exercise" (78, 124, 138, 169).

Although it seems evident that regular physical exercise contributes to the reduction of body weight, it is essential to clarify that this can happen not only because energy expenditure is increased, but also because food intake is reduced. In addition to the peripheral effects, physical exercise promotes similar actions in the CNS, which are decisive for the control of body weight. Therefore, the pathophysiological basis for the illustrious description of the Roman poet Juvenal *"mens sana in corpore sano"* has been elaborated (75). Despite the scarcity of molecular mechanistic explanations involving the control of food intake in humans, in 2010, Ropelle *et al.*, using exercise protocols in rodents, proposed a new interpretation of cytokine actions in the CNS, which could protect against hypothalamic dysfunction induced by obesity. In this elegant study, acute exercise improved insulin and leptin sensitivity in specific neuronal subpopulations in the hypothalamus, causing anorexia in obese rodents (124).

Thus, in the present review, we will discuss the acute and chronic effects of physical exercise on the control of food intake in rodents and humans. Moreover, we will address the primary physiological and molecular mechanisms involved in physical exercise-induced appetite suppression, especially in the context of obesity.

CENTRAL CONTROL OF FOOD INTAKE

The control of food intake and thermogenesis are dynamic and complex processes, which makes understanding the hypothalamic function vitally important. Specific hypothalamic neuronal populations play a central role in integrating peripheral signals, such as leptin, insulin, and nutrients, apprising whole-body energy status, and activating particular signaling routes to induce or suppress the appetite and thermogenesis (97). After sensing these signals, specific neuronal subpopulations are also activated by synaptic transducers to perform a particular action, in a coordinated manner, across accurate controls.

Firstly, the hypothalamic arcuate nucleus (ARH) is composed of at least two specialized populations of first-order neurons that recognize mainly insulin and leptin and synthesize diffuse neuropeptides (through orexigenic or anorexigenic actions). These neurons have a spatial ability, giving the subjects the first notions of their hunger state. These neurons produce neuropeptide Y (NPY) and the agouti-related protein (AgRP), with orexigenic actions. In the vicinity of the AgRP/NPY neurons, other neuronal populations synthesize and secrete anorexigenic neurotransmitters such as proopiomelanocortin (POMC), releasing the melanocyte-stimulating hormone (α-MSH), and the cocaine and amphetamine-regulated transcript (CART) (60, 97). Therefore, the POMC/CART specialized neurons have an anorexigenic/thermogenic effect, while the NPY/AgRP specialized neurons are orexigenic/anti-thermogenic.

Secondly, axonal connections are established between first-order ARH neurons (POMC/CART and NPY/AgRP) and second-order neurons located in other hypothalamic nuclei such as the paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), and lateral area of the hypothalamus (HL). These areas are involved in a definitive and robust act of seeking food or food aversion, and in starting or stopping energy expenditure by the organism (60, 167). Neuronal projections from POMC/CART in ARH to PVN neurons induce the activation and release of the thyrotropin-releasing hormone (TSH) and corticotrophin-releasing hormone (CRH), both responsible for a strong anorexic stimulus (food aversion) and energy expenditure through thermogenesis. On the other hand, neuronal projections from NPY/AgRP in ARH to the HL induce orexin and melanin-concentrating hormone (MCH) production, both of which also have substantial orexigenic and anti-thermogenic repercussions (33, 145). Finally, thermogenesis is directly controlled in the brown adipose tissue. To increase or decrease its functioning, efferent neuronal projections connect HL and PVN neurons to brown adipose tissue, blocking or releasing catecholamines directly to β3-receptors in brown adipocytes (95, 96).

Disorders in neuronal functioning/connections in these different hypothalamic nuclei circuits are associated with hypo or hyperphagia and disruptions in heat production. Modern society is marked by high consumption of ultra-processed foods, rich in calories from fat and sugar, and sedentarism, the most significant factors driving the world toward an obesity pandemic and its various associated comorbidities.

Among the peripheral signals that act in the hypothalamus to control appetite and satiety, leptin and insulin stand out as hormones with potent anorexigenic effects. The binding of leptin to its specific receptor (ObR) activates several intracellular signaling pathways, including Janus kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3). STAT3 migrates to the cellular nucleus inducing POMC gene transcription (73). Regarding insulin signaling, this hormone activates the insulin receptor tyrosine (IR) kinase activity, which phosphorylates and recruits different substrate adaptors such as insulin receptor substrate1/2 (IRS1/2) proteins. Once activated, phosphorylated IRS displays binding sites for several signaling molecules, among them, phosphatidylinositol 3-kinase (PI3K), which has a significant role in insulin action, mainly via protein kinase B (Akt) activation (134). In the hypothalamus, activated Akt induces phosphorylation and deactivation of the Forkhead Box O1 protein (FoxO1).

Interestingly, FoxO1 is a transcription factor that is located at the cellular nucleus, inducing the transcription of several genes, including NPY. Once phosphorylated by Akt, FoxO1 leaves the nucleus and stops NPY transcription (73, 134). There is also cross-talk between the leptin and insulin signaling pathways in the hypothalamus, when leptin, through JAK2, also activates PI3K. This redundant process demonstrates the ability of neurons to use different strategies to control food intake (167). The inflammation induced by obesity or by high saturated fat intake disrupts these subtle signaling interneuronal connections, affecting the energy homeostasis and increasing food intake and body adiposity.

INFLAMMATORY MECHANISMS AND HYPOTHALAMIC DISORDERS

The chronic and low-grade inflammatory process is recognized as the main link between obesity and intracellular resistance to insulin and leptin signaling in hypothalamic neurons. The unveiling of this interplay started with an experimental study demonstrating that rodents chronically exposed to a high-fat diet exhibited a consistent immune response in the hypothalamic tissue, marked with increased gene expression and protein content of proinflammatory cytokines including TNF-α (tumor necrosis factor-alpha) and interleukin 1β (IL-1β) (2). Recent studies indicate that the inflammatory process induced by saturated fatty acids is initiated in non-neuronal cells, such as astrocytes (27, 46) and microglia (162, 163), subsequently extending the damage induced by inflammatory signaling to neurons. Inflammatory signaling in hypothalamic neurons results in the activation of serine kinases, including JNK (c-Jun N-terminal kinases) and IKK (inhibitor of kappa kinase), which are capable of negatively interfering with the leptin and insulin signals and their anorexigenic actions in neurons, contributing to hyperphagia and weight gain (19, 174). In both animals and humans, a few days of consuming a high-fat diet are sufficient to trigger the inflammatory process in hypothalamic cells (151). Also, these effects can be observed in other regions of the brain, such as the hippocampus (102).

In humans, the findings on obesity-associated hypothalamic inflammation are still discreet, as post-mortem studies are scarce and do not necessarily show the molecular mechanisms mainly due to the need for fresh tissue. In the last decades, analyses of cerebrospinal fluid (CSF), which is in direct contact with the CNS subareas, and magnetic resonance imaging, have helped to highlight inflammation, hypothalamic dysfunction, and also tissue degeneration in obese individuals. In 2003, Stenlöf *et al.* showed, for the first time, the inverse correlation between IL-6 levels in CSF and the weight and body fat of overweight or obese individuals (147). Moreover, van de Sande-Lee *et al.* (164) verified an increase in IL-6 and IL-10 levels in the CSF of subjects eight months after bariatric surgery. In the same study, when compared to lean individuals, morpho-functional analyses demonstrated that the hypothalamus of obese individuals presents functional changes, such as a reduced neuronal firing rate. In response to dextrose solution ingestion, lean individuals were more sensitive and had higher neuronal activity than obese individuals before and after bariatric surgery. The significant loss of body fat after bariatric surgery partially recovered neuronal firing in regions adjacent to the hypothalamus (164). Also, individuals with higher sensitivity to cerebral insulin, which was assessed by functional magnetic resonance imaging in response to nasal spray treatment, have a better response and weight reduction to an intervention program involving lifestyle modification (66).

Besides insulin and leptin resistance, the inflammatory process can chronically trigger pro-apoptotic signals in hypothalamic neurons in rodents, which are mainly concentrated in POMC neurons (74, 92). Although the verification of apoptotic signal markers in the human hypothalamus is a non-realistic strategy, Thaler and colleagues have established an indirect way of observing the hypothalamic damage in obesity, marked by gliosis, through functional magnetic resonance imaging (fMRI) analysis (151). Interestingly, this study showed that the gliosis associated with obesity was restricted to the hypothalamus and did not occur in different CNS areas. Furthermore, hypothalamic gliosis signals have also been found in obese children, followed by less hypothalamic activation in response to glucose intake (135). It should be noted that not all forms of obesity require hypothalamic gliosis. Gao and colleagues showed that leptin-deficient *ob/ob* mice become extremely obese on a standardized chow diet without gliosis. However, exposure to a high-fat diet increased inflammation and microglial activity, suggesting gliosis (39). Altogether, these data strongly suggest that human obesity is associated with neuronal lesions in the hypothalamus.

The most recent scientific evidence has shown that neurons do not suffer inflammatory damage directly from saturated fatty acid overload. High saturated fat concentrations reach the hypothalamus by crossing the blood-brain barrier in a specific region controlled by tanycytes (4). These fatty acids are recognized by astrocytes, which, after being sensitized, project signals through dendritic connections to the native microglial cells (18). On the microglia cell surface, TLR2/4 (toll-like receptor 2/4) is activated, firing proinflammatory intracellular signaling. After that, the inflammatory process

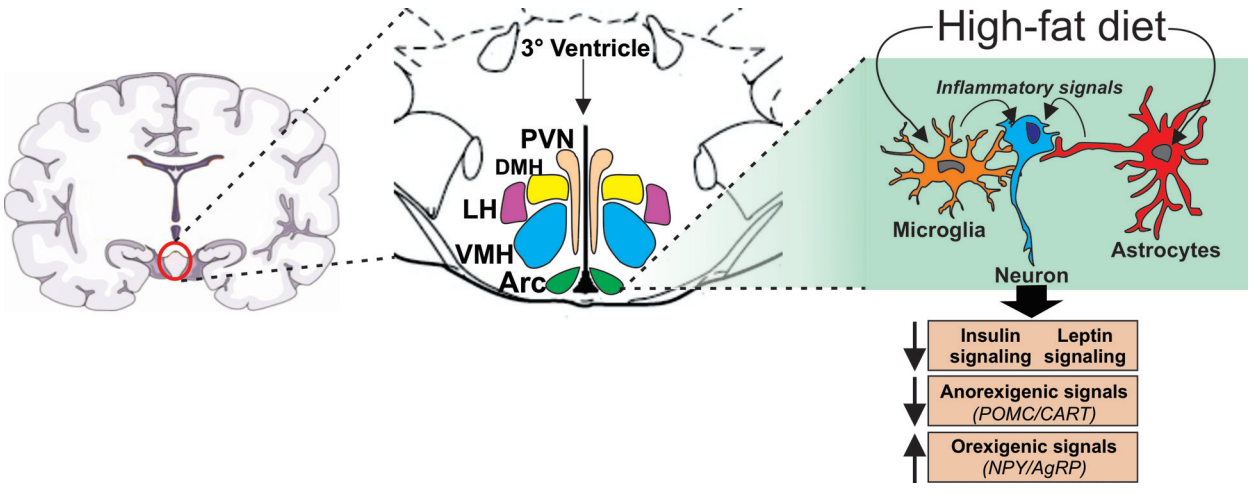

Figure 1. Schematic view of hypothalamic nuclei and the role of microglia and astrocytes in neuroinflammation. Inflammatory signals impair insulin and leptin pathways in hypothalamic neurons.

increases robustly, where microglial stimuli act directly in the neurons, beginning neuronal inflammation (4) (Figure 1). Although the neurons tolerate the inflammation for some time, the apoptotic signals in them are increased in a time-dependent manner (92).

Therefore, the development of strategies to attenuate the inflammatory process at a central level can be considered promising in the fight against obesity. In this sense, several studies have addressed the effects of physical exercise on the attenuation of inflammation, as well as insulin and leptin resistance in the hypothalamus and its impact on food intake control. Next, we will describe and discuss the main findings in this research field, involving studies with experimental and human models.

PHYSICAL EXERCISE AND FOOD INTAKE CONTROL

In the 1950s, the first studies appeared to determine the influence of physical exercise on energy expenditure and food intake. Initially, it was considered that food intake would be higher after physical activity, compensating for the exercise-induced energy expenditure (84, 156). Also, obesity, especially of a hereditary nature, was recognized as a result of voluntary activity reduction, impacting obesity etiology significantly (83). However, in 1953, Mayer showed less weight gain in genetically susceptible mice after exercise (83). These findings prompted the search for knowledge to understand whether the effects of physical exercise on body weight are exclusively achieved due to increased energy expenditure or are also related to reduced food intake. The study of King and colleagues was relevant in demonstrating that exercise has an anorexigenic effect, even in lean individuals (62). The work of Ropelle and co-authors was consistent in explaining that circulating factors from skeletal muscle in response to exercise would have an impact on reducing hyperphagia in obese rodents (124).

Although these studies were fundamental for advancing the knowledge regarding the mechanisms that regulate physical exercise-induced food intake control, we still have many challenges ahead. Indeed, several factors influence the effects of exercise on food intake, and this phenomenon is even more complicated when humans are investigated since energy intake can be mostly influenced by appetite, which is affected by numerous physiological, psychological, behavioral, and sociocultural factors (71, 142).

In the next section, we will address the acute and chronic effects of physical exercise in overweight and obese individuals. We do not intend to exhaust the subject, but to present the latest data from well-controlled research that has sought to investigate the impact of exercise on energy intake and hormonal response related to appetite suppression in overweight and obesity conditions.

EFFECTS OF ACUTE EXERCISE ON FOOD INTAKE CONTROL

Human studies: Acute effects

The real impact of acute physical exercise on energy intake and satiety in overweight individuals remains inconclusive. However, the number of investigations reporting on the effects of exercise on the regulation of hunger in obese populations has grown in recent years, and well-conducted and controlled studies have indicated that physical exercise is capable of causing a reduction in energy balance, which is transient, as a result of its anorexigenic effect in obese individuals (25).

Although the currently available research techniques do not allow for an acute assessment of sensitivity to anorexigenic and thermogenic hormones (i.e., leptin and insulin) specifically in the hypothalamus, studies with overweight or obese humans have aimed to support the evidence. Thus, several works have evaluated the serum levels of appetite-related hormones in response to physical exercise, including the orexigenic hormone (acylated ghrelin), and the anorexigenic hormones peptide tyrosine-tyrosine (PYY) and glucagon-like peptide-1 (GLP-1), as well as the circulating levels of leptin and insulin, among others (11, 25, 26, 54, 160), before, during, and after exercise. Also, fMRI allows us to understand the blood fluxes into the brain, specified by region, with realistic images in real-time.

For example, when evaluating how 30 minutes of aerobic exercise sessions could reduce stress behavior, Zschucke and colleagues showed that improved stress response was not associated with low levels of cortisol and α-amylase after exercise, but positively associated with a high bilateral hippocampus response. Both the hippocampus and the prefrontal cortex are intimately involved in hypothalamus-pituitary-adrenal (HPA) axis negative feedback (177). Several brain regions are involved in the downregulation of HPA axis activity, and when the paraventricular nucleus initiates the neuroendocrine signaling cascade of the HPA axis, it receives mostly inhibitory input from the hippocampus, the anterior cingulate cortex, and the prefrontal cortex (52). In contrast, the amygdala exerts a mostly excitatory effect on the paraventricular nucleus. All these areas are integrated by neuronal projections, which directly influences the hypothalamus (52, 86). It has been demonstrated that there is a complex circuitry at the central level that participates in the hypothalamic responses of hunger control. This research field still needs further advances.

Several studies have evaluated the impact of acute exercise sessions on eating behavior in overweight and obese subjects, from both sexes (26, 132), among adolescents (152, 155), and among young or middle-aged individuals (26, 160), in activities, such as cycling (160), running (26), or other continuous types of moderate or high-intensity exercise (54, 132, 140, 159, 160), using measurements of circulating insulin or leptin levels (79, 159). Based on this set of studies, some of which were very well controlled, we can present essential characteristics of the interference of exercise in the modulation of appetite in overweight individuals, in a debate that becomes more robust when associated with other studies with mechanistic evidence.

Ueda and coworkers showed that 1 hour of cycle ergometer exercise at 50% of maximal oxygen uptake (VO_2max) enhances the PYY and GLP-1 plasma levels in young adult obese individuals [age: 22.93 ± 4 yr; BMI (body mass index): 30.0 ± 3.1 kg.m^2], indicating that these satiety signals may play a role in acutely regulating appetite and energy homeostasis (160). Also, the effect of exercise in reducing caloric intake in overweight individuals was higher than in eutrophic individuals (160). Douglas *et al.* verified similar responses in overweight

and obese adult women and men (age: 45.0 ± 12.4 yr; BMI: 29.2 ± 2.9 $kg.m^{-2}$), who performed moderate-intensity exercise (60 min/60% peak VO_2) on a treadmill (26). The authors attributed the temporary suppressive effects of exercise on food intake to an increase in the circulating concentration of PYY and GLP1, without further compensatory changes in appetite (26).

After investigating the acute effects of continuous (1h) and intermittent (12 sessions of 5 min, totaling one hour) treadmill exercise performed at moderate intensity (60–65% VO_2 peak) in young obese women and men (age: 18–35 yr; BMI: 30.0 kg.m2), Holmstrup and coworkers reported that serum PYY levels did not change over 12 hours of continuous or intermittent exercise. However, the authors observed that intermittent exercise induced satiety more significantly than a continuous exercise in obese individuals (55). Also, Sim *et al.* compared the acute effects of continuous moderate-intensity cycling exercise (MC = 60% VO_2 peak), high-intensity intermittent cycling exercise (HI = alternating between 60 s at 100% of VO_2 peak and 240 s at 50% of VO_2 peak), and very high-intensity intermittent cycling exercise (VHI = alternating between 15 s at 170% of VO_2 peak and 60 s at 32% of VO_2 peak) in overweight and sedentary young adult men (age: 30 ± 8 yr; BMI: 27.7 ± 1.6 $kg.m^2$) and verified that food intake was lower in the HI and VHI sessions compared to the MC session (140). Furthermore, the subsequent energy intake remained lower in the participants who performed the VHI exercise compared to the control group. There was also a reduction in the levels of active ghrelin after VHI exercise when compared to other types of exercise.

Interestingly, Holliday and coworkers evaluated the effects of low-volume and high-intensity cycle ergometer exercise (4 sprints of 30 s) on appetite and gastrointestinal hormones (ghrelin and GLP1) in overweight women and men (age: 34 ± 12 yr; BMI: 27.7 ± 1.7 $kg.m^2$). The authors observed that exercise-induced appetite suppression was accompanied by a reduction in acetylated ghrelin and a modest increase in circulating GLP1 (54). The answers found regarding the variables involved in food intake control are certainly not always unanimous. For instance, Nyhoff and coworkers found no changes in GLP1 in obese women (Age: 24.3 ± 4.6 yr; BMI: 37.3 ± 7.0 $kg.m^2$) after running exercise on a moderate-intensity treadmill (55% of VO_2max) or high-intensity interval exercise (4 min at 80% of VO_2max and 3 min at 50% of VO_2max) (105). There was no difference in the concentration of GLP1 between the types of effort during the exercise, but there was during recovery. Also, the insulin concentration was reduced during the meals that followed the exercise; however, the intensity of the exercise had no impact on this response (105).

Tsofliou *et al.* evaluated the effects of moderate exercise (20 min brisk walking) in obese adult women (age: 50.0 ± 8.5 yr; BMI: 37.27 ± 6.5 $kg.m^2$) and observed a reduction in appetite and satiety (159). Interestingly, serum leptin levels were correlated with reduced hunger and satiety after the exercise intervention. Larsen and colleagues noted that acute high-intensity exercise, but not moderate-intensity exercise, reduced ghrelin levels in overweight men (69). This large body of evidence suggests that the relationship between exercise intensity and the secretion of appetite-suppressing gastrointestinal hormones requires further investigation to determine the mechanisms involved.

Regarding the effect of physical exercise on obese young people (obese adolescents), it has been reported that appetite is suppressed in subsequent meals. Thivel and colleagues elegantly showed that acute exercise does not affect energy intake in lean individuals, but reduces food intake in obese young people when performed at high intensities, without changing the macronutrient composition of the meal (153, 155). Said *et al.* demonstrated that an acute cycle ergometer session (70% of VO_2max for 40 min) reduced food consumption in the morning among obese girls (age: 13.7 ± 1.1 yr; BMI: 30.5 ± 3.4 $kg.m^2$) (125).

A recent study, which investigated the effects of high-intensity exercise (HIEX = three 10-minute sessions at 70% of VO_2peak at 60–70 rpm, with 1.5 min of active rest interposed at 25 W with 60-70 rpm) performed on a bicycle on appetite, hunger regulating hormones, and inflammation markers in obese boys (age: 10-18 yr; BMI: 15th – 85th BMI for age percentile) (56), showed a reduction in the levels of active ghrelin and an increase in IL-6. These findings were followed by an inverse correlation between IL-6 and appetite intensity. Altogether, the data suggest an association between increased levels of IL-6 through chronic exercise and reduced appetite in overweight and obese boys. However, this interpretation should be made with caution, as there was also a reduction in ghrelin levels and an increase in cortisol, which are hormones that are known to increase appetite (56).

Overall, the evidence shows that at least partial aerobic exercise acutely exerts a regulatory effect on hormones involved in food intake control, and consequently, this has been associated with a suppressive effect on appetite in overweight individuals (26, 131). On the other hand, studies using resistance exercise are limited. In one such study, Larsen *et al.* found that the levels of PYY and GLP-1 did not change after resistance exercise (10 sets of 8 repetitions of bilateral leg extension exercises at a resistance of 75% of 1RM with 150 s recovery between sets) in overweight men (age: 48 ± 5 yr; BMI: 29.9 ± 1.9 kg m^2) (70). However, more research evaluating the acute effects of resistance exercise in its different forms of execution (concentric, eccentric, and isometric) on individuals with varying degrees of obesity and age (especially older generations) is still needed.

RODENT STUDIES: ACUTE AND SHORT-TERM EFFECTS AND PROPOSED MECHANISMS

Improvement of hypothalamic insulin and leptin sensitivity

Experimental models in research have been essential for assessing the effects of acute or short-term physical exercise in controlling food intake. Some studies have contributed by elucidating the mechanisms responsible for the improvement of insulin and leptin signaling in the hypothalamus after acute exercise. Gaspar *et al.* showed that a single session of exercise increased the hypothalamic protein content of adaptor protein containing the pleckstrin homology domain, the phosphotyrosine-binding domain, and leucine zipper motif 1 (APPL1) in high-fat diet-induced obese mice (41). It is known that APPL1 can bind to Akt (41), improving its phosphorylation at serine 473, as well as enhancing leptin signaling in specific cell types (24). On the other hand, Gaspar and coworkers observed a reduction in the protein levels of the mammalian

homolog of Drosophila tribbles protein 3 (TRB3) (41), a negative regulator of Akt activity (28), in the hypothalamus of exercised obese mice. These results suggest that acute exercise can stimulate the production of proteins that interact directly with insulin and leptin signaling in the hypothalamus, increasing the anorexigenic signals of these hormones.

Another impressive effect that could explain the transient improvement in insulin and leptin signaling in the hypothalamus is the acute anti-inflammatory effect of exercise. For instance, acute moderate-intensity swimming or treadmill exercise was sufficient to reduce IKK phosphorylation and endoplasmic reticulum stress in the hypothalamus of obese rats (124). The reduction in inflammation was associated with an increased response to insulin and leptin in the hypothalamus of these rats. After the acute exercise session, a significant improvement was observed in insulin (IRS/PI3K/Akt pathway) and leptin (JAK2/STAT3) signal transductions in the hypothalamus, which was accompanied by the modulation of neuropeptides such as POMC and AgRP, resulting in less food intake (124). However, the question is: how could acute physical activity change the inflammatory status in hypothalamic neurons?

IL-6 is an intracellular signaling molecule associated with extensive immune responses in several conditions (146). Also, IL-6 can be produced and released by skeletal muscle (hence the name myokine) in response to physical exercise, performing pro- and anti-inflammatory actions depending on the concentration and circumstances of the target tissue (110, 146). Exercise-induced muscle contraction leads to IL-6 mRNA expression and its transcription (109). During physical exercise, this myokine is released into the circulation, which can influence metabolism and modify the production of other cytokines in tissues and organs (110). Interestingly, IL-6 knockout mice develop obesity prematurely, highlighting the importance of this molecule in controlling energy homeostasis (168).

Besides being released by skeletal muscle during contraction, IL-6 can be produced in response to exercise directly in the CNS. Nybo *et al.* observed increased brain levels of IL-6 in healthy subjects after two sets of aerobic exercise on a cycle ergometer (104). Similar findings were verified in animal models. Silva and coworkers showed high levels of IL-6 in the hypothalamic tissue of exercised mice (138). Matsumoto and coworkers suggested that the enhanced hypothalamic levels of IL-6 occur due to positive feedback generated by the peripheral increase in this cytokine (82). They injected recombinant IL-6 into rats intraperitoneally and observed an increase in the protein content of IL-6 in the hypothalamus (82). Goldfish performing a short exercise protocol displayed lower food intake and high levels of IL-6 in both skeletal muscle and hypothalamic samples (101), suggesting that this is a conserved physiological mechanism among different species. Furthermore, Aniszewska and colleagues clearly showed that forced acute swimming exercise led to high levels of IL-6 in the microglia, astrocytes, and neurons of mice (3). So, how can we explain the anti-inflammatory effects of IL-6?

Exercise-induced IL-6 release may lead to systemic anti-inflammatory responses (103, 110) through the modulation of soluble TNF-α receptors, as well as the increase of IL-1 receptor antagonist (IL-1Ra) and mainly IL-10, which can inhibit the phosphorylation and activity of the IKK complex, as well as nuclear factor kappa B DNA binding (130). Interestingly, the disruption of IL-10 synthesis by using an antisense oligonucleotide against IL-10 in the hypothalamus of obese rats abrogated the anti-inflammatory effects of acute exercise or those of IL-6 recombinant intracerebroventricular injection in obese animals (124). The IL-6/IL-10 axis-generated anti-inflammatory mechanism could contribute to insulin and leptin sensitivity improvement in hypothalamic neurons, reestablishing the anorexigenic and thermogenic signals, and finally, committing to the maintenance research has shown that increased IL-6/IL-10 levels can contribute to the control of energy homeostasis (124, 164), further studies are needed to expand the knowledge regarding this mechanism.

Altogether, the results obtained suggest that acute exercise induces an anti-inflammatory response, which is accompanied by an increase in insulin and leptin sensitivity in the hypothalamus of obese rodents. Also, this anti-inflammatory effect observed after acute exercise appears to be partially related to the central role of IL-6 in the hypothalamus. Figure 2 illustrates the effects of acute exercise on the hypothalamus and hunger control in humans and animals.

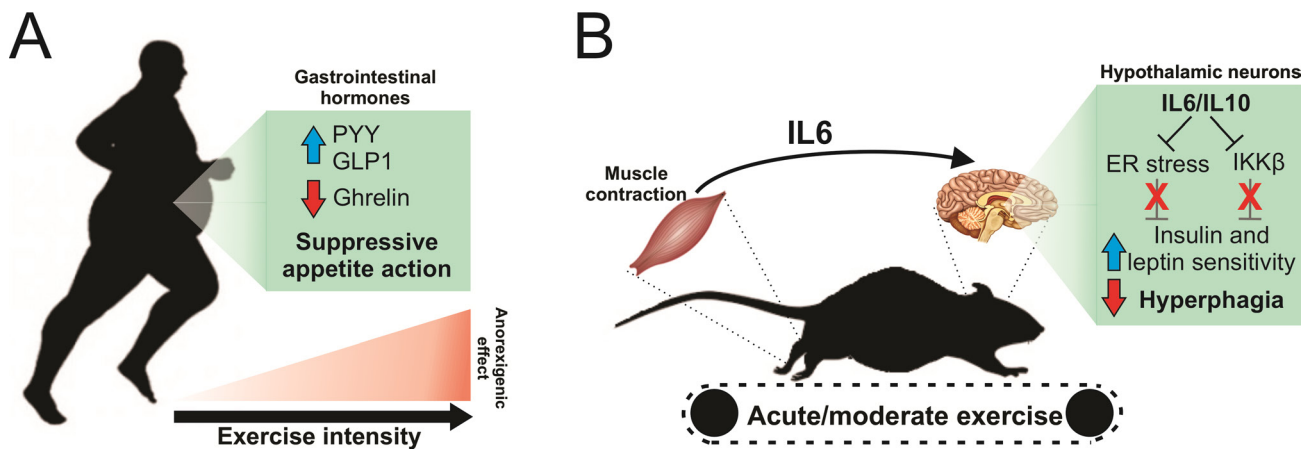

Figure 2. Effects of acute exercise in the control of food consumption. A. Acute exercise modulates gastrointestinal hormones and suppresses appetite in humans in an intensity-dependent manner. B. Muscle contraction increases circulating IL-6 levels. IL-6 elicits an anti-inflammatory action in hypothalamic neurons, improving insulin and leptin signaling in rodents.

EFFECTS OF CHRONIC EXERCISE ON HYPOTHALAMIC INFLAMMATION AND FOOD INTAKE CONTROL

Human studies: Chronic effects

There are still a limited number of studies addressing the impact of chronic physical exercise on appetite and hunger-regulating hormones in obese humans. These studies have examined the effects of physical training on sedentary, overweight, and obese individuals, highlighting different appetite responses when comparing inactive with trained subjects. The evidence regarding the impact of physical training on the plasma levels of satiety-related peptides is also controversial and relatively scarce, particularly in a population that is overweight.

Martins and coworkers investigated the effects of medium-term exercise treadmill walking or running (12-week supervised exercise program/five times per week, at 75% maximal heart rate) on body weight, fasting/postprandial levels of orexigenic and anorexigenic hormones, as well as subjective appetite sensations in sedentary overweight/obese subjects (age: 36.9 ± 8.3 yr; BMI: 31.3 ± 3.3 kg/m^2) (77). The authors verified a significant decrease in body weight and fasting insulin, as well as an enhancement in acylated ghrelin plasma levels and fasting hunger state. Also, a moderate rise in the release of GLP1 was described after exercise, as well as a significant increase in acylated ghrelin postprandial suppression. These findings suggest that exercise-induced body weight reduction can enhance the desire to eat during fasting conditions but seems to be counterbalanced by an improvement in appetite control sensitivity (77). On the contrary, obesity is associated with increased levels of plasma ghrelin (17). Overweight subjects performing aerobic and resistance exercise for 12 months significantly reduced their body weight, adiposity, and circulating leptin levels (158). According to a systematic review and meta-analysis of randomized controlled trials, chronic physical exercise lasting two weeks or more is related to reduced leptin levels in children up to the age of 18 years with a body mass index higher than the 95th percentile for age and sex (32).

Martins and colleagues analyzed obese individuals (age: 33.4 ± 10.0 yr; BMI: 32.3 ± 2.7 kg.m^2) subjected to isocaloric exercise sessions (250 kcal), on a high-intensity interval cycle ergometer (85%–90% HRmax and work/rest ratio of 8/12 s) or on a moderate-intensity continuous cycle ergometer (70% HRmax), and short-term exercise (125 kcal), performed three times per week for 12 weeks and verified that insulin levels were reduced and GLP1 levels were increased during all exercise sessions when compared to individuals at rest (60). The acetylated ghrelin values were lower for continuous and high-intensity exercise, but not for short duration exercise when compared to the control group. Plasma PYY levels and energy intake did not differ between interventions (76). In a study involving overweight and obese men and women (age: 39.6 ± 9.8; average BMI: 31.8 kg.m^2), it was shown that 12 weeks of aerobic training (approximately 70% of each individual's maximum heart rate 5 d/wk) augmented fasting hunger but also increased the satiety response to a fixed meal (63).

Adiposity reduction-induced anti-inflammatory response is another significant impact of performing physical training. A large body of evidence shows that chronically different types and intensities of physical exercise reduce serum and tissue inflammatory markers (5, 40, 103, 121). Elder and colleagues reviewed the effects of various amounts of physical activity on body fatness, energy intake, and food preferences in humans, and concluded that physical training has a consistent impact on body fat percentage, which is caused by increased energy expenditure, however, without changing eating behavior (31). Also, previous studies suggest that individuals with higher pre-training adiposity show more significant changes in appetite and gastrointestinal hormones (93, 114). Gibbons and colleagues demonstrated that overweight individuals (age: 18-55 yr; BMI: 27-34.9kg / m^2) presenting more significant reductions in adiposity after an aerobic exercise intervention of 5 sessions/ week at moderate intensity (70% of maximal heart rate) for 12 weeks also exhibited a more pronounced postprandial increase in GLP1 and PYY hormones, as well as a reduction in acetylated ghrelin levels compared to individuals who lost less weight (42).

In a recent systematic review and meta-analysis, Taylor *et al.* compared the chronic effects (i.e., four weeks or more) of high-intensity interval training and sprint interval training on energy intake with moderate-intensity continuous training or no exercise conditions (150). Despite the considerable heterogeneity of the findings, there was no compensatory enhancement of energy intake in response to high-intensity interval training and sprint interval training compared to moderate-intensity continuous training or no exercise. Nevertheless, the inclusion of the participants' adherence to exercise programs, individual food preferences, and psychological parameter measurements was highlighted by the authors as being essential methodological considerations to be included in future investigations (150).

Although the following hypothesis must be experimentally confirmed, physical training effects could also cause an anti-inflammatory response in the hypothalamic nuclei of obese individuals, restoring satiety signals. It is essential to point out the contradictions in the literature regarding how appetite is influenced by the type, duration, or intensity of exercise in humans. Also, the characteristics of the subjects (i.e., body fat percentage, initial fitness level, age, or gender) may contribute to the data discrepancies in the literature. The difference in the responses is more pronounced when analyzing the effects of exercise on appetite in athletes of different sports (44, 125, 127), thus hampering interpretations of the data. Other issues, such as the impact of concentric or eccentric contractions, have been investigated, providing more information within the scope of exercise sciences and hunger control (154). Thivel and coworkers showed that eccentric cycling during a multidisciplinary weight loss intervention might prevent adolescents with obesity from increasing their food intake (108). The amount of studies looking at the responses of food intake and metabolic parameters to physical exercise performed at different times of the day has also been increased (34, 128). It is important to note that the chronic exercise-induced body fat reduction hampers the interpretation of the isolated beneficial effects of exercise since the reevaluations are performed under indirect actions of exercise, mainly related to adiposity loss.

Another interesting point of view concerns the impact of physical exercise on energy homeostasis with advancing age. It is well established that senescence is associated with increased proinflammatory markers, such as circulating levels of IL-6 and TNF-α, a new phenomenon known as "inflammaging." Therefore, the effects of physical exercise on energy control during the aging process need to be better elucidated (1, 87). Also, inflammatory responses and cytokine secretion during

advancing age are influenced by fat mass depots and adherence or not to physical exercise (1, 45). Physical activity can contribute to metabolic health in aging through reducing body weight (48), proinflammatory cytokines (1), and TLR-4 signaling (122), as well as preventing the accumulation of senescent T-cells (90), hemodynamic improvement, anti-atherogenic actions, and oxidative stress attenuation (47, 170). However, it is necessary to assess whether the anti-inflammatory effects of exercise also occur in the hypothalamus, benefiting calorie intake during the aging process. Furthermore, the impact of physical exercise throughout life has been shown to be positive, culminating in lower levels of proinflammatory cytokines and higher levels of anti-inflammatory cytokines (89, 91).

Recently, it was reported that the success of lifestyle changes (i.e., increased physical activity, as well as reduced-fat consumption, and high fiber diet) in reducing total and visceral adiposity is more significant in obese individuals with high sensitivity to brain insulin, which was assessed by fMRI after nasal spray administration of insulin (66). Also, higher susceptibility to brain insulin is associated with lower fat regain during a 9-year follow-up period (66). Heni *et al.* reported that lean young individuals with high sensitivity to hypothalamic insulin showed an improved second phase of insulin secretion by pancreatic cells, positively impacting the organism (51). On the other hand, the obese subjects with hypothalamic insulin resistance did not present the same response and showed negative impacts on metabolic control, highlighting that the maintenance of the central response to insulin may be a pivotal point in obtaining more robust responses of physical exercise to food intake. Future research involving the obese population may lead to new insights into this phenomenon.

Although there is no evidence of a direct anti-inflammatory effect on the hypothalamic tissue of humans, there are a large number of studies reporting that physical exercise increases the circulation of anti-inflammatory factors (IL-6 and IL-10) and decreases proinflammatory factors such as TNF-α and IL-1β, among others, which are found to be elevated in obese individuals (65, 149). The anti-inflammatory effects of exercise are associated with weight loss and hormonal regulation, such as increased insulin sensitivity and increased adiponectin, with metabolic health-promoting repercussions (65, 116, 117, 157). It should be noted that responses to exercise can be different depending on health conditions, cytokine profile, the period after the stimulus when the analysis is performed, and characteristics of the effort made (type and intensity of exercise practiced) (65, 116, 117, 157).

Rodent studies: Chronic effects and hypothalamic molecular analyses

Obese rodents performing voluntary aerobic exercise for eight weeks showed a significant reduction in adiposity (8). Despite the decrease in plasma leptin concentrations, there was no increase in food intake as a compensatory mechanism. Furthermore, a reduction in NPY orexigenic peptide expression was observed in the hypothalamic ventromedial nucleus of these animals (8). Similar evidence was found in obese mice performing voluntary exercise for 12 weeks that showed increased sensitivity to insulin and leptin in the hypothalamus (68). These results were accompanied by increased expression of POMC and reduced adiposity (68). Another interesting study showed that aerobic training could mimic leptin effects by increasing Jak2 and STAT3 phosphorylation in the hypothalamus of rats, even with low levels of leptin (175).

On the other hand, Borg and coworkers administered a high-fat diet for 12 weeks to mice and subjected them to an aerobic physical training protocol in the last 6 weeks of the dietetic treatment. The authors found no change in leptin sensitivity and caloric intake in obese animals (10). It is necessary to report that these responses related to improved leptin signaling at the central level are not unanimous, reinforcing the urgency for further studies. The anti-inflammatory effects of chronic exercise on the CNS, including the hypothalamus, appear to be more consistent and long-lasting when compared to acute exercise.

Physical training can prevent hypothalamic inflammation induced by a high-fat diet. Yi *et al.* demonstrated lower microglia activity in exercised animals, marked by a decrease in Iba1 (allograft inflammatory factor 1) (172). Silva and coworkers showed that aerobic training reduced transforming growth factor-beta 1 (TGF-β1) protein content and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha (IkB-α) phosphorylation in hypothalamic tissue in middle-aged obese mice (137). These data were accompanied by an increase in energy expenditure, as well as a reduction in both body weight and accumulated food intake. However, it is important to note that, chronic excessive exercise can promote hypothalamic tissue inflammation in healthy mice, highlighted by the upregulation of IL-1β, TNF-α , and JNK phosphorylation (111). Similar findings were described by Pinto and coworkers, who observed that chronic/intense exercise led to endoplasmic reticulum stress markers in the hypothalamus (115). Therefore, the anti-inflammatory effects of physical exercise on the CNS seem to be dependent on the balance between training load and an adequate recovery period.

Another important study demonstrated that repeated bouts of high-intensity physical exercise were sufficient to promote a rapid reorganization of synaptic signals in NPY/AgRP and POMC neurons in the arcuate nucleus of the hypothalamus of mice, concomitantly with a reduction in food intake (50). Recently, the protective effect of physical exercise on neuronal apoptosis in obese animals has been addressed (74). Obese mice receiving a fat-rich diet and performing an endurance exercise protocol for eight weeks showed increased hypothalamic levels of IL-10 and B-cell lymphoma 2 (Bcl2), with more effective anti-apoptotic action, as well as reduced BCL2-associated X protein (Bax), a potent pro-apoptotic protein. These molecular responses were accompanied by increased leptin sensitivity and reduced food intake (74). Nevertheless, the number of studies evaluating the hypothalamic apoptotic process in response to physical exercise is still limited.

On the other hand, Kim and colleagues evaluated the effects of aerobic physical training on short-term memory and the hippocampus apoptotic pathways of rats with traumatic brain injury (59). The authors observed attenuation of short-term memory impairment, as well as a decrease in DNA fragmentation, marked by reduced caspase-3 protein content in the hippocampus of the mice. Also, a 10-day treadmill exercise protocol increased the Bcl-2 content in the hippocampus of rats (59), suggesting that physical exercise may prevent traumatic brain injury-induced apoptotic neuronal cell death and allow the partial recovery of short-term memory.

Altogether, the human and animal studies have demonstrated that the effect of chronic exercise on the control of food consumption is still controversial; however, exercise training is associated with lower adiposity and inflammatory markers. Animal models of obesity have demonstrated that exercise training prevents or reduces high-fat diet-induced hypothalamic inflammation and neuronal damage induced by apoptosis. Figure 3 illustrates the effects of chronic exercise on the hypothalamus and hunger control in humans and animals.

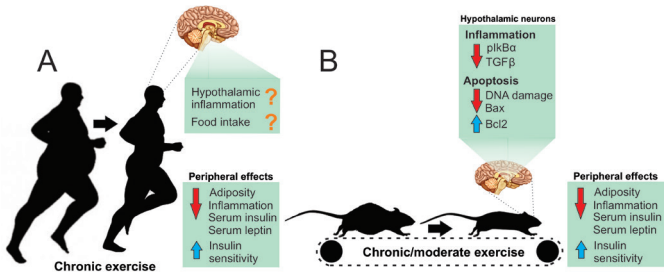

Figure 3. Effects of chronic exercise in the control of food consumption. A. Exercise training reduces the body weight and multiple benefits in peripheral metabolism, but the effects in the control of food consumption are controversial. B. Chronic exercise reduces hypothalamic inflammation and neuronal apoptosis in rodents.

OTHER MECHANISMS WITH A POTENTIAL REGULATORY EFFECT OF FOOD INTAKE AND ENERGY EXPENDITURE IN RESPONSE TO EXERCISE

In the last decades, there has been a growing understanding of the integration between organs and systems, especially among those with a crucial role in controlling energy metabolism. In this context, the intestinal microbiota (also known as the microbiome) regulates metabolic pathways of the host, with several connections established as a new axis with other tissues such as muscle and brain (36). These microbial products from the intestine also influence the endocannabinoid system (ECS). Through a vast and complex network of lipid and protein mediators, they have an important influence on the functioning of organs, tissues, and body metabolism (36). Both the microbiota and the ECS are strongly influenced by lifestyle and food patterns.

The gut-brain axis mechanistic studies showed the diversity of interactions modulating the hypothalamus through vagal nerve stimulated by GLP1, fatty acids, and, recently, agonists for ECS or its analogs, impacting the control of food intake and energy expenditure. Short-chain fatty acids (SCFAs) (64), lipopolysaccharide (LPS) (161), glutamate and γ-aminobutyric acid (GABA) (85), and other microbial products present at the intestinal lumen act as bidirectional messengers at the gut-brain axis. Some microbial products reach the brain during dysbiosis conditions, interfering with the neuronal circuits (36). High levels of LPS that cross the blood-brain barrier (BBB) (120) inducing TLR-4-dependent proinflammatory responses in the microglia, which are associated with the activation of inflammatory cytokines and resistance to leptin and insulin directly in hypothalamic neurons. Endotoxemia causes damage in both the intestine and brain, raising systemic and cerebral inflammatory mediators (IL-1β, TNFα, and IL-6), associated with gliosis signs in hypothalamic areas responsible for controlling energy balance and maintaining body weight (36, 57, 126, 171). Also, studies in recent years have shown that some commensal bacteria produce endocannabinoid (eCB)-like metabolites functioning in the same receptors and cells as the host (36).

Current data indicate an essential link between obesity development and disorders in the ECS in different tissues involved in regulating metabolism (21, 80, 106, 139). Hyperactivity of ECS is characterized by an increase in the concentration of eCB (such as N-arachidonoethylamine [anandamide, AEA] and 2-arachidonoylglycerol [2- AG]), modification of the expression of cannabinoid receptors (CB1R and cannabinoid type 2 (CB2R), and alterations in crucial enzymes involved in the biosynthesis and degradation of eCB acid amide hydrolase (FAAH, the enzyme that degrades AEA) and N-acylethanolamines (NAEs, AEA counterparts) are generally evident during the development of obesity (21, 80, 106, 139). In the hypothalamus, endocannabinoids are involved with increased orexigenic neuropeptides and decreased anorectic neuropeptides (15, 22). CB1R deficient mice are hypophagic, lean, sensitive to insulin, and are protected from diet-induced obesity (15, 119). In genetic models of obesity (Zucker fa/fa rats and ob/ob and db/db mice) with an interruption in leptin signaling, high hypothalamic levels of endocannabinoids were observed compared to wild-type animals (106). Studies with obese humans have found ECS dysregulation with higher AEA and 2-AG in plasma and adipose tissue (6, 23). The increase in 2-AG levels in obese rodents seems to be related to leptin signaling deficiency (21). Di Marzo *et al.* were pioneers in reporting that intravenous leptin injection decreases hypothalamic levels of AEA and 2-AG in rats (21). However, other hormones such as ghrelin and glucocorticoids have also been indicated as critical agents for increasing the levels of endocannabinoids and inducing hypothalamic dysregulation in obesity (16, 29).

Strategies aimed at changing lifestyle with effects on the intestinal microbiota and the ECS may represent a safer alternative than pharmaceutical approaches (20). Data obtained through experiments with rodents demonstrated that physical exercise alters the composition of the intestinal microbiota (2, 14, 100, 112, 129). Rats submitted to physical exercise showed an increase in n-butyrate. It was also observed that the total distance covered by these animals correlates inversely with the Bacteroidetes-Firmicutes ratio (81). Obese Zucker (fa/fa) rats under moderate-intensity running training had altered the composition and diversity of its intestinal bacteria, similarly to that of their non-obese congeners (113). Human studies have also shown positive effects of physical exercise on the microbiota. It was seen that an aerobic physical training program with brisk walking for 12 weeks increased the content of Bacteroides spp in sedentary older women (94). Reinforcing these findings, Allen *et al.* (2) found that obese and lean individuals had different intestinal microbiota compositions at the beginning of the study. After 6 weeks of endurance exercise training, this difference disappeared. It was further observed that SCFAs concentrations increased in lean individuals. Such an attempt at analogy and interpretation of the data can be reinforced by a study investigating the effects of the participation of overweight sedentary women in an aerobic exercise program (exercise bike, three times a week, for six weeks) (100). The authors found that physical

exercise increased the relative abundance of the genera Verrucomicrobia and Akkermansia and decreased the number of proteobacteria associated with inflammation in the intestine. A previous study found a reduction in the Firmicutes/Bacteroidetes ratio in patients with pre-diabetes and type 2 diabetes mellitus (DM2) in response to both sprint interval training and moderate-intensity continuous training program (99). Furthermore, both training programs reduced systematic and intestinal inflammatory markers (TNF-α and LPS) (99). Although the evidence points to positive effects of physical exercise on the microbiota, it is relevant to document that the adaptations obtained seem to be reversed after the discontinuity of training (2). Otherwise, forced and exhaustive exercise can have a different impact than moderate exercise (173). In an experiment that sought to assess the effects of overtraining on an animal model, it was found that the diversity of the microbiota was reduced in mice (173).

The effects of physical exercise have also been promising in ECS. Elegantly, after submitting trained male university students to an acute treadmill running or cycling session (for 50 min at 70-80% of maximum heart rate), Sparling *et al.* showed for the first time that moderate exercise activates the ECS (144). Other studies that evaluated the acute effect of physical exercise in humans also found an increase in plasma levels of endocannabinoids (i.e., AEA) (53, 118). Interestingly, a study investigating the effects of 1 year of intervention with lifestyle modification, including physical activity in obese humans, found that this strategy induced a significant decrease in plasma AEA and 2-AG levels and fasting visceral fat (20). Gamelin *et al.* demonstrated that chronic aerobic exercise attenuates the CB1R gene induced by a high-fat diet in Wistar rats (38); however, the same study showed a high-fat diet combined with physical training did not affect AEA and 2-AG levels in the rats hypothalamus. Therefore, further studies about the impact of physical exercise on ECS, especially on the central nervous system, are needed. Discoveries in this area of knowledge will undoubtedly open new paths for future approaches involving microbiota, ECS, and physical exercise, which will allow advances and perspectives to combat disorders linked to obesity.

In addition to microbiota and the endocannabinoid system, accumulating evidence has demonstrated that resistin plays a critical role in controlling neuroinflammation and hypothalamic insulin and leptin resistance. Resistin is recognized as a hormone, and high levels of this substance are strongly associated with insulin resistance, DM2, cardiovascular diseases, and other metabolic abnormalities (98). It has been demonstrated that while resistin is mainly produced and secreted from adipose tissue of rodents (148), in humans, resistin is predominantly expressed in cells derived from the immune system, including macrophages and peripheral blood mononuclear cells (133). Resistin signaling remains unclear; however, it has been proposed that TLR-4 and adenylyl cyclase-associated protein 1 (CAP1) play a critical role in the resistin signaling transduction (107). Benomar and colleagues demonstrated that resistin binds to TLR-4 and elicits hypothalamic JNK activation and insulin resistance in rodents (7).

Conversely, the disruption of hypothalamic resistin signaling reduced several inflammatory markers and improved insulin sensitivity in rodents (123). Importantly, it has been nicely demonstrated that resistin controls the expression of leptin signaling components, including the leptin receptor and SOCS-3 in the arcuate nucleus of the hypothalamus (176). These studies collectively support the hypothesis that resistin is involved in controlling hypothalamic inflammation and insulin and leptin resistance, at least in rodent obesity models.

Physical exercise effects on resistin serum levels have been extensively investigated. While several studies reported that physical exercise modulates resistin serum levels in both mice and humans, other studies showed no alterations. Shirvani and colleagues reported that 6 weeks of eccentric training increased irisin and nesfatin-1 but reduced the resistin serum levels in rats (136). Curiously, Liu *et al.* reported that 8 weeks of aerobic exercise decreased serum resistin levels in diabetic (*db/db*) mice. Still, no alterations were observed in insulin resistance or glucose tolerance (72). However, Sousa *et al.* reported that 8 weeks of physical training reduced inflammatory markers but did not change the resistin levels of obese mice (143). These discrepant findings could be associated with the different exercise protocols, for instance, aerobic vs. resistance, 6wk vs. 8 wk, and moderate vs. intense. Furthermore, the animal models used in these studies were also different, including lean, obese, and obese/diabetic. Thus, further studies are required to define the effects of different exercise protocols in controlling resistin secretion in animal models.

The effects of physical exercise on resistin serum levels in humans have been investigated in the last decades, particularly in obese and type 2 diabetic patients. In 2007, Kadoglou *et al.* reported that 16 weeks of aerobic exercise training reduced the resistin serum levels in patients with DM2 (58). Furthermore, a multicentric study revealed that moderate to vigorous exercise training reduced resistin serum levels in humans, but was not associated with alterations in central adiposity (166). A similar result was observed in overweight and obese adults in response to 12 months of moderate exercise (43). Although several studies have demonstrated that chronic exercise may reduce the resistin levels in overweight, obese and diabetic individuals, a meta-analysis study revealed that exercise did not promote a significant reduction in resistin levels (49).

Furthermore, the impact of exercise on resistin serum levels was not observed after an acute session of high-intensity interval exercise in young overweight/obese women (165) or after high-intensity training in chronic obstructive pulmonary disease (35). Conversely, Fonseca and coworkers demonstrated that an acute session of exercise until fatigue increased serum resistin levels in sedentary but not in trained individuals (9). Although all of these studies evaluated the impact of exercise on resistin content in murine and humans, the precise implications involving the resistin signaling to the hypothalamus in response to physical exercise remains unknown and deserves further investigations.

CONCLUSIONS AND FUTURE DIRECTIONS

The effects of physical exercise on food intake control have been investigated over the last 70 years. Human and animal models have shown different outcomes due to the type of exercise (resistance, aerobic), modality (running, swimming, cycling), environmental characteristics (i.e., cold, heat, and hypoxia) (4, 10, 57), training load (i.e., frequency, volume, and intensity), and recovery intervals following exercise sessions, which could

have a notable impact on food intake and even on spontaneous activity (8, 17, 35, 39, 43, 84, 126), challenging the determination of food consumption in exercised individuals. Furthermore, the host factors (i.e., gender, lean, obese, age, etc.) seem to be critical. Chronic exercise induces an extensive modification in human metabolism, changes the hormonal profile, and may promote a negative energy balance, contributing to body weight loss, but the influence on appetite control is still controversial. However, the amount of recent evidence showing that an acute session of moderate and intense exercise reduces energy intake in overweight and obese individuals has increased. Most of the advances in this field have been obtained with the use of animal models of obesity. Molecular analyses in the hypothalamic tissue have revealed that exercise improves anorexigenic signals in the hypothalamus of obese animals. Currently, the central anti-inflammatory response and myokines produced during muscle contraction are the main mechanisms explaining how physical exercise can recapitulate the hypothalamic anorexigenic signals in obese conditions. New studies and technologies are required to clarify in detail the impact of physical exercise on the control of food consumption.

ACKNOWLEDGEMENTS

In *memorian* to friend and lab technician Sr. Jósimo Pinheiro.

Funding information

The work was supported by National Council for Scientific and Technological Development (CNPq; process number 306535/2017-3; 301279/2019-5; 304771/2017-1), Coordination for the Improvement of Higher Education Personnel (CAPES; finance code 001), and São Paulo Research Foundation (FAPESP; process numbers 2019/00227-1; 2018/20872-6; 2018/07634-9; 2019/2179-4).

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Effect of exercise-conditioned human serum on the viability of human cancer cell cultures: A systematic review and meta-analysis

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ABSTRACT:

Numerous epidemiological studies have shown the existence of a relationship between exercise and a reduced risk of different types of cancer. In vitro studies have identified a direct effect of exercise-conditioned human serum on cancer cell lines of the lung, breast, prostate, and colon. The aim of this systematic review with meta-analysis (SRM) was to estimate the magnitude of the effect that exercise-conditioned human serum produced on the viability of human cancer cell cultures. The design followed the PRISMA guidelines and the TREND statement to assess the quality of information (QoI) in each study. Nine in vitro studies were included in the SRM, involving a total of nine cancer cell lines and serum from 244 individuals from different countries, including namely healthy sedentary individuals, at risk of prostate cancer individuals and cancer patients, with ages ranging from 18 to 73 years. The impact of acute exercise-conditioned human serum on the viability of cancer cell cultures was analysed by a variety of assays, using pre-exercise human serum for comparison purposes. Globally, cultures of cancer cell lines exposed to human serum conditioned by acute exercise of various intensities exhibited a reduced viability, when compared with control cultures, with an overall effect size (ES) of -1.126 (95% CI; -1.300 to -0.952; $p < 0.001$). When the analysis only included human serum conditioned by acute high-intensity exercise, the effect became more pronounced (ES -1.350; -1.522 to -1.179 (95% CI); $p < 0.001$). These results are in line with the hypothesis that changes in human serum induced by exercise might play a role in the beneficial effects of physical activity in cancer prevention and management and that these effects depend on exercise intensity.

Keywords: physical activity; anticancer activity; cancer prevention; cancer management; cellular studies; tumor

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1. Introduction

Cancer is a major public health problem worldwide, with a significant impact on people's quality of life and health costs (11, 53). Significantly, a large proportion of new cancer cases and cancer deaths are preventable by eliminating or reducing exposure to environmental risk factors and/or modifying lifestyles, as revealed by recent studies (13, 63). In terms of lifestyle factors, consistent data from epidemiological studies suggest that physical activity reduces risk for various cancers and improves cancer survival rates (24, 25, 38, 45, 48). According to the 2018 Physical Activity Guidelines Advisory Committee (PAGAC), there is strong evidence that increased physical activity reduces the risk of several major types of cancer by 10% to 21%, including breast, colon, endometrium, bladder, stomach, oesophagus, and kidney cancers (42), moderate evidence for a reduced risk of lung cancer 21%-25% (25, 42), and limited evidence for a reduced risk of prostate cancer (42). On the contrary, an increased risk of melanoma has been observed in some cases (48). The evidence for an association between physical activity and survival after cancer is more limited, although emerging data suggest a 40% to 50% reduction in mortality from breast, colon and prostate cancer (4, 42), as well as a decrease in disease recurrence (4). Epidemiological evidence also suggests that the benefits of physical activity in terms of cancer prevention, particularly in the case of lung cancer, are also present in smokers (12). Of note, levels of cardiorespiratory fitness and the primary incidence of various forms of cancer have been found to be inversely correlated (3). Unfortunately, the optimal amount of physical activity (namely in terms of frequency, type, intensity, and duration) required to produce the above-mentioned benefits is still unknown (48).

It is believed that the effects of physical activity on prevention are mediated by multiple systemic responses, namely by reducing inflammation and improving immune system function and surveillance (30, 62). In addition, physical activity can also have a direct effect on cancer cells. In order to understand whether and to which extent cancer cells are affected by exercise, i.e., by the subset of physical activity that is planned, structured, repetitive, and performed with the purpose of improving or maintaining physical fitness, several research groups investigated the impact of acute

and/or chronic exercise-conditioned serum from animals or humans in human cancer cell lines (5-7, 15-17, 31, 33, 37, 39, 46, 50, 55, 57). Although using different endpoints, all of these studies ultimately assessed the effects of acute and/or chronic exercise on cell culture viability under a variety of experimental contexts, which were then taken as a measure of transformation status. Endpoints included, but were not restricted to, number of live or viable cells in culture, ability for anchorage-independent growth, clonogenic potential, and levels of apoptosis. These studies differed in various other aspects of their design, such as in number and characteristics of participants recruited, type and intensity of the exercise intervention, time points of serum collection, cell line and exposure regimen (serum concentration and duration of exposure). Also, in some of these studies, the intervention involved not only exercise, but a combination of exercise and other factors (e.g., dietary modification). While most studies showed that exercise-conditioned serum affected cancer cell culture viability, the magnitude of the effects varied across the studies, even for those employing a similar design or the same cancer cell lines (33, 50). Altogether, the results suggest that the observed effects are dependent on exercise intensity, suggesting also that they might be modulated by other factors.

One major aim of this systematic review with meta-analysis was to quantify the magnitude of the effect of acute exercise-conditioned human serum on the viability of human cancer cell cultures and to verify if this effect was dependent on the intensity of the exercise performed. Our hypothesis is that human serum conditioned by acute exercise reduces the viability of cancer cells and that this effect is dependent on exercise intensity, when compared to the corresponding pre-exercise serum.

2. Methods

2.1 Search Strategies

Search strategies followed the PRISMA guidelines (44) and were based on the following descriptor terms and keywords defined by the authors and indexed in the Medical Subject Headings (MeSH, U.S.National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894): ((exercise* OR "physical activity" OR sport* OR training OR "resistance training" OR "aerobic training" OR "high intensity interval training" OR "physical exercise") AND (neoplasm* OR tumor* OR malignant* OR cancer* OR carcinoma) AND (cell* OR "cell culture" OR "*in vitro*")). This combination was used to search the following academic journal databases: PubMed, Web of Science, SPORTDiscus and Scopus. The advanced options were carried out using the filter by title into each database. Research procedures were carried out in July 2020.

2.2 Data Extraction

Data from search were imported into EndNote X7 (Thomson Reuters EndNote X7). After this, the following screening procedures were implemented to select the relevant articles for the study: (a) all duplicates were removed; (b) articles whose title and abstract did not provide enough information on the topic were removed; (c) articles whose full texts did not meet the inclusion criteria were removed; (d) quality of information (QoI) from each study was checked using the TREND statement guidelines (14). If the total score for TREND items and sub-items was below 50% of the maximum score, publications were excluded from the study due to lack of QoI. Additionally, if the article's full text did not provide complete data, authors were contacted by e-mail requesting the missing information. If no response was obtained, the article was excluded from the study.

2.3 Criteria for Study Selection

The inclusion criteria used to select the articles for the present study were: a) the effects of exercise-conditioned serum were assessed *in vitro* using human cancer cell lines; b) the exercise-conditioned serum was of human origin; c) exercise was the only intervention, i.e., no combined interventions (e.g., diet and exercise) were involved; d) written in English.

2.4 Methodological Design

The PRISMA Statement (40) positioning guidelines were followed to assist the design of this SRM. These guidelines describe the four stages (identification, screening, eligibility, and final selection) required to search and select manuscripts for a systematic review and feature the option of illustrating procedures in a flowchart (40). The PRISMA presents the PICOS acronym ("patient, problem or population", "intervention", "control or comparison" and "outcomes"), which helps making research questions and systematic searches more effective (47). Qualitative data from the different articles were selected, extracted, and organized in a specific table, following the PRISMA method, i.e., including authors, year and country, number of participants included, their age and gender, cancer type, intervention characteristics, central outcomes, and the existence of a control group. The protocol for this systematic review and meta-analysis has been registered at the International Platform of Registered Systematic Review and Meta-Analysis Protocols (INPLASY) under the registration number: INPLASY2020120096.

2.5 Quality of Information (QoI)

The Quality of Information (QoI) of the articles included in the systematic review was evaluated with application of the TREND statement guidelines (Transparent Evaluation Report with Nonrandomized Designs) (14). The method requires the evaluation of a list of 22 items (general criteria), subdivided into 59 sub-items (specific criteria) to quantitatively assess the QoI (14). One point is assigned to each reported sub-item. All studies with QoI $\geq$50% were included in the meta-analysis, since this qualification considers them as highly relevant for the topic under study.

2.6 Publication Bias

The publication bias was calculated using the software Comprehensive Meta-Analysis (CMA) (Biostat, Englewood, NJ, USA, version 3.3.070) creating a funnel plot by the standard error (y-axis) and the standard difference in means (x-axis) to determine whether the plot was balanced. Funnel plots are either symmetrical or asymmetrical (18). Studies without publication bias are distributed symmetrically around the mean effect size, since the sampling error is random. Studies with publication bias are expected to follow the model with symmetry at the top of the funnel plot, a few studies missing in the middle, and more studies missing near the

bottom of the plot. If the direction of the effect is toward the right, then near the bottom of the funnel plot we expect a gap on the left, where the non-significant studies would have been if we had been able to locate them. Because the interpretation of the funnel plot is sometimes subjective, different tests such as the Begg, and Mazumdar, and the Egger's tests have been proposed to quantify bias and test the relationship between sample size and effect size (8, 19). In the present study, the Egger´s test was used to check publication bias as suggested by Borenstein et al. (10).

2.7 Effect-Size Calculations

This meta-analysis was conducted to quantify the magnitude of the effects of acute exercise-conditioned human serum on the viability of cancer cell cultures. The meta-analysis took into account the intensity of the exercise performed before blood collection. Effect size was calculated using the software Comprehensive Meta-Analysis (CMA) (Biostat, Englewood, NJ, USA, version 3.3.070). The effect-size metric selected was the standardized difference in means (Std diff in means), since all studies evaluated the same outcome variable, but with different criteria. In such circumstances, it is necessary to standardize the results from each study using a uniform scale before they can be combined (20).

Data extracted for effect-size calculations from the different studies included sample size (N), statistical significance (p value) and effect direction. A random-effects model was used for the present meta-analysis, as it combines sampling error and between-study variance to estimate effect size (20). The following thresholds were used to interpret the effect sizes: trivial ($d < 0.20$), small ($0.21 < d < 0.50$), moderate ($0.51 < d < 0.79$), and large ($d > 0.80$) (10).

2.8 Heterogeneity of Variance

We followed the assumption that there would be variability in the true effect sizes between studies due to the expected differences in sampling error and between-study variance. The following statistics were used to quantify between-study heterogeneity: Q-value, I-squared (I^2), tau-squared (τ^2), and tau (τ). The Q Cochran statistic was used as a significance test to verify the null hypothesis and assess if all publications involved in this SRM share common effect sizes. Any variation would be due to the sample error within the studies. If all studies share the same effect size, the expected Q value will be equal to the degrees of freedom (df), e.g., the number of studies minus one. The I^2 statistic corresponds to the ratio between the true heterogeneity and the total variation of the observed effects. It shows the proportion (percentage) of the observed variance that reflects the differences in the true effect size rather than in the sample error (2). The τ^2 is the variance of the true effect sizes (in log units) among studies, while the τ value refers to the standard deviation of the true effects (20).

3. Results

3.1 Study Selection

A total of 888 publications were identified through an electronic database search and one additional publication was identified through cross-referencing (*Figure 1*). After duplicate removal, 389 publications were screened by title and abstract. Of these, 363 publications were excluded, because they did not reflect the research question or were meeting abstracts. At the eligibility stage, the full texts of the remaining 26 publications were read and we excluded: ten studies that were conducted on animals, rather than on cell lines; five studies where interventions involved not only exercise, but also diet; one study that focused on electrostimulation intervention; and one study that evaluated the methods used, rather than the effects of exercise-conditioned human serum in cancer cells. The TREND methodology guidelines were applied to the nine publications that fulfilled all eligibility criteria. All were accepted and regarded as satisfactory for the inclusion in the SRM, as their QoI scores were higher than 50% (*Table 1*).

3.2 Characteristics of the Studies and Participants

This SRM included nine publications of in vitro studies: four publications on prostate cancer, two publications on breast cancer, one publication on prostate cancer and breast cancer, one publication on lung cancer and one publication on colon cancer. All studies followed the same research hypothesis, testing the effect of acute and/or chronic exercise-conditioned human serum on the viability of human cancer cell cultures. The samples tested were obtained from a total of 244 participants from different countries, with ages varying between 18 to 73 years. Participants' characteristics varied significantly across the studies and included healthy sedentary women, breast cancer patients, women after cancer treatment, healthy sedentary men, men considered to be at risk of prostate cancer, and male colorectal cancer survivors.

Altogether, nine cell lines were used: two prostate cancer cell lines (LNCaP and PC3), two breast cancer cell lines (MCF-7 and MDA-MB-231), three lung cancer cell lines (A549, H460 and H1299) and two colon cancer cell lines (CaCo and LoVo). The different studies employed a variety of complementary viability assays (*Table 2*) and some of them also assessed apoptotic cell death (*Table 3*). Culture medium was supplemented with either 5% (v/v) or 10% (v/v) human serum. The types of exercise (one single session) or training (repeated exercise sessions performed periodically) performed were: (1) integrative (two or more activities by exercise (e.g., strength exercise plus cycling exercise) at intensities between 50% to 95% of their VO_2peak or one maximal repetition at workload resistance); (2) high intensity (cycling intervals with active rest periods at 85% – 95% of their VO_2peak); (3) moderate intensity (ergometer cycling at 50% – 65% of their VO_2peak).

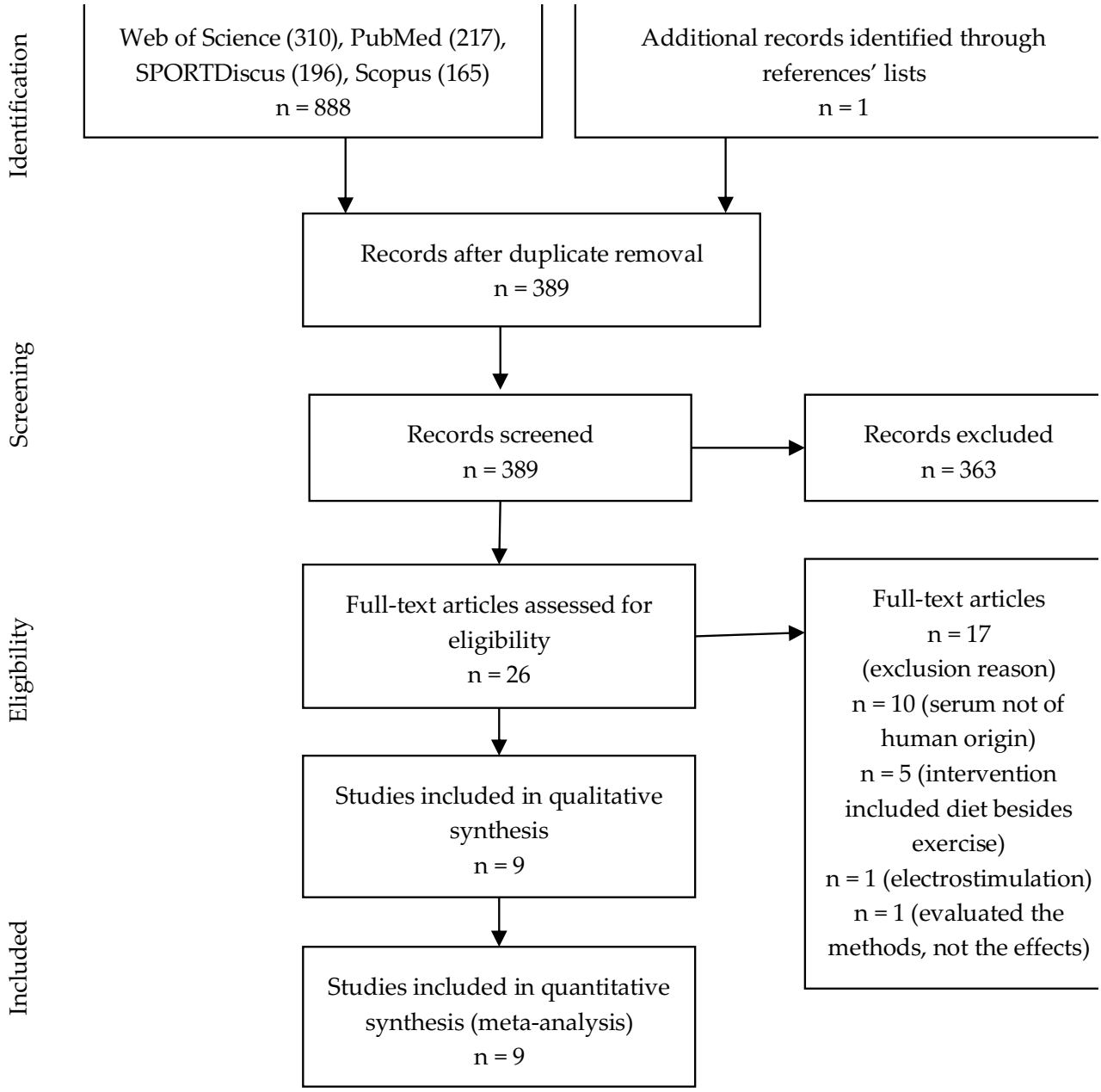

Figure 1. Schematic description of the different phases of the systematic search performed according to the PRISMA statement guidelines.

Study	TREND Assessment Protocol																							
	Title and abstract	Introduction	Participants	Interventions	Objectives	Outcomes	Sample size	Assignment method	Blinding (masking)	Unit of analysis	Statistical methods	Participant flow	Recruitment	Baseline data	Baseline equivalence	Numbers analysed	Outcomes and estimation	Ancillary analyses	Adverse events	Interpretation	Generalizability	Overall evidence	Total item	Total percentage
Items (paper sections)	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	22	100%
Sub-items (descriptor) per items	3	2	4	8	1	3	1	3	1	2	4	7	1	4	1	2	3	1	1	4	1	1	58	100%
Barnard et al. 2003 (7)																								
Item	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	18	81.8%
Sub-item	3	2	2	3	1	3	0	1	0	1	3	2	0	3	1	2	4	1	0	4	1	1	38	65.5%
Leung et al. 2004 (39)																								
Item	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	18	81.8%
Sub-item	2	2	3	5	1	3	0	1	0	2	1	3	0	3	1	2	3	1	0	3	1	1	38	65.5%
Rundqvist et al. 2013 (50)																								
Item	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	19	86.4%
Sub-item	2	2	1	5	1	3	0	1	0	2	2	2	0	2	1	2	3	1	1	3	1	1	36	62.1%
Dethlefsen et al. 2016 (16)																								
Item	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	19	86.4%
Sub-item	2	2	4	7	1	3	1	0	0	2	3	4	0	4	1	2	3	1	1	3	1	1	46	79.3%
Dethlefsen et al. 2017 (15)																								
Item	1	1	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	1	0	1	1	1	16	72.7%
Sub-item	2	2	2	5	1	3	0	0	0	1	3	3	0	2	0	2	3	1	0	3	1	1	35	60.3%
Kurgan, et al. 2017 (37)																								
Item	1	1	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	1	0	1	1	1	16	72.7%
Sub-item	2	2	1	5	1	3	0	0	0	2	3	2	0	2	0	2	3	1	0	2	1	1	33	56.9%
Devin et al. 2019 (17)																								
Item	1	1	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	1	0	1	1	1	16	72.7%
Sub-item	3	2	2	5	1	3	0	0	0	2	3	2	0	2	0	2	2	1	0	3	1	1	35	60.3%
Hwang, et al. 2020 (33)																								
Item	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	18	72.7%
Sub-item	1	1	2	3	1	3	0	1	0	1	2	4	0	3	1	2	3	1	0	3	1	1	34	58.6%
Baldelli et al. 2020 (5)																								
Item	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	18	81.8%
Sub-item	3	2	2	5	1	3	0	1	0	1	3	2	0	3	1	2	3	1	0	3	1	1	38	65.5%

Table 1. Categories and subcategories that emerged from the results in the nine studies selected for meta-analysis.

Note: Roman numerals refer to the 22 categories of the TREND assessment protocol. Arabic numerals indicate the number of sub-items reported for each item.

Table 2. Synthesis of the systematic search of articles assessing the effects of acute and chronic exercise-conditioned human serum on the viability[1] of human cancer cell cultures.

Study	Country	Cancer	Sample N	Subjects	Age	BMI	Design	Exercise	Systemic marker	In vitro assay[1]	Outcomes Main results by cell line	*p value*	Output
Acute													
Baldelli et al. 2020 (5)	Italy	Breast cancer	12	Healthy sedentary women	21 (0.8)	21 (1.3)	Non-randomized *in vitro* study Control Group (5% pre-exercise serum) and Exercise Group (5% serum collected immediately, 4 hours and 24 hours after exercise)	High Intensity Endurance Cycling (HIEC; 5 min warm-up + four 5 min incremental stage (50%, 55%, 60%, 70%) + ten 90 sec sprints at 90% interspersed by a 180 sec recovery) before(I) and after(II) 9 weeks period training	CK levels ↑ In post-HIEC (t1) and then decreased steadily, returning to basal values after 24 hours	Trypan blue dye exclusion assay Three- dimensional (3D) cell culture assay (soft agar) Trypan blue dye exclusion assay Three- dimensional (3D) cell culture assay (soft agar)	a) MDA-MB-231 (I 0 hours) 12.1% b) MDA-MB-231 (I 4 hours) 24.0% c) MDA-MB-231 (I 24 hours) 24.9% d) MDA-MB-231 (II 0 hours) 15.7% e) MDA-MB-231 (II 4 hours) 30.6% f) MDA-MB-231 (II 24 hours) 35.3% g) MDA-MB-231 (I 0 hours) 17.6% h) MDA-MB-231 (I 4 hours) 33.7% i) MDA-MB-231 (I 24 hours) 30.0% j) MDA-MB-231 (II 0 hours) 10.1% k) MDA-MB-231 (II 4 hours) 18.2% l) MDA-MB-231 (II 24hours) 15.6% m) LNCaP (I 0 hours) 13.8% n) LNCaP (I 4 hours) 21.8% o) LNCaP (I 24 hours) 22.8% p) LNCaP (II 0 hours) 14.0% q) LNCaP (II 4 hours) 22.9% r) LNCaP (II 24 hours) 27.2% s) LNCaP (I 0 hours) 15.95% t) LNCaP (I 4 hours) 21.45% u) LNCaP (I 24 hours) 25.94% v) LNCaP (II 0 hours) 14.44% x) LNCaP (II 4 hours) 20.31% z) LNCaP (II 24 hours) 25.79%	p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001	Reduction in breast cancer cell viability and prostate cancer cell viability, stimulated with all human serum conditioned by HIEC. Result were remarkably similar between human serum obtained immediately after the HIEC and the Human serum collected after 4hours and 24hours.
		Prostate cancer	18	Healthy sedentary men	21 (1.4)	22 (2.8)							
Hwang et al. 2020 (33)	Australia Australia	Prostate cancer	12	Healthy young men	28.2 (2.6)	22.8 (2.1)	Non-randomized experimental *in vitro* study Control Group (5% serum collected pre-exercise) and Exercise Group (5% serum collected post-exercise)	65 min exercise electro-dynamically loaded cycle ergometer (20 min 50% VO2peak + 40min 65% VO2peak)	Granulocyte ↑ Lymphocyte ↑ Testosterone ↑ (young group) osteonectin (SPARC) ↑ oncostatin M (OSM) ↑ IL-6, IL-15 and Irisin (not consistently detectable or did not change)	DNA quantification assay Alamar blue assay DNA quantification assay Alamar blue assay	a) LNCaP (no sig. difference) b) LNCaP (no sig. difference) c) PC3(no sig. difference) d) PC3 (no sig. difference) e) PC3 (no sig. difference) f) LNCaP (no sig. difference) g) LNCaP (sig. reduction) h) PC3 (no sig. difference)	p > 0.05 p > 0.05 p > 0.05 p > 0.05 p > 0.05 p > 0.05 p < 0.01 p > 0.05	Pre-exercise serum, collected from older men after a single bout of exercise reduced the metabolic activity of (androgen-responsive) LNCaP prostate cancer cells. No such effect was observed after treatment of the cancer cells with pre- versus post-exercise serum collected from young men.
			10	Healthy old man	63.1 (6.9)	24.9 (1.92)							
Devin et al. 2019 (17)	Australia	Colon cancer	10	Male colorectal cancer survivors	66.9 (8.4)	27.7 (3.6)	Non-randomized experimental *in vitro* study Control Group (10% serum collected at rest) and Exercise Group (10% serum collected immediately after exercise or 120 min after exercise)	Acute trial - HIIE 38 min (10 min warm-up; 4x4 minutes bouts of cycling 85-95% Hr peak with 3 minutes active recovery)	*IL-6 ↑ *IL-8 ↑ *TNF-α ↑ *(returned to baseline levels at 120 min post-exercise) **Insulin ↑ ** (significantly lower than baseline at 120 min)	Alamar blue assay	a) CaCo2 (0 hours at 24 hours of incubation) ES= -1.3 b) CaCo2 (0 hours at 48 hours of incubation) ES= -1.7 c) CaCo2 (0 hours at 72 hours of incubation) ES= -1.1 d) LoVo (0 hours at 24 hours of incubation) ES= -1.2 e) LoVo (0 hours at 48 incubation hours) ES=-0.8 f) LoVo (0 hours at 72 hours of incubation) ES= -1.1 g) CaCo2 (2 hours at 24 hours of incubation) h) CaCo2 (2 hours at 48 hours of incubation) i) CaCo2 (2 hours at 72 hours of incubation) j) LoVo (2 hours at 24 hours of incubation) k) LoVo (2 hours at 48 hours of incubation) l) LoVo (2 hours at 72 hours of incubation)	p=0.002 p<0.001 p=0.035 p=0.001 p≥0.05 p=0.032 p≥0.05	The serological changes associated with acute HIIE transiently reduce colon cancer cell number.

Table 2. Synthesis of the systematic search of articles assessing the effects of acute and chronic exercise-conditioned human serum on the viability[1] of human cancer cell cultures (Continuation).

Study	Country	Cancer	Sample N	Subjects	Age	BMI	Design	Exercise	Systemic marker	Outcomes In vitro assay[1]	Main results by cell line	*p value*	Output
							Acute						
Kurgan et al. 2017 (37)	Canada	Lung cancer	23	Recreationally active male university students	21.8 (2.4)	23.9 (2.5)	Non-randomized experimental *in vitro* study Control Group (10% FBS treatment) and Exercise Group (10% serum collected 5min, 1h, 24h after exercise)	High Intensity Exercise Trial 90% (4 min warm-up; 6x(1min High Intensity cycling+1min active rest + 2-3 min cool-down)	Insulin - levels were not significantly changed - (pre-exercise, 5 minutes, 1 hour, or 24 hours post-exercise: 34.9 ± 3.6, 32.5 ± 5.6, 42.4 ± 6.3, 42.2 ± 3.6 pmol/L, respectively)	Crystal violet proliferation assay Clonogenic survival assay	a) A549 (5 minutes) 90.1 (4.2) % b) A549 (1 hour) 91.0 (2.1) % c) A549 (24 hours) 84.1(1.9)% d) A549 (5 minutes) 21.5 (2.9) % e) A549 (1 hour) 33.9 (3.5)% f) A549 (24 hours) 35.8(6.7)% g) H460 (5 minutes) 37.7 % h) H460 (1 hour) 38.4% i) H460 (24 hours) 39.3% j) H1299 (5 minutes) 33.5% k) H1299 (1 hour) 41.9% l) H1299 (24 hours) 37.7%	p < 0.05 p < 0.05 p < 0.05 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001 p < 0.001	The treatment of lung cancer cells with serum taken post-exercise leads to significant inhibition of cell survival and proliferation. Post-exercise serum did not have any significant effect on normal lung fibroblasts.
Dethlefsen et al. 2017 (15)	Denmark	Breast cancer	7	Healthy women	24.6 (1.0)	23.3 (2.7)	Non-randomized experimental *in vitro* study Control Group (10% pre-exercise serum) and Exercise Group 1 (10% 1 hour of exercise) or Exercise Group 2 (10% Serum collected after the end of exercise)	2h - 55% VO2peak cycling ergometer	Epinephrine ↑ Norepinephrine ↑ Lactate ↑	CellTiter-Fluor cell viability assay	a) MCF-7 (1 hour) 10%; b) MDA-MB-231 (1 hour) 14%; c) MCF-7 (2 hours) 19%; d) MDA-MB-231 (2 hour) 13%	p < 0.01 p < 0.05 p < 0.001 p < 0.05	Systemic changes occurring during exercise can reduce breast cancer cell viability.
			20	Breast cancer patients	48.5 (7.3)	26.0 (5.2)	Non-randomized experimental *in vitro* study Control Group (pre-exercise serum) and Exercise Group (serum collected immediately after exercise)	2hour (30 min warm-up +60 min whole body training + 30 min High Intensity)	IL6 ↑		e) MCF-7 11%; f) MDA-MB-231 9%;	p < 0.05 p < 0.05	
Dethlefsen et al. 2016 (16)	Denmark	Breast cancer	20	Breast cancer patients	49.5 (7.3)	26.0 (5.2)	Non-randomized experimental *in vitro* study Control Group (10% pre-exercise serum) and Exercise Group (10% serum after 2 hours acute exercise)	2hour (30 min warmup +60 min resistance training + 30 min High Intensity on stationary bicycles)	Insulin ↓ IL-6 ↑ IL-8 ↑ IL-10 ≃ TNF-α ↑ Lactate ↑ Epinephrine ↑ Norepinephrine ↑	CellTiter-Fluor cell viability assay	a) MDA-MB-231 -9.4%; b) MCF-7 -9.2%;	p < 0.001 p = 0.04	The marked systemic response occurring acutely during performance of exercise reduces breast cancer viability.
Rundqvist et al. 2013 (50)	Sweden	Prostate cancer	10	Healthy male	25		Non-randomized experimental *in vitro* study Control Group (5% serum collected pre-exercise) and Exercise Group (5% serum collected post exercise or 120 min after exercise)	65 min exercise electro-dynamically loaded cycle ergometer (20 min 50% VO2peak + 40min 65% VO2peak)	EGF ↓ IGFBP-1 ↑	Click-iT EdU microplate assay kit	a) LNCaP - reduce 9/10 individual serum b) LNCaP -31% (pool of 10 serum)	p < 0.05	Serum obtained directly after a bout of strenuous exercise, despite its mitogenic potential, reduced proliferation of prostate cancer tumor cells.

Table 2. Synthesis of the systematic search of articles assessing the effects of acute- and chronic exercise-conditioned human serum on the viability[1] of human cancer cell cultures (Continuation).

Study	Country	Cancer	Sample N	Sample Subjects	Sample Age	Sample BMI	Design	Exercise	Outcomes Systemic marker	Outcomes In vitro assay[1]	Outcomes Main results by cell line	Output *p value*	Output
Chronic													
Baldelli et al. 2020 (5)	Italy	Breast cancer	12	Healthy sedentary women	21 (0.8)	21 (1.3)	Non-randomized experimental *in vitro* study Control Group (5% serum collected at rest before training period) and Exercise Group (5% serum collected at rest after training period)	9 weeks training period (36 indoor cycling training sessions; 3 sessions per week - first 3 weeks - and 4 sessions per week from de fourth to the sixth week)	-----	Trypan blue dye exclusion assay Three-dimensional (3D) cell culture assay (soft agar)	w) LNCaP (no sig. difference) y) MDA-MB-231 (no sig. difference) aa) LNCaP (no sig. difference) bb)MDA-MB-231 (no sig. difference)	... ...	No significant difference between the proliferative and tumorigenic stimulatory capacity of human serum obtained at rest, before and after the training period.
		Prostate cancer	18	Healthy sedentary men	21 (1.4)	22 (2.8)							
Devin et al. 2019 (17)	Australia	Colon cancer	10	Male colorectal cancer survivors	64.9 (6.0)	30.2 (3.9)	Non-randomized experimental *in vitro* study Control Group (10% serum collected at rest before intervention) and Exercise Group (10% serum collected at rest after 4 weeks intervention).	Short-term training (4 Weeks - 3x HIIE/week)	IL-6 ≈ IL-8 ≈ TNF-α ≈ Insulin ≈ resting serum after 4 weeks of HIIE	Alamar blue assay	o) CaCo (no sig. difference) p) LoVo (no sig. difference)	p= 0.223 p= 0.375	Short-term HIIE training was not associated with changes in cell number or common metabolic factors related to the risk of colorectal cancer when measured at rest.
Dethlefsen et al. 2016 (16)	Denmark	Breast cancer	37	Women after cancer treatment (PA - physical activity group)	46.0 (9.6)	24.0 (3.8)	Non-randomized experimental *in vitro* study Control Group (10% rest serum) and Exercise Group (10% serum collected at rest after 6 months of intervention)	Exercise ≥ 3 hours/week Health evaluation without exercise counselling	Insulin ↑ Leptin ≈ Glucose ≈ LDL/HDL ↓ IL-6 ↓ IL-8 ≈ IL-10 ↓ TNF-α ↓	CellTiter-Fluor cell viability assay	c) MCF-7 no effect d) MDA-MB-231 no effect e) MCF-7 no effect f) MDA-MB-231 no effect	... ...	The systemic adaptations to 6 months of training had no effect on breast cancer cell.
			37	Women after cancer treatment (HE - health evaluation group)	48.2 (7.8)	24.8 (3.7)							
Leung et al. 2004 (39)	EUA	Prostate cancer	10	Men considered to be at risk of (CG)	62 (2)	31.5 (1.6)	Non-randomized experimental *in vitro* study Control Group (10% serum of sedentary lifestyle at rest) and Exercise Group (10% serum collected at rest from adult with Exercise >10 years)	Sedentary lifestyle 5 days/wk for 1 hour (warm-up + 45-50 min of continuous, strenuous exercise	Insulin ↓ IGF-I ↓ IGFBP-1 ↑	CellTiter 96AQ assay	a) LNCaP 27%;	$p < 0.05$	Serum obtained from men after a regular exercise program for >10 years reduced the growth rate.
			12	Adult Fitness Program at least 10 years (EG)	60 (3)	26.5 (1)							
Barnard et al. 2003 (7)	EUA	Prostate cancer	14	Men considered to be risk (CG)	60 (3)	38 (2)	Non-randomized experimental *in vitro* study Control Group (10 % serum of sedentary lifestyle at rest) and Exercise Group (10% serum collected at rest from adult with Exercise >10 years)	Sedentary lifestyle 5 days/wK for 1 hour (warm-up + 45-50 min of continuous, strenuous exercise	Insulin ↓ IGF-I ↓ IGFBP-1 ↑	MTS assay	a) LNCaP 65 (4)%	$p < 0.01$	Intensive exercise training can favourably alter the IGF axis and reduce LNCaP prostate cancer cell growth.
			12	Men considered to be risk (EG)	62 (2)	26.5 (1)							

[1] For the majority of the studies, the viability assays employed do not allow to distinguish between changes in viability due to alterations in proliferation rates and those due to increased/decreased cell death.

Abbreviations: BMI, body mass index; EGF, epidermal growth factor; HDL, high-density lipoprotein; HIIE, high-intensity intermittent exercise; HR, heart rate; IGFBP-1, insulin-like growth factor binding protein 1; IGF-I, insulin-like growth factor-1; IL-6, interleukin-6; IL-8, interleukin-8; N, number of subjects; TNF-α, tumor necrosis factor alpha; VO_2peak, peak oxygen uptake.

Study	Country	Cancer	Sample N	Subjects	Age	BMI	Design	Exercise	Systemic marker	Outcomes In vitro assay	Main results by cell line	*p value*	Output
Acute													
Devin et al. 2019 (17)	Australia	Colon cancer	10	Male colorectal cancer survivors	66.9 (8.4)	27.7 (3.6)	Non-randomized experimental *in vitro* study Control Group (10% serum collected at rest) and Exercise Group (10% serum collected immediately after exercise or 120 min after exercise)	Acute trial - HIIE 38 min (10 min warm-up; 4x4 minutes bouts of cycling 85-95% HR peak with 3 minutes active recovery)	IL-6 ↑ IL-8 ↑ TNF-α ↑ Insulin ↑	Annexin-V Kit - flow cytometry (apoptosis)	m) CaCo2 (0 hours)-0.04% n) LoVo (0 hours) 0.64%	p=0.702 p=0.395	Distribution of apoptotic cells incubation with serum post HIIE was not significantly different.
Rundqvist et al. 2013 (50)	Sweden	Prostate cancer	10	Healthy male	25		Non-randomized experimental *in vitro* study Control Group (5% serum collected pre-exercise) and Exercise Group (5% serum collected post exercise or 120 min after exercise)	65 min exercise electro-dynamically loaded cycle ergometer (20 min 50% VO2 peak + 40min 65% VO2 peak)	EGF ↓ IGFBP-1 ↑	Annexin-V Kit (apoptosis)	c) LNCaP fraction of apoptotic cells did not differ between groups	-----	Acute exercise serum had no apoptotic effect on the tumours cells.
Chronic													
Leung et al. 2004 (39)	EUA	Prostate cancer	10	Men considered to be risk (CG)	62 (2)	31.5 (1.6)	Non-randomized experimental *in vitro* study Control Group (10%serum of sedentary lifestyle at rest) and Exercise Group (10%serum collected at rest from adult with Exercise >10 years)	Sedentary lifestyle 5 days/week for 1 hour (warm-up + 45-50 min of continuous, strenuous exercise	Insulin ↓ IGF-I ↓ IGFBP-1 ↑	Cell death detection ELISA plus (apoptosis)	b) LNCaP +371%;	p < 0.01	Serum obtained from men after a regular exercise program for >10 years induced apoptosis of LNCaP prostate cancer cell line.
			12	Adult fitness program at least 10 years (EG)	60 (3)	26.5 (1)							
Barnard et al. 2003 (7)	EUA	Prostate cancer	14	Men considered to be risk (CG)	60 (3)	38 (2)	Non-randomized experimental *in vitro* study Control Group (10% serum of sedentary lifestyle at rest) and Exercise Group (10% serum collected at rest from adult with Exercise >10 years)	Sedentary lifestyle 5 days/week for 1 hour (warm-up + 45-50 min of continuous, strenuous exercise	Insulin ↓ IGF-I ↓ IGFBP-1 ↑	Annexin-V / TUNEL assay (apoptosis)	b) LNCaP apoptotic cells increased significantly at culture with exercise-conditioned serum	--	Intensive exercise training can favourably alter the IGF axis and inducing apoptosis.
			12	Adult fitness program at least 10 years (EG)	62 (2)	26.5 (1)							

Table 3. Synthesis of the systematic search of articles assessing the effects of acute and chronic exercise-conditioned human serum on levels of apoptosis in human cancer cell cultures.

Abbreviations: BMI, body mass index; EGF, epidermal growth factor; HDL, high-density lipoprotein; HIIE, high-intensity intermittent exercise; HR, heart rate; IGFBP-1, insulin-like growth factor binding protein 1; IGF-I, insulin-like growth factor-1; IL-6, interleukin-6; IL-8, interleukin-8; N, number of subjects; TNF-α, tumor necrosis factor alpha; VO_2peak, peak oxygen uptake.

3.3 Meta-Analysis Outcomes

The nine publications under consideration presented separate results for more than one cell line and/or assay. The results of all samples were used in the meta-analysis for the overall effect outcomes, except in the case of a single study, in which cultures were exposed for different times to the same serum, in which case only the result from one incubation time was considered (17). Meta-analysis outcomes were: effect of acute exercise-conditioned human serum on the viability of cancer cell cultures (section 3.3.1); effect of high-intensity acute exercise-conditioned human serum on the viability of cancer cell cultures (section 3.3.2); effect of moderate-intensity exercise-conditioned human serum and of human serum conditioned by an integrative exercise of moderate and high-intensity on the viability of cancer cell cultures (section 3.3.3).

Due to limited data, we did not perform a quantitative analysis on the effects of acute exercise-conditioned human serum on levels of apoptosis and on the effects of chronic exercise-conditioned human serum on the viability of cancer cell cultures.

3.3.1 Effect of Acute Exercise-Conditioned Human Serum on the Viability of Human Cancer Cell Cultures

The meta-analysis on the effect of acute exercise-conditioned human serum on the viability of cancer cell cultures included the data from all samples and assays from seven studies. The high number of entries was due to the high number of samples and assays in each study. A random effects model was used to run the meta-analysis, the results showing, based on the standard difference in means, that acute exercise-conditioned human serum exhibited an overall effect size of -1.126 in reduction of the viability of cancer cell line cultures (*Figure 2*), when compared to the same cultures exposed to at pre-exercise human serum. The confidence interval for the standard difference in means was -1.300 to -0.952 (95% CI) with a corresponding p value < 0.001 (*Figure 2*). Of note, this interval does not include a zero effect. Similarly, z-values obtained to test the null hypothesis, that the standard difference in means is zero, showed a $z = -12.694$ and a corresponding value of $p < 0.001$ (*Figure 2*). Thus, the null hypothesis was rejected and the alternative hypothesis was accepted in all analysed studies, i.e., upon exposure of cancer cells to acute exercise-conditioned human serum there is a reduction in their viability, with a standard difference in means higher than 1 point.

When verifying the homogeneity of the effects, a Q-value of 130.350 was obtained, with 59 degrees of freedom and a $p < 0.001$ indicating a true effect-size, which was not identical in all studies. The I^2 value was 54.737, meaning that about 54.737 % of the variance in the observed effects reflects variance in the true effects. The τ^2 value had a value of 0.244. The τ value, i.e., the standard deviation of the true effects in this SRM was equal to 0.494. The funnel plot (Figure 5a) for the distribution of the observed studies was not entirely symmetrical, with a little trend of the studies distributed towards the left side of the mean effect size. Additionally, the Egger´s test was performed and the intercept value was -3.23286, with a 95% confidence interval between −4.61752 and -1.84819, t value = 4.67354. The recommended p value (2-tailed) was 0.00002. These statistical results show the lack of studies on the right side where the non-significant studies would be if there were any or if we had managed to locate them.

3.3.2 Effect of High-Intensity Acute Exercise-Conditioned Human Serum on the Viability of Human Cancer Cell Cultures

The meta-analysis of the effect of high-intensity acute exercise-conditioned human serum on the viability of cancer cell cultures was restricted to those four studies where the exercise intervention consisted of high-intensity exercise (>80% VO_2peak) and it included data from all samples and assays. A random effects model was used to run the meta-analysis and the results showed, based on the standard difference in means, that cancer cells cultured in the presence of acute high intensity exercise-conditioned human serum exhibited a reduction of -1.350 in their viability (*Figure 3*). The confidence interval for the standard difference in means was -1.522 to -1.179 (95% CI) with a corresponding value of $p < 0.001$ (*Figure 3*). Once again, this interval does not include a zero effect. Similarly, z-values obtained to test the null hypothesis i.e., that the standard difference in means is zero, showed a $z = -15.428$, and a corresponding value of $p < 0.001$ (*Figure 3*). Thus, the null hypothesis, was rejected and the alternative hypothesis accepted in all analysed studies, i.e., cultures of cancer cells exposed to acute high-exercise-conditioned human serum exhibited a lower viability than cultures of cancer cells exposed to at rest human serum.

When verifying the homogeneity of the effects, a Q-value of 59.006 was obtained, with 41 degrees of freedom and a $p < 0.05$ indicating that true effect-size was not identical in all studies. The I^2 value was 30.516, meaning that about 30.516% of the variance in the observed effects reflects variance in the true effects. The τ^2 value was 0.094. The τ value, i.e., the standard deviation of the true effects in this SRM, is equal to 0.306. The funnel plot (Figure 5b) for the distribution of the observed studies is not entirely symmetrical, with a trend towards the distribution of the studies on the left side of the mean effect size. Additionally, the Egger´s test was performed and the intercept value was -2.99967, with a 95% confidence interval between −4.19201 and -1.80734, t value = 5.08461. The recommended p value (2-tailed) was 0.00001. These statistical results show the lack of studies on the right side where the non-significant studies would be if there were any or if we had managed to locate them.

Study name	Std diff in means	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Baldelli et al 2020 a)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 b)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 c)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 d)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 e)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 f)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 g)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 h)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 i)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 j)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 k)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 l)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 m)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 n)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 o)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 p)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 q)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 r)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 s)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 t)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 u)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 v)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 x)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 z)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Devin et al 2019 a)	-1,615	0,515	0,265	-2,624	-0,605	-3,136	0,002
Devin et al 2019 d)	-1,754	0,526	0,277	-2,785	-0,722	-3,333	0,001
Devin et al 2019 g)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Devin et al 2019 j)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Hwang et al 2020 e)	0,000	0,408	0,167	-0,800	0,800	0,000	1,000
Hwang et al 2020 f)	0,000	0,408	0,167	-0,800	0,800	0,000	1,000
Hwang et al 2020 a)	0,000	0,408	0,167	-0,800	0,800	0,000	1,000
Hwang et al 2020 b)	0,000	0,408	0,167	-0,800	0,800	0,000	1,000
Hwang et al 2020 g)	-1,287	0,491	0,241	-2,250	-0,324	-2,620	0,009
Hwang et al 2020 h)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Hwang et al 2020 c)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Hwang et al 2020 d)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Kurgan et al 2017 a)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Kurgan et al 2017 b)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Kurgan et al 2017 c)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Kurgan et al 2017 d)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 e)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 f)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 g)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 h)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 i)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 j)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 k)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 l)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Rundqvist et al 2013 a)	-0,940	0,471	0,222	-1,863	-0,016	-1,994	0,046
Dethlefsen et al 2016 a)	-1,128	0,340	0,116	-1,795	-0,460	-3,312	0,001
Dethlefsen et al 2016 b)	-0,673	0,325	0,106	-1,310	-0,035	-2,069	0,039
Dethlefsen et al 2017 a)	-1,633	0,617	0,381	-2,842	-0,423	-2,645	0,008
Dethlefsen et al 2017 b)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Dethlefsen et al 2017 c)	-2,308	0,690	0,476	-3,660	-0,956	-3,345	0,001
Dethlefsen et al 2017 d)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Dethlefsen et al 2017 e)	-0,640	0,324	0,105	-1,276	-0,005	-1,974	0,048
Dethlefsen et al 2017 f)	-0,640	0,324	0,105	-1,276	-0,005	-1,974	0,048
Devin et al 2019 m)	-0,174	0,448	0,201	-1,052	0,704	-0,388	0,698
Devin et al 2019 n)	-0,390	0,451	0,204	-1,275	0,495	-0,863	0,388
Rundqvist et al 2013 c)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
	-1,126	0,089	0,008	-1,300	-0,952	-12,694	0,000

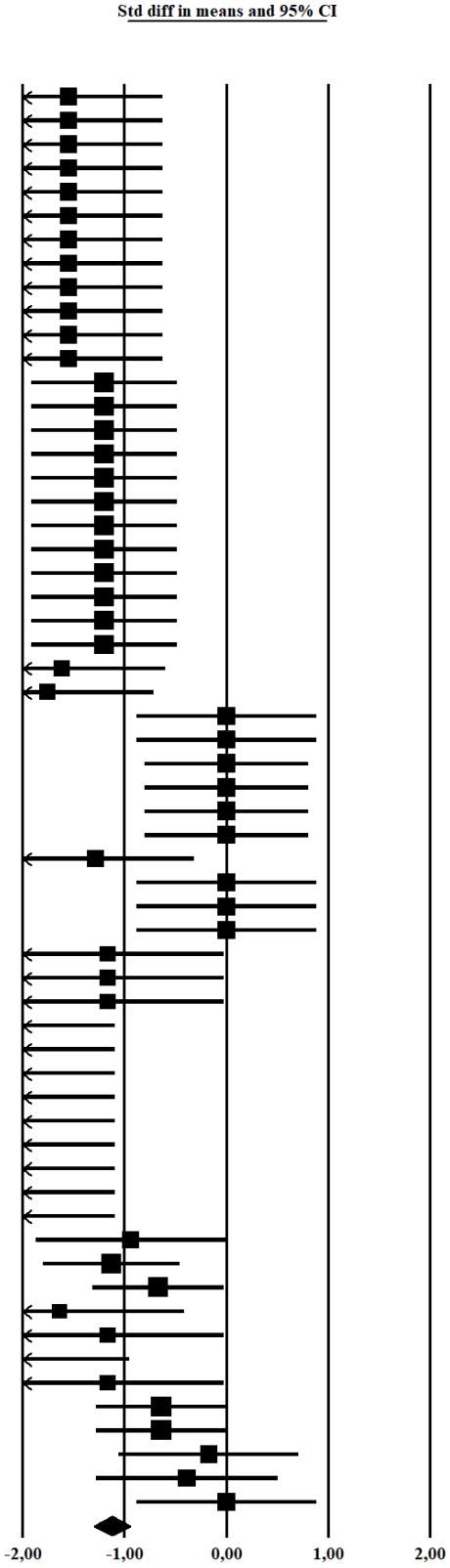

Figure 2. Summary of descriptive and inferential statistics of results for each study and overall effect size of the effects of the acute-exercise-conditioned human serum in human cancer cell viability.

Study name	Std diff in means	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Baldelli et al 2020 a)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 b)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 c)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 d)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 e)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 f)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 g)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 h)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 i)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 j)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 k)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 l)	-1,548	0,465	0,217	-2,460	-0,636	-3,326	0,001
Baldelli et al 2020 m)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
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Baldelli et al 2020 o)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 p)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 q)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 r)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 s)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 t)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 u)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 v)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 x)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Baldelli et al 2020 z)	-1,200	0,362	0,131	-1,910	-0,491	-3,315	0,001
Devin et al 2019 a)	-1,615	0,515	0,265	-2,624	-0,605	-3,136	0,002
Devin et al 2019 d)	-1,754	0,526	0,277	-2,785	-0,722	-3,333	0,001
Devin et al 2019 g)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Devin et al 2019 j)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Kurgan et al 2017 a)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Kurgan et al 2017 b)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Kurgan et al 2017 c)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Kurgan et al 2017 d)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 e)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 f)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 g)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 h)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 i)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 j)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 k)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Kurgan et al 2017 l)	-2,648	0,791	0,626	-4,198	-1,098	-3,348	0,001
Devin et al 2019 m)	-0,174	0,448	0,201	-1,052	0,704	-0,388	0,698
Devin et al 2019 n)	-0,390	0,451	0,204	-1,275	0,495	-0,863	0,388
	-1,350	0,088	0,008	-1,522	-1,179	-15,428	0,000

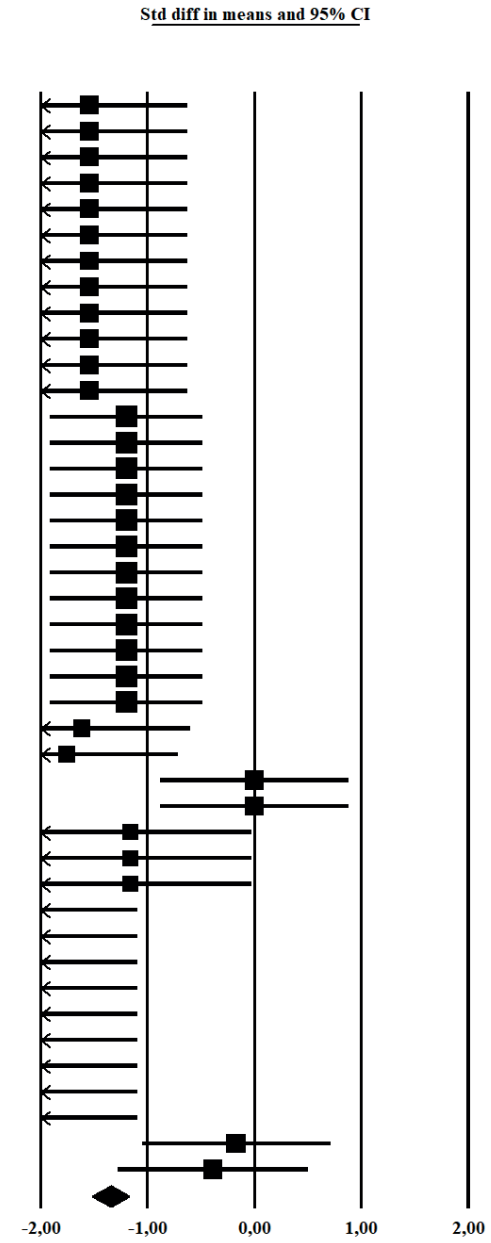

Figure 3. Summary of descriptive and inferential statistics of results for each study and overall effect size of the effect of the acute high-intensity exercise-conditioned human serum in human cancer cell viability.

3.3.3 Effect of Serum Conditioned by Moderate-Intensity Acute Exercise or by an Acute Integrative Exercise of Moderate- and High-Intensity on the Viability of Human Cancer Cell Cultures

The meta-analysis on the effects of serum conditioned by moderate intensity acute exercise or by acute integrative exercise of moderate and high-intensity on the viability of cancer cell cultures included four studies. These studies used moderate-intensity exercise or the combination of exercise with different intensities. This meta-analysis, performed with a random effects model, showed, based on the standard difference in means, that cultures of cancer cells exposed to serum conditioned by acute moderate-intensity or by acute integrative exercise of moderate and high-intensity exercise exhibited a reduction of -0.559 in their viability (*Figure 4*). The confidence interval for the standard difference in means was -0.830 to -0.287 (95% CI) with a corresponding value of $p < 0.001$ (*Figure 4*). Once again, this interval does not include a zero effect. Similarly, z-values obtained to test the null hypothesis i.e., that the standard difference in means is zero, showed a $z = -4.036$, and a corresponding value of $p < 0.001$ (*Figure 4*). Thus, the null hypothesis was rejected and the alternative hypothesis accepted in all analysed studies, i.e., after exposure of cancer cell cultures to serum conditioned by acute moderate-intensity or by an acute integrative exercise of moderate and high-intensity exercise, there was a reduction in their viability.

In the analysis of the homogeneity of the effects, the Q-value was 32.234 with 17 degrees of freedom and $p = 0.019$. Although the values of Q and the degrees of freedom show that all studies may not share the same effect size, the value of p indicates that the null hypothesis must be accepted since the true effect-size is identical in all studies. The I^2 value is

Study name	Std diff in means	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Hwang et al 2020 e)	0,000	0,408	0,167	-0,800	0,800	0,000	1,000
Hwang et al 2020 f)	0,000	0,408	0,167	-0,800	0,800	0,000	1,000
Hwang et al 2020 a)	0,000	0,408	0,167	-0,800	0,800	0,000	1,000
Hwang et al 2020 b)	0,000	0,408	0,167	-0,800	0,800	0,000	1,000
Hwang et al 2020 g)	-1,287	0,491	0,241	-2,250	-0,324	-2,620	0,009
Hwang et al 2020 h)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Hwang et al 2020 c)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Hwang et al 2020 d)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
Rundqvist et al 2013 a)	-0,940	0,471	0,222	-1,863	-0,016	-1,994	0,046
Dethlefsen et al 2016 a)	-1,128	0,340	0,116	-1,795	-0,460	-3,312	0,001
Dethlefsen et al 2016 b)	-0,673	0,325	0,106	-1,310	-0,035	-2,069	0,039
Dethlefsen et al 2017 a)	-1,633	0,617	0,381	-2,842	-0,423	-2,645	0,008
Dethlefsen et al 2017 b)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Dethlefsen et al 2017 c)	-2,308	0,690	0,476	-3,660	-0,956	-3,345	0,001
Dethlefsen et al 2017 d)	-1,165	0,578	0,334	-2,298	-0,032	-2,015	0,044
Dethlefsen et al 2017 e)	-0,640	0,324	0,105	-1,276	-0,005	-1,974	0,048
Dethlefsen et al 2017 f)	-0,640	0,324	0,105	-1,276	-0,005	-1,974	0,048
Rundqvist et al 2013 c)	0,000	0,447	0,200	-0,877	0,877	0,000	1,000
	-0,559	0,138	0,019	-0,830	-0,287	-4,036	0,000

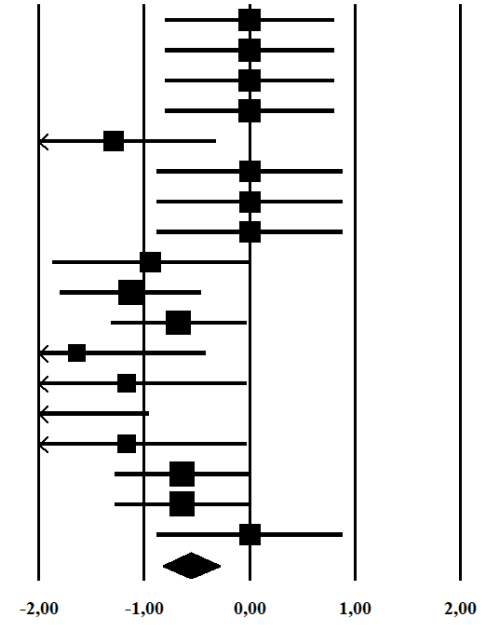

Figure 4. Summary of descriptive and inferential statistics of results for each study and overall effect size of the effect of serum conditioned by moderate-intensity acute exercise or by an integrative exercise of moderate- and high-intensity acute exercise on the viability of human cancer cell cultures.

45.573, meaning that about 45.573% of the variance in the observed effects reflects variance in the true effects. The τ^2 value was 0.152. The τ value, i.e., the standard deviation of the true effects in this SRM was 0.389. The funnel plot (Figure 5c) for the distribution of the observed studies was symmetric, with the majority of the studies distributed symmetrically around the mean effect size, since the sampling error is random, providing no subjective evidence of publication bias. Additionally, the Egger´s test was performed and the intercept value was -2.11767, with a 95% confidence interval between −5.30865 and 1.07331 and a t value = 1.40686. The recommended p value (2-tailed) was 0.17860. Thus, there was also no statistical evidence for publication bias.

4. Discussion

A major aim of the present systematic review and meta-analysis was to determine the magnitude of the effect of exercise-conditioned human serum on the viability of cancer cell cultures. Quantitative analyses could only be performed for the effects of serum conditioned by exercise bouts (acute exercise), due the scarcity the studies and data on the effects of serum conditioned by long-term exercise training (chronic exercise). Our analyses revealed significant effects of acute exercise-conditioned human serum in reducing the viability of cancer cell cultures (ES = -1.126; -1.300 to -0.952 (95% CI); $p < 0.001$). Moreover, it was found that the effect was more pronounced when human serum was conditioned by high-intensity exercise (ES = -1.350; -1.522 to -1.179 (95% CI); $p < 0.001$) than when it was conditioned by moderate-intensity exercise or by an integrative exercise of moderate- and high-intensity exercise (ES = -0.559; -0.830 to -0.287 (95% CI); $p < 0.001$).

Two of the studies simultaneously assessed the effects of exercise-conditioned human serum on the viability of cancer cell cultures and cultures of normal fibroblasts (37, 50). In both cases, no effect was observed in the latter, suggesting that exercise-conditioned human serum does not affect cell culture viability in general, but the viability of cancer cell cultures specifically. It must be noted, though, that in one of these studies, the fibroblasts used (NIH 3T3 cell line) were not of human origin (37, 50).

While the effect of exercise on cancer cell culture viability had the same direction in all studies, regardless of cancer type (breast (5, 15, 16), prostate (5, 33, 39, 50), lung (37), or colon (17)), the magnitude of the effect differed among the studies, likely due to considerable heterogeneity in study designs, namely in terms of type and duration of the exercise intervention (ranging from short exercise bouts to 10 years); population (e.g., healthy sedentary subjects, patients at risk of cancer and cancer patients or survivors); cell line; exposure regimen to exercise-conditioned human serum (5% (v/v) or 10% (v/v) human serum; 24 h to 7 days); endpoints of viability (e.g., total cell numbers, proliferation rates, clonogenic potential). Unfortunately, this considerable heterogeneity in multiple aspects of study design does not allow, at present, the establishment of any trends regarding the influence of any of these parameters. In spite of this limitation, some aspects are worth a brief discussion, as detailed below.

One of the aspect that needs clarification is the duration of the serological changes produced by bouts of exercise. In the study by Devin et al., the marked reduction in the viability of colon cancer cell cultures produced by serum prepared from

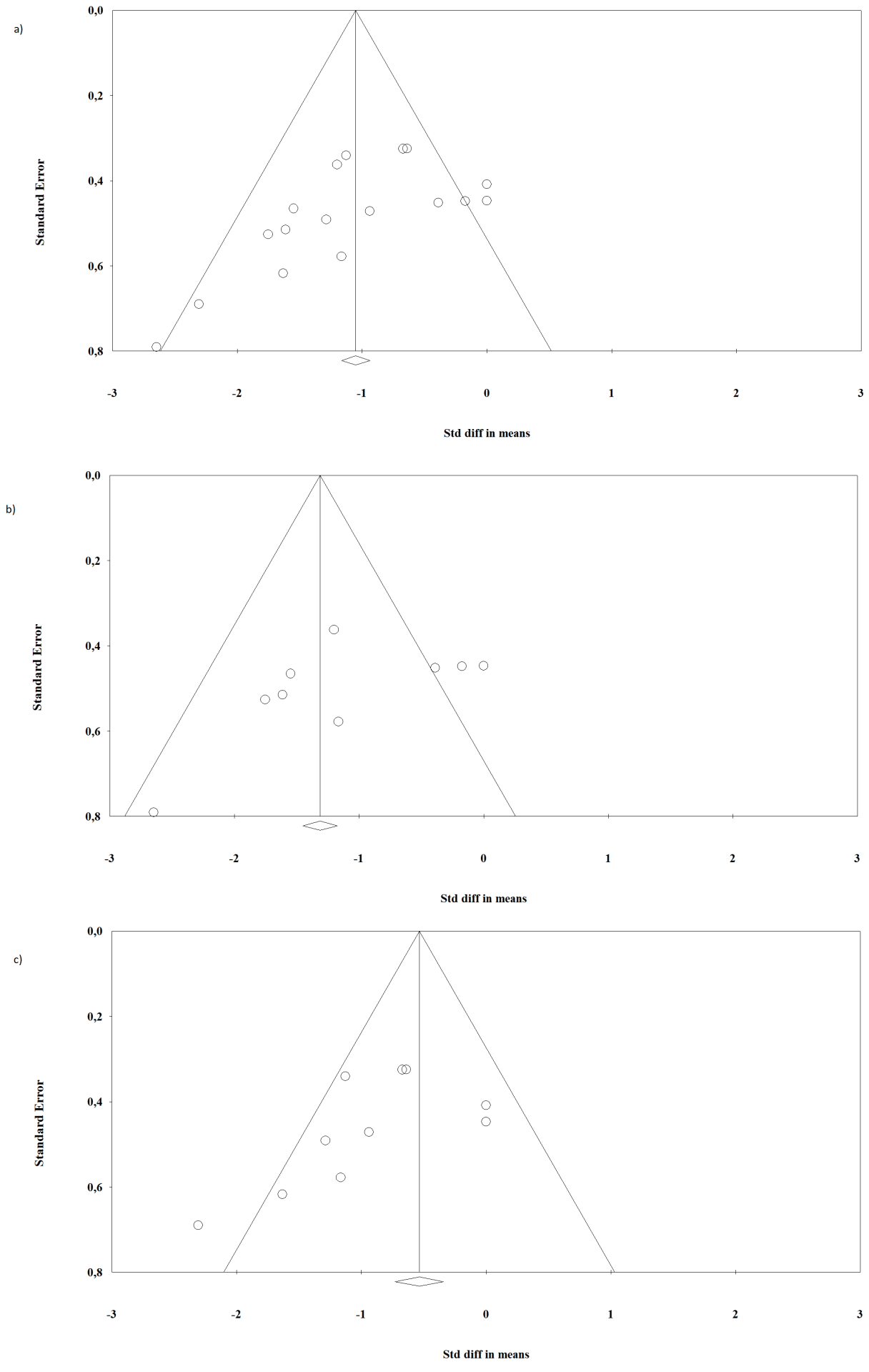

Figure 5. Effect of acute exercise-conditioned human serum in human cancer cell –funnel plot of standard error by std diff in means: a) acute exercise-conditioned human serum; b) acute high-intensity exercise-conditioned human serum; c) acute moderate-intensity or integrative exercise-conditioned human serum.

blood collected immediately after exercise was not replicated by serum prepared from blood collected 2 h after exercise (17). In the study of Rundqvist et al. (17, 50), on the other hand, serum prepared from blood collected 2 h post exercise reduced the viability of prostate cancer cell culture. Unfortunately, as blood was not collected immediately after exercise, so it is not known whether the effect decreased over time post-exercise. In some studies, such as those of Baldelli et al. and Kurgan et al., blood samples were collected several times, up to 24 h post-exercise. In both cases, it was found that the reduction in viability produced in, respectively, prostate and breast cancer cell cultures and lung cancer cell cultures did not decrease over time (5, 37).

Regarding long-term exercise training, five studies reported that it did not induce any changes in baseline serum (i.e., prepared from blood collected at rest) in terms of effects on the viability of the cancer cell cultures (5, 15-17, 33). This contrasts with the findings of Leung et al. and Barnard et al., who did observe higher effects for baseline serum collected after training (7, 39). This apparent contradiction might be due to the fact that the long-term exercise training interventions in the former studies were much shorter than the latter (months versus years).

Differences in outcomes could be found even among studies employing the same cell line, exercise protocol and exposure time. This can be exemplified by the results obtained in two studies employing the prostate cancer cell line LNCaP. In one of those studies, which aimed to determine whether the effects of exercise-conditioned human serum are influenced by aging, Hwang et al. recruited individuals from two distinct age groups (young age group, aged 20 to 33 years; old age group, aged 60 to 73 years) and compared the effects of a 96 h exposure to exercise-conditioned serum from the two groups on cell culture viability. Their results showed that exercise-conditioned serum from the old age group, but not from the young age group, reduced the viability of cancer cell cultures (33). Rundqvist et al., on the other hand, found a reduction in cell culture viability for 9 out of the 10 sera assessed, from participants aged 18 to 37 years, i.e., roughly within the young age group defined by Hwang et al. (17, 50). It must be mentioned that the two studies employed different endpoints of cell culture viability, but it is unlikely that this difference alone could account for the strikingly different outcomes in what concerns the effects of exercise-conditioned serum from young adults.

It is interesting to note that the studies of Devin et al. (17) and of Rundqvist et al. (50) assessed the effects of exercise-conditioned serum on the proportion of apoptotic cells in culture. Considering that this proportion was found unchanged in both studies, the observed reduction in total cell numbers might safely be assigned to lower proliferation rates, suggesting a lower tumorigenicity. Leung et al. and Barnard et al., on the other hand, observed an upregulation on the levels of apoptosis induced by serum collected at rest from regular exercising individuals in prostate cancer cell lines (7, 39). Taking into consideration that the same cell line (LNCaP) was used in three of these studies (7, 17, 39, 50), it is possible that the different outcomes are due, at least partially, to the duration of the exercise intervention.

Our analysis suggests that changes in serum composition promoted by exercise affect the viability of cancer cell cultures and may interfere with some hallmarks of cancer (26, 27), such as: sustentation of proliferative signalling and evasion of growth suppressors.

In the human model, the effects of exercise on tumorigenesis can occur through direct actions on cancer cells and through changes in the immune response (32, 56, 66). These effects may result from changes in serum levels of biomolecules such as epinephrine, norepinephrine, lactate, myokines, cytokines (IL-6, IL-8, TNF-α), SPARC and oncostatin M. Acute high-intensity exercise and integrative exercise (strength and high-intensity) increased IL-6, IL-8, TNF-α, epinephrine, norepinephrine, lactate, but not IL-10 (15-17), while moderate intensity exercise only increased SPARC and oncostatin M levels and did not change IL-6, IL-15 and Irisin levels (33). These changes promoted by high-intensity exercise tended to disappear 120 min post-exercise (17). Long-term exercise training elicited a reduction of IL-6, IL-8 and TNF-α at the end of 6 months (16), but not at the end of four weeks (17). However, the precise mechanisms linking these changes to cancer cell proliferation are still unclear.

Leung et al. (39) and Barnard et al. (7) suggested that exercise promoted changes in the IGF axis, reducing IGF-I (which was directly correlated with tumor cell growth) and increasing IGFBP-1 (which was inversely correlated with tumor cell growth), increasing p53 protein expression, reducing cell growth, and increasing apoptosis, in prostate cancer cell lines (LNCaP) (7, 39). The results obtained by Rundqvist et al. (50) support this hypothesis and showed that reduction of EGF by exercise could be another factor linking it to the inhibition of LNCaP cell growth (50, 51).

To explain the observed reduction in breast cancer cell viability and tumorigenesis, Dethlefsen et al. (15) suggested that exercise regulated the Hippo signalling pathway through the action of catecholamines (epinephrine and norepinephrine). The increase of catecholamine secretion during exercise stimulates Hippo signalling through β-adrenergic receptors inactivating the oncoproteins YAP/ZAP (35, 64, 65) by induction of YAP phosphorylation and YAP cytoplasmic retention (15). Kurgan et al., on the other hand, suggested that serum changes promoted by exercise may target directly Akt, mTOR/p70S6K, PI3K or PDK1/2 (37). This hypothesis was supported by their results, showing inhibition of Akt and ErK1/2 by exercise, ultimately leading to inhibition of cell proliferation and decreased survival (37).

Exercise can also reduce tumorigenesis through the regulation of the immune and metabolic networks (36), with muscle-to-tumor cross-talk playing an important role. Exercise induced myokines may affect immune cell activity through the release of immune regulatory cytokines like IL-6, IL-7 and IL-15 (32). Regular exercise has been shown to mitigate immunosenescence and low-grade inflammation (9, 43). It also has direct effects on cytotoxic NK and T cells, whose mobilization and redistribution into circulation depend on epinephrine and IL-6 release during exercise (49, 62). These changes in the mobilization and infiltration of specific immune cell populations (mostly NK and T cytotoxic cells) and inflammatory cytokines that occur during exercise could directly affect cancer cell formation and progression (30, 34, 49, 61). Exercise can also target the specific energy metabolism of cancer cells, which is highly reliant on lactic acid fermentation, a phenotype often described as the Warburg

effect (1, 58). High-intensity exercise, which can inhibit lactic acid fermentation in places away from the lactate-producing muscles, may neutralize tumor fermentation and affect this metabolic predominance (29, 59, 60), decreasing the inhibitory effect that lactate has on cytotoxic immune cells (22, 52).

This systematic review with meta-analysis has a high methodological value due to the quality of the studies included and the large number of samples tested. However, it is possible to identify some limitations, namely the employment of a search strategy that only included studies with defined terms in English on the title from specific selected databases, thereby potentially overlooking other relevant publications, namely in other languages, even if the included studies were from different countries and continents. Another possible limitation is the reduced number of articles published in the literature that analysed the effect of exercise-conditioned human serum in human cell lines. Lastly, the imbalance on the number of studies between cancer types and exercise types can minimize the inferential power over the real effects of exercise in general cancer cell viability.

Future studies should explore whether the observed effects on cancer cell viability are also observed in other cancer cell lines and for serum conditioned by other activities (e.g., running, swimming, and football). It would also be important to gather more information regarding the influence of exercise intensity on the observed effects and to determine whether there is a causal relationship between serum factors (e.g., cytokines, myokines and catecholamines) modified by exercise (acute and chronic) and type of activities. It is also important to consolidate the research carried out regarding the hypothesized mechanisms here discussed, as well as to investigate other mechanisms that may contribute to the anti-cancer effect of exercise, namely the impact that serum factors may have on oncometabolism, specifically on the "*Warburg effect*", and on immunosurveillance (e.g., energy alterations in NK cell via metabolism) (21).

Exercise can be safely implemented and is feasible in cancer patients (28, 54) with its benefits in preventing cancer and survival rates widely recognized (23, 25, 32, 41). Data on exercise-conditioned human serum effects on the viability of cancer cell cultures support the importance of exercise as a prevention strategy and support therapy in the treatment of cancer patients.

5. Conclusions

This systematic review with meta-analysis provides evidence that exercise promotes a large overall effect in reducing the viability of human culture cancer cells, also showing that this effect is more pronounced for high-intensity exercise-conditioned human serum. This effect is promoted by molecular and cellular mechanisms that can be triggered by acute or systematic changes in serum factors which are possibly dependent on exercise type, regularity and intensity. More research is needed to better understand the mechanisms underlying this effect, as well as the chronic effects of exercise and sport, in order to improve the prescription of exercise as a prevention strategy and/or as an adjuvant to the treatment of cancer.

Author Contributions:
Conceptualization, C.M.S.; J.P.F.; A.M.T.; H.S.; Methodology, C.M.S.; J.P.F.; A.M.T; H.S.; Software, C.M.S.; F.M.S.; M.F.; M.C.R.; P.R.N.; M.A.F.; J.P.F.; Formal Analysis, C.M.S. and J.P.F; Writing – C.M.S; Writing – Review & Editing, J.P.F.; A.M.T.; H.S.; A.M.U.; P.R.N.; M.A.F.; Visualization, J.P.F; A.T; H.S; Supervision, J.P.F; A.M.T.; H.S; All authors approved the final version of the manuscript.

Funding:
This research received no external funding.

Conflicts of Interest:
The authors declare no conflict of interest.

ACKNOWLEDGMENT:
F.M.S acknowledge a grant by Portuguese Foundation for Science and Technology (2020.08759.BD).
Research in A.M.U.'s laboratory is currently funded by Fundação para a Ciência e a Tecnologia (FCT, grant UIDB/00070/2020) and Associação de Apoio ao Centro de Investigação em Meio Ambiente, Genética e Oncobiologia (ACIMAGO, grant 16/12).

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Regular physical exercise mediates the immune response in atherosclerosis

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ABSTRACT

Atherosclerosis is a chronic inflammatory cardiovascular disease, which results from lipid accumulation in the blood vessel wall, forming a plaque, and ultimately restricting blood flow. The immune system plays a vital role in progression to plaque rupture. While recent evidence clearly indicates the anti-inflammatory function of regular exercise, the mechanisms by which regular exercise can modulate its pathophysiology is not well understood. In this review, we discuss how regular exercise can lower systemic inflammation directly via modulation of the immune system or indirectly via altered myokine concentrations and metabolites. We describe the exercise-induced responses of various myokines (such as IL-6, adiponectin, and FGF21), and how cell function in the innate immune system can be modulated via regular exercise, with the aim to modulate plaque formation in atherosclerosis.

Keywords: atherosclerosis, exercise, myokines, cytokines, IL-6, adiponectin, FGF21

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INTRODUCTION

Atherosclerosis is a chronic inflammatory disease, characterized by the formation of a plaque inside the smooth muscle wall of blood vessels [33; 141]. It is the major contributing factor to coronary heart disease [116], which is responsible for ~7 million annual deaths worldwide [139].

The inflammatory profile of atherosclerosis [137] is marked by slow, progressive structural changes within the intima, particularly in the medium and large arteries in the body [16]. If left untreated, blood vessels occlude [137], as a result of atherosclerotic plaque formation [40]. Atherosclerosis is a complex, multifactorial disease, as well as a precursor of cardiac ischemia-reperfusion, and a main risk factor for several other chronic cardiovascular diseases [48].

The response-to-retention model of atherogenesis [136] suggests that the atherogenic process initiates with subendothelial retention of lipids and lipoproteins (e.g. Apolipoprotein B, ApoB), consisting of cholesteryl fatty acyl esters and triglycerides, and low-density-lipoprotein (LDL) cholesterol. These can bind to proteoglycans in the arterial wall, particularly at arterial branch points and bifurcations [77, 128]. Increased retention rate of lipoproteins promotes fatty streaks formation inside the intima, eventually progressing into a plaque [48]. If lipids continuously accumulate in the already thickened intima [85], fibrous cap (or sclerosis) gradually forms around the lipidic core, narrowing the arterial lumen [91,48], which reduces blood flow, oxygen and nutrient supply, and, eventually, causes downstream tissue death [4, 94]. Also inside the plaque, cell apoptosis and necrotic core formation lead to thinning of the fibrous cap and plaque instability. If this plaque ruptures, it can cause cardiovascular complications such as intravascular thrombosis and myocardial infarctions.

Current therapies mainly rely on low-density-lipoprotein (LDL)- and triglyceride-lowering treatments through statins and others [10]. Other therapies aim at increasing expression of LDL receptors, in order to prevent degradation, and inhibit cholesterol absorption [15]. Bile acid sequestrants and high-density-lipoprotein (HDL)-increasing therapies have also been used. However, the side effects of the former and the lack of significant improvements of the latter have made these alternatives less popular [10; 15].

While pharmacological approaches have been widely used in a clinical context, alternative therapies, such as exercise training have clinical potential to modulate the disease progression. Regular physical exercise has been shown to improve metabolic health, cardiovascular function and improves insulin sensitivity, all factors that are known to be implicated in the onset and progression of atherosclerosis [99].

One factor that has obtained considerably less attention is the observation that regular moderate physical activity potently

lowers systemic inflammation in many cardiovascular diseases [98], and increases the concentration of circulating peripheral blood mononuclear cells (monocytes and lymphocytes). Additionally, regular exercise stimulates the release of adrenaline and cortisol, both of which with strong anti-inflammatory effects [88]. The role of small molecules secreted by skeletal muscle (myokines) and their subsequent effects on the immune response and lipid metabolism in atherosclerosis is currently unknown [36]. How exactly exercise exerts its therapeutic and anti-inflammatory effects on the prevention of plaque formation and treatment of atherosclerosis remains enigmatic [54], with just few molecular mechanisms being identified so far. Although current evidence suggests a strong anti-inflammatory effect of exercise, research on exercise-induced secretion of anti-inflammatory products is scarce [132]. In this review we provide an overview of the latest advances in exercise immunology related to atherosclerosis, and discuss whether the progression to severe atherosclerosis can be delayed by exercise, and what the underlying mechanisms are.

A literature search was performed on PubMed and Google Scholar, with search terms, such as 'exercise', 'atherosclerosis', 'immune system', 'myokines' and 'immunology' either individually or combined. Article selection was extended to cited papers considered important, as well as searches for specific myokines (i.e. FGF21), exercise and atherosclerosis.

2. IMMUNE RESPONSE AND INFLAMMATION IN ATHEROSCLEROSIS

A graphical overview of the contribution of various immune cells is provided in Figure 1. Endothelial cell injury within the blood vessels is considered to be the first step towards an atherosclerotic lesion [47]. Classic obesity markers, such as high levels of circulating lipids, particularly LDL, and metabolic dysregulation [77] are implicated in the initial stages. LDL infiltration and retention in the intima cause endothelial cells to manifest a dysfunctional phenotype [77], particularly in arterial branches and curves. These atheroprone areas are characterized by low endothelial shear stress, higher circulating levels of vasoconstrictors (e.g. endothelin-1), production of reactive oxygen species (ROS), and imbalances between nitric oxide (NO) and prostacyclin (PGI2)-mediated vasorelaxation [18]. Oxidative stress in the endothelial cells speeds LDL oxidation in the subendothelial space, and promotes a pro-atherogenic environment [147]. A lower endothelial-derived NO production reduces vasorelaxation, endothelial regeneration and integrity [86], and facilitates the accumulation and retention of atherogenic LDL in the subendothelial layer [110]. This vicious cycle ultimately activates the nuclear factor kappa B (NF-κB)-pathway that triggers the expression of pro-inflammatory cytokines in the injured cells [70], further causing endothelial dysfunction [145].

Stressed endothelial cells express higher levels of immunoglobulin-G adhesion molecules (e.g. VCAM-1, P-selectin, ICAM-1), proinflammatory receptors, and cytokines (e.g. IL-8, MCP-1 [53]). Together they are responsible for immune cell recruitment into the vascular tissue [75]. Particularly, monocytes are recruited [39] and converted into proinflammatory macrophages, which proliferate within the intima layer [106]. Figure 1 describes how the interactions between dysfunctional endothelial cells, adipocytes found at the atheroma and pro-inflammatory T-cells (Th1 and Th17) contribute towards monocyte differentiation.

2.1. Immune cells in atherosclerosis

Leukocytes are of major importance in the onset of atherosclerosis [125]. Under normal conditions, circulating monocytes are uncapable of proliferating [4, 43]. However, atherogenesis alters monocyte behaviour. Lipid accumulation inside endothelial cells increases expression levels of cellular adhesion molecules, such as E-selectin, P-selectin, VCAM-1 and ICAM-1 [24], promoting a local vascular inflammation

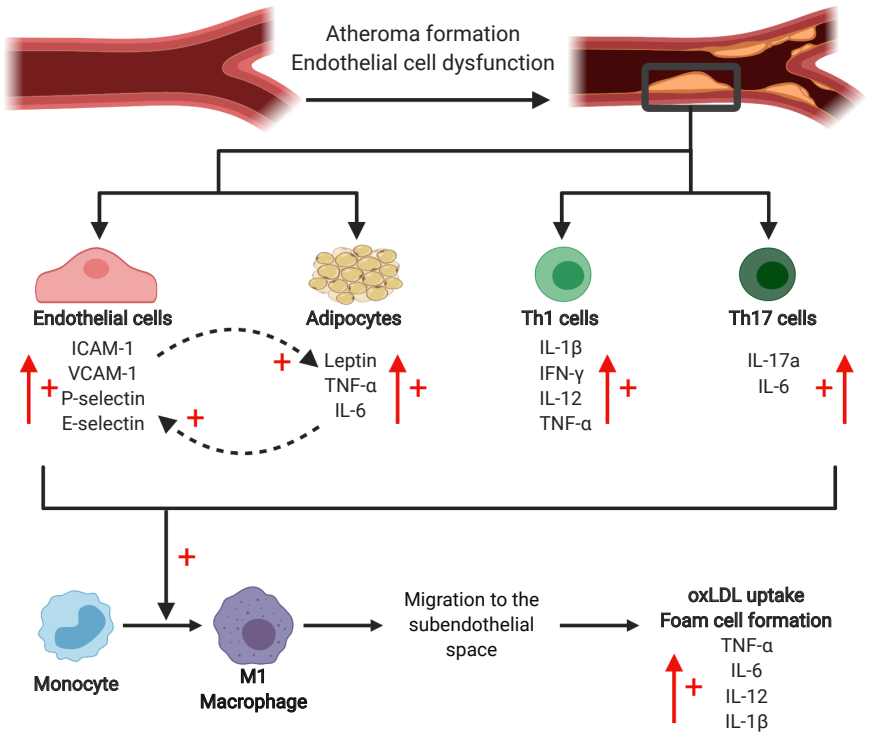

Figure 1. Interactions between endothelium, atheroma and immune system. LDL retention at the intima gives rise to atherosclerotic plaque formation. Consequently, endothelial cells become dysfunctional and express higher levels of adhesion molecules (ICAM-1, VCAM-1, P-selectin and E-selectin), contributing to monocyte recruitment towards the intima. Simultaneously, adipocytes from the accumulated LDL secrete pro-inflammatory cytokines, such as leptin, TNF-α and IL-6, whose expression stimulates, and is enhanced by endothelial cells-mediated secretion of adhesion molecules. Th1 and Th17 immune cells also found in the intima express a wide range of pro-inflammatory cytokines. Monocyte recruitment to the blood vessel is favoured by permanent expression of adhesion molecules, differentiation into pro-inflammatory M1 macrophages. Subsequent foam cells formation is facilitated by the pro-inflammatory products released by adaptive immune cells, adipocytes and endothelial cells.

response by attracting and deposition of monocytes and T-cells [42]. These cells contribute to a continuous inflammatory state via secretion of pro-inflammatory cytokines (e.g. leptin, TNF-α and IL-6) [38], causing a vicious circle by further increasing the expression of adhesion molecules and recruitment of leukocytes to the intima [55, 103].

2.1.1. Monocytes and macrophages

The interaction between monocytes and adhesion molecules, and continuous secretion of lipoprotein-binding proteoglycans [83] cause lesions to the endothelium, further facilitating the transmigration of monocytes in the intima and allowing monocytes to differentiate into macrophages [57]. Monocyte differentiation into either classically activated atherogenic M1 macrophages, or alternatively activated anti-inflammatory M2 macrophages strongly depends on the microenvironment at the artery [42]. M1 differentiation is favoured due to the exposure of monocytes TO pro-inflammatory cytokines cytokines secreted by the dysfunctional endothelium [24] and neutrophil-mediated secretion of TNF-a, IL-8 and IFN-y [60]. Pro-inflammatory macrophages regulate plasma lipoprotein metabolism and express several receptors with high-affinity to oxidized LDL (e.g. LOX-1; [25]). These will take up on oxidized LDL molecules and other lipids at the lesion site [50]. Macrophages, therefore, accumulate at the subendothelial space, due to continuous monocyte recruitment and LDL oxidation, and eventually turn into foam cells, one of the key markers in atherosclerotic plaque formation [25]. Foam cells release a series of pro-inflammatory cytokines, particularly CD40, IL-1, IL-3, IL-6, IL-8, IL-18 and TNF-α [122]. The cholesterol that has been collected and accumulated in macrophage foam cells enhances activation of inflammatory receptors [129], therefore exacerbating the inflammatory state at the atheroma. The pro-inflammatory phenotype of foam cells contributes towards plaque progression and instability, necrotic core formation, and degradation of the extracellular matrix [71].

2.1.2. T-cells

Other key players in monocyte differentiation into macrophages are T-cells. Just like monocytes, T-cells are recruited to the endothelial wall via cellular adhesion molecules (CAMs)-signalling [89], with both Th1 and Th2 cells contributing to the immune response. Th1 cells are primed by interactions between LDL molecules and oxidized-LDL-specific T-cells, found at the injured endothelium [55]. Th1 cells have an atherogenic function, as they secrete pro-inflammatory cytokines, such as IL-1β, IFN-γ, IL-2, IL-12 and TNF-α, worsening local inflammation [45]. IFN-γ, particularly, contributes to a continuous cycle of monocyte recruitment to the injured site and their subsequent differentiation into pro-inflammatory M1 macrophages [131].

Conversely, Th2 cells produce an array of anti-inflammatory cytokines, particularly IL-4, IL-5, IL-9, IL-10, IL-13 and IL-33 [60; 112]. These cytokines have a strong anti-atherogenic effect, but their exact role in the progression of atherosclerosis remains unclear, due to spatial and temporal uncertainties [79]. Early studies showed that Th2 cells prevent fatty streak formation and IL-5 lowers plasma levels of IFN-γ, possibly preventing atherosclerosis progression [79]. On the other hand, IL-4 contributes to plaque formation at later stages [79].

Th17 cells, commonly found in the atheroma, are also part of the effector T-cell subset, but with a different lineage than that of Th1 and Th2 [80]. Th17 cells secrete IL-17a, IL-17f and IL-6 [143]. Although IL-17f is known to exert an anti-atherogenic effect by increasing plaque stability [143], the overall role of Th17 cells is believed to promote disease progression due to pro-inflammatory IL-17a and IL-6 activity [2]. Their exact role is still under debate due to its ambiguous function in atherosclerosis.

Treg cells, another subset of T-cells, have a clear inhibitory effect on Th1 cells, thus reducing the pro-inflammatory milieu. Treg cells suppress the activity of CD8+ T-cells [44], which are commonly found in human atherosclerotic plaques, particularly in a more advanced stage of the disease [46, 91]. Treg cells secrete IL-10 and TGF-β, which are considered to promote plaque stability [4]. Lower Treg subpopulation are linked with atherosclerosis progression. Therefore, possible therapeutic strategies involve increasing Treg number either by cell transfer or expansion [95]. However, little is known about Treg survival upon transfer or activation/expansion, thus limiting the long-term effects on atherosclerosis progression. Additionally, these cells might behave differently on distinct stages of atherosclerosis, which makes it difficult to define a specific treatment strategy. Although promising, future studies and clinical trials should focus on the long-term contribution of the anti-inflammatory effects of Tregs [95]. Physically active individuals often have higher frequencies of circulating Treg cells, likely due to an exercise-induced increase in TGF-β, which is essential to the anti-inflammatory function of Treg cells. Current data, therefore, suggests a link between physical activity, TGF-β and optimal Treg cells function [135].

2.2. Atherosclerotic plaque

Plaque formation initiates with lipid deposition at the intima, after lipid accumulation occurs in the intima due to the injured endothelium [5], giving rise to fatty streaks. Recruited monocytes, subsequent monocyte-derived macrophages and T-lymphocytes will also accumulate at the intima [74]. As macrophages take up on oxidized LDL molecules and turn into foam cells, they secrete an array of growth factors and cytokines which stimulate migration and proliferation of vascular smooth muscle cells (VSCMs) at the intima, followed by production of extracellular matrix (ECM) and related components (e.g. collagen) to form the fibrous cap [64]. Ultimately, the atheroma is an agglomeration of leukocytes, foam cells, VSCMs, ECM and lipids, and the fibrous cap is what provides stability to the plaque.

The plaque becomes unstable once foam cells undergo apoptosis. Apoptotic cell removal is mediated by M2 macrophages [127]. However, if phagocytosis occurs at an exacerbated rate, the endoplasmic reticulum of M2 macrophages becomes stressed, culminating in cell death [134]. Combined, both apoptotic M1 and M2 macrophages make up the necrotic core. Apoptosis of M2 macrophages leads to release of lipids, inflammatory and thrombotic factors, and metalloproteinases (MMPs) [134]. Continuous recruitment of pro-inflammatory leukocytes to the atheroma causes TNF-α-induced VSMCs death [11], inhibiting VSMCs-mediated production of matrix components [26]. Particularly, CD8+ T-cells, also found at the plaque, are highly cytotoxic and promote plaque instability by inducing apoptosis of macrophages and VSMCs [67]. MMPs,

on the other hand, erode the ECM, which together with lower numbers of VSMCs contributes towards plaque susceptibility to rupture [134]. An important thrombotic mediator is tissue factor (TF), which is found. It is found in high quantities within the plaque. As its expression is enhanced in apoptotic foam cells and VSCMs. Once the plaque is ruptured, TF mediates thrombus formation [108], as well as other life-threatening ischemic cardiovascular events [77].

3. EXERCISE AS A THERAPEUTIC INTERVENTION FOR ATHEROSCLEROSIS

It is widely accepted that moderateintensity aerobic exercise improves cardiovascular, respiratory and skeletal muscle function, with little to no severe complications, and can prevent, treat and delay the development of various chronic diseases [98]. As such, regular aerobic exercise has been implicated in the prevention of the metabolic derailment, obesity, endothelial dysfunction, and insulin resistance. Generally, these effects are contributed to improved blood lipid levels, glucose signalling and skeletal muscle adaptations.

One aspect that has received little attention is that exercise also has immune-modulatory effects [88]. All skeletal muscle-derived factors that are secreted into the blood stream are called myokines [34], and more than 600 have been discovered to date. Exercise triggers the secretion of various substances including cytokines (particularly IL-6, IL-15, IL-1ra, IL-19 and sTNF-R; [121]), fostering a potent anti-inflammatory environment, and modulates immune responses [28]. Physical activity also influences leukocyte behaviour. For instance, circulating levels of non-classical, anti-inflammatory monocytes increase following exercise training [120]. High intensity exercise enhances CCR5 expression on monocytes and T-cells [6], and CCR5+ Treg cells greatly increase their expression of the anti-inflammatory cytokine IL-10, indicating potent anti-inflammatory effects of augmented CCR5 expression on the cell membrane of leukocytes [32], ameliorating inflammation in the atheroma. In addition, physical activity also alters T-cell balance by increasing circulatory levels of Treg cells [112] and inducing Th2 cell polarization, subsequently releasing higher quantities of immunosuppressive cytokines [92]. Important to note that an effective exercise protocol, capable of eliciting is optimal anti-inflammatory responses, is yet to be determined [132]. Figure 2 shows how an overview of how regular exercise affects the immune system directly, and indirectly via the release of various compounds.

3.1. Indirect effects of exercise via altered circulating metabolites

3.1.1. Endothelial shear stress-mediated substances

Physical exercise increases whole-body blood flow and endothelial shear stress [101]. Endothelial shear stress causes the endothelium to secrete vasodilators (such as NO), growth factors and others [82]. The interaction between blood flow and shear stress regulates gene expression contributing to its optimal function [148].

Angiographic studies showed a 200% higher vasodilation capacity in ultra-distance runners versus sedentary individuals [56]. This endothelium-dependent relaxation of coronary arteries and arterioles is accompanied with higher endothelial nitric oxide synthase (eNOS) expression and, subsequently, higher levels of NO [68]. Endothelial cells on the vascular lumen secrete more NO into the bloodstream [51], maintaining endothelial function and preventing local LDL accumulation. Regular exercise also improves endothelial function in ApoE-deficient mice fed on a high fat diet [37]. The areas of atherosclerotic lesions in the aortic sinus and thoracoabdominal aorta were significantly lower in the exercise group, and correlated with the amount of daily exercise. This was associated with a reduction in inflammatory cell markers in the aorta. Serum IL-6 and macrophage chemoattractant protein-1 levels were significantly lower and those of adiponectin were significantly higher in the exercise group compared to the control group [37].

While aerobic exercise increases endothelial function,

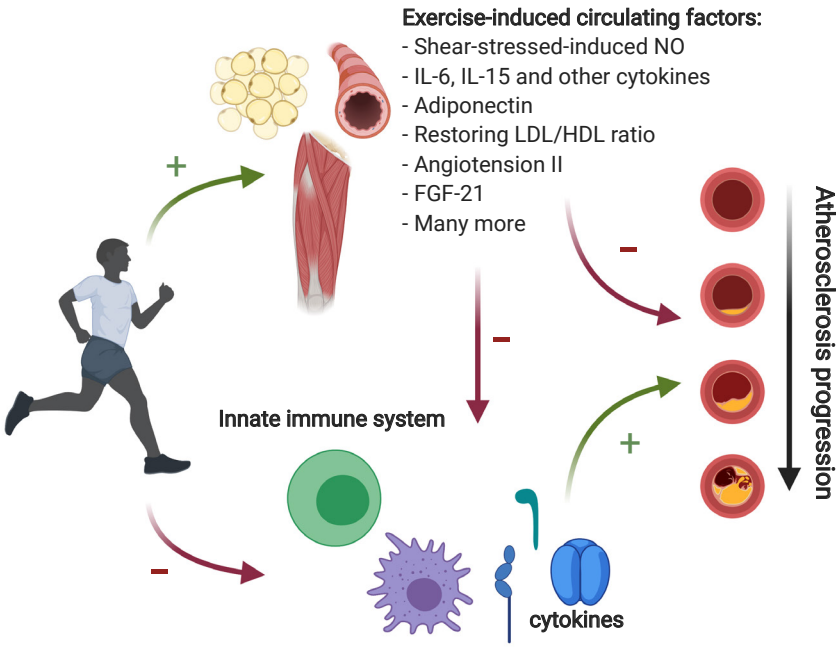

Figure 2. Schematic overview of how exercise alters the immune response in atherosclerosis. Acute exercise results altered endothelial cell function (via increased shear-stress-mediated cell signalling), and in the secretion of circulating factors, such as IL-6, IL-15, adiponectin, FGF-21 and many others. These factors, by themselves, or via the suppression of the innate immune system, are thought to alter endothelial and smooth muscle function. Exercise on the other hand also modulates macrophage and T-cell function, resulting in an additional pathway by which exercise delays the progression of plaque formation in atherosclerosis.

physical inactivity reduces overall blood flow and endothelial shear stress. Endothelial cells become dysfunctional and produce pro-inflammatory cytokines [3], changing the endothelial cells towards an atheroprone phenotype [58].

In summary, optimal endothelial function is jeopardized under conditions of inactivity or a high fat diet, which are associated with a low NO bioavailability, local attraction of inflammatory cells, causing an atheroprone environment. Regular exercise increases endothelial shear stress and endothelial-derived NO, and has the potential to revert endothelial dysfunction and reduce the local immune response.

3.1.2. Interleukin-6

Interleukin-6 (IL-6) is a key regulator of inflammation in the atherosclerotic plaque, synthesized by various cells at the vessel wall, such as macrophages, endothelial cells and smooth muscle cells [105]. IL-6 is known for exerting both anti- and pro-inflammatory properties in the course of atherosclerosis development [105]. However, when mentioned in the context of exercise training, IL-6 is a potent anti-inflammatory myokine and one of the most important biomarkers secreted by skeletal muscles released during physical activity [35]. Its inflammatory profile is more evident through chemotaxis, which brings monocytes to the vessel wall, increases chemokine production and induces higher expression levels of adhesion molecules, thus inducing an inflammatory milieu that promotes atheroma formation [140]. In contrasting, exercise-derived IL-6 triggers the production of IL-1ra and IL-10 cytokines [124], which have anti-inflammatory properties.

IL-6-mediated production of IL-10 suppresses activation and circulation levels of Th1 cells, known for exacerbation of the inflammatory state and markedly present in the atherosclerotic plaque [69]. Studies have shown that inactivation of IL-10, either genetically or via blockade of IL-6, fosters a pro-atherogenic environment, as it allows for increased transmigration of cells into the subendothelial space of the blood vessel and enhanced production of pro-inflammatory cytokines [13].

Other targets of muscle-derived IL-6 are Th1 and Th2 cells. The former is the most commonly found subset of T-cells in the atherosclerotic plaque [131]. Th1 cells have a pro-inflammatory, atherogenic profile, and worsens atherosclerosis [45 and section 2.1.2]. Opposingly, Th2 cells, which counteract inflammation via secretion of anti-inflammatory cytokines such as IL-4, are not often detected in atherosclerotic lesions [79]. However, when released during moderate endurance exercise, IL-6 acts directly on Th1 cells, via enhanced cortisol release, and suppresses its activity, whilst promoting Th2 cells differentiation [119], and higher production rates of anti-inflammatory cytokines, such as IL-4 [123].

The IL-6 signalling pathway mediates the atheroprotective effect of physical activity [126]. Exercise-induced secretion of IL-6 from skeletal muscles suppress the classical pro-inflammatory cytokines TNF-α and IL-1β that are found in high concentrations in the atheroma [93]. It is therefore likely that IL-6 can halt atherosclerosis progression by targeting pro-atherogenic markers. Although its anti- and pro-inflammatory characteristics are well documented, the exact trigger of each signaling pathway is not yet fully understood. Likely, the combination of IL-6 with other (unknown) myokines provides the key to unlock the anti-inflammatory characteristics typically seen after exercise.

3.1.3 Adiponectin

The cytokine adiponectin is mainly released by adipocytes [107], and modulates whole body lipid and glucose metabolism [107], with important anti-atherogenic and anti-inflammatory properties [90]. A study conducted by Hotta and colleagues [59] concluded that adiponectin is positively correlated with insulin sensitivity and that its serum levels decrease with obesity. Binding of adiponectin to its receptor AdipoR1 activates AMPK signalling pathway [31], increasing eNOS expression, NO synthesis and vasodilation [19], whilst its binding to AdipoR2 enhances fatty acid oxidation via PPAR-α upregulation [144].

Low circulating adiponectin levels have been implicated in the onset of type 2 diabetes mellitus and cardiovascular diseases, such as atherosclerosis [133]. The atheroprotective role of adiponectin is thought to be of indirect nature. Atherosclerotic-induced endothelial dysfunction, at its earliest stages, is mostly caused by impaired expression and activity of eNOS and poor secretion of NO, which contribute towards arterial vasoconstriction [22]. Adiponectin-knockout mice had impaired aortic vasodilation, lower NO synthesis and increased oxidative stress, accentuating even further the endothelial cell dysfunction at the intima [20]. Therefore, adiponectin might eventually emerge as a promising approach aimed at increasing NO synthesis, ameliorating endothelial dysfunction and promoting vasodilation of occluded vessels.

The atheroprotective role of adiponectin is also observed via acceleration of the reverse cholesterol transport system, a process through which high density lipoproteins (HDL) remove excess cholesterol from foam cells at the atherosclerotic plaque [107]. Adiponectin also fosters an anti-atherogenic microenvironment, via reduced expression of adhesion molecules in endothelial cells, which reduces monocyte attachment [97], and inhibits foam cell formation [96]. Altogether, these effects prevent and reduce plaque dimensions and formation, and diminish the inflammatory state in which atherosclerosis is involved.

Moderate intensity aerobic exercise increases serum adiponectin levels to the same level as several anti-diabetic drugs [1]. This would, at least in theory, improve glucose and lipid metabolism [76]. Nevertheless, exercise-induced circulating adiponectin levels are not always associated with a reduced risk of atherosclerosis and atherosclerotic burden [17]. Clearly more work needs to be performed in order to establish a causal link between increased levels of exercise-mediated adiponectin and atherosclerosis.

3.1.4 Interleukin-15

IL-15, an anti-inflammatory myokine secreted during moderate physical activity, contributes to lipid metabolism [87]. Overexpression of IL-15 is linked to a lower visceral fat mass [104]. By enhancing adipose tissue metabolism, exercise-induced induction of IL-15 possibly diminishes the concentration of circulating LDL and is a determinant factor in the treatment of atherosclerosis. More work should be performed to demonstrate a mechanistic link between exercise-induced IL-15, lipid metabolism and the onset of atherosclerosis.

3.1.5 Angiotensin II

Another key mediator of inflammation is the endothelial angiotensin II pathway, which is highly active in patients with atherosclerosis [109]. Angiotensin II promotes

vasoconstriction, fosters the progression of atherosclerosis and reduces plaque stability [100]. Angiotensin II also activates circulating immune cells and facilitates their adhesion to the endothelium and subsequent transmigration by synthesizing adhesion molecules, chemokines and cytokines [126].

Regular exercise blocks the activity of endothelial angiotensin II, and as a result inhibits these processes. Consequently, lowering angiotensin II activity reduces circulating levels of oxidized LDL and lowers macrophage recruitment to the subendothelial space. How exercise-induced alterations in angiotensin II activity ultimately (in)directly reduces atherosclerosis risk should be studied in mechanistic (animal) models.

3.1.6 Apolipoprotein E

Apolipoprotein E (ApoE) is a key, polymorphic, anti-inflammatory lipoprotein involved in plasma cholesterol homeostasis [115]. ApoE mediates the binding of VLDL to LDL- and VLDL-receptors, hence facilitating triglyceride clearance. ApoE inhibits endothelial cell activation [122], enhances HDL efflux, inhibits activation and proliferation of monocytes, and its expression on the surface of macrophages prevents foam cell formation [41]. Its interplay with the immune system is observed through suppression of NF-κB-driven inflammation [72], proliferation of CD4+ and CD8+ T-cells involved in a pro-atherogenic environment, and induction of major histocompatibility complex (MHC) II expression on macrophages, thus expanding the population of anti-inflammatory M2 macrophages [111].

Plasma triglyceride and cholesterol levels are increased in ApoE-knockout (ApoE-KO) mice, and this knock-out model is a popular model to study atherosclerosis. Shimba and colleagues [113] studied whether skeletal muscle-specific peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) overexpression alters the progression to atherosclerosis in ApoE-KO mice. PGC-1α is known to be upregulated during endurance exercise training by approximately 3-fold [81]. Indeed, a higher expression of PGC-1α resulted in a significantly smaller atherosclerotic plaques than ApoE-KO mice with normal levels of PGC-1α. Whether this is mediated by a PGC-1α-dependent inhibition of VCAM-1 and MCP-1 mRNA expression from dysfunctional endothelial cells in the atheroma is unknown, as it is unknown whether PGC-1α can have endocrine effects. Likely, an unknown paracrine factor, released from high-oxidative muscles, contributes to the observed effect. More work should be performed to better understand the athero-protective effects overexpression of PGC-1α in skeletal muscle.

The polymorphic profile of ApoE was first described by Davignon, Gregg & Sing [29], who identified six genotypes that give rise to three isoforms. ApoE2 has an atheroprotective effect, and is linked with the highest circulating levels of HDL out of all phenotypes, as well as the lowest plasma values of total cholesterol and LDL [7,73]. ApoE3 has similar functions to ApoE2, but to a lesser extent. However, following exercise training, ApoE3 causes great reductions in circulating triglyceride and LDL/HDL levels by increasing lipid metabolism. ApoE3 also removes cholesterol from macrophages and inhibit T-cell differentiation and endothelial cell proliferation [52], through inhibition of the pro-inflammatory IL-2 signalling pathway. ApoE4, on the other hand, has a strong affinity to both VLDL and LDL, which leads to a marked competition for the LDL receptor, therefore delaying its clearance from plasma [73]. Sedentary ApoE4 carriers have a 1.4-fold greater risk of developing cardiovascular diseases, and particularly atherosclerosis [49]. Nevertheless, physically active individuals who express the ApoE4 isoform present similar lipid profiles to ApoE2- and ApoE3-carrying individuals, contributing to an enhanced atheroprotective environment [30]. Whether this exercise-mediated protection in ApoE4 carriers is due to a reduced affinity to LDL and VLDL molecules due to myokine secretion is still unknown. Similarly, what the interaction is between other ApoE isoforms and exercise training responses is currently not studied in detail.

3.1.7 Fibroblast growth factor 21 (FGF21)

Fibroblast growth factor 21 (FGF21) is a metabolic growth factor responsible for controlling glucose levels and lipid metabolism [138]. It is secreted from multiple organs across the human body in obesity-derived type 2 diabetes mellitus and cardiovascular diseases [63]. Animal studies revealed improved lipid profiles upon administration of FGF21 [21]. Muise and colleagues [84] concluded that high-fat diets induce hepatic secretion of FGF21 in mice, which resulted in an enhanced lipid metabolism. Higher serum levels of FGF21 were associated with elevated pericardial fat in people without cardiovascular disease at baseline and its reduced accumulation over time, thus highlighting its cardioprotective properties [78]. In addition to overall improvement of lipid profiles, FGF21 also has anti-inflammatory and anti-oxidant functions [65]. Therefore, FGF21 has beneficial metabolic effects which contribute towards metabolic homeostasis [63].

In humans, however, augmented serum levels of FGF21 have been linked to the development and incidence of a wide range of pathologies, including atherosclerosis [138]. Circulating levels of FGF21 increase as a response to pericardial fat accumulation, the latter being a strong determinant of atherosclerosis. Therefore, FGF21 can be used as a biomarker of cardiovascular disease, as its synthesis is markedly enhanced under conditions of adipose tissue accumulation [130]. Additionally, evidence on administration of recombinant and/or analogues of FGF21 showed several preventive effects on atherosclerosis development and progression [21]. This suggests that FGF21 in fact exerts a protective effect that contributes to reduced risk to cardiovascular diseases, likely as a compensatory cardioprotective mechanism [142].

Current research on the role of FGF21 in atherosclerosis is still scarce. The mechanisms though which FGF21 operates, likely direct or indirect via adjusting whole body metabolic homeostasis, are not fully understood yet.

4. FUTURE DIRECTIONS

Regular exercise has the potential to improve endothelial function and reduce the progression to atherosclerosis via a combination of factors. Here, we focused on the immune-modulating role of various myokines that are released during exercise. Since the field of exercise immunology are still in its relative infancy, it is important to realize that a lot of future research is needed to fully understand the cellular and molecular pathways.

One clear requirement for future understanding of the beneficial effects of exercise on atherosclerosis is the type and duration of exercise. From what is currently known, it is likely that the required exercise has to induce enough shear stress to allow endothelial function to be improved. One could argue that aerobic exercise, with a long duration and low intensity would result in more improvements compared to short-duration resistance training. However, how myokine profiles differ between various types of exercise (duration and intensities) is only marginally understood. Clearly, this is something that future research should focus on.

Regular exercise has a multitude of effects on cardiovascular function. One of them is angiogenesis which is the process of the formation of new blood vessels, via the exercise-mediated release of vascular endothelial growth factor (VEGF). It is known for being central to the pathogenesis of a wide range of diseases [14]. In atherosclerosis, however, the role of angiogenesis remains a highly unresolved issue. It has been suggested that therapeutic angiogenesis replaces dysfunctional or occluded vessels with new fully functional capillaries, and thus revascularize cardiac and peripheral tissues [146]. While atherosclerosis is mainly thought to affect larger vessels with a layer of smooth muscle, the ultimate tissue damage occurs downstream in the occlusion of capillaries by ruptured plaques. Therefore, the use of VEGF has emerged as a way to stimulate blood vessel formation in cardiovascular disease [117], but its role in atherosclerosis is highly contradictory. Whilst some studies have suggested VEGF-induced angiogenesis exerts atheroprotective effects and enhances cardiovascular health [114], others demonstrated that VEGF promotes the development of atherosclerosis [118]. As there is still no consensus on the possible benefits of angiogenesis on atherosclerosis regression and/or progression, it is highly recommended that future research needs to focus on how angiogenesis (possibly via the release of certain myokines) can alter the time course of atherosclerosis.

As atherosclerosis mainly occurs in older individuals, the development of atherosclerosis could, at least in part, overlap the process of biological ageing. Immunosenescence refers to the deterioration of the (adaptive) immune system with increasing age. Recent insights have suggested immunosenescence to play a contributing role in atherosclerosis [9]. Immunosenescence is marked by an active, pro-inflammatory subpopulation of T-cells, namely CD4+, CD8- and terminally differentiated effector memory CD45RA+ T (TEMRA) cells, which secrete high amounts of TNF-a and INF-g. TEMRA cells are highly cytotoxic and contribute to vascular inflammation, plaque disruption and worsening of atherosclerosis in older patients [12]. The risk of atherosclerosis is further enhanced by shortening of telomeres in leukocytes of elderly individuals [8] and accumulation of senescent endothelial and vascular smooth muscle cells [62]. Approximately 60% of women diagnosed with atherosclerosis, and 88% in men can be attributed to ageing-related immunosenescence [61]. Nearly all senescent cells secrete the same pro-inflammatory cytokines (IL-1, IL-1b, IL-6, IL-8, IL-18, TNF-a and IFN-γ) which have been linked with atherogenesis and its subsequent progression [23]. Physical activity is known to induce apoptosis of senescent T cells, possibly stimulating production and maintenance of naïve T-cells with ageing [66]. A 3-week endurance exercise training programme significantly reduced the number of CD8+ TEMRA cells, acting as a countermeasure to immunosenescence [102]. Therefore, as immunosenescence heavily depends on age-associated sedentarism, maintaining a physically active lifestyle throughout adulthood prevents immunosenescence. How this affects the course of atherosclerosis, independent of other lifestyle factors, remains however, an unexplored topic.

CONCLUSION

Atherosclerosis is an inflammatory chronic cardiovascular disease, caused by injury to the intimal wall within the blood vessel. Its progression is marked by a pro-inflammatory milieu induced by monocyte transmigration and differentiation into M1 macrophages, which secrete pro-inflammatory cytokines. Physical exercise is an effective non-pharmacological approach to delay and prevent atherosclerosis, likely through positive adaptations in the inflammatory pathways. These effects are mostly observed upon release of myokines IL-6 and IL-10, which block Th1 cell activity, and activate Th2 cell function. The practical link between physical exercise and atherosclerosis treatment and prevention is clear, although specific exercise prescriptions to optimally alter immune cell function are yet to be developed. Also, the direct and indirect interaction between various myokines and the immune system make future work in the field of exercise immunology exciting. We look forward to more mechanistic, therapeutic and clinical studies to better understand the beneficial effects of exercise in the development of atherosclerosis.

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Higher levels of physical activity are associated with reduced tethering and migration of pro-inflammatory monocytes in males with central obesity

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ABSTRACT

Despite evidence that monocyte migration is accentuated by central adiposity, the impact of physical activity (PA) and exercise, particularly in the post-prandial state, on limiting migration are not established. We hypothesised that PA and a single bout of walking exercise would be associated with reduced ex vivo monocyte tethering and migration in middle-aged males with central obesity (CO). Objective levels of PA were measured for 7 days in lean males (LE, N=12, mean (SD) age 39 (10) years, waist circumference 81.0 (6.3) cm) and males with CO (N=12, mean (SD) age 40 (9) years, waist circumference 115.3 (13.9) cm), followed by donation of a fasted blood sample. On the same day, CO undertook a bout of walking exercise, before donation of a second fasted blood sample. An ex vivo assay, coupled to flow cytometry, determined tethering and migration of classical, intermediate, and non-classical monocytes. C-C and CXC chemokine receptor (CCR2, CCR5 and CX3CR1) expression were also determined on total and classical monocytes. Monocyte subsets (total, classical, intermediate and CCR2+ monocytes), metabolic (glucose and lipids) and inflammatory (C-reactive protein) markers were greater in CO vs. LE (lower high-density lipoprotein); however, adjustments for PA mitigated group differences for glucose, lipids, and monocyte subsets. Ex vivo tethering and migration (absolute and relative) of most monocyte subsets was greater in CO vs LE. Relative monocyte tethering and migration was largely not influenced by PA; however, higher PA was associated with reduced absolute migration and tethering of CD16 expressing monocytes in CO. Prior walking had no impact on these variables. These results highlight that regular PA, not single exercise bouts may limit the migration of pro-inflammatory monocytes in CO. These changes may relate to physiological parameters in blood (i.e. number of cells and their adhesion), rather than differences in chemokine receptor expression.

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INTRODUCTION

Cardiovascular, metabolic, and neurological diseases are threatening to reach epidemic proportions, presenting an enormous health, economic and societal challenge. Common to all these conditions is their chronic inflammatory aetiology (40). Increased central adiposity is known to significantly elevate the risk of developing these diseases, with a heightened migration of immune cells from blood into various tissues, namely monocytes into adipose tissue (44). An increase in adipose tissue-resident macrophages can initiate a perpetual inflammatory cycle whereby proteins that tether (e.g. cellular adhesion molecules) (34), attract (e.g. chemokines) (32), and subsequently cause damage (e.g. pro-inflammatory cytokines) (35) are chronically elevated, thus drawing more cells into tissues. Over time, excessive monocyte migration in individuals with central obesity (CO) drives a dysfunctional interaction that promotes metabolic dysfunction and an increased risk of chronic disease (44).

This risk of developing cardiovascular, metabolic, and neurological diseases is exaggerated by multiple factors, including physical inactivity and diet (41). For the latter, prolonged elevations of triglycerides in the bloodstream after high fat meals, termed postprandial lipaemia (PPL), is a known independent predictor of cardiovascular disease (41). There is evidence that PPL is also associated with higher systemic inflammation (8, 41), with monocyte activation (29), adhesion and propensity for foam cell formation higher following a high fat meal (28). Given the central role of monocytes in the aetiology of chronic inflammatory disease, monocyte blood profiles have been used to predict current and future cardiovascular (22), metabolic (37), and neurological health (38). Recent evidence has documented that individuals with obesity have a more pro-inflammatory and pro-migratory monocyte profile than individuals who are lean (15, 33). This is reflected by higher concentrations, and an altered composition of the three monocyte sub-populations; e.g. decreased classical (CM: CD14++CD16-), higher intermediate (IM: CD14++CD16+), and higher non-classical (NCM: CD14+CD16++) monocytes (15, 20, 33). A reduced percentage of CM occurs as a result of preferential tissue migration in response to cues from inflamed tissues, whereas IM and NCM proportions increase due to their role in patrolling the circulation by adhering to the endothelium (31). These functions are governed by higher expression of C-C and CXC chemokine receptors (e.g. CCR2, CCR5 and CXC3R1), which positively correlate with body weight, body mass index, fat mass, and insulin insensitivity on total monocytes (6). Chemokine receptors have high affinity for complementary chemokine ligands (e.g. CCL2, CCL5 and CX3CL1), which are released from cells or tissues under metabolic and inflammatory stress. Obesity-associated inflammation also causes an increase in circulating cellular adhesion

molecule concentration (34), which enhances the potential for monocytes to tether to the surface of these cells and tissues. A higher number of circulating monocytes with a higher capacity to tether and migrate therefore generates a favourable gradient for the progressive movement of monocytes towards metabolically active tissues or the endothelial layer in individuals with CO, increasing the risk of cardiovascular diseases (14). Given the substantial economic and social impact of managing and treating these diseases, cost-effective interventions to prevent monocyte tethering and migration are urgently required.

Increased levels of physical activity are associated with reduced blood markers of chronic inflammation (e.g. inflammatory cytokines and adhesion molecules), and a reduced risk of chronic inflammatory disease, compared with sedentary individuals (16, 24). Studies in adults with obesity indicate that regular structured exercise can reduce the pro-inflammatory monocyte phenotype in blood (13, 42), as well as the expression of chemokine receptors on total monocytes (2). It is well documented that single sessions of exercise result in acute elevations in chemokine concentrations in blood plasma (12, 17, 39). It has been suggested that this response may result in the internalisation of chemokine receptors on the surface of immune cells that with regular physical activity, drive a reduction in expression level (2, 6, 24). Indeed, recent evidence has highlighted that moderate intensity exercise lowered the expression of CCR2 on intermediate monocytes within an hour of exercise cessation in individuals with CO (6). When considering how this transient internalisation of CCR2 on monocytes may translate into chronic changes in expression that have been reported separately (2), the impact of PPL-induced inflammation must also be considered, particularly in individuals with CO as this is associated with unfavourable metabolic and inflammatory profiles (14). A large body of evidence supports a role for single sessions of exercise being an effective strategy to attenuate PPL within 24 hours of a high-fat meal (23). Despite this, there is limited available data to support a reduction in PPL-associated inflammation over this 24 hour period with prior exercise. At present, our collective understanding of how regular physical activity and single sessions of exercise, particularly in the PPL state, independently impact chemokine expression on monocyte populations in individuals with CO is lacking. Furthermore, how these changes then relate to the tethering and migration of monocytes is yet to be established.

We, and others, have previously quantified the *ex vivo* migratory capacity of peripheral blood mononuclear cells (PBMCs) towards a fixed chemokine gradient over time (3, 33). Using this method, there is evidence that monocyte migration is greater in individuals who are obese than those who are lean (33). Evidence from our group indicates that a single session of exercise can reduce the *ex vivo* migration of T-helper lymphocytes towards chemokine-rich supernatants in healthy individuals (3). To our knowledge, there is no data indicating that regular physical activity or single sessions of exercise in a state of PPL, can reduce monocyte migration in individuals with CO. Importantly, previous investigations into monocyte migration are limited by only quantifying the number of cells that migrate in response to a fixed chemokine gradient (33), rather than their phenotype, as done in our laboratory previously with lymphocytes (3). Adopting this approach would indicate more about the inflammatory characteristics of monocytes and potential to cause damage within metabolically active tissues (i.e. CM) or the vasculature (i.e. IM and NCM). Furthermore, it has been highlighted that mimicking conditions of physiological blood flow better maintains monocyte phenotype when implementing *ex vivo* models (45), and this is often overlooked (3, 33).

By validating a dynamic experimental platform, which coupled *ex vivo* monocyte tethering and migration under conditions of physiological blood flow to digital flow cytometry, the aims of this study were to: 1) cross-sectionally determine the association of central adiposity and physical activity levels with monocyte tethering and migration in middle-aged males and 2) determine whether a single session of exercise can impact the tethering and migration of monocytes in middle-aged males with CO under conditions of PPL.

MATERIALS AND METHODS

Participants

This project involved cross-sectional assessments in males who were lean (LE, N=12) and males with central obesity (CO, N=12), followed by a walking intervention in CO only. Using our preliminary data, we based our sample size calculation on mean resting (and SD) differences in total monocytes between CO and LE. Using GPower 3.1.9.7, we calculated we would need 12 participants in each group to detect similar differences in the present study, with an effect size of 1.1, 80% power and α of 5%. Age and ethnicity-matched participants (White European (WE), N=6 and South Asian (SA), N=6 in both LE and CO) gave their informed written consent and the investigation was approved by the ethical review committee at Loughborough University (ethics code: R18-P120) in accordance with the Declaration of Helsinki. Central obesity was defined as a waist circumference $\geq$ 94cm in White European men and $\geq$ 90cm in South Asian men according to the International Diabetes Federation cut-off points (1). Males who were lean were classified based on a waist circumference under the aforementioned boundaries. All participants had stable weight for the preceding 3 months, were non-smokers and had not taken any anti-inflammatory drugs (e.g. NSAIDs) for 4 weeks prior to taking part. Participants were screened for diabetes using an HbA1c test, and a health screen questionnaire was used to screen for other underlying health conditions, plus lifestyle factors that may influence the results such as smoking. In addition, participants were required to refrain from any strenuous physical activity or consumption of alcoholic or caffeine-based beverages in the 48 hours prior to or during the experimental session.

Experimental Procedures

All participants first visited the laboratory for screening of height and weight using a fixed wall stadiometer with a digital weighing scale built in (Seca Ltd, Hamburg, Germany). Hip and waist circumference were measured using a flexible, non-elastic tape (Hokanson, Washington, USA) whilst adhering to established measurement guidelines (1). An assessment of peak oxygen uptake ($\dot{V}O_2$peak) was undertaken in CO only using the modified Bruce treadmill test (43). Heart rate was continuously measured using short-range telemetry (*Polar T31; Polar Electro, Kempele, Finland*) and subjective effort measured using the rating of perceived exertion scale (7). At

the end of the first visit, participants were fitted with an accelerometer (*ActiGraph GT3X, ActiGraph corporation, Florida, USA*) to quantify levels of habitual physical activity for a period of 7 days. Data were analysed over 15 second epochs using specialised software (*Actilife, Actigraph corporation, Florida, USA*). Accelerometer data were screened for wear time using standard methods (10). Time spent in a defined intensity of activity was determined by summing together counts per minute and categorising this based on widely used cut points (19).

Prior to the experimental period, all participants were asked to maintain their normal habitual diet for a period of 7 days. The day before, both groups undertook an overnight fast from 22:00 (except plain water). The next morning (08:00), both groups returned to the laboratory and consumed 250mL of water prior to bioelectrical impedance analysis (*Seca mBCA 515, Seca Ltd, Hamburg, Germany*) to measure body fat percentage. Participants then donated a blood sample via venepuncture to an antecubital vein. After this, CO remained rested in the laboratory throughout the day, with standardised meals (57% fat, 32% carbohydrate, 11% protein, 14.2 kcal per kg of body mass) provided, before undertaking a 60-minute session of walking exercise at 60-65% of their $\dot{V}O_2$peak (15:00-16:00). Walking intensity was confirmed using a portable metabolic cart which analysed breath-by-breath gases (*Metalyzer 3B, Cortex, Leipzig, Germany*) and subjective measures of perceived exertion were obtained using the Borg Scale (6-20) (7). Participants were then free to leave the laboratory and were provided with a standardised evening meal to consume before 22:00. After this, participants fasted overnight (except plain water) and returned to the laboratory the next morning (08:00) to donate a second rested blood sample.

Blood collection and analysis

Whole blood (40.9mL) was collected via venepuncture into EDTA (4.9mL) and sodium heparin-coated (36mL) monovettes. Heparinised blood was used for the isolation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation. Briefly, whole blood was diluted 1:1 with D-PBS and layered on top of Histopaque 1077 (2:1). Blood was centrifuged at 400g for 30 minutes at 21 degrees (brake off) and the PBMC layer aspirated and washed in PBS and RPMI. EDTA monovettes were centrifuged at 3500g for 10 minutes at 4 degrees and plasma isolated for future analysis of triacylglycerol (TAG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), non-esterified fatty acids (NEFA), glucose, and C-Reactive Protein (CRP).

Ex vivo Migration Assay

The *ex vivo* migration assay was adapted from previously published protocols in our laboratory (3) and others (33), as well as internal validation experiments. Whole blood cell counts were initially performed using a haematology analyser (*Yumizen H500, Horiba, Northampton, UK*) to determine the circulating monocyte count (≈ 0.2 – 1.0 x 10^6/mL). PBMC counts were then determined by using CountBright™ Absolute Counting Beads on a BD C6 Accuri Flow Cytometer (*Becton Dickinson, Oxford, UK*). The seeding density of monocytes for the migration assay matched the monocyte concentration in whole blood, thus mimicking physiological conditions. Peripheral blood mononuclear cells (in 2mL RPMI) were added in duplicate to fibronectin-coated polyester (PET) inserts (5µm pore size) and placed into 6-well non-tissue culture treated plates containing 3mL of heat-inactivated 10% fetal bovine serum (FBS) or RPMI (background migration control). Monocytes were then allowed to migrate for 3 hours at 37 degrees (5% CO_2). To further generate conditions that closely mimic moderate physiological flow within the circulation (3.2 dyn/cm^3) (9) and to maintain monocyte phenotype, the PBMC suspension was oscillated on a 2-dimensional orbital shaker at 77 rpm during this 3 hour period (45). Fetal bovine serum concentration and incubation times were based on in-house validation experiments from our laboratory. After 3 hours, non-adherent cells were removed from the upper side of the PET insert, and this was then washed twice with 1mL of D-PBS. *Tethered* PBMCs were then removed from the upper side of the PET inserts by adding 1mL of enzyme free, EDTA-based dissociation media (4 degrees, 30 minutes), followed by five washes with D-PBS (1ml). The underside of the PET inserts and the wells of the tissue culture plate beneath were treated identically to collect cells that had migrated across the PET insert. *Tethered* and *migrated* cells were collected into separate tubes and washed in D-PBS ready for counting and phenotyping using flow cytometry.

Flow Cytometry

Pre-migration (baseline) and collected tethered and migrated PBMCs were counted as above. For each sample, 1.75 x 10^5 PBMCs were then stained using fluorescently conjugated antibodies for identification of monocytes subsets and chemokine receptor expression (e.g. CCR2, CCR5 and CX3CR1) using four-colour flow cytometry. Cells were incubated with CD14-FITC, CD16-PE, CX3CR1-APC, CCR5-APC, CCR2-Alexa Fluor-647 antibodies, and 7-AAD for 30 minutes at 4°C in the dark. Cells were then twice washed with FACS buffer (D-PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA; pH=7.2) for 5 minutes at 300 x g. Compensation was adjusted weekly by using single stained controls and gates established using fluorescence minus one controls. Confirmation of non-specific antibody binding was determined by using isotype-matched controls.

Flow cytometry data were analysed using BD C6 Accuri software (*Becton Dickinson, Oxford, UK*). Briefly, monocytes were gated on forward versus side scatter. Doublets were discriminated using FSC-A vs FSC-H plots, and non-viable monocytes (i.e. 7-AAD+) excluded. CD14+ and CD16+ positive populations were then used to determine classical (CD14++CD16-), intermediate (CD14++CD16+), and non-classical (CD14+CD16++) monocyte proportions (%) as previously described (18). Histogram plots of the cells in the total monocyte, and CM regions that positively expressed CCR2, CCR5, and CX3CR1 were subsequently used to calculate the percentage of chemokine receptor positive cells. Mean fluorescent intensity (MFI), an indicator of the density of chemokine receptor expression on each cell, was also determined for total monocyte and CM populations. A sub-population of cells was defined as ≥1000 positive cells in a gate. For baseline, the percentage of monocyte subsets and CCR2+, CCR5+, and CX3CR1+ monocytes were used with whole blood cell counts to determine the circulating number of chemokine receptor positive monocytes for each sub-population.

Similarly, the percentages of these cells following tethering and migration were used with the total number of tethered and migrated cells to determine absolute changes at each assay stage.

Metabolic Parameters

Concentrations of TC, HDL, LDL, TAG, glucose, CRP (Horiba Medical, Montepellier, France), and NEFA (*Randox Laboratories Ltd., County Antrim, UK*) were determined spectrophotometrically using commercially available kits (high detection for CRP) and a benchtop analyser (*Pentra 400, Horiba Medical, Montpellier, France*). Concentrations of HbA1c were determined using a bench top analyser (Quo-lab HbA1c POC, EKF Diagnostics, Penarth, UK).

Data and Statistical Analyses

Data were analysed using the statistical package for social sciences (SPSS version 24). Residuals for the outcomes were explored using histograms. Normally distributed data are presented as the arithmetic mean (95% CIs). Skewed data underwent natural log-transformation and were back transformed which gave a similar variance to non-logged data and a reasonable estimate which is presented as the geometric mean (95% CI) (4). Log-transformed data are presented as the ratio of geometric means and 95% CIs for the ratio difference between geometric means (5). An effect size of 0.2 was considered the minimal value for a meaningful difference, 0.5 for moderate and 0.8 for large (11). Statistical significance was accepted as $P<0.05$.

Physical and behavioural characteristics were compared using linear mixed models (LMM) with waist circumference category (CO vs LE) and ethnicity (WE vs. SA) as fixed factors. Blood and *ex vivo* migration variables were compared using LMM with the same fixed factors mentioned above. These variables were then compared in CO only using LMM with day (day 1 vs day 2) and ethnicity as fixed factors. Chemokine receptor expression was compared before (baseline) and after the *ex vivo* assay (tethered and migrated) using LMM with stage (baseline vs migrated vs tethered) as a fixed factor. Adjusted models were performed to account for differences in moderate-vigorous PA (MVPA) and daily steps between CO and LE. From here on adjustment refers to the adjusted model for daily steps and MVPA, and no difference refers to a statistical difference.

RESULTS

CROSS-SECTIONAL COMPARISON OF CO VS. LE

Participant characteristics are displayed in Table 1. CO participants demonstrated higher BMI, waist circumference, hip circumference, waist-to-hip ratio, body fat percentage, and lean mass than the LE group (ES≥2.30, P≤0.001). CO demonstrated lower daily steps and MVPA (ES≥1.47, P≤0.001).

There were no differences in participant characteristics between WE and SA within or between CO and LE groups. Due to low numbers of participants and inconclusive data on ethnic differences between CO and LE participants for the remaining variables, ethnic data are not presented throughout the rest of the manuscript.

Table 1. Participant characteristics and fasting metabolic marker and monocyte subset concentrations (unadjusted) between men with central obesity (CO) and men who were lean (LE).

Variable	LE (*n* = 12)	CO (*n* = 12)	Mean difference (95% CI centrally obese vs. lean)
	Mean	Mean	
Age (years)	38.8 (33.0 to 44.6)	40.4 (34.7 to 46.2)	1.6 (-6.6 to 9.7)
Body mass index (kg/m^2)	22.5 (19.5 to 25.5)	36.2 (33.2 to 39.3)	13.7 (9.4 to 18.0)*
Waist circumference (cm)	81.0 (72.5 to 87.7)	115.3 (108.5 to 122.1)	35.1 (24.9 to 45.3)*
Hip circumference (cm)	95.0 (88.7 to 101.3)	117.8 (112.2 to 123.5)	22.9 (14.4 to 31.3)*
Waist to hip ratio	0.85 (0.82 to 0.87)	0.98 (0.95 to 1.00)	0.13 (0.10 to 0.17)*
Body fat (%)[1]	19.6 (15.5 to 23.3)	36.4 (32.9 to 40.0)	17.0 (11.8 to 22.2)*
Lean mass (kg)	27.9 (19.6 to 36.0)	75.9 (68.5 to 83.2)	48.1 (37.0 to 59.1)*
MVPA (mins/day)	61.8 (50.0 to 73.7)	30.9 (18.6 to 43.4)	-30.9 (-48.0 to -13.7)*
Daily steps	9215 (7969 to 10461)	5993 (4666 to 7280)	-3243 (-5048 to -1437)*
TC (mmol/L)	4.5 (3.9 to 5.2)	5.8 (5.0 to 6.8)	29% (5% to 60%) *
HDL (mmol/L)	1.4 (1.2 to 1.5)	1.1 (1.0 to 1.2)	-20% (-31% to -8%) *
LDL (mmol/L)	2.2 (1.8 to 2.7)	3.3 (2.7 to 4.1)	50% (14% to 99%) *
TAG (mmol/L)	1.1 (0.7 to 1.5)	1.8 (1.4 to 2.2)	0.7 (0.1 to 1.2) *
Glucose (mmol/L)	5.5 (5.0 to 6.0)	6.4 (5.8 to 7.1)	17% (2% to 34%) *
HbA1c [a] (mmol/mol)	33.2 (31.5 to 35.0)	35.2 (33.6 to 36.8)	2.0 (-0.4 to 4.4)
CRP (mg/L)	0.3 (0.2 to 0.5)	1.8 (1.1 to 3.1)	542% (208% to 1241%) *
NEFA (mmol/L)	0.5 (0.4 to 0.5)	0.6 (0.5 to 0.6)	0.1 (0.0 to 0.2) *
CM Conc. (cells/uL)	277.1 (229.3 to 334.8)	373.2 (308.9 to 450.9)	35% (3% to 76%) *
IM Conc. (cells/uL)	17.6 (7.8 to 27.5)	34.0 (24.1 to 43.9)	16.4 (2.4 to 30.3) *
NCM Conc. (cells/uL)	21.2 (14.5 to 30.8)	32.1 (22.0 to 46.7)	52% (-11% to 158%)
CCR2+ monocyte Conc. (cells/uL)	253.6 (211.1 to 304.7)	350.1 (291.4 to 420.7)	38% (7% to 79%) *
CCR2+ CM Conc. (cells/uL)	234.9 (195.3 to 282.4)	313.4 (260.6 to 376.9)	33% (3% to 73%) *
CCR5+ monocyte Conc. (cells/uL)	8.9 (3.5 to 14.4)	16.1 (10.7 to 21.5)	7.1 (-0.5 to 14.8)
CX3CR1+ monocyte Conc. (cells/uL)	36.4 (18.3 to 54.5)	51.7 (33.6 to 69.8)	15.2 (-10.3 to 40.8)

Table 1. Groups were ethnicity matched, with a 1:1 ratio of WE and SA males. Data were analysed using linear mixed models with the ethnic specific waist circumference category (CO vs LE) as a fixed factor. Values are mean and standard deviations. 95% CI, 95% confidence interval of the difference between the groups. *Main effect of central obesity (P≤0.032). [1]Body fat % determined by bioelectrical impedance. [a]For HbA1c, n=10 for the LE group. MVPA, moderate to vigorous PA; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; TAG, triacylglycerol; HbA1c, glycated haemoglobin; CRP, C-reactive protein; NEFA, non-esterified fatty acids; CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes; CCR, C-C chemokine receptor; CX3CR1, CX3C chemokine receptor-1

Metabolic markers

Unadjusted fasting metabolic marker concentrations are presented in Table 1. Concentrations of HDL were lower (ES=1.22, P=0.003), and concentrations of TC, LDL, TAG, glucose, CRP, and NEFA were higher in CO vs LE (ES≥0.93, P≤0.025). There was no difference for HbA1c (ES-=0.43, P=0.100). Adjustment eliminated the difference for TC, TAG, glucose, and NEFA (P≥0.101). No difference for HbA1c remained (P=0.265). HDL was still lower, and LDL and CRP remained higher in CO vs. LE (P≤0.048).

Monocyte phenotype in blood

A representative gating strategy for monocyte subsets and their respective chemokine receptor expression are presented in Figure 1. Unadjusted fasting blood monocyte subset concentrations are presented in Table 1. Higher concentrations of monocytes, CM, IM, CCR2+ monocytes, and CCR2+CM subsets were seen in CO vs. LE (ES≥0.90, P≤0.037). No differences for NCM, CCR5+, and CX3CR1+ monocytes were seen (ES≤0.77, P≥0.066). Adjustment eliminated all differences for monocyte subset concentrations (P≥0.155). There were no differences in the unadjusted or adjusted models for the percentages of monocyte subsets (P≥0.154).

Figure 1:

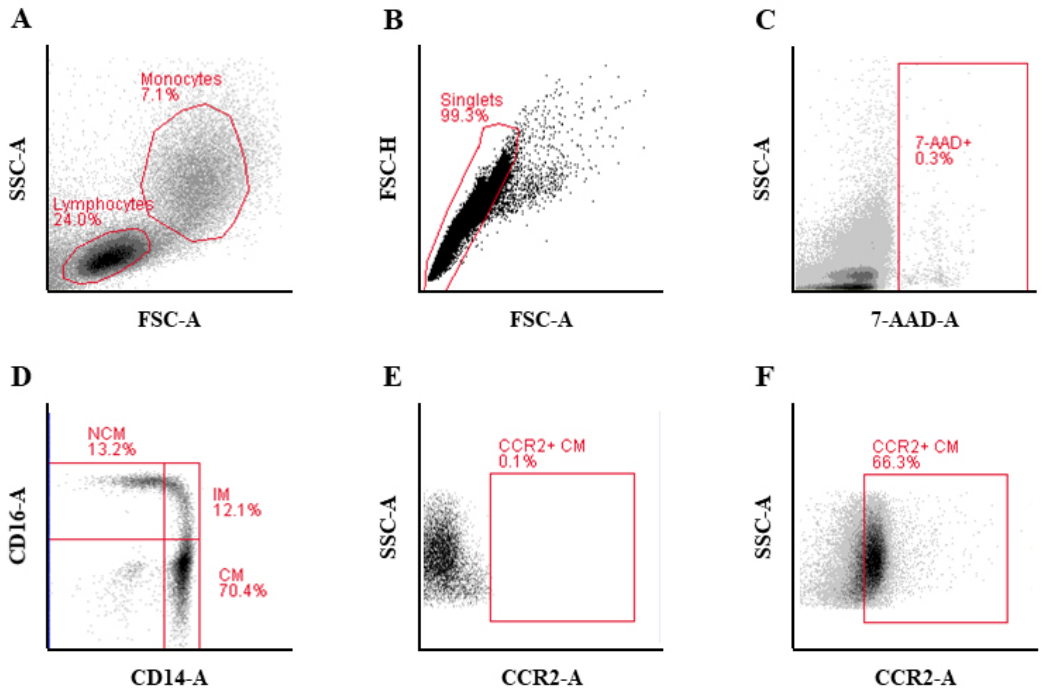

Figure 1: Representative gating strategy for monocyte subsets and their respective chemokine receptor expression levels. The example presented includes data from a male with central obesity on Day 1 of the study. The example data indicate how monocyte subsets and then CCR2+ classical monocytes were determined: (A) Monocytes were gated on forward light scatter (FSC) vs. side light scatter (SSC); (B) doublets were discriminated using FSC-Area vs. FSC-Height; (C) non-viable cells were excluded using a 7-AAD; (D) monocyte subsets were identified using a CD14-Area vs. CD16-Area bivariate plot; (E) the positive gate for CCR2+ classical monocytes was determined using a fluorescence minus one control; (F) CCR2+ classical monocytes (%) were determined on CCR2-Area vs. SSC-Area.
7-AAD, 7-Aminoactinomycin D; CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes; CCR2+ CM, C-C chemokine receptor-2+ classical monocytes.

Absolute and relative ex vivo migration

Unadjusted and adjusted absolute migrated monocyte subsets are presented in Figure 2 panel A. Higher absolute migration of monocytes, CM, IM, NCM, CCR2+ monocytes, CCR2+CM, and CX3CR1+monocytes were seen in CO vs. LE (ES≥0.90, P≤0.046). No difference was seen for the absolute migration of CCR5+ monocytes (ES=0.02, P=0.953). Adjustment maintained higher absolute migration of monocytes, CM, CCR2+ monocytes, and CCR2+ CM subsets (P≤0.023), eliminated the differences for IM, NCM, and CX3CR1+ monocytes (P≥0.465), and maintained no difference for CCR5+ monocytes (P=0.763).

Unadjusted and adjusted percentages of migrated monocyte subsets are presented in Figure 2 panel B. Higher relative migration of CX3CR1+ monocytes were seen in CO vs. LE (ES=1.01, P=0.017). No difference was seen for the relative migration of the other subsets (ES≤0.85, P≥0.058). Adjustment eliminated the difference for CX3CR1+ monocytes (P=0.091), but revealed higher relative migration of monocytes, CM, CCR2+ monocytes, and CCR2+CM in CO (P≤0.040).

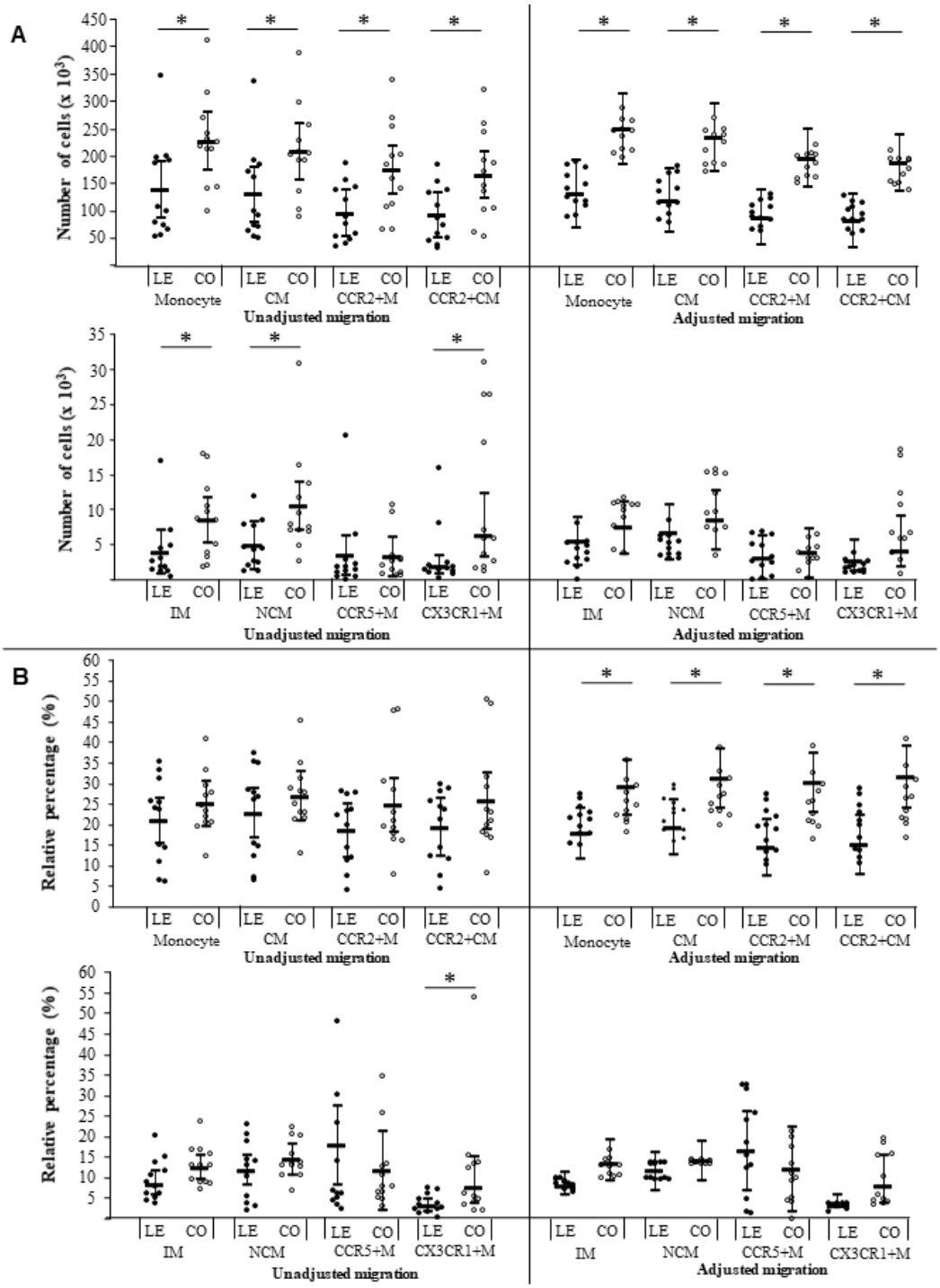

Figure 2. Unadjusted and adjusted absolute (Panel A) and relative (Panel B) monocyte subset migration between men with central obesity (CO, N=12) and men who were lean (LE, N=12). Linear mixed models were used with the ethnic specific waist circumference category (CO vs LE) as a fixed factor (unadjusted). The adjusted model accounted for group differences in moderate-vigorous physical activity (MVPA) and daily steps. Data are presented as the arithmetic / geometric mean (95% confidence intervals). CM, classical monocytes; CCR2+M, C-C chemokine receptor-2+ monocytes; CCR2+CM, C-C chemokine receptor-2+ classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes; CCR5+M, C-C chemokine receptor-5+ monocytes; CX3CR1+M, CX3C chemokine receptor-1+ monocytes. *Main effect of central obesity (P≤0.046).

Absolute and relative ex vivo tethering

Unadjusted and adjusted absolute tethered monocyte subsets are presented in Figure 3 panel A. Higher absolute tethering of monocytes, CM, IM, NCM, CCR2+ monocytes, and CX3CR1+ monocytes were seen in CO vs. LE (ES≥0.92, P≤0.029). No difference was seen for CCR5+ monocytes (ES=0.65, P=0.125). Adjustment maintained higher absolute tethering of monocytes, CM, NCM, CCR2+ monocytes, and CCR2+CM in CO vs. LE (P≤0.039). The differences for IM and CX3CR1M were eliminated (P≥0.070), and there was still no statistical difference for CCR5+ monocytes (P=0.238).

Unadjusted and adjusted percentages of tethered monocyte subsets are presented in Figure 3 panel B. Higher relative tethering of IM, NCM, and CX3CR1+ was seen in CO vs. LE (ES≥1.11, P≤0.008). No difference was seen for the relative tethering of other subsets (ES≤0.78, P≥0.057). Adjustment maintained a higher relative tethering of IM, NCM, and CX3CR1+ monocytes (P≤0.003), and revealed a higher relative tethering for monocytes, CM, CCR2+ monocytes, and CCR2+CM in CO vs. LE (P≤0.019). No difference was maintained for CCR5+ monocytes (P=0.437).

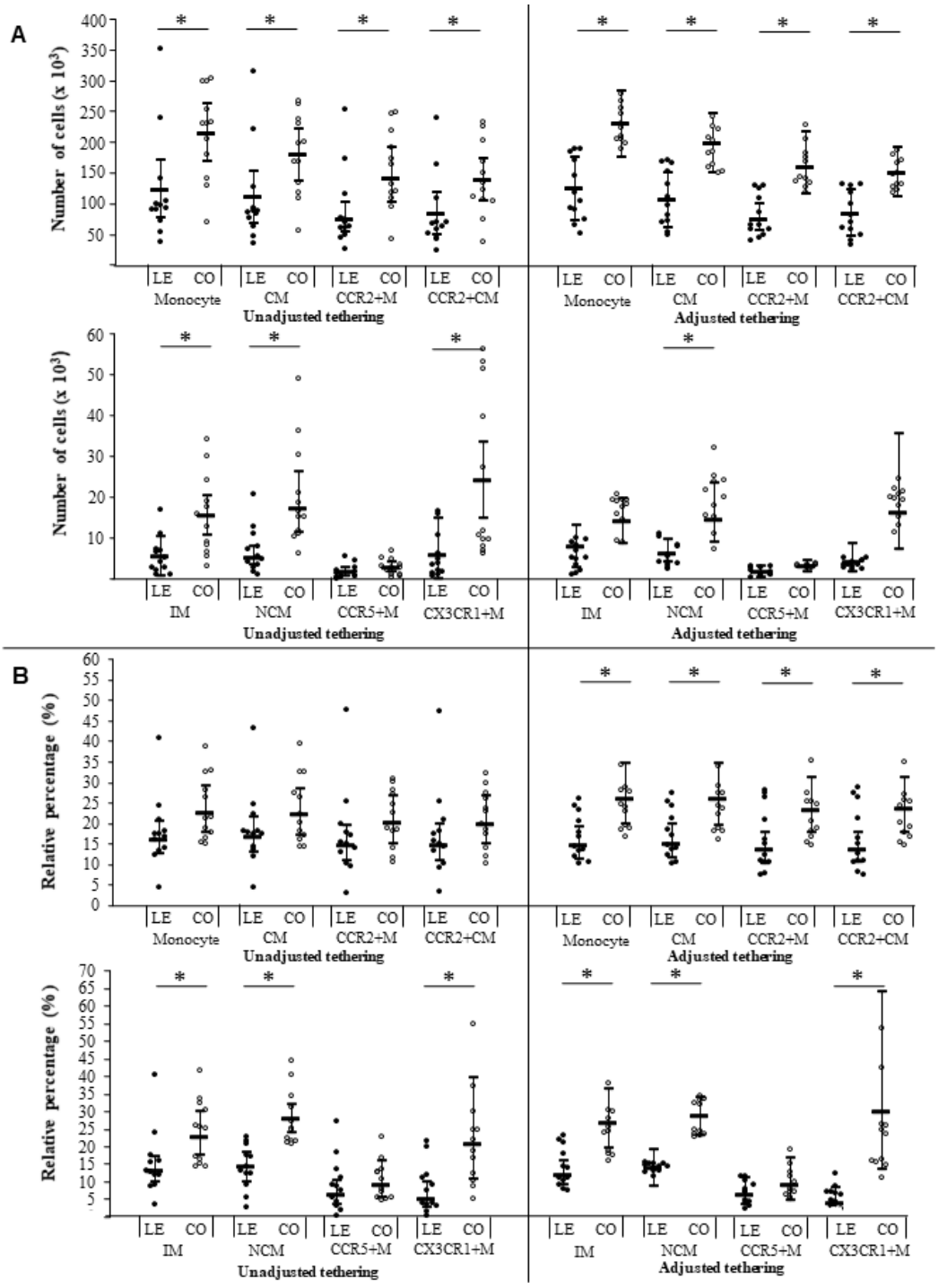

Figure 3. Unadjusted and adjusted absolute (Panel A) and relative (Panel B) monocyte subset tethering between men with central obesity (CO, N=12) and men who were lean (LE, N=12). Linear mixed models were used with the ethnic specific waist circumference category (CO vs LE) as a fixed factor (unadjusted). The adjusted model accounted for group differences in moderate-vigorous physical activity (MVPA) and daily steps. Data are presented as the arithmetic / geometric mean (95% confidence intervals). CM, classical monocytes; CCR2+M, C-C chemokine r eceptor-2+ monocytes; CCR2+CM, C-C chemokine receptor-2+ classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes; CCR5+M, C-C chemokine receptor-5+ monocytes; CX3CR1+M, CX3C chemokine receptor-1+ monocytes. *Main effect of central obesity (P≤0.039).

Chemokine receptor expression

Unadjusted expression of CCR2, CCR5, and CX3CR1 on total monocytes between CO and LE at baseline, and at stages of the *ex vivo* assay (baseline, tethered and migrated) are presented in Table 2. No difference for CCR2, CCR5, or CX3CR1 at baseline was found in the unadjusted (ES≤0.82, P≥0.060) or adjusted model (P≥0.081). When comparing CO vs. LE, no central obesity x stage interactions for CCR2, CCR5, or CX3CR1 were detected for the unadjusted (ES≤0.34, P≥0.743) or adjusted (P≥0.612) models.

To explore changes in chemokine receptor expression during different stages of the *ex vivo* assay in isolation, we evaluated changes in both groups combined. Unadjusted expression of CCR2, CCR5, and CX3CR1 on total monocytes between stages of the assay for CO and LE combined (N=24) revealed lower CCR2 receptor expression on migrated (mean difference = -610, 95% CI, -1023 to –196) and tethered (mean difference = -644, 95% CI, -1058 to -231) monocytes compared to baseline. No difference was found between the other stages for the monocyte subsets (ES≤0.41, P≥0.073). Adjustment maintained the differences for CCR2 (P≤0.011), CCR5 and CX3CR1 receptor expression (P≥0.083).

Table 2. Unadjusted receptor expression in men with central obesity (CO) and men who were lean (LE) in response to *ex vivo* migration. Expression in monocytes isolated at baseline, after tethering and after migration are indicated for CCR2, CCR5 and CX3CR1.

Variable	Chemokine receptor expression on total monocytes		Receptor expression
	LE ***(n=12)***	**CO** ***(n=12)***	**CO vs LE (95% CI / CL)**
CCR2			
Baseline	5194 (4743 to 5645)	5448 (4991 to 5905)	254 (-388 to 897)
Tethered	4465 (4067 to 4863)	4901 (4498 to 5305)	436 (-130 to 1003)
Migrated	4424 (4002 to 4847)	5000 (4571 to 5429)	576 (-27 to 1178)
CCR5			
Baseline	4029 (3386 to 4794)	3915 (3282 to 4670)	-3% (-24% to 24%)
Tethered	4594 (3670 to 5751)	3850 (3065 to 4835)	-16% (-39% to 15%)
Migrated	4722 (3602 to 6191)	4373 (3322 to 5755)	-7% (-37% to 36%)
CX3CR1			
Baseline	13469 (9190 to 17748)	13696 (9356 to 18036)	227 (-5868 to 6322)
Tethered	10757 (7384 to 14130)	11555 (8134 to 14976)	799 (-4005 to 5603)
Migrated	9304 (6507 to 12102)	10357 (7520 to 13194)	1053 (-2932 to 5037)

Table 2. Values for CCR2 and CX3CR1 are arithmetic means (95% confidence intervals) and between group differences are absolute differences (95% confidence intervals). Values for CCR5 are geometric means (95% confidence intervals) and between group differences are 95% confidence limits of the ratio difference between geometric means.

WALKING EXERCISE INTERVENTION

Physiological Responses

Participants walked at 59% (SD=3) $\dot{V}O_2$ peak and consumed 1.87 (SD=0.67) litres of O_2 per minute. Average heart rate was 126 beats per minute (SD=9) and a subjective rating of perceived exertion score of 12 (SD=2).

Metabolic and monocyte phenotype in blood

Unadjusted concentrations of TAG, NEFA, and CRP were similar between day 1 and day 2 (ES≤0.33, P≥0.481). Unadjusted concentrations of glucose were lower on day 2 (ES=1.43, P=0.003). Adjustment maintained no difference for TAG, NEFA, and CRP between day 1 and day 2 (P≥0.457), and lower concentrations of glucose on day 2 (P=0.006).

Fasting concentrations of monocyte subsets and monocytes positive or CCR2, CCR5, and CX3CR1 were similar between day 1 and day 2 for the unadjusted (ES≤0.21, P≥0.653) and adjusted models (P≥0.615). There were no differences in the percentages of monocyte subsets for the unadjusted (ES≤0.23, P≥0.600) and adjusted models (P≥0.533).

Absolute and relative ex vivo migration and tethering

The number of migrated monocyte subsets were similar between day 1 and day 2 for the unadjusted (ES≤0.37, P≥0.446) and adjusted models (P≥0.363). Percentage of migrated monocyte subsets and monocytes positive for CCR2, CCR5M, and CX3CR1 were similar between day 1 and day 2 for the unadjusted (ES≤0.45, P≥0.314) and adjusted models (P≥0.244). The number of tethered monocyte subsets were similar between day 1 and day 2 for the unadjusted (ES≤0.49, P≥0.293) and adjusted models (P≥0.319). The percentage of tethered monocyte subsets and monocytes positive for CCR2, CCR5M and CX3CR1 were similar between day 1 and day 2 for the unadjusted (ES≤0.58, P≥0.130) and adjusted (P≥0.147) models.

Chemokine receptor expression

Unadjusted expression of CCR2, CCR5, and CX3CR1 on day 1 and day 2, and at different stages of the *ex vivo* assay (baseline, tethered and migrated) are presented in Table 3. No difference between days was seen for CCR2, CCR5, or CX3CR1 expression (ES≤0.17, P≥0.718). Adjustment maintained no difference (P≥0.749). No day x stage interactions were detected for the unadjusted (ES≤0.15, P≥927) or adjusted (P≥878) models

Table 3. Unadjusted receptor expression in men with central obesity (CO) between day 1 and day 2 in response to *ex vivo* migration. Expression in monocytes isolated at baseline, after tethering and after migration are indicated for CCR2, CCR5 and CX3CR1.

Variable	Chemokine receptor expression on total monocytes		Receptor expression day 2 vs day 1 (95% CI / CL)
	Day 1 *(n=10)*	Day 2 *(n=10)*	
CCR2			
Baseline	5378 (4689 to 6067)	5502 (4813 to 6190)	123 (-851 to 1098)
Tethered	5022 (4498 to 5547)	5128 (4571 to 5684)	106 (-659 to 870)
Migrated	5035 (4527 to 5543)	5095 (4556 to 5634)	60 (-681 to 800)
CCR5			
Baseline	3957 (3360 to 4659)	3866 (3283 to 4552)	-2% (-22% to 23%)
Tethered	3908 (3271 to 4669)	3991 (3341 to 4768)	2% (-21% to 31%)
Migrated	4108 (3414 to 4944)	4115 (3419 to 4952)	0% (-23% to 30%)
CX3CR1			
Baseline	14267 (9242 to 19292)	13685 (8660 to 18710)	-582 (-7688 to 6525)
Tethered	12044 (7975 to 16113)	13070 (8754 to 17386)	1026 (-4906 to 6958)
Migrated	10833 (7282 to 14385)	11361 (7594 to 15128)	527 (-4650 to 5704)

Table 3: Values for CCR2 and CX3CR1 are arithmetic means (95% confidence intervals) and between group differences are absolute differences (95% confidence intervals). Values for CCR5 are geometric means (95% confidence intervals) and between group differences are 95% confidence limits of the ratio difference between geometric means.

DISCUSSION

The results of the current study provide novel evidence for the independent associations of adiposity, physical activity levels, and single sessions of exercise on aspects of obesity-driven monocyte tethering and migration. By validating a dynamic methodological approach that quantified the movement of monocytes towards chemokine-rich serum under conditions of physiological blood flow, for the first time we established that both absolute and relative monocyte tethering and migration were greater in CO vs. LE for most monocyte subsets. Higher levels of physical activity (i.e. daily MVPA and step count) were associated with reduced absolute tethering and migration of CD16 expressing monocytes, but not classical monocytes. Under controlled laboratory conditions, a single bout of walking exercise had no impact on monocyte tethering and migration in males with CO 16 hours after the exercise bout. Taken together, these data indicate that regular physical activity was associated with reduced movement of pro-inflammatory monocytes towards chemokines in males with CO. These findings have important implications for the potential anti-inflammatory effects of physical activity independent of weight status.

The movement of immune cells (e.g. monocytes, lymphocytes, and dendritic cells) from blood into vascular walls and metabolically active tissues is enhanced by increased adiposity (44). In particular, monocytes are metabolically plastic cells that can migrate into tissues (i.e. CM) and transendothelial sites (i.e. IM and NCM) to form macrophages in individuals with CO, which increases the risk of several chronic diseases (25, 31). The enhanced movement of monocytes stimulates haematopoiesis in the bone marrow, resulting in higher numbers of these monocytes in the circulation (30). In the present study, most circulating monocyte counts were higher in CO vs. LE (Table 1). To subsequently determine physiologically relevant differences in *ex vivo* monocyte tethering and migration (absolute change), our method examined how monocytes at this concentration tethered and migrated towards a fixed chemokine stimulus under conditions mimicking physiological blood flow (45). This approach controlled for important systemic variables that are known to differ between individuals who are lean and obese, e.g. the number of monocyte subsets and receptors that govern their adhesion and subsequent migration. Our data demonstrate that absolute total monocyte tethering and migration were greater in CO vs LE, independent of levels of physical activity (Figures 2A and 3A). Within the monocyte pool, CM and those expressing CCR2 appeared to be the main subsets driving these group differences. This supports previous work indicating that monocytes expressing CCR2 have the greatest propensity to migrate via chemokine gradients towards inflamed tissues (31), and specifically adipose tissue in individuals with obesity (36). Importantly, adjustments for MVPA and step count removed group differences for the absolute migration of IM, NCM and monocyte expressing CX3CR1. Group differences for the absolute tethering of IM and CX3CR1+ monocytes were also lost. CD16 expressing monocytes, such as IM primarily migrate towards transendothelial sites and are a major source of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 (46). These results therefore highlight an association between higher physical activity levels and reduced IM tethering and migration. Adjustments for PA also mitigated group differences in the blood concentration of IM, suggesting that reduced tethering and migration may be driven by a reduced number of cells within the circulation. This provides some support for other work highlighting reduced percentages of IM and NCM after structured exercise training in individuals who are lean (42) and obese (13). In addition, an impact of PA on the number of CX3CR1+ monocytes that tethered and migrated also indicates reduced monocyte adhesion. These results therefore highlight that although PA does not impact the migration of CM with high tissue homing-potential, it is associated with reduced transendothelial migration of IM and NCM populations, a key step in the development of chronic systemic inflammation.

In addition to the notable *absolute* differences between groups, *relative* tethering and migration of most monocyte subsets were also higher in CO vs. LE (Figures 2B and 3B). This suggests that differences at the cellular level are also important in driving monocyte migration. With regards to chemokine receptors however, we observed no difference in the protein expression of CCR2, CCR5, and CX3CR1 between LE and CO. Previous studies conducted in females with obesity have reported higher expression of CCR2, CCR5, and CX3CR1 on monocytes at both the mRNA (15) and protein level (15, 33). Despite similar screening (i.e. no metabolic disease) and anthropometric measures in these studies, serum CRP levels were markedly higher (mean: 7.0 (33) and 9.8 (15) mg/L) when compared to the present study (mean: 1.8 mg/L), indicating a much more heightened state of systemic inflammation. Furthermore, these studies noted differences in the relative numbers of blood monocyte subsets between obese vs. lean (i.e. higher percentage of IM), also indicative of systemic inflammation. To further interrogate our data, we explored changes in chemokine receptor expression after *ex vivo* tethering and migration between CO and LE (Table 2). After binding to their cognate chemokines, chemokine receptors are typically desensitized and internalized via endocytosis to limit the magnitude and duration of the stimulus (21). In support of this, CCR2 receptor expression was lower after *ex vivo* tethering and migration. This indicates that receptor internalisation may have played a role in driving monocyte migration in our *ex vivo* model; however, no statistical differences were noted between CO and LE. Collectively, we can therefore speculate that in individuals with CO that have relatively low levels of systemic inflammation, chemokine receptor expression and internalisation does not explain the higher relative rates of tethering and migration vs. lean individuals. Although not measured in our study, this again suggests that properties of monocyte adhesion could explain these differences. Importantly, there were no associations between physical activity and chemokine receptor expression and internalisation.

On the same day as the cross-sectional analysis, CO undertook a singlebout of walking exercise under controlled lifestyle and dietary conditions, with *ex vivo* monocyte tethering and migration measured the morning afterwards (i.e. 24 hours after the first sample). This experimental model has been previously used in obese populations to demonstrate that prior brisk walking can lower postprandial lipaemia (PPL) after intake of a high fat meal (23). Acute elevations in soluble inflammatory markers (i.e. IL-6 and TNF-α) also accompany PPL; however there is limited evidence to support the notion that prior walk-

ing reduces PPL-associated inflammation (8, 41). By applying our *ex vivo* assay to this experimental model, we explored the impact of prior walking on changes in functional immunity for the first time. We report no differences in circulating monocyte counts, *ex vivo* monocyte tethering and migration, or chemokine receptor expression (absolute or migration mediated loss, Table 3) after prior brisk walking. Previous studies have reported elevated chemokine concentrations in the circulation after exercise (12, 17, 39). It has been suggested that these changes may drive internalisation of chemokine receptors that lower their surface expression over time (24), in turn reducing migration. Despite a recent study reported lower expression of CCR2 on IM immediately after (but not 1h and 2h after) a bout of cycling exercise (6), our data importantly highlight that any acute loss of monocyte chemokine receptor expression was not maintained the morning afterwards.

The current results support an association between higher levels of physical activity and reduced migration of specific pro-inflammatory monocytes, as well as important markers of metabolic health (TC, TAG, glucose and NEFA, Table 1). Epidemiological studies report that being more physically active is associated with reduced blood markers of inflammation (26, 27); however it is unclear whether this is directly related to changes in adiposity (16, 24). Our data provide further support for the notion that the movement of specific populations of monocytes (i.e. IM) may be reduced independently of adiposity (2, 13). Given that our data reveals no short-term impact of walking exercise on monocyte migration, future studies should examine the impact of regular PA on immune cell migration, in the context of energy balance and weight loss.

We must acknowledge some limitations to the current study. Our *ex vivo* method when coupled to flow cytometry permits much more detailed phenotypic analysis of monocyte sub-populations; however, analysis of rarer cell populations was challenging. For example, we did not present data on chemokine receptor positive cells for IM and NCM due to low event counts during acquisition (< 500). This was a limitation of the number of monocytes that tethered and migrated, which was lower in LE vs. CO. Repeated blood measures after the bout of walking exercise collected before 16 hours post-exercise may have been beneficial, but given assay logistics and ethical considerations of blood volume, this was not possible. Finally, we must acknowledge that the walking intervention was only carried out in CO, and therefore a direct comparison with LE was not made; however, this would not have influenced the interpretations of the present study.

CONCLUSION

The current data adds to the growing body of evidence highlighting that central adiposity is a major driver of monocyte migration in individuals with CO. By exploring both absolute and relative changes, we established that the concentration of monocytes in the circulation of middle-aged males with CO may drive their heightened tethering and migration towards an *ex vivo* chemokine stimulus, compared to lean individuals. Cellular level changes likely also contribute (e.g. adhesion); however, we highlight that this was not chemokine receptor-mediated in individuals with a relatively low level of systemic inflammation. Regular physical activity did not impact the capacity of classical monocytes to tether and migrate; however, reduced tethering and migration of specific pro-inflammatory subsets was associated with MVPA and step count. Further research is needed to establish the significance of these changes by monitoring individuals who are lean and centrally obese over time and under controlled lifestyle interventions.

FUNDING

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

AUTHOR CONTRIBUTION STATEMENT

AJW, MR, AET, DJS, and NCB were involved in the conception and design of the study. AW and NCB developed the laboratory methods. MR undertook recruitment and participant testing. AJW, JC and MR carried out all data acquisition. MR, AJW, and NCB carried out statistical analysis and data presentation. Drafting of the article for important intellectual content was undertaken by AJW and MR and all authors undertook revision and final approval of the manuscript.

Conflict of Interest

None of the authors declare a conflict of interest.

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Improvement in the anti-inflammatory profile with lifelong physical exercise is related to clock genes expression in effector-memory CD4+ T cells in master athletes.

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ABSTRACT

Purpose: *Ageing is associated with alterations in the immune system as well as with alterations of the circadian rhythm. Immune cells show rhythmicity in execution of their tasks. Chronic inflammation (inflammaging), which is observed in the elderly, is mitigated by lifelong exercise. The aimed this study was to determine the acute effect of a maximal exercise test on clock genes, regulatory proteins and cytokine expression, and evaluate the effect of lifelong exercise on the expression of clock genes in subpopulations of effector-memory (EM) CD4+ and CD8+T cells and the association of these processes with the inflammatory profile. Therefore, this study aimed to investigate the expression of clock genes in subpopulations of effector memory (EM) CD4+ and CD8+ T cells in master athletes and healthy controls and further associate them with systemic inflammatory responses to acute exercise.*

Methods: *The study population comprised national and international master athletes (n = 18) involved in three sports (athletics, swimming and judo). The control group (n = 8) comprised untrained healthy volunteers who had not participated in any regular and competitive physical exercise in the past 20 years. Anthropometric measurements and blood samples were taken before (Pre), 10 min after (Post) and 1 h after (1 h Post) a maximal cycle ergometer test for the determination of maximum oxygen consumption ($VO_{2\,max}$). The subpopulations of EM CD4+ and CD8+ T cells were purified using fluorescence-activated cell sorting. RNA extraction of clock genes (CLOCK, BMAL1, PER1, PER2, CRY1, CRY2, REV-ERBα, REV-ERBβ, RORa, RORb and RORc) in EM CD4+ and EM CD8+ T cells as well as regulatory proteins (IL-4, IFN-γ, Tbx21, PD-1, Ki67, NF-kB, p53 and p21) in EM CD4+ T cells was performed. The serum concentration of cytokines (IL-8, IL-10, IL-12p70 and IL-17A) was measured.*

Results: *The master athletes showed better physiological parameters than the untrained healthy controls (P<0.05). The levels of cytokines increased in master athletes at Post compared with those at Pre. The IL-8 level was higher at 1 h Post, whereas the IL-10 and IL-12p70 levels returned to baseline. There was no change in IL-17A levels (P< 0.05). The clock genes were modulated differently in CD4+ T cells after an acute session of exercise in a training status-dependent manner.*

Conclusion: *The synchronization of clock genes, immune function and ageing presents new dimensions with interesting challenges. Lifelong athletes showed modified expression patterns of clock genes and cytokine production associated with the physical fitness level. Moreover, the acute bout of exercise altered the clock machinery mainly in CD4+ T cells; however, the clock gene expressions induced by acute exercise were different between the master athletes and control group.*

Keywords:
Circadian Rhythm, Cytokines, Ageing, Lymphocytes and Exercise Immunology

INTRODUCTION

The decline in numerous physiological processes due to ageing, widely reported in previous studies, results in increased vulnerability to infectious and inflammatory diseases(1). Ageing also induces perturbations of the circadian rhythm (2), including disruption of the sleep/wake cycle, body temperature, food intake and metabolism, secretion of hormones, glucose homeostasis, cell cycle regulation and immune response(3). The circadian rhythm is controlled by a central (suprachiasmatic nucleus) and peripheral clocks (organs and tissues)(3). At the cellular level, the circadian rhythm is controlled by a set of genes known as clock genes, which are transcriptional regulators that maintain rhythmic expression of their target genes over approximately 24-h cycles(4).

As a master regulator, CLOCK (circadian locomotor output cycles kaput) is a transcription factor that forms heterodimers with BMAL1 (brain and muscle Arnt-like protein), forming a transcriptional activator complex that stimulates the transcription of the Period (PER1, PER2 and PER3) and Cryptochrome (CRY1 and CRY2) genes (negative feedback) in various tissues, cells and subsets of immune cells. In turn, the cytoplasmic PERs and CRYs proteins migrate to the nucleus after dimerization to inhibit the transcription of CLOCK-BMAL1. The regulation of the circadian cycle occurs with the degradation of PER/CRY complexes in the proteasome, which

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withdraws the inhibition of CLOCK and BMAL1, as well as the restart of this feedback loop (5–7). Another regulatory mechanism is induced by the CLOCK/BMAL1 heterodimeric complex that activates the transcription of orphan nuclear receptors (REV-ERB and ROR) (8–10). REV-ERB (α and β) and ROR (a, b and c) subsequently compete for ROREs (retinoic acid-related orphan receptor response elements), present in the BMAL1 promoter. ROR activates BMAL1 transcription (10–12), whereas REV-ERB suppresses it (8,12). Therefore, BMAL1 circadian oscillations are both positively and negatively regulated by RORs and REV-ERBs.

Organisms present either a constant circadian rhythm or an endogenous rhythm. The environment operates only as a synchronizing agent (Zeitgeber)(13). Some environmental conditions adjust the oscillators that generate circadian rhythm, resulting in a continuous cycle. These external factors also serve as indicators of time and they are thus referred to as Zeitgeber (synchronizers). Daily light which restarts the central clock is the most important Zeitgeber. Therefore, the central clock in collaboration with other peripheral clocks, ensures the maintenance of the circadian cycle and can be modified by external factors such as light, social activities, diet (14,15) and physical exercise (16–18).

The rhythmic expression of clock genes has been previously described in innate and adaptive immune cells, including macrophages (19–21), microglia (22), monocytes (23), mast cells (24,25), dendritic cells (DCs) (21), B cells (21), natural killer cells (26), neutrophils (27), eosinophils (24) and CD4+ and CD8+ T cells (28,29). Furthermore, the disruption of clock gene signalling induces chronic inflammation or increases the severity of symptoms in the elderly (30).

Apart from circadian rhythm, clock genes are implicated in inflammatory response through activation of NF-κB signalling in response to various immunomodulators that are regulated by CLOCK, which is capable of regulating NF-κB-mediated transcription in the absence of BMAL1. Moreover, BMAL1 counteracts the CLOCK-mediated activation of NF-κB responsive genes. To substantiate this, Spengler et al. demonstrated the anti-inflammatory role of BMAL-1, and the activation of pro-inflammatory cytokines by CLOCK (31).

Interestingly, Nobis et al. (2019) demonstrated that clock genes in CD8+T cells modulate the response to vaccination by programming the transcriptional activities of these cells, making them more prone to efficient activation and proliferation depending on the time of day. However, deletion of BMAL1 in DCs or CD8 lymphocytes abrogated the circadian rhythm response to vaccination (32).

It is established that the ability to generate and maintain memory T cells is critical for lifelong immune health. Upon antigen encounter (after infection or vaccination), the number of antigen-specific T cells increase by several magnitudes and they differentiate into effector cells. While most of these effector cells die after the peak of their response, a subset of the subpopulation survives as memory cells that are long-lived and thereby providing protection against reinfection (33). However, the generation and maintenance of these memory T cells are compromised with ageing (33,34).

Chronic exercise may alter the profile and expression of the circadian rhythm clock genes (17). Programmed exercise has been demonstrated to alter behavioural rhythms in mammals kept in constant darkness. Previous studies highlighted that voluntary exercise influences how quick an animal synchronises a new phase of a light/dark cycle (35,36).

Physical inactivity, sedentary lifestyle and poor nutrition induce a disruption of the circadian rhythm, promoting an increase in the levels of pro-inflammatory factors and leading to chronic low-grade inflammation (37,38). Physical exercise is known to decrease TNF-α expression levels, reduce pro-inflammatory adipokine levels, and lower the expression of Toll-like receptors on monocytes and macrophages, and increase the circulating levels of anti-inflammatory cytokines in healthy populations (38–41).

Evidence has shown that the practice of lifelong regular exercise may decelerate the ageing process (42–48). Cellular senescence is a physiological process that occurs throughout life and is characterized by shortening of telomeres, damage to nuclear and mitochondrial DNA and, on immune cells, it induces the secretion of pro-inflammatory cytokines (49).

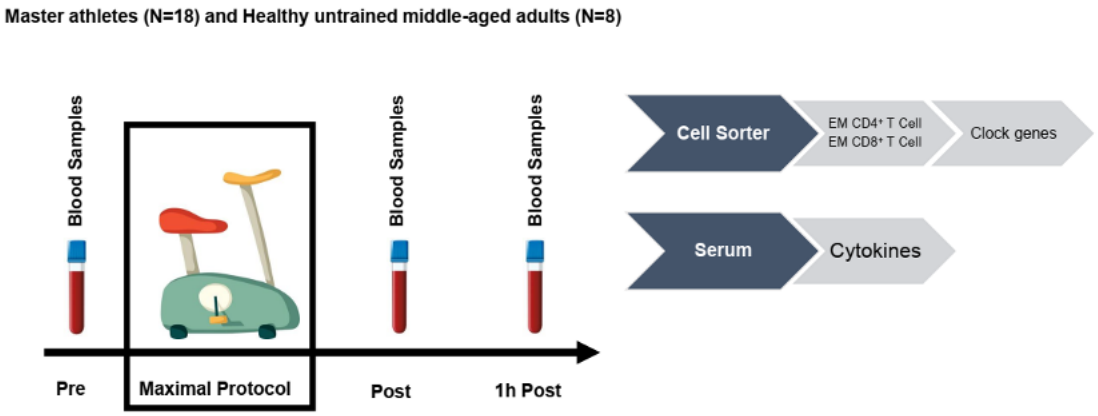
In terms of the immune system, lifelong athletes with consistent and regular years (≥20 years) of exercise practice (42,43,50,51) show reduced senescent T cell numbers and increased plasma levels of anti-inflammatory cytokines, with IL-10 levels similar to those of young adults (42–44).

A recent study demonstrated that sleep deprivation induces a robust modification in the transcriptome of cytokine and interleukin pathways as well as in clock genes (52), thereby instigating the need to clarify the inter-relationship between immunosenescence (declining of the immune system function observed during ageing) exercise and clock genes. Therefore, we hypothesised that lifelong training can be useful as a low cost intervention to improve quality of life and promote healthy ageing by reducing the number of senescent cells through regulation of the circadian rhythm. This study aimed to determine the acute effect of a maximal exercise test on clock genes, regulatory proteins and cytokine expression and evaluate the effect of lifelong exercise on the expression of clock genes in subpopulations of effector-memory (EM) CD4+ and CD8+ T cells and the association of these processes with the inflammatory profile.

METHODS

Participants

Eighteen master athletes (MA) (53.56±9.25 years) and a control group (CG) of eight untrained middle-aged adults (52.88±5.64 years) consented to participate in this study. All participants were healthy, non-smokers without any form of illness, as determined by a health and medical questionnaire. The inclusion and exclusion criteria for both groups were as previously described (42). Briefly, MA are athletes who currently participate in competitive sports and have done so for more than >20 years. We did not include athletes who trained or competed sporadically and/or older competitors who resumed training after a long period of physical inactivity. The MA group comprised athletes currently participating in national and international competitions in three sports (athletics, swimming and judo). Participants in the CG had not participated in regular physical training for the past 20 years, as determined by a questionnaire. All participants provided their written, informed consent to participate in the study, which was approved by the Ethics and Human Subjects Review Board at the Faculty of Sports Science and Physical Education, University of Coimbra.



Study Flowchart

Anthropometric measures

The stature and body mass of each participant was determined using a Harpenden stadiometer model 98,603 (Holtain Limited) and a calibrated digital scale Seca model 770 (Seca, Birmingham, UK), respectively. Measurements of weight and height were used to calculate the body mass index.

Determination of VO_{2max}

The physiological response to an incremental exercise test was determined by a maximal progressive exercise on a cycle ergometer (Lode, Groningen, Netherlands). In brief, participants began cycling with a 75 W load for 3 min (warm-up stage) followed by increments of 25 W every 3 min until volitional exhaustion. The participants cycled at a constant rate of 80 - 85 rpm.

Breath-by-breath measurements of O_2 and CO_2 were continuously recorded throughout cycling using a gas analyser with a breath by breath recording system (Quark CPET, COSMED, Rome, Italy). Participants breathed continuously through a facemask. The O_2 and CO_2 analysers were calibrated with known gases following the manufacturer's guidelines (COSMED, Rome, Italy). The adopted criterion to define if VO_{2max} was attainted was previously describe (53). Heart rate was monitored throughout the cycling by short-wave telemetry (COSMED, Rome, Italy).

Collection of blood samples

Venous blood samples (16 ml) were obtained through venepuncture in the antecubital vein and collected before exercise (Pre), 10 min after exercise (Post) and 1h after exercise (1h Post). The blood samples were collected in tubes containing EDTA and in a serum collection tube. EDTA tubes were used for flow cytometry and cell separation (cell sorting). The blood samples were centrifuged at 2000 rpm (4°C) for 10 min. Serum and plasma after the centrifugation were stored at −80°C until use.

Isolation of purified CD4+ and CD8+ T-cells

Highly purified CD4+ and CD8+ T cells were isolated by fluorescence-activated cell sorting using a FACSAria II cell sorter (BD). A five-colour combination of fluorochrome-conjugated monoclonal antibodies was used:CD3-PB (Pacific blue, clone UCHT1, Pharmingen, San Diego, CA EUA); CD4-APC (allophicocyanin, Clone 13B8.2, Beckman Coulter, Miami, FL, EUA); CD8-KO (Krome Orange, Clone 5MZ.332, Beckman Coulter, Miami, FL, USA); CD27-PECy5 (phycoerythrin-cyanine 5, clone R.8.01, Beckman Coulter) and CD45RA-PECy7 (phycoerythrin-cyanine 7, clone L48, BD Bioscience). This labelling enabled the identification EM CD4+ T cells (CD3+CD4+ CD27-CD45RA) and EM CD8+ T cells (CD3+CD8+CD27-CD45RA-). Furthermore, mRNA was extracted from EM CD4+ and EM CD8+ T cells.

RNA extraction of purified cells

The purified cells were transferred into a 1.5mL Eppendorf tube and centrifuged at 300g for 5 min; the pellet was resuspended in 350µL of RLT lysis buffer (Qiagen, Hilden, Germany).

The RNA was reverse transcribed into complementary DNA (cDNA) using reverse transcriptase. The iScriptTM Reverse Transcription Supermix for RT-qPCR kit (BIO-RAD) was used for reverse transcription of total mRNA.

The RT-qPCR SYBR® green method was used for cDNA synthesis. In brief, this method uses a fluorochrome SYBR® green which binds to the amplification product (that is, between the double strands of cDNA) and emits fluorescence that is proportional to the amplification product. This enables the monitoring of the kinetics and efficiency of the amplification process. Gene expression was determined using real-time PCR (54) using LightCycler® 480 apparatus (Roche Diagnostics, Rotkreuz, Switzerland) and SYBER green as a fluorescent marker. Quantification of gene expression was performed using the comparative Ct method (Ct = threshold cycle; number of cycles in which the PCR product reaches a detection threshold). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as the internal standard (55). Table 1 shows the sequences of the genes analysed.

Table 1. List of primers for the clock genes

Genes	Forward sequence	Reverse sequence
CLOCK	AGACCCTTCCTCAACACCAA	TGATGACCTTCTTTGCACCA
BMAL1-ARNTL	GCAGCTCCACTGACTACCAAG	TGTGAGCTTCCCTTGCATTT
PER1	TCTGCCGTATCAGAGGAGGT	CCCGGATCTTGGTCACATA
PER2	AGCTGCTTGGACAGCGTCATCA	CCTTCCGCTTATCACTGGACCT
CRY1	TGTTGAAGCAAGGAAGAAGC	TGCTCTGTCTCTGGACTTTAGG
CRY2	GGGGACTACATCAGGCGATAC	ATGCACTTGGCTGCCTTCT
REV-ERBα	CCCCAATGACAACAACACCT	CATAGGACATGCCAGCAGAAC
REV-ERBβ	ATGTCAGCAATGTCGCTTCA	CACGCTTAGGAATACGACCAA
RORa	AGGCTGCAAGGGCTTTTT	GCAGCGGTTTCTACTGGTTC
RORb	ATCAAAGCAAGTCCAGGGAAG	TGCAAACTCCACCACGTATT
RORc	GTCCCGAGATGCTGTCAAGT	TGGTTCCTGTTGCTGCTGTT
P21	TCCTCTTGGAGAAGATCAGCCG	TCCTCTTGGAGAAGATCAGCCG
IL-2	AGAACTCAAACCTCTGGAGGAAG	GCTGTCTCATCAGCATATTCACAC
IL-4	CCGTAACAGACATCTTTGCTGCC	GAGTGTCCTTCTCATGGTGGCT
P53	CCTCAGCATCTTATCCGAGTGG	TGGATGGTGGTACAGTCAGAGC
IFN-gamma	GAGTGTGGAGACCATCAAGGAAG	TGCTTTGCGTTGGACATTCAAGTC
TBX21	ATTGCCGTGACTGCCTACCAGA	GGAATTGACAGTTGGGTCCAGG
PD1	AAGGCGCAGATCAAAGAGAGCC	CAACCACCAGGGTTTGGAACTG
NF-kB p65	TGAACCGAAACTCTGGCAGCTG	CATCAGCTTGCGAAAAGGAGCC
Ki67 (MKI67)	GAAAGAGTGGCAACCTGCCTTC	GCACCAAGTTTTACTACATCTGCC
GAPDH	AGAAGGCTGGGGCTCATTT	GGTTCACACCCATGACGAAC

Serum cytokine levels

Quantification of the levels of cytokines including IL-10, IL-12p70, IL-17A and IL18 were performed using LEGENDplexTM multiplex kit (Human Inflammation Panel 1 Mix and Match Subpanel LEGENDplexTM - 5-plex; Biolegend) according to the manufacturer's instructions. The assays were performed in duplicates. Analysis was performed using BD FACSCalibur ™ flow cytometer. Data obtained were analysed using Legendplex V8.0 software (Biolegend) and the results were expressed as pg/mL.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.4.2 for windows (GraphPad Software, San Diego, California USA). The significance level was set at $P<0.05$. Descriptive statistics were presented as mean±standard deviation. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test. Correlations between parameters and between changes in variables were analysed using Spearman's rank correlation. To estimate the strength of the exercise, Cohen's effect size was calculated and categorised by convention as small (d=0.2), medium (d=0.5) and large effect (d=0.8) using G*Power version 3.1.9.2.

RESULTS

The anthropometric data and physiological parameters are summarised in Table 2. As expected, MA showed better physiological parameters in response to maximal exercise than CG, as well as a higher value for the time test (min; P=0.005), maximum power produced by the test (watts; P=0.005), maximum ventilation (VE, ml/min; P=0.03), maximum oxygen consumption (VO_2max, ml/min and ml/min/Kg; p=0.003 and P=0.021, respectively) and carbon dioxide production (VCO_2 max, ml/min; P=0.006) (Table 2).

Table 2. Participants characteristics at baseline.

	Master Athletes (n = 18)	Control group (n = 8)	*p* value	Effect size (r)
	Mean (SD)	Mean (SD)		
Age (years)	53.56 (9.25)	52.88 (5.64)	0.849	0.08
Weight (kg)	75.45 (15.90)	72.03 (13.65)	0.602	0.22
Height (cm)	171.4 (5.31)	169.3 (0.07)	0.438	0.47
Body Mass Index (kg/m2)	25.62 (4.78)	24.99 (3.53)	0.743	0.14
Time of test	17.06 (5.38)	11.04 (2.08)	**0.005**	**1.29**
Power (watts)	209.7 (44.67)	156.3 (29.12)	**0.005**	**1.31**
VE (ml/min)	111.9 (28.90)	85.13 (23.10)	**0.030**	**0.98**
VO_{2max} (ml/min)	2997 (638.20)	2198 (362.4)	**0.003**	**1.40**
VO_{2max} (ml/min/Kg)	40.89 (10.79)	30.98 (5.14)	**0.021**	**1.04**
VCO_{2max} (ml/min)	3335 (632.2)	2614 (337.0)	**0.006**	**1.28**
VE/VO_{2max}	37.55 (6.19)	39.36 (6.85)	0.595	0.28
VE/VCO_{2max}	33.51 (5.25)	32.48 (6.85)	0.679	0.18
FC_{max}	160.4 (15.53)	160.1 (15.84)	0.968	0.02

Note: Unpaired t test. Data are Mean (SD). Values of significance ($p < 0.05$) are highlighted in bold. Large effect size ($r > 0.8$) are highlighted in bold.

The proportion of total lymphocytes, T cells, CD4+ and CD8+ T cells and EM CD4+ and CD8+ T cells in MA and CG in response to a maximum effort protocol is summarised in Figure 1. At baseline, there were no significant differences between the total lymphocyte population and subsets (T cells, CD4+ and CD8+ T cells and EM CD4+ and CD8+ T cells) in MA and in untrained individuals. The percentage of lymphocytes increased at Post only in MA, indicating that lymphocytosis is dependent of intensity and duration of exercise as opposed to the CG, whose exercise test duration was much lower. Subsets of EM CD4+ and EM CD8+ T cells were also mobilised, with a higher response in MA, as substantiated by a high percentage of EM CD4+ and EM CD8+ T cells at Post (P=0.000 and P=0.007, respectively), whereas only EM CD4+T cells in the CG were increased at Post(P =0.023) (Figure 1; Suppl. Table 1).

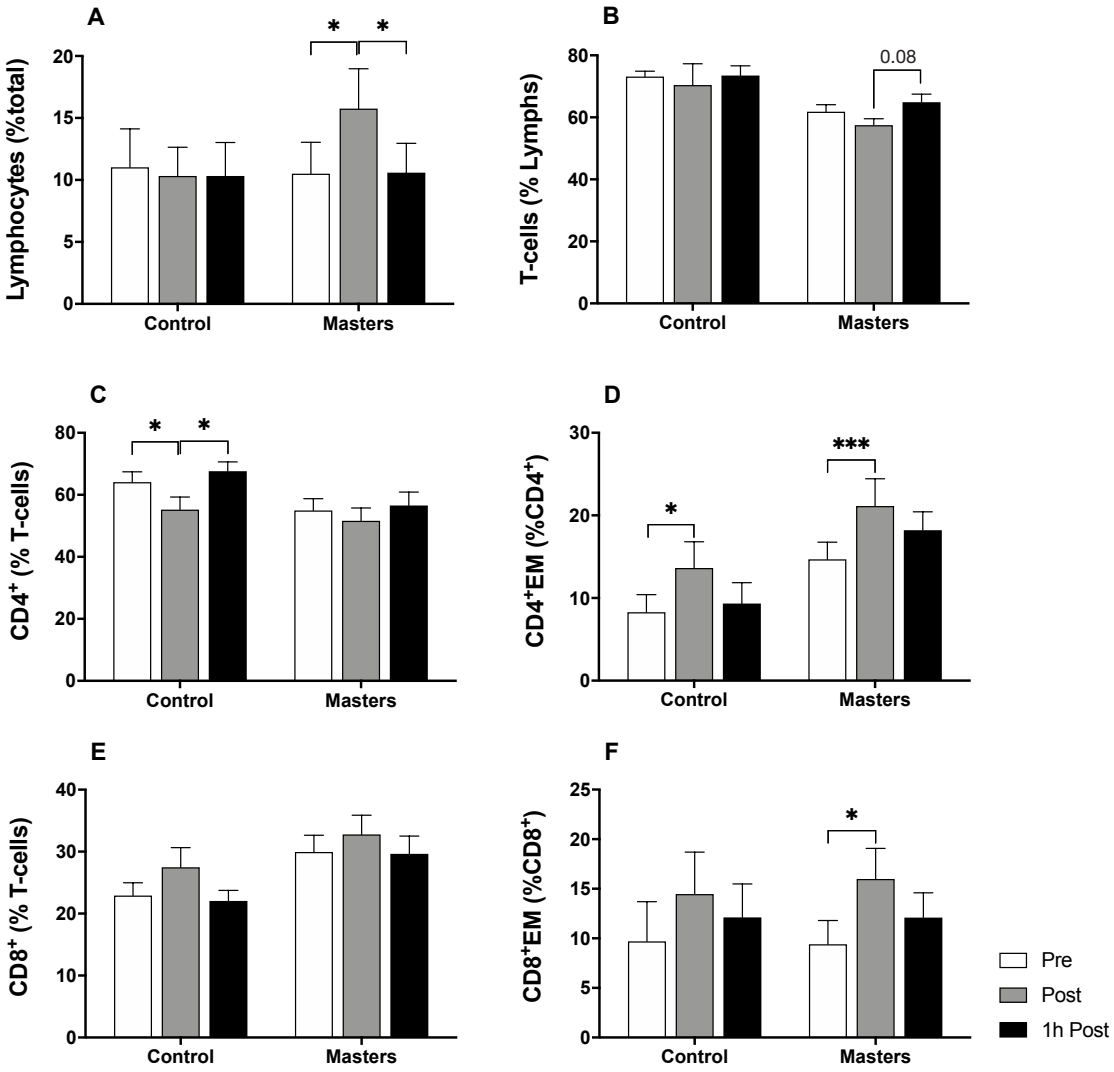


Figure 1. Proportion of total lymphocytes, T-cells, CD4+ and CD8+ T-cells, and effector-memory subpopulations in master athletes and control groups in response to a maximum effort protocol. Data represent the mean ± standard error of the mean. * $p < 0.05$; *** $p < 0.001$. Proportion of total lymphocytes (% total cells) (A), T-cells (% total lymphocytes) (B), CD4+ T-cells (% of T-cells) (C), effector-memory CD4+ T-cells (% of CD4+ T-cells) (D), CD8+ T-cells (% of T-cells) (E), effector-memory CD8+ T-cells (% of CD8+ T-cells) (F). Concentrations before (white columns = Pre), 10 min after (grey columns = Post), and 1h after (black columns = 1h Post) the exercise protocol in Control Group and Master Athletes group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

There was no effect of lifelong training on the level of pro-inflammatory cytokines (IL-12p70 and IL-17A), anti-inflammatory cytokine (IL-10) and regulatory cytokine (IL-8) at Pre (Figure 2; Suppl. Table 2). However, the levels of these cytokines were increased in MA at Post compared with that at Pre. The IL-8 level remained high at 1h Post, whereas the IL-10 and IL-12p70 levels returned to their baseline value. The IL-17A levels showed no statistical differences at 1h post compared with that at baseline and Post ($P<0.05$, Figure 2; Suppl. Table 2).

We observed a more pronounced modulation of clock genes in EM CD4+ T cells. The expression of CLOCK and BMAL1 in EM CD4+T cells was significantly increased in the CG at 1h Post compared with those at Pre and Post (Figure 3A and B; Suppl. Table 3). In the MA group, there was a significant increase in the expression of REV-ERBα and CRY1 at 1h Post compared with those at Pre and Post ($P<0.05$, Figure 3E and G; Suppl. Table 3). In EM CD8+ T cells, the expression of REV-zERBβ decreased after exercise (Figure 4H; Suppl. Table 4), with no statistical significant differences noted for other clock genes in terms of groups and moments (Figure 4; Suppl. Table 4).

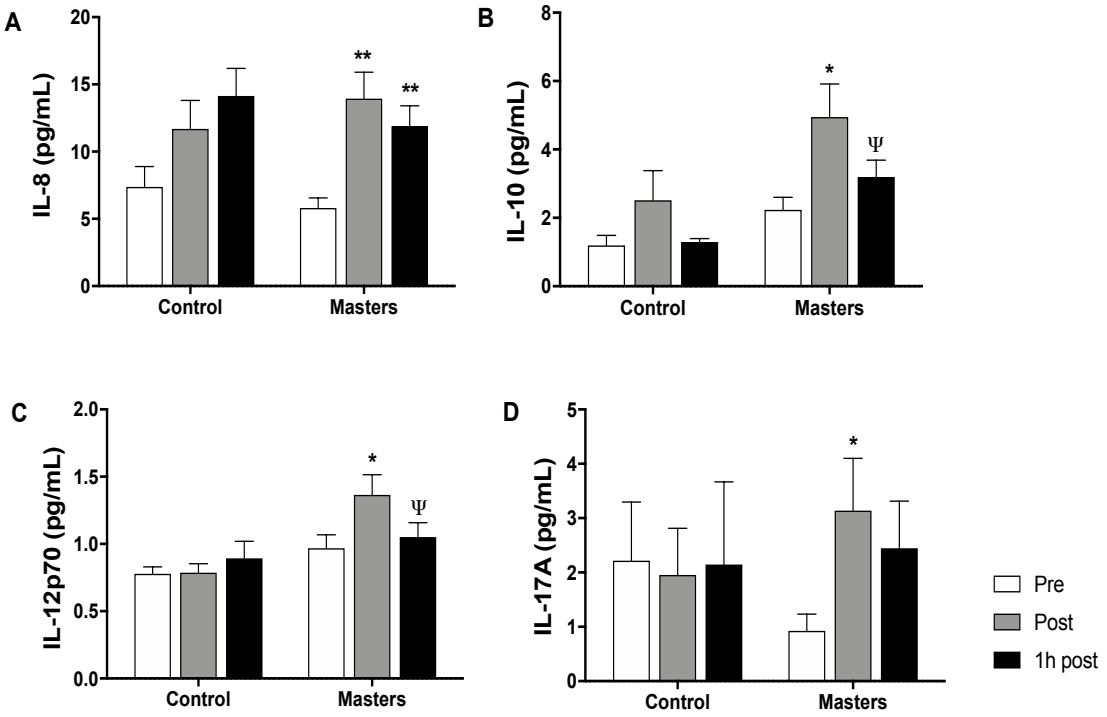


Figure 2. Differences in Cytokine levels in master athletes and control groups in response to a maximum effort protocol. Data are presented as mean with standard error of mean (SEM) bars. *$p < 0.05$ and **$p < 0.01$ compared to Pre; Ψ $p < 0.05$ compared to Post. (A) Interleukin 8; (B) Interleukin 10, (C) Interleukin 12p70; (D) Interleukin 17A. Concentrations before (white columns = Pre), 10 min after (grey columns = Post), and 1h after (black columns = 1h Post) the exercise protocol in Master Athletes group and Control Group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

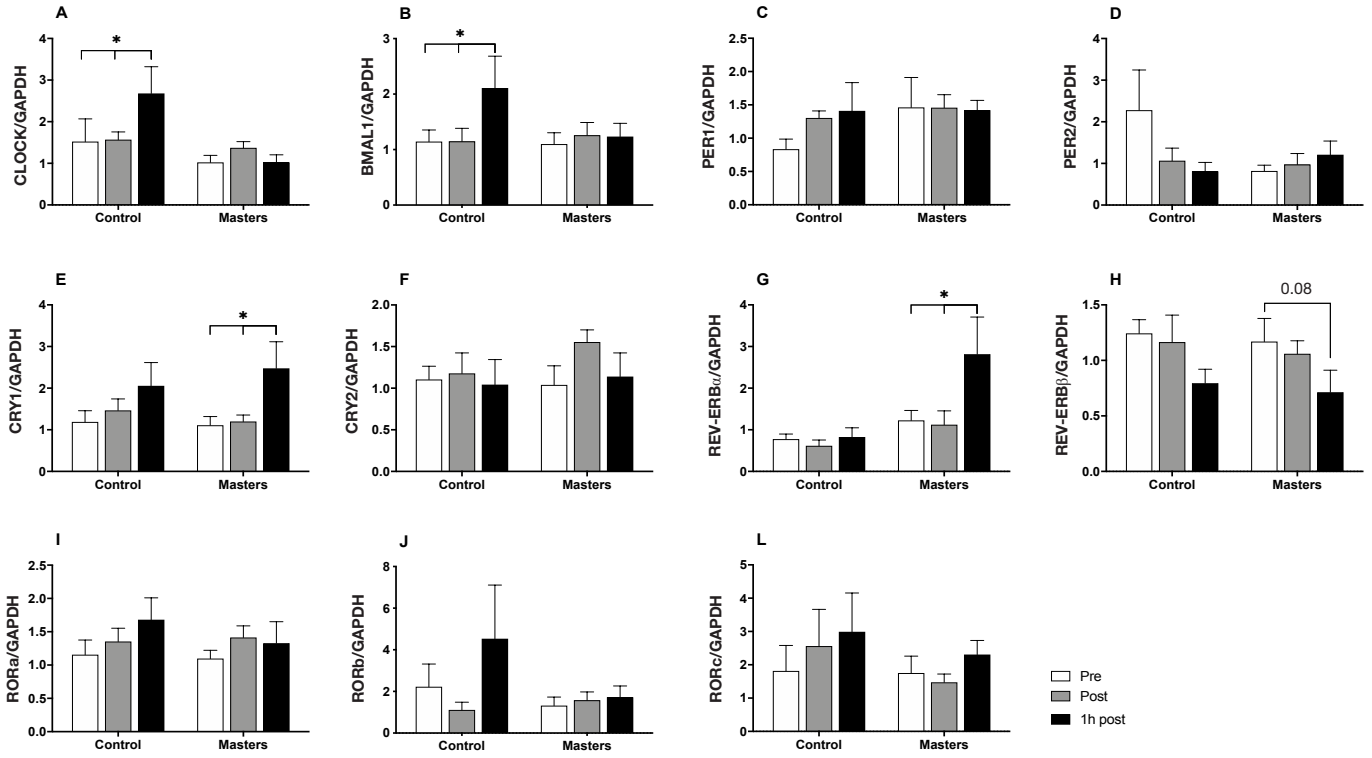


Figure 3. Differences in gene expression of clock genes in purified effector-memory CD4⁺ T-cells in master athletes and control groups in response to a maximum effort protocol. Data are presented as mean (SEM) bars. *p < 0.05 and **p < 0.01 compared to Pre; Ψ p < 0.05 compared to Post. Concentrations before (white columns = baseline), 10 min after (grey columns), and 1h after (black columns) the exercise protocol in Master Athletes group and Control Group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

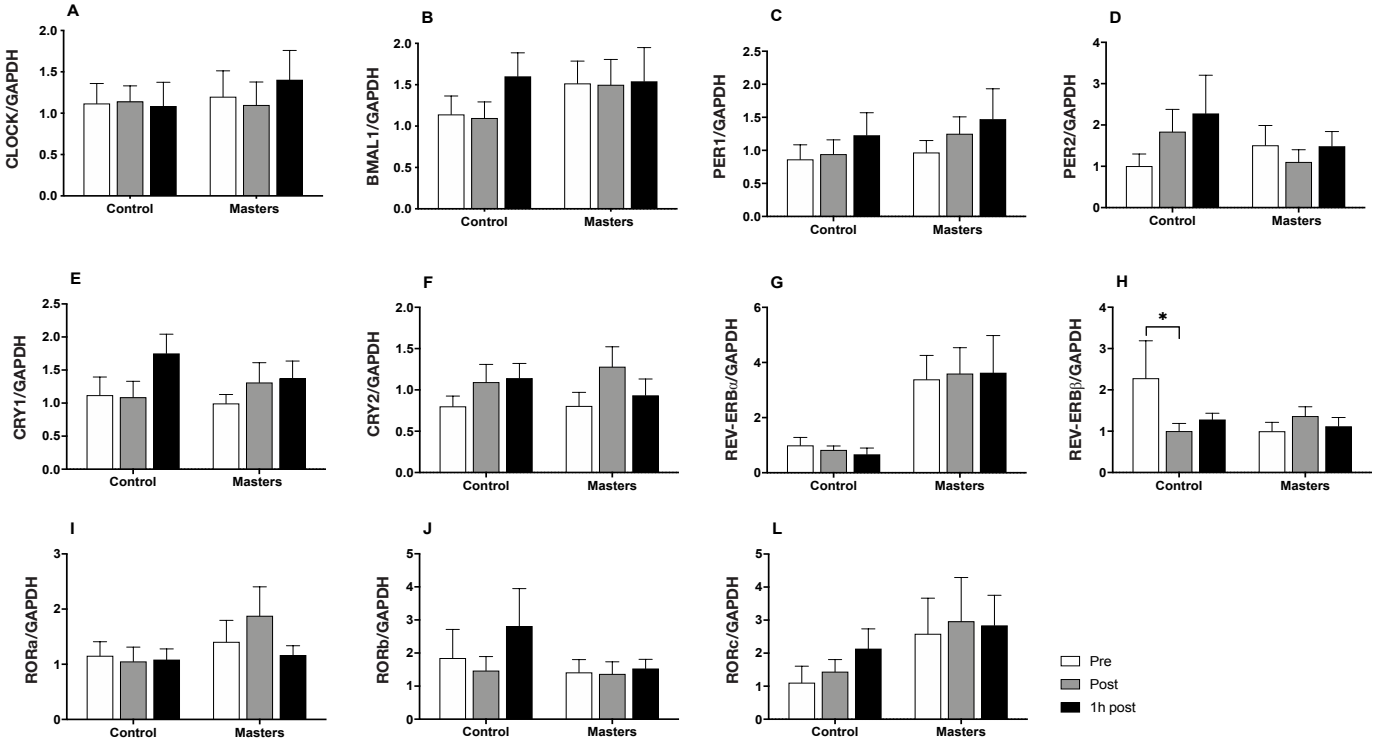


Figure 4. Differences in gene expression of clock genes in purified effector-memory CD8⁺ T-cells for master athletes and control groups in response to a maximum effort protocol. Data are presented as mean with standard error of mean (SEM) bars. *p < 0.05 and **p < 0.01 compared to Pre; Ψ p < 0.05 compared to Post. Concentrations before (white columns = baseline), 10 min after (grey columns), and 1h after (black columns) the exercise protocol in Master Athletes group and Control Group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

The expression of regulatory genes including IL-4, IFN-γ, TBX21, PD-1, Ki67, NF-kB, p53 and p21, were also analysed in the purified EM CD4+ T cells (P<0.05, Figure 5; Suppl. Table 5). In MA, only TBX21 expression increased at Post compared with that at Pre (P<0.05, Figure 5C; Suppl. Table 5).

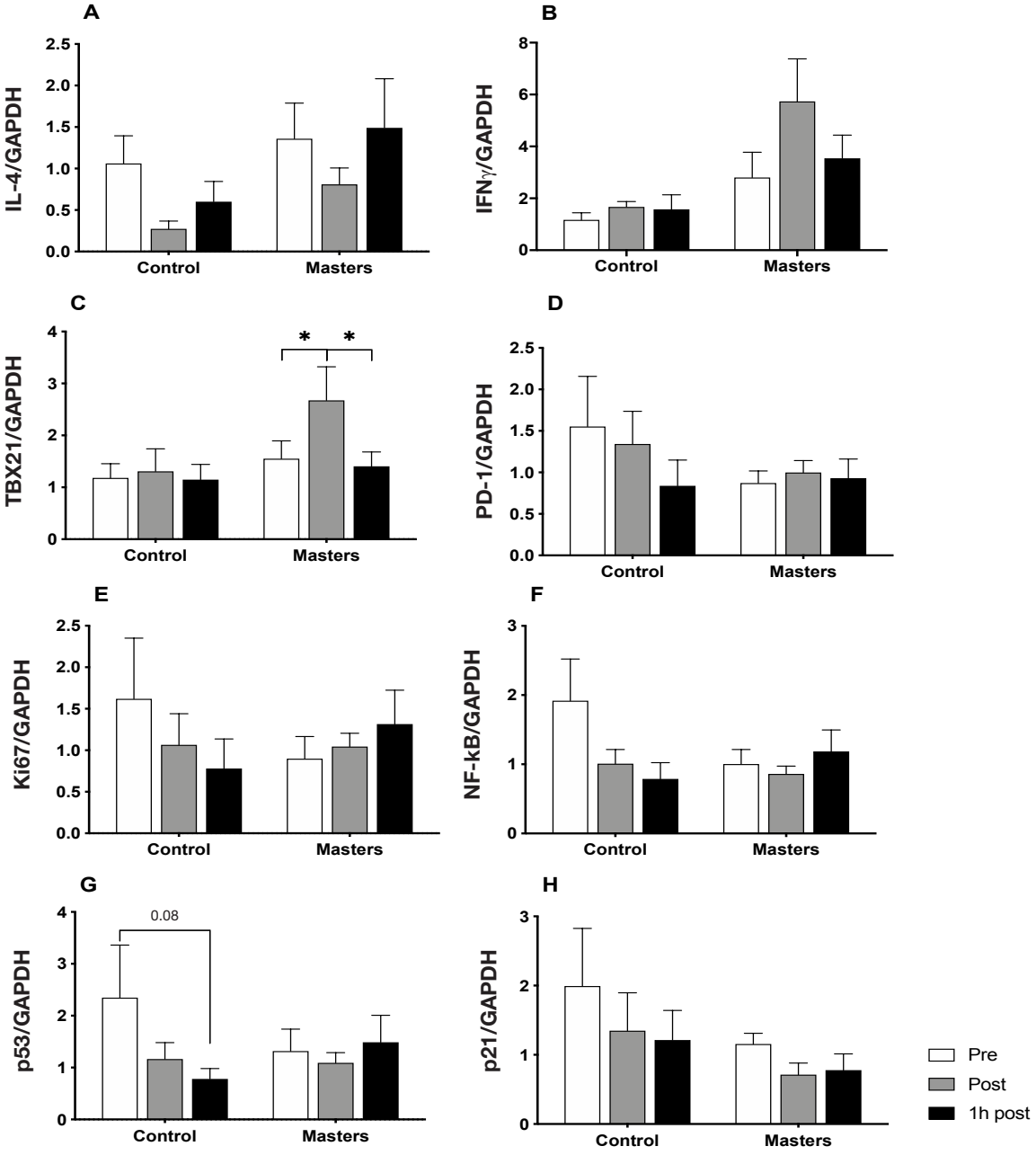


Figure 5. Differences in gene expression of regulatory proteins in purified effector-memory CD4$^+$ T-cells in master athletes and control groups in response to a maximum effort protocol. Data are presented as mean with standard error of mean (SEM) bars. *p < 0.05 and **p < 0.01 compared to Pre; Ψ p < 0.05 compared to Post. Concentrations before (white columns = baseline), 10 min after (grey columns), and 1h after (black columns) the exercise protocol in Master Athletes group and Control Group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

There was an inverse correlation between IL-8 and $VO_{2\ max}$ in MA after acute exercise (Post: r=−0.675, P=0.013; 1h Post: r=−0.774, P=0.002; Table 3). The IL-17 level was inversely correlated with test time, power and $VO_{2\ max}$ in MA at 1h Post (Pre: r=−0.701, P=0.013; Post: r=−0.762, P=0.005; 1h Post: r=−0.640, P=0.029; Table 3). There were no correlations between any cytokines and physiological parameters in the CG.

Table 3. Correlations between the cytokines and physiological parameters in master athletes and control group.

Cytokines	Physiological parameters	Master Athletes PRE		Master Athletes POST		Master Athletes 1h POST		Control group PRE		Control group POST		Control group 1h POST	
		r value	p value	r value	p value	r value	p value	r value	p value	r value	p value	r value	p value
Interleukin 8	Time of test (min)	0.168	0.598	–0.266	0.375	–484	0.095	0.485	0.355	–0.036	0.934	0.800	0.333
	Power (Watts)	0.073	0.827	–0.255	0.399	–0.511	0.077	0.470	0.344	0.079	0.876	0.632	0.500
	VO_{2max}	–0.195	0.543	–0.675	**0.013**	–0.774	**0.002**	0.371	0.497	–0.234	0.619	0.200	0.916
Interleukin 10	Time of test (min)	–0.145	0.632	–0.037	0.900	0.104	0.732	–0.436	0.327	–0.720	0.077	0.500	0.666
	Power (Watts)	–0.113	0.711	0.113	0.699	0.138	0.649	–0.519	0.233	–0.709	0.085	0.272	>0.999
	VO_{2max}	–0.329	0.271	–0.204	0.482	–0.362	0.224	–0.306	0.500	–4643	0.302	–0.632	0.500
Interleukin 12p70	Time of test (min)	0.000	>0.999	0.018	0.949	–0.060	0.837	0.449	0.333	0.102	0.450	0.458	0.450
	Power (Watts)	–0.207	0.491	0.042	0.885	–0.047	0.871	0.490	0.285	0.2874	0.600	0.750	0.300
	VO_{2max}	–0.206	0.494	0.035	0.906	–0.013	0.966	0.356	0.476	0.270	0.666	0.335	0.600
Interleukin 17	Time of test (min)	–0.440	0.151	–0.563	0.059	–0.701	**0.013**	–0.467	0.286	–1.911	0.672	–0.500	0.666
	Power (Watts)	–0.672	**0.017**	–0.576	0.053	–0.762	**0.005**	–0.563	0.209	–0.222	0.766	–0.544	0.750
	VO_{2max}	–0.626	0.033	–0.546	0.069	–0.640	**0.029**	0.259	0.590	0.753	0.105	0.632	0.500

Note: Correlations were analysed using Spearman's rank correlation.

There was a strong positive correlation between REV-ERBα expression and VO_{2max} in EMCD4+ T cells in MA after the acute exercise (Post: r=0.927, P=0.000; Table 4). In addition, REV-ERBα expression was positively correlated with test time and power in MA (Post: r=0.632, P=0.049; r=0.723, P=0.022, respectively; Table 5). Interestingly, after exercise, REV-ERBα expression in EM CD8+ T cells was negatively correlated with test time in MA and CG(Post: r=−0.701, P=0.028; r=−1.000, P=0.016; Table 3).Furthermore, the clock genes (PER1, CRY1 and CRY2)in EM CD4+ T cells at 1h Post and RORc in EM CD8+ T cells at Post were positively correlated with power (1h Post: r=0.617, P=0.035; r=0.744,

Table 4. Correlations between the Clock genes in purified effector-memory $CD4^+$ T-cells and physiological parameters in master athletes and control group.

Clock genes	Physiological parameters	Master Athletes PRE r value	PRE p value	POST r value	POST p value	1h POST r value	1h POST p value	Control group PRE r value	PRE p value	POST r value	POST p value	1h POST r value	1h POST p value
CLOCK	Time of test (min)	0.457	0.183	0.518	0.124	0.073	0.840	0.600	0.241	0.257	0.658	0.885	**0.033**
	Power (Watts)	0.685	**0.034**	0.598	0.073	0.249	0.483	0.706	0.144	0.206	0.711	0.794	0.077
	VO_{2max}	0.248	0.491	0.127	0.733	-0.478	0.166	0.771	0.102	0.142	0.802	0.314	0.563
BMAL1	Time of test (min)	0.255	0.449	-0.009	0.978	0.478	0.136	0.571	0.200	-0.285	0.556	0.071	0.906
	Power (Watts)	0.403	0.217	0.032	0.929	0.575	0.067	0.636	0.128	-0.374	0.414	-0.056	0.928
	VO_{2max}	-0.118	0.734	-0.300	0.371	0.409	0.214	0.607	0.166	-0.428	0.353	-0.321	0.497
PER1	Time of test (min)	0.449	0.143	-0.026	0.935	0.505	0.093	0.200	0.783	0.900	0.083	0.700	0.233
	Power (Watts)	0.520	0.085	0.023	0.943	0.617	**0.035**	0.410	0.500	0.820	0.133	0.564	0.366
	VO_{2max}	-0.160	0.691	-0.465	0.128	0.531	0.079	0.800	0.133	0.600	0.350	0.300	0.683
PER2	Time of test (min)	-0.012	0.973	-0.486	0.154	-0.492	0.148	0.571	0.200	0.000	>0.999	0.357	0.444
	Power (Watts)	-0.075	0.838	-0.574	0.105	-0.390	0.260	0.486	0.266	-0.093	0.852	0.187	0.700
	VO_{2max}	0.139	0.707	-0.551	0.104	-0.078	0.838	0.107	0.839	-0.214	0.661	-0.250	0.594
CRY1	Time of test (min)	0.284	0.425	0.090	0.802	0.553	0.097	0.178	0.713	-0.071	0.906	-0.214	0.661
	Power (Watts)	0.436	0.207	0.099	0.787	0.744	**0.017**	0.168	0.738	0.000	>0.999	-0.355	0.442
	VO_{2max}	0.054	0.891	-0.260	0.469	0.522	0.124	0.035	0.963	0.214	0.661	-0.428	0.353
CRY2	Time of test (min)	-0.047	0.910	-0.023	0.955	0.523	0.182	0.750	0.066	-0.428	0.353	-0.285	0.556
	Power (Watts)	0.358	0.370	0.173	0.675	0.778	**0.031**	0.673	0.114	-0.467	0.295	-0.505	0.247
	VO_{2max}	-0.166	0.703	0.095	0.840	0.381	0.359	0.142	0.782	-0.285	0.556	-0.714	0.088
REV-ERBα	Time of test (min)	0.176	0.626	0.632	**0.049**	0.376	0.283	0.500	0.4500	0.400	0.516	0.600	0.350
	Power (Watts)	0.302	0.393	0.723	**0.022**	0.478	0.162	0.223	>0.999	0.223	>0.999	0.223	>0.999
	VO_{2max}	0.187	0.607	0.927	**0.000**	0.842	**0.003**	-0.500	0.450	-0.300	0.683	-0.700	0.233
REV-ERBβ	Time of test (min)	0.803	**0.009**	0.451	0.222	0.083	0.830	-0.300	0.683	-0.102	0.900	-0.700	0.233
	Power (Watts)	0.935	**0.001**	0.623	0.081	0.356	0.342	-0.102	0.900	-0.263	0.783	-0.820	0.133
	VO_{2max}	0.466	0.212	-0.066	0.880	-0.466	0.212	0.400	0.516	-0.461	0.433	-0.900	0.083
RORa	Time of test (min)	-0.091	0.778	-0.452	0.139	-0.084	0.794	0.750	0.066	-0.428	0.353	-0.250	0.594
	Power (Watts)	0.096	0.764	-0.409	0.185	0.100	0.756	0.711	0.081	-0.430	0.347	-0.467	0.295
	VO_{2max}	-0.083	0.800	-0.587	**0.048**	0.062	0.851	0.428	0.353	-0.142	0.782	-0.714	0.088
RORb	Time of test (min)	0.203	0.525	-0.017	0.956	-0.189	0.553	0.600	0.350	0.800	0.133	0.500	0.450
	Power (Watts)	0.190	0.551	0.035	0.914	-0.326	0.297	0.368	0.533	0.579	0.333	0.368	0.533
	VO_{2max}	0.440	0.154	0.062	0.851	0.069	0.834	-0.200	0.783	-0.100	0.950	0.000	>0.999
RORc	Time of test (min)	0.134	0.697	-0.178	0.600	0.132	0.697	0.571	0.200	0.500	0.266	0.250	0.594
	Power (Watts)	0.163	0.629	-0.243	0.468	0.168	0.619	0.542	0.209	0.486	0.266	0.224	0.638
	VO_{2max}	-0.509	0.114	-0.263	0.434	-0.281	0.402	0.357	0.444	0.357	0.444	0.142	0.782

Note: Correlations were analysed using Spearman's rank correlation.

Table 5. Correlations between the Clock genes in purified effector-memory $CD8^+$ T-cells and physiological parameters in master athletes and control group.

Clock genes	Physiological parameters	Master Athletes PRE r value	PRE p value	POST r value	POST p value	1h POST r value	1h POST p value	Control group PRE r value	PRE p value	POST r value	POST p value	1h POST r value	1h POST p value
CLOCK	Time of test (min)	-0.560	0.122	-0.577	0.110	-0.585	0.103	0.428	0.353	0.071	0.906	0.714	0.088
	Power (Watts)	-0.519	0.157	-0.570	0.115	-0.604	0.090	0.318	0.495	-0.074	0.895	0.654	0.123
	VO_{2max}	-0.466	0.212	-0.566	0.120	-0.583	0.108	-0.071	0.906	-0.285	0.566	0.357	0.444
BMAL1	Time of test (min)	-0.443	0.274	-0.335	0.413	-0.323	0.434	-0.464	0.302	-0.142	0.782	0.107	0.839
	Power (Watts)	-0.414	0.319	-0.244	0.566	-0.414	0.319	-0.467	0.295	-0.018	0.990	0.018	0.990
	VO_{2max}	-0.023	0.976	-0.285	0.500	-0.452	0.267	-0.321	0.497	0.285	0.556	-0.214	0.661
PER1	Time of test (min)	-0.418	0.262	-0.276	0.470	-0.527	0.149	-0.428	0.419	0.085	0.919	0.600	0.241
	Power (Watts)	-0.290	0.445	-0.316	0.404	-0.461	0.213	-0.576	0.283	0.030	>0.999	0.394	0.466
	VO_{2max}	-0.216	0.580	-0.400	0.291	-0.416	0.269	-0.828	0.058	0.085	0.919	-0.085	0.919
PER2	Time of test (min)	-0.242	0.470	-0.018	0.959	0.191	0.570	0.085	0.919	0.028	>0.999	-0.314	0.563
	Power (Watts)	-0.264	0.429	-0.095	0.778	0.051	0.885	-0.147	0.777	-0.117	0.844	-0.382	0.477
	VO_{2max}	0.145	0.673	-0.091	0.790	0.118	0.734	-0.771	0.102	-0.428	0.419	-0.485	0.355
CRY1	Time of test (min)	-0.500	0.144	-0.469	0.172	0.024	0.952	-0.312	0.497	0.107	0.839	-0.107	0.839
	Power (Watts)	-0.417	0.229	-0.274	0.441	0.118	0.745	-0.467	0.295	0.074	0.895	-0.224	0.638
	VO_{2max}	-0.030	0.946	-0.090	0.811	-0.297	0.406	-0.642	0.138	0.071	0.906	-0.428	0.353
CRY2	Time of test (min)	-0.503	0.210	0.192	0.654	-0.457	0.255	-0.028	>0.999	0.028	>0.999	-0.142	0.802
	Power (Watts)	-0.531	0.174	0.049	0.918	-0.356	0.390	-0.235	0.677	0.000	>0.999	-0.029	0.977
	VO_{2max}	-0.239	0.564	-0.333	0.427	-0.595	0.132	-0.771	0.102	0.085	0.919	0.257	0.658
REV-ERBα	Time of test (min)	-0.597	0.073	-0.701	**0.028**	0.597	0.073	0.100	0.950	-1.000	**0.016**	-0.600	0.350
	Power (Watts)	-0.549	0.103	-0.543	0.108	0.668	**0.040**	-0.118	>0.999	-0.894	0.100	-0.335	0.600
	VO_{2max}	-0.466	0.178	-0.260	0.469	0.406	0.247	-0.100	0.950	-0.500	0.450	0.100	0.950
REV-ERBβ	Time of test (min)	-0.816	**0.010**	-0.233	0.551	-0.616	0.085	0.314	0.563	0.116	0.838	-0.428	0.419
	Power (Watts)	-0.886	**0.002**	-0.320	0.396	-0.540	0.137	0.151	0.816	-0.092	0.875	-0.637	0.183
	VO_{2max}	-0.850	**0.006**	-0.566	0.120	-0.216	0.580	-0.314	0.563	-0.579	0.233	-0.771	0.102
RORa	Time of test (min)	-0.900	**0.009**	-0.126	0.797	-0.234	0.619	-0.285	0.556	-0.035	0.963	0.000	>0.999
	Power (Watts)	-0.778	0.052	-0.111	0.840	-0.259	0.597	-0.299	0.519	0.000	>0.999	-0.149	0.761
	VO_{2max}	-0.035	0.963	-0.035	0.963	-0.285	0.556	-0.285	0.556	0.107	0.839	-0.428	0.353
RORb	Time of test (min)	-0.214	0.523	0.041	0.904	0.438	0.178	-0.464	0.302	0.071	0.906	-0.464	0.302
	Power (Watts)	-0.246	0.463	0.011	0.974	0.334	0.312	-0.523	0.223	-0.074	0.895	-0.598	0.161
	VO_{2max}	0.163	0.633	-0.177	0.599	0.018	0.967	-0.500	0.266	-0.285	0.556	-0.642	0.138
RORc	Time of test (min)	-0.383	0.346	-0.263	0.524	-0.646	0.091	-0.600	0.350	0.100	0.950	-1.000	0.333
	Power (Watts)	-0.494	0.216	-0.337	0.412	-0.783	**0.026**	-0.564	0.366	0.205	0.766	-1000	0.333
	VO_{2max}	-0.190	0.664	0.000	>0.999	-0.595	0.132	-0.700	0.233	0.700	0.233	-0.500	>0.999

Note: Correlations were analysed using Spearman's rank correlation.

There was a strong positive correlation between REV-ERBα expression and VO_{2max} in EMCD4+ T cells in MA after the acute exercise (Post: r=0.927, P=0.000; Table 4). In addition, REV-ERBα expression was positively correlated with test time and power in MA (Post: r=0.632, P=0.049; r=0.723, P=0.022, respectively; Table 5). Interestingly, after exercise, REV-ERBα expression in EM CD8+ T cells was negatively correlated with test time in MA and CG(Post: r=−0.701, P=0.028; r=−1.000, P=0.016; Table 3). Furthermore, the clock genes (PER1, CRY1 and CRY2) in EM CD4+ T cells at 1h Post and RORc in EM CD8+ T cells at Post were positively correlated with power (1h Post: r=0.617, P=0.035; r=0.744, P=0.017; r=0.778, P=0.031, respectively; Table 4; r=0.783, P=0.026; Table 5).

DISCUSSION

We demonstrated that EM T cells in MA exhibit different peripheral and cellular inflammatory responses after acute exercise compared with untrained healthy individuals. We observed increased levels of cytokines (IL-8, IL-10, IL-12p70 and IL-17A) after 10 min of acute exercise (with IL-8 and IL-10 levels remaining elevated until 1h Post) and augmented CRY1, REV-ERBα and TBX21 expression in EM CD4+ T cells in MA. Physiological parameters, including test time, power and $VO_{2\ max,}$ were negatively correlated with IL-17A levels (a pro-inflammatory cytokine) in MA at 1h Post. In untrained healthy individuals, CLOCK and BMAL1 expression was increased in EM CD4+ T cells and REV-ERBβ expression was decreased in EM CD8+ T cells after exercise.

Clock genes, inflammation and exercise

We found that the expression of CRY1 and REV-ERBα in EM CD4+ T cells was increased after 1h of acute exercise in MA. CRY1 and CRY2 are closely linked to the anti-inflammatory profile. A study using a mice model of arthritis with CRY1 and CRY2 knockout showed an increase in serum levels of IL-1β, IL-6, MMP-3 and TNF-α, with aggravated pathological changes in the arthritis score observed (56).

The kinetics of IL-17A is also very interesting because it shows an interplay with the clock family. At 10 min after ex-

haustive exercise, the circulating levels of this cytokine were increased and at the same time an elevation of TBX-21 expression was observed. Perhaps these findings may be explained by the fact that Th17 cells contain a dynamic subset of CD4 T cells that are able to develop into other subset lineages, including Th1-like Th17 cells. These cells co-express RORγt and the transcription factor TBX21 and produce IL-17 (57); however, the increase in IL-17A levels and TBX21 expression may be related to the increase in the percentage of EM $CD4^+$ cells.

On the other hand, we observed the restoration of IL-17 levels to the Pre levels 1 h after the exhaustive exercise and at this time, REV-ERBα expression was elevated 1h after the exercise in MA. REV-ERBα also has a close relationship with anti-inflammatory profile. In a study in which macrophages were stimulated with LPS and cultured with a pharmacological agonist of REV-ERBα, a decreased release of IL-6 was observed. Moreover, the deletion of REV-ERBα increased a subset of inflammatory genes in macrophages (58). In Th17 cells, a study demonstrated that REV-ERBα binds to ROR response elements to inhibit the expression of RORγt-dependent genes including IL-17A and IL-17F. Furthermore, elevated REV-ERBα expression or treatment with a synthetic REV-ERBα agonist significantly delayed and mitigated the progression of experimental autoimmune encephalomyelitis (59). Similarly, REV-ERBα negatively regulated pro-inflammatory Th17 responses in vivo (60). These results explain some of our findings, such as the negative correlation between REV-ERBα and IL-17A levels in MA. Another interesting finding was the positive correlation between REV-ERBα and the physiological parameters in MA immediately after the maximal test. Overexpression of REV-ERBα in myocytes induces an increase in the number of mitochondria and improves the respiratory capacity (61). These results have been validated in vivo via pharmacological activation of REV-ERBα as conditional loss of this gene impairs the aerobic capacity and oxidative metabolism in muscle tissue of mice through deactivation of Lkb1-Ampk-Sirt1–Ppargc-1α signalling(61). Because an imbalance between Treg and Th17 is observed in sustained inflammaging observed in senescent lymphocytes (62), the result of the anti-inflammatory regulation of clock genes in MA could be related to their better anti-inflammatory profile. Moreover, a better Treg response was observed in MA previously (44).

It is interesting to note that the clock genes altered by acute exhaustive exercise in the sedentary group were different from those in MA. In untrained healthy individuals, there was an increase in the expression of CLOCK and BMAL1 at 1h Post. A recent study revealed a change in the expression of clock genes in human leukocytes due to exercise, with BMAL1 expression increasing with exercise performed in the morning and afternoon and CRY1 expression increasing only during morning exercise (63). In healthy untrained individuals, there was a decrease in the expression of REV-ERBβ in EM CD8+ T cells. REV-ERBβ is best known for regulating genes involved in metabolism and circadian rhythm(64).

However, its effect on immune cells is still unclear, although one study showed a strong expression of REV-ERBβ in macrophages(65). In general, acute physical exercise promoted major changes in the expression levels of clock genes of EM $CD4^+$ T cells. Therefore, the effects of clock genes in lymphocytes after acute exercise and the difference between the MA and sedentary group should be better explored.

Production of cytokines

Acute exercise induces psychological and physiological stress as well as production of immunological mediators such as cytokines, chemokines and growth factors. The type, intensity and duration of these mediators depend on many factors, including pre-conditioned physical fitness, age, sex and mode of exercise (66,67).

It is interesting to note that CG did not show any difference in Pre values compared with the MA group. In our study, although CG comprised healthy volunteers, we believe that this group is not necessarily a representation of the elderly in the occidental society. However, ageing modified the number and function of circulating immune cells as well as the pattern of cytokines circulating in serum (34). Ageing, per se, induces increases in the levels of pro-inflammatory cytokines (IL-1, TNF-α, C reactive protein and IL-12)(47).

However, we observed that MA exhibit augmented cytokine levels after the maximal exercise test, but these alterations were not seen in the untrained individuals. The increase in the levels of serum cytokines during and after exercise is closely related to the intensity, type, mode and workload of the exercise (68–74). In addition, it also depends on physical fitness (75). We observed that there were no changes in the plasma IL-6 levels 10 min after a maximal exercise in both MA and age-matched untrained healthy individuals. In this study, increased serum levels of IL-10 (120% post-exercise and 48% 1h post), IL-8 (140% post-exercise and 105% 1h post), IL-12p70 (41% post-exercise and 9,4% 1h post) and IL-17A (240% post-exercise and 165% 1h post) were observed after maximal exercise in MA but not in the untrained healthy individuals (for IL-10: 110% post-exercise and 8% 1h post; for IL-8: 58% post-exercise and 91% 1h post; for IL-12p70: 1,2% post-exercise and 15% 1h post; for IL-17A: -11% post-exercise and -3% 1h post, $p>0.05$ for all). This finding may be, at least in part, due to physical fitness.

Because the production of cytokines induced by acute exercise is dependent on the duration/volume of acute exercise, Pedersen and Febbraio (2008) and Cabral-Santos et al (2019) demonstrated that the production of IL-6 and IL-10 during exercise depends on the exercise duration(74,76). In the present study, the time of maximal exercise was different between the groups: CG performed the exercise for ~11 min and MA for ~17 min (~54%). Several studies have reported that the duration of exercise exerts a major impact on the level of cytokines. Cabral-Santos et al. (2016) demonstrated that high-intensity intermittent exercise (such as 1.25km and 2.50 km treadmill runs, which have the same intensity as 1:1 min at 100% of VO_{2max} with passive recovery) effectively increased brain-derived neurotrophic factor (BNDF) and IL-6 levels immediately after exercise. Only the IL-10 response was associated with the duration of exercise, indicating the importance of exercise prescription(72). Jankord and Jemiolo (2004) demonstrated that older men that perform higher volume of lifelong exercise have higher IL-10 levels, suggesting that exercise may play a vital role in regulating the production of inflammatory markers(77).

MA exhibited a negative correlation between the levels of pro-inflammatory cytokines (IL-8 and IL-17) and physiological parameters (VO_{2max}, time of test and power) and a positive correlation between clock genes expression in EM CD4+ T cells and physiological parameters.

Pro-inflammatory cytokines (IL-17 and IL-8) have been shown to induce catabolic effects such as lipolysis and glycogenolysis (78,79). In the present study, a negative correlation between IL-8 and IL-17 levels and performance parameters was observed in MA, suggesting that lifelong exercise protects against degenerative diseases as documented in the literature(80).

Rhythmicity imposed by routine exercise, nutritional habits and sleep behaviour promote an anti-inflammatory and healthy metabolic environment. In a recent review, Parr et al. (2020), explored the deleterious effects of modern-day lifestyles on circadian biology. They suggested that the organisation of a nutritional and exercise training routine could have a potential beneficial effect on metabolic health(81).

CONCLUSION

The synchronization of clock genes, immune function and ageing presents new dimensions with interesting challenges. Acute sessions of exercise altered the clock machinery in CD-4+ T cells, but the expression of clock genes was different between MA and untrained individuals. Moreover, cytokine production was dependent on the fitness level of the elderly population. MA showed a negative correlation between inflammatory cytokine levels and physiological parameters.

LIMITATIONS

Our results do not allow the determination of a cause effect response. It is necessary to perform additional studies to clarify the relation between each clock gene and the cytokine response after exercise in the elderly.

Our sample size was limited because of the molecular approach (cell isolation by cell sorting and PCR). In addition, the nature of the control group may be a limitation in our study, because these were healthy elderly, and most of the elderly population usually as some form of disease. The lack of differences between CG and MA groups in resting conditions may be because of this.

The different modalities of exercise training could also present a limitation, because the physiological adaptations are more heterogeneous, but may also represent a strength since we showed that clock genes were modulated in lymphocytes after acute exercise independent of the exercise training modality.

STRENGTHS

This is the first work that shows a difference in clock genes expression modulated by physical fitness and acute exercise sessions in older persons. Since exercise could be a Zeitgeber it may be an excellent tool to improve the biological rhythms that are altered by ageing, and be a player in improving the inflammatory status observed during aging.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

ACKNOWLEDGEMENTS

To the funding sources, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil) process number 2015/16777-0, 2016/01409-8, 2018/24187-6, 2019/10187-7, 2019/09854-9, 2019/25626-6, 2018/19678-0 and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

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A systematic literature review on the effects of exercise on human Toll-like receptor expression

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ABSTRACT

Background

Toll-like receptors (TLRs) are a family of transmembrane pattern recognition receptors that are mainly expressed on immune cells. Recognition of various exogenous and endogenous molecular patterns activates the TLR signalling cascade, which orchestrates an inflammatory immune response. Dysfunctional immune responses, including aberrant TLR signalling, are increasingly implicated in the associations between sedentarism, chronic low-grade systemic inflammation and various non-communicable diseases. Conversely, exercise exerts anti-inflammatory effects, which could be conferred through its immunomodulatory properties, potentially affecting TLRs. This study aims to systematically review the effects of exercise on human TLR expression.

Method

A systematic literature search of Pubmed, Embase, The Cochrane Library and SPORTDiscus for articles addressing the impact of exercise (as isolated intervention) on TLRs in humans was conducted, ending in February 2020.

Results

A total of 66 articles were included. The publications were categorised according to exercise modality and duration: acute resistance exercise (4 studies), acute aerobic exercise (26 studies), resistance training program (9 studies), aerobic training program (16 studies), combined (i.e. resistance and aerobic) training program (8 studies) and chronic exercise not otherwise classifiable (9 studies). Five articles investigated more than one of the aforementioned exercise categories.

Several trends could be discerned with regard to the TLR response in the different exercise categories. Acute resistance exercise seemed to elicit TLR upregulation, whereas acute aerobic exercise had less activating potential with the majority of responses being neutral or, especially in healthy participants, downregulatory. Chronic resistance and combined exercise programs predominantly resulted in unaltered or decreased TLR levels. In the chronic aerobic exercise category, mixed effects were observed, but the majority of measurements demonstrated unchanged TLR expression.

Conclusion

Currently published research supports an interplay between exercise and TLR signalling, which seems to depend on the characteristics of the exercise. However, there was large heterogeneity in the study designs and methodologies. Therefore, additional research is required to further corroborate these findings, to define its pathophysiological implications and to elucidate the mechanism(s) linking exercise to TLR signalling.

Keywords: Exercise, Toll-like receptor, immunity, inflammation, systematic review

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INTRODUCTION

Low-grade chronic inflammation has been implicated in the pathogenesis of numerous chronic diseases, including atherosclerosis, heart failure, diabetes mellitus, obstructive pulmonary diseases, rheumatoid arthritis, dementia and particular types of cancer.(9, 27, 38, 68, 69, 115) Physical inactivity seems to contribute significantly to the development of a state of systemic inflammation, which has led to the paradigm of 'inflamm-inactivity'.(57) Conversely, physical activity (*i.e.* exercise) elicits anti-inflammatory adaptations, which would confer a protective effect against chronic inflammation-associated diseases.(22, 68, 129, 130) However, the mechanisms underlying the anti-inflammatory effects of exercise are not fully understood.(35) It is known that physical exercise can modulate an array of immunological responses.(9) The possibility that exercise mediates these anti-inflammatory effects by affecting Toll-like receptor (TLR) signalling has gained increasing attention in the last decade.(9) Nevertheless, the literature still lacks clarity.

Toll-like receptors

TLRs are a family of evolutionarily conserved transmembrane glycoprotein receptors.(17, 49) To date, 13 members of the TLR family have been identified in mice, and 10 (TLR1-10) in humans.(69) TLRs are widely distributed and expressed in various cell types and tissues, but primarily in/on immune cells.(4, 9, 51, 118, 159, 211) They recognise a variety of exogenous and endogenous signals. More specifically, TLRs respond to distinct molecular patterns and are therefore designated as pattern recognition receptors (PRRs). The recognised molecular motifs can be divided into pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are invariant molecular structures shared by a large spectrum of microbial pathogens. As such, TLRs play an important role in the defence against Gram-negative and Gram-positive bacteria, DNA and RNA viruses, fungi and protozoa.(17) DAMPs, on the other hand, are endogenous signals released by stressed or injured cells. In physiological conditions, DAMPs are hidden from recognition by the host immune system.(154) The known ligands for each of the TLRs are summarised in Supplementary Table 1.

The conserved nature of the TLRs is related to their central role in both innate and adaptive immunity.(43) TLR activation typically generates a pro-inflammatory environment, potentially leading to the aforementioned disease states.(34, 38, 43, 65, 118, 153) Therefore, TLRs are a focus of investigation in the associations between sedentarism, inflammation and disease.(39)

An overview of the TLR signalling pathways and their differential expression in/on monocyte and dendritic cell subtypes can be found in the Supplementary Material.

Exercise and Toll-like receptors

It has been hypothesised that exercise exerts its anti-inflammatory effects through modulation of TLR signalling. Several viable mechanisms exist to substantiate this proposed relationship. Especially serum factors whose kinetics are influenced by exercise have been put forward as candidates to explain such relationship. These include circulatory cytokines, translocated lipopolysaccharide (LPS) from the gastrointestinal tract, fatty acids, hormones (glucocorticoids, catecholamines, insulin-like growth factor 1 and growth hormone) and heat-shock proteins (HSPs).(128) Also DAMPs, muscle derived microRNAs (miRNAs) (potentially DAMP-induced), autophagy-related proteins, oxidative stress and acidosis have been suggested as mediators.(17, 46, 65, 119, 128, 174, 190) A commonly cited mechanism of action is tolerance or cross-tolerance by (low-dose) exposure.(94, 141, 153, 174) More recently, soluble forms of some TLRs have been identified in various body fluids. Consequently, it was suggested that TLR downregulation could also be mediated by receptor shedding.(39, 43, 68, 98, 216)

Aims of this systematic review

The concept that exercise modulates TLR signalling has gained attention in the last decade. However, conflicting results have been reported, suggesting that the modality and duration of the physical stimulus may be a strong determinant of the outcome. Previous reviews on the relationship between exercise and TLRs were limited to patients with the metabolic syndrome(145), not systematic in design(39, 68), not focused on TLRs (but on inflammatory markers in general)(67, 68) or only concentrated on specific TLRs(27). The aim of this review was to present a systematic overview of the literature on the effect of exercise on TLRs to date. In addition, special attention is paid to the impact of the health status and age of the subjects, and to concurrent alterations in leukocyte populations, which predominantly harbour TLRs.

METHODS

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.(123)

Search strategy and selection criteria

We searched the electronic databases Pubmed, Embase, The Cochrane Library and SPORTDiscus for articles addressing the impact of exercise on Toll-like receptors. Electronic searches were conducted from inception to 21 February 2020. There were no restrictions with regard to publication year unless stated otherwise. Pubmed was searched with the following Medical Subject Heading (MeSH) terms: ("Toll-Like Receptors"[Mesh]) AND "Exercise"[Mesh]. In order not to disregard very recent articles without MeSH indexation, we also ran a query with free-text fields, which was restricted to articles published since 1 January 2019: ("exercise"[MeSH Terms] OR "exercise"[All Fields]) AND ("toll-like receptors"[MeSH Terms] OR ("toll-like"[All Fields] AND "receptors"[All Fields]) OR "toll-like receptors"[All Fields] OR ("toll"[All Fields] AND "like"[All Fields] AND "receptor"[All Fields])). For Embase, we used the query: ('exercise'/exp OR exercise) AND ('toll like receptor'/exp OR 'toll like receptor'). The "/exp" indicates that this is an "explosion" in Emtree and that related terms are also included. SPORTDiscus was consulted with the query: (exercise or physical activity) AND toll-like receptors. For The Cochrane Library the following keywords were used: exercise in Title Abstract Keyword AND toll-like receptor in Title Abstract Keyword. In addition, references from previous articles were hand-searched.

Studies describing the (direct) impact of exercise (without restriction on modality, intensity or load) on Toll-like receptors (both gene and protein level) in human subjects were eligible for inclusion. There were no predefined criteria with regard to age, sex and health status of the study subjects. Both observational and interventional designs were accepted.

Animal studies were excluded. This review was restricted to articles written in English or Dutch. Results published in abstract form were not included as this prohibits adequate evaluation of study quality and bias. Commentaries, letters to the editor, editorials and project proposals were also excluded. Studies containing insufficient information, or with no full-text of the manuscript available were to be excluded if no response was received after contacting the authors through e-mail.

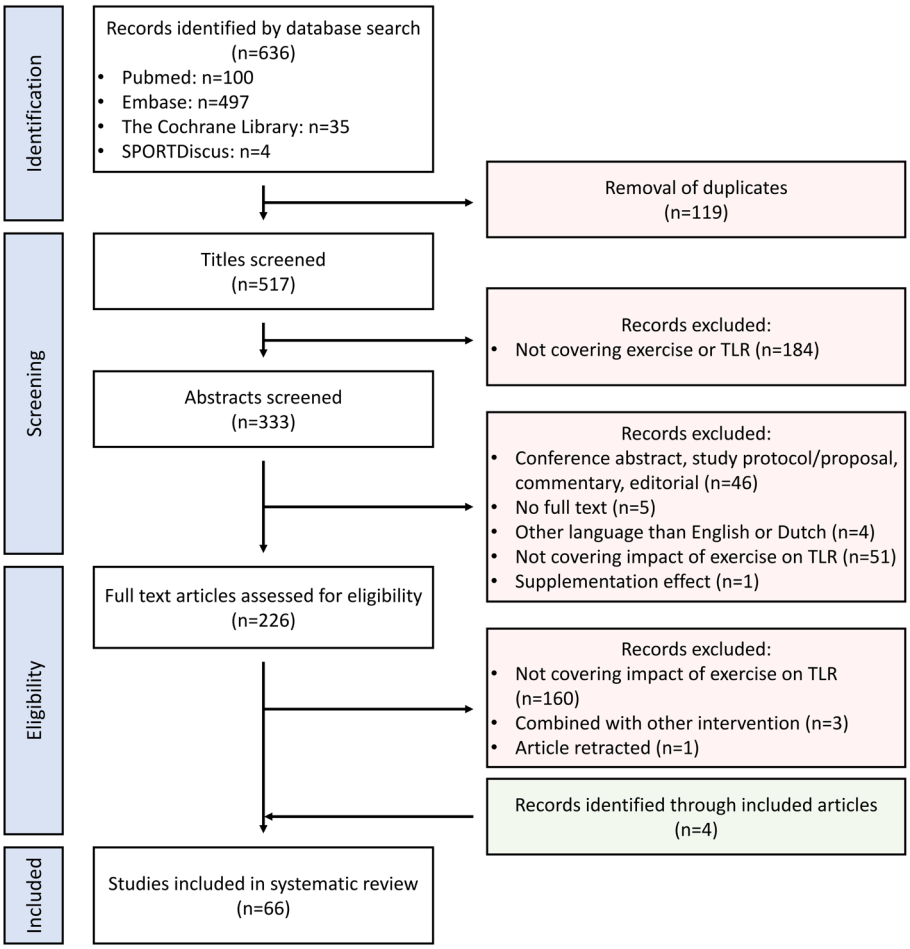
Study selection and data extraction

The searches were exported to EndNote X9. Duplicates were removed. First, KF assessed titles and abstracts for eligibility. Next, two independent researchers (KF and MB) checked the full text of the articles that were considered relevant in a first phase against the predefined inclusion and exclusion criteria.

A data extraction spreadsheet was created. KF extracted information on study design, study population, exercise characteristics (modality, intensity, duration), specimen collection, analytical technique and outcomes.

Simultaneously to the data extraction, the study quality and the risk of bias were assessed. Disagreements between both researchers regarding study inclusion, and any ambiguities regarding data extraction, and quality and risk of bias assessment were discussed within the wider team (KF, MB, HH and PJG).

Figure 1 | Flow diagram of the study selection process.



RESULTS

Literature search

Figure 1 summarises the study selection process. The search of the 4 electronic databases identified 636 articles with 517 articles remaining after removal of duplicates. Of these, 184 articles were excluded after screening of the titles and 107 after screening of the abstract. The main reason for exclusion was the subject matter (*i.e.* not investigating the impact of exercise on TLRs), but also articles that combined exercise with another intervention (hereby obscuring the exercise impact) were removed at this stage. Forty-six articles were excluded based on article type (*i.e.* conference abstract, project protocol or proposal, commentary or editorial). Of the 226 retrieved articles, 165 were excluded after full text review. Subject matter not fitting the scope of this review and combined interventions were the main reasons. Eventually, 62 articles were eligible for inclusion in this review. Through detailed review of the reference lists of the included articles, an additional 4 references were found eligible and were included.(35, 108, 112, 189) For scientific substantiation, an additional 150 trials were consulted without complying with the predefined inclusion and exclusion criteria.

Characteristics of the included trials

The characteristics of the included trials are presented in Supplementary Tables 2-7. The majority of studies (55%) was published in the time period 2015-2020. Sixty-two percent of the articles studied healthy subjects (we considered overweight and obesity to be a pathological state). Regarding the exercise modality, 40 (58%) articles studied the effects of endurance exercise, 13 (19%) the effect of resistance exercise and the remainder (8 studies, 12%) evaluated combined forms of exercise. Twelve percent of the exercise forms were not classifiable. The majority of trials (55%) evaluated the effects of chronic exercise training, 41% focused on the impact of an acute exercise bout and 5% combined the effects of acute and chronic exercise.

Eight trials did not mention the sex distribution of the subjects studied. (54, 115, 119, 126, 150, 154, 155, 180) Among the 12 randomised controlled trials (RCTs), the main issues regarding bias were lack of blinding of participants and study personnel, and lack of blinding of outcome assessment.(5, 40, 51, 52, 94, 119, 124, 142, 154, 155, 172, 174) Information regarding the allocation process was not present in 83% of the studies.

Impact of exercise on Toll-like receptors

To study the impact of exercise on TLRs, we have categorised the exercise protocols according to their modality (resistance versus endurance) and duration (acute versus chronic). We acknowledge that the dichotomisation of exercise into resistance and endurance effort is very artificial and unphysiological, however, the heterogeneity of the study protocols did not allow for a valuable alternative.(56) Chronic exercise (exercise training) can be defined as a repeated amount of exercise bouts during a short or long period of time, while acute exercise can be defined as a single bout of exercise.(165) The effects of chronic exercise should be evaluated after sufficient exercise abstinence to avoid interference from delayed acute effects. The findings per exercise category are summarised in Figure 2.

For each exercise category, we first discuss the findings in healthy subjects, and subsequently in participants with a pathological condition. We conclude each section with data on the impact of age and physical activity status.

Acute resistance exercise

Only four studies investigated the effect of a bout of resistance exercise on Toll-like receptors.(51, 52, 58, 116) All were performed in apparently healthy subjects. Contrary to the previous review by Cavalcante *et al.*, we chose to classify the staircase running exercise of Millard *et al.* as an acute aerobic stimulus.(27, 121)

In 2003, Flynn *et al.* subjected elderly resistive-trained women to a resistance training program and evaluated the response to a subsequent acute bout. No difference in TLR4 mRNA was noted in response to the exercise bout, although the trained group did show lower resting TLR4 mRNA levels compared to a sedentary control group at the end of the program.(58) One year later, McFarlin *et al.* compared the response of trained and untrained elderly women to a series of upper and lower body resistance exercises. The untrained group showed higher levels of TLR4 expression on monocytes at baseline, but no exercise-induced alterations in TLR4 (both at gene and cell surface level) were observed (in contrast to the interpretation of Cavalcante *et al.* of these study results).(27) *Ex vivo* LPS-stimulation of the blood samples did show increased cytokine production in the post-exercise samples.(116) Fernandez-Gonzalo *et al.* first studied a group of healthy young and moderately active male students. An eccentric exercise bout induced increased gene expression and protein levels of TLR4 in peripheral blood mononuclear cells (PBMCs). Also other proteins of the TLR signalling pathway (*i.a.* myeloid differentiation primary response gene 88 (MyD88), TNFR-associated factor (TRAF) 6, extracellular signal-regulated protein kinase (ERK) 1/2, TIR-domain-containing adaptor protein-inducing interferon-β (TRIF)) and tumor necrosis factor alpha (TNF-α) were upregulated. Next, he repeated the exercise bout after half of the students had completed a 6-week eccentric training program. Remarkably, the trained students showed decreased protein levels of TLR4 after the second bout and stable levels of pathway proteins and TNF-α, whereas the response of the control group was identical to the first bout.(51) They later corroborated these findings in female students where TLR4 protein levels were elevated after the first bout and remained stable after the second bout. It is worth mentioning that in both studies the TLR4 mRNA remained elevated with a different response at protein level as discussed above.(52)

Acute aerobic exercise

Twenty-three articles evaluated the effects of acute aerobic exercise. Study subjects varied from young endurance-trained athletes and professional soccer players to sedentary and diseased patients.(22, 69) No elderly were included in any of the trials. There was large variation between studies in every aspect of the aerobic stimulus. The exercise intensity was between moderate (50% VO_2max) and maximal.(12) Exercise duration varied from very brief (68.6 seconds) to long-lasting (263 minutes).(121, 129) Lastly, environmental conditions ranged from cold (1°C) outdoor conditions to an environmental chamber with room temperature of 37°C and relative humidity of 25%.(2)

One study evaluated TLR expression in (vastus lateralis) muscle tissue, the other study groups focused on venous blood samples. Monocytes were the most frequently studied cell fraction within the blood samples (8/23). The most commonly used analysis methods were PCR-based techniques to evaluate gene expression (11/23 studies) and flow cytometry (9/23 studies). Western blot was less frequently applied (4/23). Only one study simultaneously evaluated the TLR gene and protein level.(129)

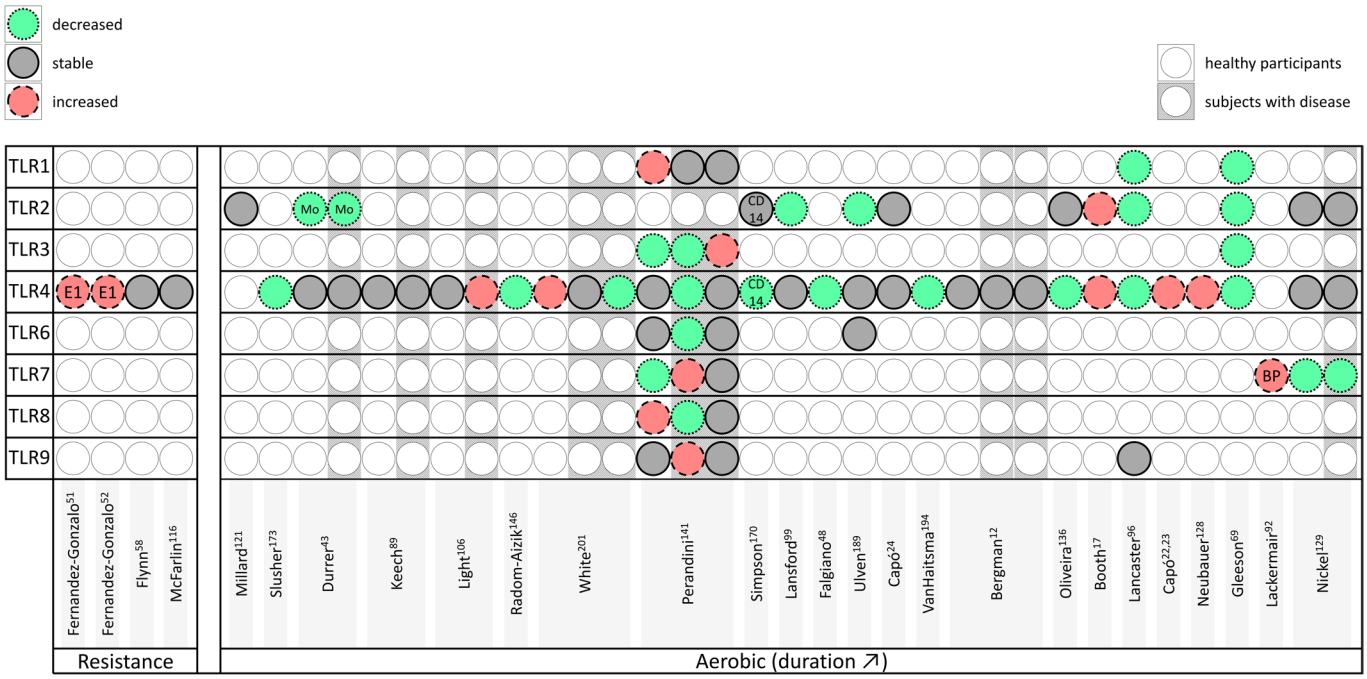
In healthy participants, the majority of studies showed downregulated (19/45; 42%) or stable (18/45; 40%) levels of TLR gene or protein expression, with only a minority recording increased levels (8/45; 18%). Studies that evaluated different TLR family members demonstrated that they can react differently to the same exercise stimulus. The predominant response(s) of the different TLRs was decrease for TLR1 (2/3), TLR2 (5/11), TLR3 (2/2) and TLR7 (2/3), stable levels for TLR2 (5/11), TLR4 (9/21), TLR6 (2/2) and TLR9 (2/2), and increase for TLR8 (1/1).

Five studies with an initial response of TLR to exercise performed a re-evaluation after several hours of recovery and all demonstrated normalisation to baseline levels (after 4-48 hours). Only one subgroup in the study of VanHaitsma *et al.*, that was subjected to cycling in a hot environmental chamber, showed persistent decrease in TLR4 expression after 48 hours.(194) Five studies simultaneously determined the levels of several TLR signalling pathway molecules.(24, 48, 128, 141) In three of these studies, the response of these molecules corresponded to the TLR response. The results of *ex vivo* stimulation assays, although difficult to interpret for reasons explained in the discussion section on the methodological approach, were in agreement with the TLR response in 4 out of 6 studies. Cytokine levels were assessed in 11 studies, with responses matching to TLR changes in 7 studies.

As already discussed in the introduction, we know that certain pathological conditions are associated with an inflammatory state. This inflammatory milieu at baseline could affect the TLR response to exercise. In addition to healthy participants, seven studies also included one or more patient groups. The majority of observations showed unchanged TLR levels (14/25; 56%), followed by decreased (7/25; 28%) and increased (4/25; 16%) levels. In one of the two studies that simultaneously assessed the response of the TLR signalling pathway molecules, the response could be considered congruent to the TLR response.(12, 141) Durrer *et al.* performed

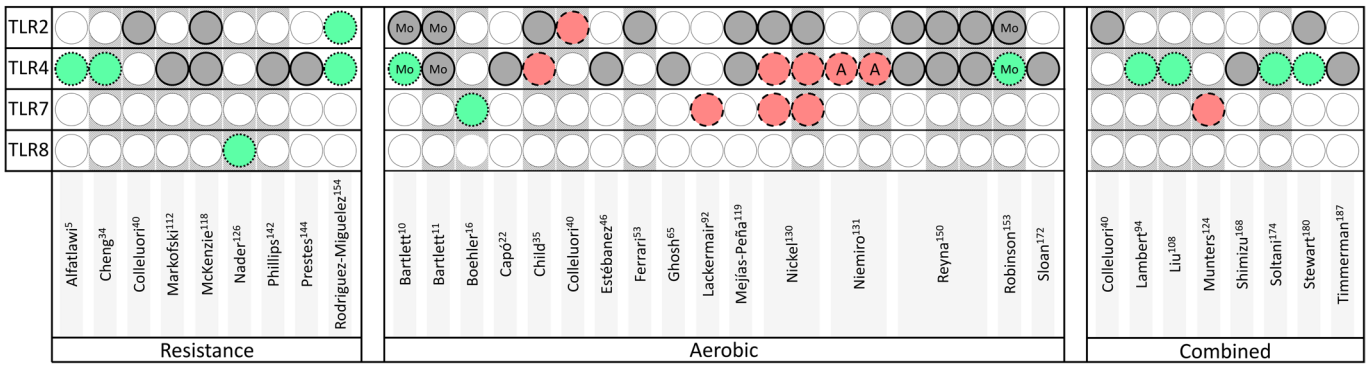
Figure 2 | Impact of exercise on individual TLR receptors according to exercise category.

A) Acute exercise bout.



The X-axis denotes exercise modality, first author and corresponding reference. Studies examining the impact of acute aerobic exercise were ranked according to duration of the exercise bout from left (short duration) to right (long duration). Responses have been categorised as increased (red colour; dashed outline), stable (grey colour; solid outline) or decreased (green colour; speckled outline). If both gene and protein level were available, only protein expression was considered. In healthy participants, the circles corresponding to the TLR responses are placed on a white background. In subjects with disease, the circles are superimposed on a striped background. E1, only first exercise bout was taken into account. Mo, only monocytes were considered. CD14, response of total $CD14^+$ cells. BP, biphasic response with increase more pronounced than decrease.

B) Exercise training program.



The X-axis denotes exercise modality, first author and corresponding reference. Responses have been categorised as increased (red colour; dashed outline), stable (grey colour; solid outline) or decreased (green colour; speckled outline). If both gene and protein level were available, only protein expression was considered. In healthy participants, the circles corresponding to the TLR responses are placed on a white background. In subjects with disease, the circles are superimposed on a striped background. Mo, only monocytes were considered: CD14+ monocytes (153) or non-classical monocytes (10, 11). A, ambiguous response, interpreted as increase.

ex vivo stimulation of blood cultures of type 2 diabetes patients and noticed lower TNF-α production after exercise, which is in line with findings regarding TLR expression.(43) Cytokine levels were evaluated in 10 studies, and in 5 of them the response was in agreement with the TLR response.

When directly comparing healthy controls and patient groups, 4 out of 6 studies showed identical TLR responses to exercise. In the two studies that showed differences, there were also differences between the different patient groups. One of these studies reported increased TLR expression after exercise in healthy controls, unchanged TLR levels in chronic fatigue syndrome (CFS) patients and decreased levels in multiple sclerosis (MS) patients.(201) Perandini *et al.* compared gene expression in response to exercise of patients with active or inactive systemic lupus erythematosus (SLE) and healthy controls, and although innate and adaptive immunity was downregulated in all groups, differences in the kinetics of the expression changes of the different TLRs were noticed.(141)

Also age and baseline physical activity status could influence the TLR response to exercise. All of the upregulatory responses occurred in young and physically active individuals, although overall, far more stable or downregulatory responses were recorded in these subjects. Only two studies included subjects of middle age, and no elderly were studied in any of the trials. Capó *et al.* found similar results in young and 'senior' athletes.(24) Bergman *et al.* and Nickel *et al.* compared sedentary subjects to athletes and non-elite to elite athletes respectively, and no differences in TLR response to exercise were observed.(12, 129) In the trial of Bergman *et al.*, the predefined intensity level was expressed as a percentage of the VO_2max and thus corrected for the aerobic capacity of the participants.

Two studies were not considered in the aforementioned evaluation due to their particular design. Sureda *et al.* recruited professional divers to perform an immersion to a depth of 50 metres. Stable expression of TLR2 and increased expression of TLR4 and NF-κB were reported, but possible effects of hyperoxia and hyperbaria must be taken into account.(181) Fuller *et al.* subjected the participants to an acute cycling bout, but blood sampling was only performed 16-18 hours later (to assess the impact of a high fat meal). They demonstrated stable levels after 16-18 and 20-22 hours, which could correspond to the recovery of TLR within hours after exercise as mentioned above.(62)

Resistance exercise program

Nine studies assessed the impact of a resistance exercise program. The status of the patient groups varied from healthy to sedentary, obese, frail or even diseased (*e.g.* auto-immune inflammatory myopathy or recovering from a hip fracture). The majority of patients were elderly, with only 2 groups being middle-aged. The duration of the programs ranged from 4 to 26 weeks, all with 2-3 sessions per week. Seven out of nine programs consisted of upper and lower body exercises. The training intensity is difficult to estimate given the differences in number of sets and repetitions.

The tissues subjected to analysis included whole venous blood, serum, peripheral blood cells, PBMC, lymphocytes, vastus lateralis muscle and subcutaneous adipose tissue. The analytical techniques were mainly gene expression based (6/9). In the different studies together, only three receptors (TLR2, TLR4 and TLR8) were evaluated.

Three trials recruited healthy participants to assess the impact of their training program. In total, 4 responses were recorded, of which 3 showed decreased and 1 unchanged TLR levels. Rodriguez-Miguelez *et al.* looked into molecules involved in the TLR signalling pathway, and similar to the TLR response, downregulation was observed.(154) No *ex vivo* stimulation assays were performed. In all three studies, cytokine assays of peripheral blood were in agreement with the TLR response.

Six studies (solely) focused on patients with various pathologies. Of the 7 TLR responses recorded, 5 were stable and 2 downregulated. Four studies simultaneously determined TLR pathway molecule levels, and these were in accordance with the TLR response in 3 of them.(34, 40, 118, 126) Two studies performed *ex vivo* stimulation assays and in both the results were in accordance with the TLR response.(112, 142) Five studies determined cytokine levels, and these were in agreement with the TLR response in two studies.(34, 40, 118, 126, 142)

Insufficient information is available to make any statements regarding the impact of the patient characteristics. The exercise programs were limited to middle-aged or elderly subjects with little physical activity (although five trials did not provide exact information on the baseline physical activity status). As obesity is associated with a state of meta-inflammation, one would expect these patients to have additional 'room for improvement' with regard to TLR lowering. Remarkably, the trials that studied overweight/obese subjects all reported absence of TLR alterations.(40, 112, 142)

In addition to the effects of the resistance exercise program itself, such program can also modulate the effects exerted by acute exercise bouts. As previously mentioned, Fernandez-Gonzalo and colleagues demonstrated that an eccentric exercise program attenuated the TLR-mediated pro-inflammatory response after a bout of eccentric exercise.(51, 52)

Aerobic exercise program

Sixteen studies were included to evaluate the effect of chronic aerobic exercise on TLRs. The study population varied from young sports professionals to sedentary, obese elderly and patients with chronic diseases (myositis, rheumatoid arthritis). The exercise program duration ranged from very brief (2 weeks) to long-lasting (26 weeks). Session load was situated between 2 sessions per week and daily. Again, exercise intensity is difficult to quantify, especially given the fact that most programs gradually increased the intensity throughout their program.

Most studies focused on PBMCs or blood monocytes. Three studies examined vastus lateralis muscle tissue. The use of flow cytometry, Western blot and gene-based techniques were equally distributed. Only TLR2, TLR4 and TLR7 were examined.

In healthy participants, the majority of assessments in 10 studies found no impact of exercise training on TLR expression (10/15; 67%). Four of the evaluations (4/15; 27%) showed TLR upregulation, and in one occasion (1/15; 7%), the TLR level was decreased. Pathway molecules were assessed in 4 studies, with accordance with the TLR response in 3 of them.

Sloan *et al.* evaluated inducible cytokine production in whole blood and found no effect of exercise training, corresponding to the observed TLR response.(172) Regarding *ex vivo* assays, Bartlett *et al.* reported increased monocytic phagocytosis and oxidative burst compared to pre-exercise, while stable TLR2 levels and reduced TLR4 levels were reported.(10) Of the 4 trials that investigated cytokine responses, 3 showed analogous responses to TLR.

In nine studies, one or more patient groups were included. Ten of the 17 responses recorded (10/17; 59%) showed unchanged TLR levels. TLR expression was increased in 5 of the 17 assessments (5/17; 29%) and decreased in two assessments (2/17; 12%). Pathway molecules showed concordance to the TLR response in 2 out of the 3 studies that performed the assessment.(40, 130, 150) Robinson *et al.* assessed inducible cytokine production in whole blood cultures, and found no effect of exercise training, corresponding to the observed TLR response.(153) On the contrary, Bartlett *et al.* reported increased *ex vivo* monocyte phagocytosis with stable levels of TLR2 and TLR4 expression on classical and non-classical monocytes.(11) Regarding the cytokine response, accordance to the TLR response was described in 4 out of 5 studies.(11, 40, 53, 130, 153)

No distinguishable patterns were found regarding the influence of age and health status on the TLR response. Three studies compared lean and obese subjects with a comparable physical activity status and reported similar responses to an aerobic exercise program.(130, 131, 150) Only three studies included non-sedentary subjects and no study included both sedentary and non-sedentary groups. Nickel *et al.* compared non-elite and elite athletes and no significant group differences were reported.(130)

Combined exercise program

Eight studies were considered eligible for the evaluation of combined exercise programs. All of the subjects were physically inactive. Some of the participants suffered from obesity or type 2 diabetes mellitus and one group was diagnosed with inflammatory myopathies. Patient age varied from college-aged to elderly. The shortest program spanned only two weeks, the longest 26 weeks. In the program of 2 weeks, 5 sessions per week were planned, the other programs scheduled 2-3 sessions per week. All of the programs combined aerobic and resistance exercises within the same session, although this was not clearly mentioned in one of the research articles. Three research groups studied the effects on vastus lateralis muscle tissue, the other groups analysed PBMCs or blood monocytes. Three of the 8 studies used flow cytometry, the other studies used gene expression analysis. One study performed mRNA and protein analysis simultaneously.(108) Only TLR2, TLR4 and TR7 were evaluated.

Only three studies investigated the effects of a combined exercise program in healthy subjects. The TLR levels were unchanged in three out of four assessments (TLR2 1/1 and TLR4 2/3), and one time decreased (TLR4 1/3). The result of the *ex vivo* stimulation assay matched the observed TLR alterations in 1 of the 2 studies performing this analysis.(180, 187) No TLR pathway molecules or cytokine values were assessed.

Five studies were conducted in participants with disease. Three downregulatory TLR responses (TLR4 3/3), one stable (TLR2 1/1) and one upregulatory (TLR7 1/1) response were reported. Pathway member alterations were concordant with TLR responses in 2 out of 3 studies (one with downregulatory and one with unaltered responses).(40, 108, 174) No *ex vivo* stimulation assays were performed. With regard to cytokine determination, the response was in accordance with the TLR response in two of the four trials determining the cytokine levels.(40, 94, 108, 124)

As mentioned above, all of the subjects were physically inactive. Therefore, it is unclear if baseline physical activity status influences the TLR response to a combined exercise program. Only two studies included subjects of young age. One study performed the comparison with an elderly group and an identical TLR response was reported.(180) No direct comparisons between healthy and diseased patients were made.

Interestingly, Colleluori *et al.* compared the effects of an aerobic, resistance and combined training program in obese and frail older adults (together with weight management). There was no difference in vastus lateralis nuclear factor kappa B (NF-κB), TNF-α or interleukin (IL) 6 expression compared to baseline in any of the groups, but TLR2 was significantly upregulated in the aerobic group only, albeit to a minimal degree (1.25-fold).(40)

Chronic exercise program – not otherwise classifiable

Lundeland *et al.* and Shimizu *et al.* organised training camps (a 7-day ranger training course and a 6-day kendo camp respectively) for the (trained) participants with repeated blood sampling. In the trial by Lundeland *et al.*, no significant changes in flow cytometric TLR4 expression were recorded. *Ex vivo* stimulation assay, however, did show increased cytokine production at day 3, followed by normalisation at day 5.(110) Shimizu *et al.* reported higher counts of TLR4-positive monocytes after 3 days, which persisted 7 days after ending of the camp.(167)

Rodriguez-Miguelez *et al.* assessed the impact of a whole body vibration program in seniors and demonstrated decreased protein levels of TLR2 and TLR4 in PBMC, together with lowered levels of signalling pathway molecules (MyD88, TRIF, p65) and concordant cytokine alterations (lowered TNF-α and raised IL-10).(155)

McFarlin *et al.* observed lower TLR4 expression in young and elderly subjects with an active lifestyle in comparison to their inactive counterparts, but no impact of age was recorded. (115) In a different trial, the same group compared baseline TLR4 expression in trained and untrained elderly women (before subjecting them to an exercise bout), and reported lower TLR4 expression in the trained group.(116) In a similar fashion, Flynn *et al.* subjected resistive-trained elderly women to a resistance training program, and used sedentary women who continued their normal activities as comparison. Lower TLR4 expression was reported in the trained group, although it is unclear which proportion can be attributed to the program and which to the preceding active lifestyle.(58) However, contrary results have been published. Timmerman *et al.* found that self-reported physical activity was not significantly correlated with muscle TLR4 protein level.(186) Also Ferrer *et al.* used questionnaires to assess physical activity and demonstrated higher TLR2 and comparable TLR4 levels in the most active elderly compared to the most sedentary, albeit with lower

plasma IL-6 and higher IL-10 gene expression.(62) The latter observations match those of Zheng *et al.* who compared physically active and sedentary students and reported higher mRNA levels of TLR2, TLR7 and MyD88 in the exercise group. TLR4 expression did not differ between both groups.(212)

DISCUSSION

General conclusion regarding the impact of exercise on TLR

Based on the aforementioned findings, a number of propositions can be made. Firstly, resistance exercise bouts seem to have an activating effect on TLR signalling in healthy individuals. This is in line with expectations as resistance exercise is classically considered a trigger of a robust inflammatory response, both local and systemic.(51) Repeated eccentric contractions cause damage to muscle and connective tissue with leakage of intracellular proteins. The exercise-induced muscle damage not only causes the typical delayed onset muscle soreness, but also underlies the ensuing hypertrophic response.(3) The two studies that did not demonstrate alterations in TLRs in response to acute resistance exercise were conducted in elderly women, which makes it tempting to refer to age-related blunting of the immune system. This contrasts with the review of Cavalcante *et al.*, who concluded that acute resistance exercise generally provokes a decrease in TLR expression.(27)

Secondly, an aerobic exercise bout seems to have less activating potential with regard to the TLR-system. In non-healthy subjects, generally unchanged TLR levels were observed. In healthy participants, also downregulatory responses, with even reduction of pathway molecules and cytokine end products, were reported in a significant number of articles. However, especially after intense bouts of longer duration, also upregulatory responses were recorded. These findings are in agreement with a recent systematic review on exercise and inflammation (without looking into the role of TLRs), which concluded that intensity and duration determine the magnitude of the inflammatory response that ensues a bout of exercise. Varying degrees of tissue damage could be a plausible explanation for the impact of these exercise characteristics. However, the same systematic review reported that the increase in inflammatory markers was not accompanied by a parallel increase in creatine kinase (CK) activity, which is considered a marker of muscle damage.(28)

Lastly, it seems that chronic exercise programs involving resistance training predominantly result in unaltered or decreased expression levels of TLRs and associated pathways and cytokines. In aerobic training programs, relatively more increased expression levels were recorded, although the majority of assessments still showed stable levels.

Cross-sectional analyses produced more ambiguous data regarding the effects of chronic physical activity. However, inherent to the trial design, these studies face difficulties with reliable quantification of physical activity and are highly susceptible to confounding factors in relation to an active lifestyle.(209)

Factors such as population studied (including baseline training status), exercise characteristics, the heterogeneous nature of exercise itself, environmental conditions (which could be related to induction of HSP synthesis), time of measurement and analytical method are likely responsible for the disparities in the reported results.(51, 67)

Animal studies may provide additional insight. In the systematic review of Rada *et al.*, it was reported that exercise training globally resulted in TLR downregulation, whereas acute interventions tended not to affect TLR expression.(145) In the present literature study, we observed equivocal responses following aerobic exercise training, with no predominant downregulatory trend present. The response after an acute exercise bout was more in line with the previously mentioned systematic review, as mainly unaltered and downregulatory responses were recorded.

Are alterations in TLR expression a bystander phenomenon of cell shifts?

Most investigators have focused on TLR expression on circulating leukocytes, and more specifically on monocytes. However, it seems often forgotten that exercise has a profound impact on the composition of the monocyte subsets present in the peripheral blood. Booth *et al.* already criticised the fact that the majority of studies do not take the altered monocyte composition into account when reporting on TLR expression.(17) Of note, the blind-sided focus on CD14-expressing cells does neglect the fact that TLRs are also expressed on other cells, for instance neutrophils, circulating progenitor cells, B-, T-, natural killer and dendritic cells.(130) These cell populations are also subject to exercise-induced cell shifts.(121) The relative importance of differences in TLR expression across different immune cells is currently not known.(153) However, it is certain that TLRs also fulfil important functions in these cell types, for instance in neutrophils were TLRs are implicated in cytokine production and cell survival.(43, 153) In the following paragraphs, we discuss the exercise-mediated alterations in leukocyte populations in the studies considered in this review.

Acute exercise

It is well known that acute exercise induces leukocytosis.(43) Factors as blood shear forces, body temperature, catecholamines, corticosteroids and cytokines are held responsible for the recruitment of cells into the blood stream.(23, 43, 48) This is considered an evolutionary conserved response to physical stress, preparing the body for potential injury or infection.(128) Typically, 'recovery' to the baseline leukocyte counts is observed within 24 hours.(117) Although the leukocyte shifts strongly varied between studies, a general trend of increased counts with normalisation within 24 hours could be discerned.

Figure 3 summarises the results of the 4 studies that evaluated shifts in monocyte subsets in response to an acute exercise bout. Exercise seems to increase the proportion of $CD14^{++}$/$CD16^{+}$, and especially $CD14^{+}$/$CD16^{++}$ cells. However, already after 1 hour of recovery, a reverse shift occurs. A fifth study showed similar results, but exact values were not available for inclusion.(146) The preferential mobilisation of pro-inflammatory monocytes to the circulation complicates the evaluation of exercise-induced alterations in TLR expression. However, this also means that reports on stable or lowered TLR levels are not merely the result of shifts in subpopulations, and this actually strengthens the relevance of these findings. In addition, some of the expression changes clearly exceed the magnitude of the parallel shifts in cell composition.(2)

Four groups evaluated the effects of exercise on TLR2 and TLR4 expression on individual monocyte subsets. The results are summarised in Figure 4. The majority of studies showed no changes of expression per subset.(17, 43, 170, 173) However, the few TLR expression changes that did occur, strengthen the assumption that there are more profound effects of exercise on TLR than just cell shifts.(2)

Two studies looked into dendritic cell shifts after exercise bouts (in both cases a marathon run). Running times were comparable and both studies expressed dendritic cells as a proportion of the total leukocyte count. The results were similar with conventional dendritic cell (cDC) proportion increasing and plasmacytoid dendritic cell (pDC) proportion decreasing immediately after the run. Re-measurement after 24 hours showed a tendency towards normalisation.(92, 129)

Chronic exercise

With regard to studies that investigated the effects of chronic exercise programs, generally no differences in total leukocyte count or leukocyte subpopulations (granulocytes, lymphocytes and monocytes) were described.(10, 11, 35, 41, 52, 58, 115, 128, 130, 142, 153, 167, 180) Only three studies reported (relatively minor) alterations for which we refer to Supplementary Tables 5-7.(22, 54, 168)

Five studies looked into the impact of chronic exercise on the distribution of monocyte subsets. Bartlett *et al.* performed two endurance regimen studies with assessment of monocyte distribution. In both cases, no alterations in total monocyte counts were observed. However, endurance exercise training increased the proportion of $CD14^{+}/CD16^{-}$ monocytes and reduced $CD14^{++}/CD16^{+}$ and $CD14^{+}/CD16^{++}$ cells.(10, 11) Similarly, Markofski *et al.* and Timmerman *et al.* reported reduction of the proportion of $CD14^{+}/CD16^{+}$ cells after a resistance training program and combined training program, respectively.(112, 187) On the contrary, Child *et al.* failed to find differences in monocyte subpopulations after their 2-week high-intensity training program.(35)

One study assessed TLR expression on monocyte subsets and reported no change of TLR2 or TLR4 expression on $CD14^{+}/CD16^{-}$ monocytes, but decreased TLR2 expression on $CD14^{++}/CD16^{+}$ and (limited) decrease in TLR4 expression on $CD14^{+}/CD16^{++}$ monocytes after the training program.(10) Only one group studied the impact of a resistance training program and demonstrated that the increased baseline proportion of $CD14^{+}/CD16^{+}$ monocytes in physically inactive, overweight individuals were reduced (to levels observed in active individuals) after the program.(112)

Regarding combined regimens, also Timmerman *et al.* reported higher baseline $CD14^{+}/CD16^{+}$ counts and proportions among physically inactive subjects, with normalisation after completion of their program.(187)

Reductions of inflammatory monocytes are most likely not indicative of increased infiltration into the tissues or migration towards lymphoid organs as murine studies have reported reduced leukocyte infiltration after exercise training.(87, 90, 174)

The impact of chronic exercise on DC subsets was evaluated by Lackermair *et al.* who noted stable cDC counts but lower pDC counts after 4-week preparation on a marathon run.(92) Nickel *et al.* saw similar trends in their obese subgroup, with higher baseline pDCs and lower cDCs compared to the lean groups and increase in cDC and decrease in pDC after the endurance exercise program.(130)

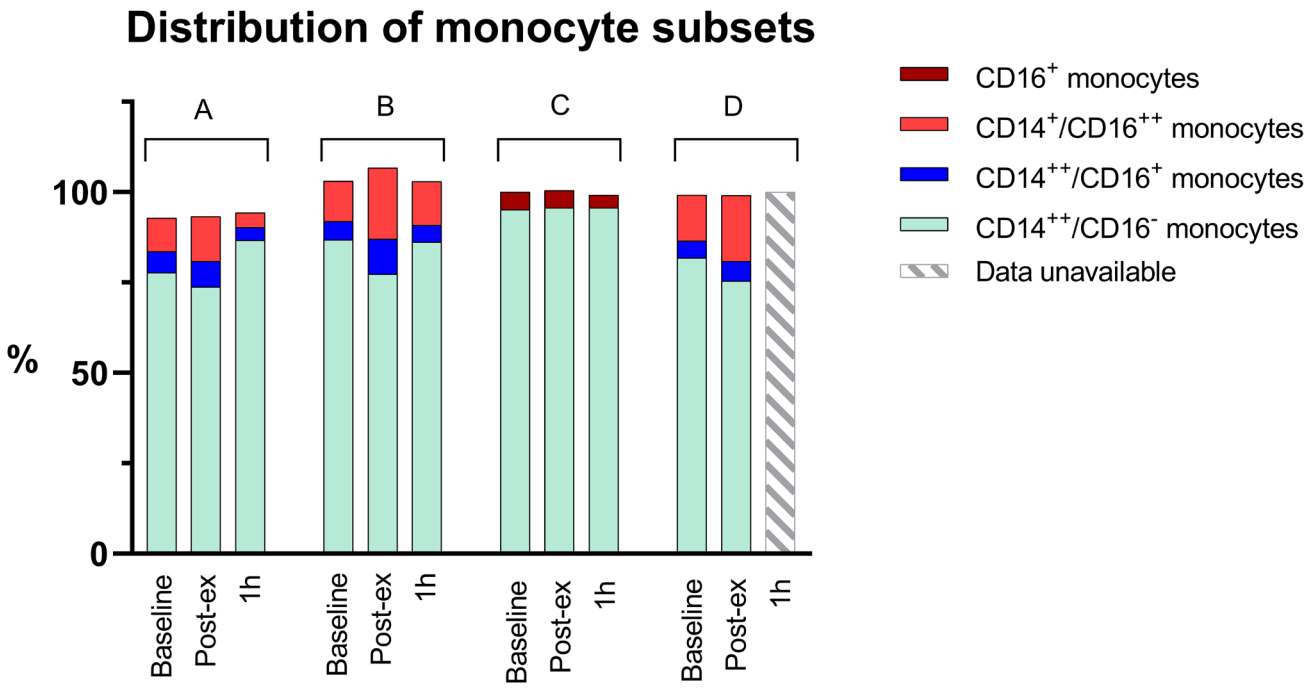
Mechanistic insights into the modulating effects of exercise on TLRs from the included human studies

As mentioned in the introduction, numerous factors have been proposed to explain the relationship between exercise and TLRs. Nevertheless, sound evidence *pro* or *contra* is lacking for the majority of factors. In the next paragraphs, we summarise current hypotheses linking exercise and TLR signalling. For each hypothesis, the most important findings of the articles included in this review are discussed. As this concerns several of the hypotheses, we would like to mention that Booth *et al.* provided evidence against an important role for serum soluble factors in general. They reported increased TLR2 and TLR4 expression on monocytes after exercise, but incubation of resting monocytes with post-exercise serum did not affect TLR expression.(17) However, a trend for elevated TLR4 was observed and the serum kinetics of soluble factors are not always accurately reflected when samples are taken at predefined time points.(110)

Myokines: it is well established that skeletal muscle acts as an endocrine organ with secretion of cytokines and small muscle-derived proteins, collectively termed 'myokines'.(2, 22, 27) In fact, cytokines released in the context of exercise are mainly produced and secreted by skeletal muscle. In contrast, cytokines released during chronic inflammatory diseases are supposed to originate from activated immune cells.(22) According to a recently introduced concept, skeletal muscle-induced anti-inflammatory myokines could even alter the inflammatory status of circulating immune cells.(174) It was suggested that cytokines released into the circulation are capable of exerting feedback on the TLR pathway.(96, 129) As such, a regulatory loop between TLRs and cytokines would exist.(154) For example, *in vitro* work has shown that IL-4 can downregulate monocyte TLR2 and TLR4 expression and that interferons can upregulate several TLR genes.(69, 120, 156, 176) Also IL-6 (which is typically elevated after exercise) and IL-1 (as the IL-1 receptor and Toll-like receptors share a similar cytoplasmic signalling domain) have been mentioned as candidates to explain the exercise-TLR interaction.(58, 136) None of the included articles could provide further information on the role of myokines in the relationship between exercise and TLRs.

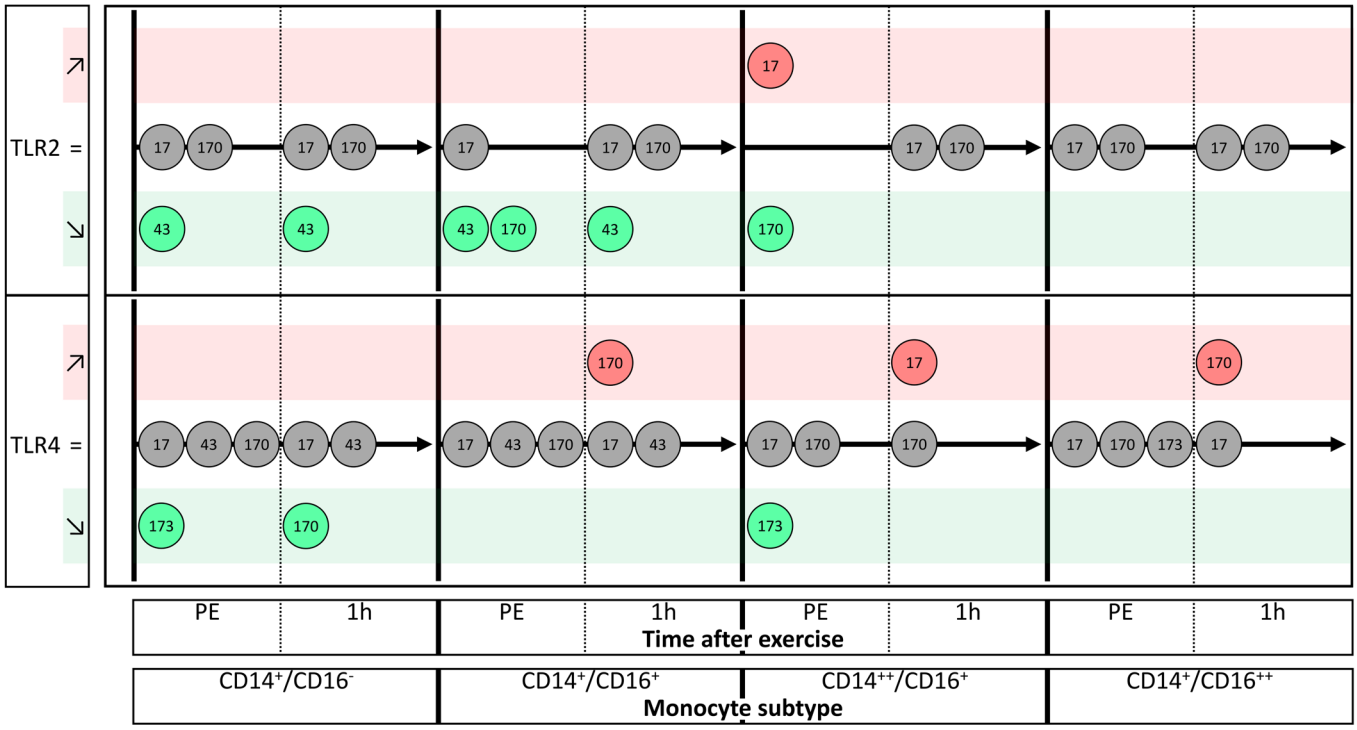
Obesity and hyperglycaemia: obesity and type 2 diabetes mellitus are associated with a pro-inflammatory status, both at cellular, TLR (including signalling pathway) and cytokine level.(10, 12, 38, 43, 108, 112, 115, 116, 129, 130, 142, 150, 174, 175, 187) A central role for adipose tissue has been proposed, related to the secretion of a variety of cytokines ('adipokines'). Increased levels of inflammatory adipokines, and reduced secretion of anti-inflammatory adipokines (*e.g.* adiponectin) would contribute to the observed systemic inflammation.(68, 103, 142) In addition, hyperglycaemia and fatty acids have been linked to TLR activation and increased TLR surface expression.(25, 43, 153, 174, 182) It has been argued that the anti-inflammatory effects of exercise are mediated by a change in body composition.(43, 112) However, numerous studies have

Figure 3 | Acute exercise-induced shifts in monocyte subsets.



The X-axis denotes time post exercise, with "post-ex" signifying immediately after (aerobic) exercise. The Y-axis represents the total monocyte proportion expressed as percentage. Deviations from 100 % are due to mathematical rounding. A) Simpson *et al.* 2009 (170); B) Booth *et al.* 2010 (17); C) Durrer *et al.* 2017.(43) The distinction between $CD14^{++}/CD16^{+}$ (intermediate) and $CD14^{+}/CD16^{++}$ (non-classical) monocytes was not made. Only the data of the healthy controls were considered; D) Slusher *et al.* 2018.(173)

Figure 4 | Impact of acute aerobic exercise on TLR expression on individual monocyte subsets.



The X-axis denotes the monocyte subpopulation and time after exercise, with post-exercise (PE) signifying immediately after exercise. The Y-axis indicates the change over time: increase (arrow up, red zone), stable levels (centre, white zone) or decrease (arrow down, green zone). Note that the $CD14^{+}/CD16^{+}$ category comprises both $CD14^{++}/CD16^{+}$ and $CD14^{+}/CD16^{++}$ cells. Each circle represents one study. The numbers in each circle are the corresponding reference.

demonstrated that the salutary actions of exercise are not (or at least not entirely) dependent on its effect on adipose tissue.(95, 174) Stewart *et al.* and Timmerman *et al.* have demonstrated that their combined training programs did not affect body fat percentage, but did significantly reduce the number of inflammatory monocytes.(180, 187) Child *et al.* reported that 2 weeks of high-intensity interval training (HIIT) did not change BMI or waist-to-hip ratio, but monocyte TLR4 expression was increased.(35) Markofski *et al.* subjected physically inactive adults to a resistance exercise program with one of the groups also receiving an energy restriction diet. The BMI and body fat percentage did not change in the resistance training only group, in contrast to the group that also received the diet intervention. Remarkably, only the group without diet intervention demonstrated decreased pro-inflammatory monocytes and LPS-stimulated TNF-α and IL-6 production.(112) Lambert and colleagues reported similar findings. In their study, muscle tissue TLR4 expression was reduced in the group that underwent a combined training program, but not in the group that received an energy-deficit diet (although both groups had reduction of the fat mass).(94)

The study of Robinson *et al.* provided information on the role of hyperglycaemia in the exercise-TLR interaction. Obese, sedentary prediabetic patients were subjected to short-term training, aimed to minimise alterations in body composition. Their moderate-intensity continuous training (MICT) protocol significantly reduced fasting plasma glucose, whereas the high-intensity training protocol failed to do so. The fact that they found reduced TLR4 expression on monocytes and lymphocytes after both training protocols, argues against plasma glucose as important mediator.(153)

Endotoxemia: obesity, type 2 diabetes mellitus and high-fat feeding are associated with increased circulating endotoxin levels, which is referred to as 'metabolic endotoxemia'.(18, 61) Also strenuous exercise can increase translocation of LPS from gut bacteria into the circulation.(17) It was demonstrated that even low-dose circulatory LPS can trigger TLR pathways.(208) On the contrary, it seems that chronic exercise decreases the gut permeability, hereby reducing TLR signalling activation.(39) At present, insufficient data is available to evaluate the significance of endotoxemia in the proposed relationship between exercise and TLRs. Jin *et al.* studied 20 obese middle-aged women and concluded that their combined training program suppressed the peak postprandial endotoxemia, but TLRs were not evaluated.(82)

Fatty acid (FA) composition: another mechanism relates to the effect of exercise on fatty acid composition of blood and tissue lipids. In their extensive review, Nikolaidis and Mougios concluded that exercise leads to an acute increase in unsaturated (especially monounsaturated), non-esterified fatty acids (NEFAs) in the plasma.(132) This is not surprising as NEFAs are an important metabolic fuel. Noteworthy, NEFAs are able to activate TLR2 and TLR4, although direct binding remains uncertain.(198, 211) Zhou *et al.* reported that *in vitro* free fatty acid treatment of bone marrow-derived macrophages of diet-induced obese (DIO) mice downregulated TLR2 expression, which could fit the proposed mechanism of downregulation by exposure.(214) Chronic exercise seems to increase the proportion of polyunsaturated FA (PUFA) and omega-6 FA and decrease the relative amount of monounsaturated FA in adipose tissue.(132) PUFAs can inhibit agonist-induced TLR4 activation.(110) Of the included trials, only four determined plasma NEFA level.(11, 12, 24, 65) In none of the studies, significant differences in baseline or post-exercise values between young and old or between obese, diabetic or athlete participants were recorded. Two trials evaluated the response to acute exercise, both of which observed higher levels in conjunction with an aerobic bout, as would be expected.(12, 24) One of the aforementioned trials also determined NEFA level during recovery (2 hour post-exercise), and recorded even higher values. In contrast, muscle TLR4 protein (and other molecules involved in TLR signalling) levels remained unaltered during and after the exercise bout.(12) Two groups that evaluated the effects of an endurance exercise program did not detect significant changes in NEFA level after completion.(11, 65) Currently, there is no evidence to support the hypothesis that exercise causes cascade activation of TLRs through increased levels of NEFAs.(95, 198) Moreover, it was very recently demonstrated that long-chain saturated fatty acids are not TLR4 agonists after all.(97)

Corticosteroids: physical activity elicits endocrine responses, including elevated levels of corticosteroids (gluco- and mineralocorticoids).(31, 109) It was shown that this upregulation involves IL-6 mediated stimulation of the adrenal glands.(27) Corticosteroids have far-reaching effects on the immune system, including selective depletion of inflammatory monocytes and modulation of NF-κB transcriptional activity.(96, 187) In human corneal fibroblasts, hydrocortisone-mediated reduction of mRNA and protein expression of TLR2 and TLR4 has been demonstrated.(83) As such, they have been considered as candidates to explain the effects of exercise on the TLR system.(96) However, there are several arguments that argue against an important role for glucocorticoids in the regulation of TLR expression *in vivo*. Lancaster *et al.* could not demonstrate circadian rhythmicity in TLR1, 2, 4 or 9 expression on $CD14^+$ monocytes, despite plasma cortisol concentration being more than two times higher in the morning compared to the evening. In addition, the plasma cortisol concentration immediately after exercise was not different from the pre-exercise value, which contrasts with already significant alterations in TLR expression immediately after exercise. In the same study, it was also demonstrated that LPS and zymosan stimulation differentially affected intracellular IL-6 expression in monocytes taken from post-exercise blood samples. This finding contrasts with the suppressive effect of dexamethasone in physiological concentrations on LPS- and zymosan-stimulated intracellular monocyte IL-6 production.(96) *Ex vivo* research from other investigators using dexamethasone incubation produced inconsistent results regarding TLR expression, with differences between receptors and within a single receptor.(69) The training program of Bartlett *et al.* reduced the expression of TLR2 and TLR4 on monocytes, but plasma cortisol was unchanged.(10) These arguments of course do not exclude the possibility that corticosteroids exert their effects indirectly, *e.g.* via cytokines or alterations in leukocyte subpopulations.(69)

Heat shock proteins: HSPs are a family of intracellular proteins that function as molecular chaperones by supporting the folding, unfolding and transport of other proteins.(58, 66, 180)

They are upregulated in stress conditions to protect the cell.(58) However, HSPs can also act as cytokines ('chaperokines') when released from damaged tissue, *e.g.* by activating TLR2 and TLR4.(58, 66, 180) HSP60 and HSP70, two highly conserved and expressed members, are among the many DAMPs recognised by TLR2 and TLR4.(76, 154) Nevertheless, increased HSP levels might actually be beneficial. Repeated exposure to HSP60 induces a tolerance to HSP and cross-tolerance to LPS stimulation, presumably through downregulation of TLR4.(76, 94, 136, 180) In addition, HSP70 seems to block NF-κB activation at different levels.(76, 136) So perhaps HSP shouldn't be considered as DAMPS, but rather as DAMPERs. It remains to be investigated whether HSPs can also transmit anti-inflammatory signals through (instead of by blocking) the TLR signalling cascade.(193) Exercise transiently increases HSPs in an intensity- and frequency dependent manner.(124, 128, 141, 154, 156) Both glucocorticoids and catecholamines could be involved in this HSP response.(139) The available evidence suggests that HSPs are potential candidates to explain the effects of exercise on TLRs, but further research is warranted.(180) In the study of Falgiano *et al.* TLR4 and HSP70 expression showed similar kinetics, with both protein levels being downregulated one hour after exercise compared to 4 hours after.(48) Also chronic exercise can modulate HSP levels. An 8-week training program in healthy seniors led to increased protein levels of HSP70 and decreased levels of HSP60. TLR2 and TLR4 protein content was decreased after the end of the program.(154) Young female handball players showed no increase in extracellular HSP70 throughout their training season, but the lymphocyte HSP70 content was higher at the middle and end of the season compared to the beginning.(199) There are, however, also (low-intensity) training programs that failed to see changes in HSP expression.(169) Exertional heat stress is thought to have a direct effect on TLRs, independent from HSPs.(194) However, Gleeson *et al.* observed surface downregulation of TLR1-TLR4 after a cycling bout without alterations in body core temperature.(69) The same group also incubated $CD14^+$ monocytes at different temperatures (22°C, 37°C and 40°C) and found no effect on CD14, TLR1, TLR2 or TLR4 expression.(69) This is in line with the results of Zhou *et al.* who demonstrated that prolonged incubation at 42°C did not change cell surface expression of CD14, TLR2 or TLR4 on human monocytes (despite upregulation of TLR2 and TLR4 mRNA and cytoplasmic HSP70 content).(213) Capó *et al.* reported that their maximal exercise test increased core (up to 39°C) and skin (up to 34.8°C) temperature in young and old athletes, but no differences in TLR gene expression in PBMC were observed.(24) On the other hand, VanHaitsma *et al.* demonstrated that acute exercise in temperate or hot conditions both reduced TLR4 expression, but the suppression persisted substantially longer in the group that exercised in hot conditions.(194)

Nucleic acids: cell-free DNA (cfDNA) and microRNA: it has been well established that circulating cfDNA concentrations increase immediately after exercise, with a rapid return to baseline. Circulating DNA is one of the known DAMPs for TLRs.(19) Growing evidence suggests that miRNAs can modulate immune functions in response to exercise.(39) MiRNAs readily appear in plasma and leukocytes following an exercise bout. Further, chronic exercise training can modulate circulating miRNA responses.(2, 8, 39) For the role of cfDNA in the exercise-TLR interaction, data are currently lacking, but for miRNAs, scarce evidence is available. Radom-Aizik *et al.* evaluated the effects of 30 min of strenuous exercise on miRNA expression in young healthy men in two different trials.(146, 147) In the first trial, exercise altered expression of 34 miRNAs in PBMCs, of which many were involved in inflammatory processes.(147) In the second trial, the expression of 19 miRNAs was altered in monocytes, again including miRNAs related to inflammation.(146) Among others, exercise alters expression of miRNA-132, miRNA-125b and let-7e, which are known to regulate TLRs in monocytes.(147) In addition, TLRs have also been proposed as receptors for circulating miRNAs, although the significance of this finding remains unclear.(47)

Reactive oxygen species (ROS): it is well known that oxidative stress levels are modulated by physical activity. Regular exercise downregulates oxidative stress.(46) On the contrary, unaccustomed and/or exhaustive exercise can generate excessive ROS.(27, 75) ROS have the potential to activate NF-κB and mitogen-activated protein kinase (MAPK) pathways and to modulate the intracellular TLR signalling cascades which converge to these same pathways.(65, 161, 181, 204) In addition, it has been reported that ROS production can upregulate TLR expression.(167, 181)

Three studies looked into oxidative stress levels. Cheng *et al.* reported downregulation of TLR4 mRNA, upregulation of superoxide dismutase and catalase, and reduced levels of hydrogen peroxide (H_2O_2) after their lower back training program. They suggested that upregulation of the inflammation related gene sirtuin-1 may be the link between exercise and the beneficial effects on oxidative stress.(34) On the other hand, Falgiano and colleagues reported downregulation of TLR4 and sirtuin-1 immediately after exercise, with similar kinetics.(48) Ferrer *et al.* categorised elderly volunteers according to self-reported physical activity and found higher TLR2 levels in active participants compared to their sedentary counterparts, but without differences in myeloperoxidase (MPO) levels.(54) The aerobic training program of Estébanez *et al.* did not change TLR4 protein levels, or oxidative stress biomarkers.(46)

Matrix metalloproteinases (MMPs)-mediated TLR shedding: MMPs are a class of enzymes that participate in ectodomain shedding.(39) The limited data available suggests that exercise modulates MMP levels.(127, 160) MMP-mediated ectodomain shedding could contribute to decreased TLR expression on immune cells after exercise.(39, 43) Additionally, the TLR ectodomain can negatively regulate TLR activation by acting as a decoy receptor.(98) However, at present, no evidence is available linking exercise to MMPs and soluble and membrane-bound TLR levels. The hypothesis of exercise-induced TLR shedding remains to be tested.

Mechanistic insights into the modulating effects of exercise on TLRs from animal studies

The majority of animal research on TLRs has been conducted in rodents. In general, comparisons of animal and human TLRs is complicated by substantial interspecies differences (includ-

ing transcriptional regulation and cellular expression).(69, 77, 149) To the best of our knowledge, no animal research with the specific purpose to elucidate the link between exercise and TLRs has been performed. However, regarding the aforementioned hypotheses, several observations are worth mentioning.

Firstly, some studies have reported that changes in TLR expression in response to exercise may be tissue specific, while others failed to find such differences.(104, 105, 107, 159) Regarding the mechanism(s) underlying the exercise-TLR interaction, a regulatory feedback loop through myokines remains a viable hypothesis. Several studies have simultaneously compared myokine and TLR expression, thereby supporting this association, but no conclusive information on causality was provided.(37, 81, 86, 105, 107, 114, 135, 156, 183, 210) Ropelle *et al.* reported that injection of recombinant IL-6 did not change TLR4 expression in rats with diet-induced obesity, although only hypothalamic tissue was evaluated.(158)

Animal research has provided additional arguments against the hypothesis that changes in body composition mediate the effects of exercise. Carpenter *et al.* demonstrated that sedentary DIO mice with weight loss showed increased monocyte TLR expression, in contrast to voluntary running DIO mice with weight loss who showed decreased TLR expression.(25) Similarly, two studies demonstrated that (forced) exercise training did not induce loss of body weight or adipose tissue in high-fat diet-fed mice, while exercise did reduce the higher TLR4 levels in these mice.(45, 87) Interestingly, it has been shown that TLR4 itself influences murine body composition. TLR4-deficient mice fed a high-fat diet gained less body fat compared to wild-type mice.(84)

Further, studies in rodents have generated evidence against a driving role of endotoxin and corticosterone. In the rat study by Liao *et al.*, downhill running resulted only in a very late (24 hours after exercise) increase in muscle endotoxin levels. In contrast, TLR4 mRNA changes were already observed 1 hour after exercise.(105) Lira *et al.* confirmed in a murine model that overtraining increases circulating endotoxin levels. Also corticosterone levels were elevated in the overtrained group. Nevertheless, hepatic and adipose tissue TLR4 protein content was not altered.(107) Oliveira *et al.* demonstrated in rats that exercise reduced TLR4 mRNA levels in adipose tissue, but no impact on serum corticosterone was observed.(135)

As in humans, HSP70 concentrations in animals increase in an intensity- and frequency-dependent way after exercise.(39, 154, 211) No further information on the validity of the hypothesis that HSPs plays an important role in the effect of exercise on TLRs is available from animal research.

In addition to the aforementioned miRNAs, Wu *et al.* reported that aerobic exercise increases miRNA-126 and miRNA-146a, while reducing TLR4 protein expression.(203) Interestingly, it has been shown that miRNA-146a can negatively regulate TLR4.(206) Further research has revealed that miRNA-146a can interact with the TRAF6 gene (which is involved in the non-MyD88-dependent TLR signalling pathway), negatively modulating its expression.(203)

Animal research has provided mixed results regarding the role of ROS. In the trial by Li *et al.* in rats, malondialdehyde (MDA) and superoxide anions showed a similar response to exercise compared to TLR2 and TLR4 mRNA and protein levels.(104) Similarly, in the trial by Rodriguez-Miguelez, MPO showed a parallel pattern after exercise as the TLR4 level.(156) On the contrary, Liao *et al.* observed a delayed increase in MPO and hydrogen peroxide, whereas TLR4 mRNA rapidly decreased after exercise, and remained decreased.(105)

Lastly, *in vitro* work by Chen *et al.* using C2C12 myoblasts has shown that mechanical stretch inhibited TLR3 expression. The precise molecular mechanism by which mechanical strain regulates TLR3 levels is not yet clear, but this could be very relevant in the context of exercise and TLRs.(32)

In summary, the limited data available in animals and humans make it difficult to draw definite conclusions regarding the underlying mechanisms of the observed TLR changes. However, as discussed above, it currently seems unlikely that these are mediated by changes in body composition, serum glucose, serum fatty acids or serum corticosteroids. Further research is required to determine the factors underlying the relationship between exercise and TLRs. Several mechanisms are likely to be involved simultaneously.

Heterogeneity in the methodological approach

Critical remarks need to be made regarding some of the diagnostic methods and reported results.

Only 7 studies simultaneously applied different analytical techniques to assess the same TLR molecules.(51, 52, 54, 108, 116, 129, 130) Although the majority of results were concordant or trending towards concordance, completely opposite results at transcript and protein level were also reported. This was also the case for molecules involved in the TLR signalling cascade and cytokines in other studies.(52, 156) In general, correlations between mRNA and protein expression levels are notoriously poor, although this is also dependent on the gene class. It is known that genes that are differentially expressed by experimental manipulation are more likely to show concordant protein expression across the same experimental conditions.(91) Interestingly, Guo *et al.* performed a mRNA-protein correlation study in human circulating monocytes. They concluded that genes belonging to the extracellular region of cell components and genes with signal transducer activity showed the highest correlation, however, expression at mRNA level was found to be generally informative but not predictive for that at protein level.(71) From this perspective, it is problematic that 25 studies solely determined TLR expression at transcript level, as proteins should still be regarded as the major direct executors. Perhaps, TLR internalisation and/or shedding are in part responsible for the observed discrepancies.

Most researchers studied repetitive venous blood samples. However, as plasma cytokines originate from 'spill over' from various organs and tissues, important changes at the cellular level may not be detected in the plasma.(153) For example, Lambert *et al.* showed decreased expression of IL-6 and TNF-α in muscle tissue in response to a combined training program, but not in the serum.(94) Therefore, peripheral blood findings cannot be generalised to tissue responses.(48) Immune responses may also vary in different parts in the body.(110) Nevertheless, assessment of blood monocytes may be a good proxy/convenience measure of TLR expression and inflammatory capacity of macrophages found in different tissues (*e.g.* adipose tissue and skeletal muscle). These peripheral tissue macrophages are deemed to be to a large extent responsible for whole body chronic inflammation.(69, 95)

Whole blood stimulation assays (frequently using LPS

as TLR-stimulant) is a commonly used technique to correlate findings on expression level with functional alterations. However, when making such correlations, it should be considered that also other cells besides monocytes can respond to LPS stimulation.(112, 173) Some of the effects of exercise only became evident after stimulation assay (and were not visible in native plasma or unstimulated cultures), which raises the question whether these results are reflective of (the complex) *in vivo* responses.(1, 2, 68, 96, 110, 121) In addition, large heterogeneity was found regarding incubation times, cell preparation and stimulant dose.(172)

The unit of expression is of critical importance for reporting of the results. For example, in the study of Niemiro *et al.*, the proportion (%) of progenitor cells expressing TLR4 was unchanged, but the concentration (cells/μL) of progenitor cells with surface TLR4 decreased, and the TLR4 expression level (median fluorescence intensity) increased.(131) Capó *et al.* used a PBMC stimulation assay. Acute exercise increased cytokine production in response to LPS. However, no differences were observed when results were expressed per monocyte, but these results were not shown.(23) Also Durrer *et al.* reported different results between absolute and leukocyte-corrected cytokine release following a stimulation assay.(43) Another study noted that the number of monocytes that responded to LPS stimulation by producing interleukines was increased, but that the stimulated cells produced less interleukines.(178) This is in contrast to the observations of Markofski *et al.* who saw no differences in stimulated cytokine production when corrected for the number of monocytes, but did find lower numbers of inflammatory monocytes after exercise.(112)

We would like to make a concluding remark regarding physiological variation that can potentially influence the results. The research group of Lancaster investigated diurnal rhythmicity of TLR expression and activation. Monocyte surface expression of TLR1, 2, 4 and 9 showed no difference between morning and evening samples. Neutrophil expression of TLR1, 2 and 4 also remained constant, but TLR9 was expressed at lower levels in the evening. Upon *ex vivo* stimulation, monocyte expression of costimulatory molecules was significantly greater in the evening samples.(96)

It was also demonstrated that monocytes of normal healthy subjects show a decline in LPS-stimulated TNF-α production from summer to autumn.(125)

In summary, we believe that the ideal methodological design should not solely focus on Toll-like receptors, but also take into account downstream pathway molecules and end products. Changes at the transcript level should be correlated to the protein level and *vice versa*. Localisation and trafficking of TLRs could provide insight into the fate of membrane-bound TLRs after exercise. It would also be interesting to compare changes in peripheral blood with those in skeletal muscle. Additional stimulation assays can provide valuable functional information. When reporting the results, researchers should present absolute numbers rather than relative measures.

Functional relevance of the observed alterations

Monocyte surface expression of TLR seems to have clinical relevance as this is known to differ and/or predict outcomes in the setting of sepsis, tuberculosis and chronic liver failure.(17) Furthermore, monocytes from donors who are homozygous for certain TLR4 mutant alleles are clearly hyporesponsive for LPS.(163) In addition, a significant number of studies have found an association between cytokine production in stimulation assays and TLR expression.(22, 43, 96, 115) However, regarding the stimulation assays, also the opposite holds true.(43, 112, 121, 153, 172, 173, 180, 187) Assessment of molecules involved in TLR signalling has varied from consistent regulation of each of the steps to completely conflicting results. This is also the case for plasma or serum cytokines. Cavaillon has dedicated an entire review to the debunking of the simplistic dichotomy of pro- and anti-inflammatory cytokines. Actually, a given cytokine may behave as a pro- and anti-inflammatory cytokine.(26) For example, elevated IL-6 is considered a marker of a pro-inflammatory disease pathology, but systemic injection of IL-6 increases plasma IL-10 concentrations and inhibits TNF-α release after LPS injection in healthy humans.(173, 177)

Furthermore, TLRs are just one of the many factors that determine the immune system functionality. For example, Bartlett *et al.* demonstrated enhanced neutrophil and monocyte phagocytic capacity and oxidative burst, despite decreased and unchanged expression of CD16, TLR2 and TLR4 on monocytes and neutrophils respectively.(10)

It should be noted that also other mechanisms can confer the effects of exercise on TLRs besides gene or protein expression. For example, stimulation by certain ligands could eventually lead to tolerance with reduced TLR responsiveness.(39, 95, 142) In addition, it was recently demonstrated in blood cultures that exercise can upregulate expression of genes involved in the negative regulation of TLR signalling. (2, 43) Overall, it remains however highly conceivable that the exercise-induced alterations in TLRs do have functional consequences.

Transient TLR activation following exercise may actually reflect a positive response that is necessary to facilitate debris clearance from muscle damage and regulate vascular function. (172, 173) Acute bouts of exercise are also held capable of selective clearance of dysfunctional immune cells, improving the functionality of the remaining pool.(10) From an evolutionary perspective, a robust response to a potential threat could actually be beneficial, as long as the response does not persist beyond clearance of the danger.(172) Short-term acute inflammation even allows the body to survive progressive tissue destruction by promoting healing.(27) The impact of acute exercise on TLRs can also be linked to the controversial and highly debated theory that exercise (especially if strenuous or prolonged) leads to a temporary decrease in immune competence.(129) This is referred to as an 'open window' of increased susceptibility for infection.(92, 117, 156) As such, downregulation of TLRs after exercise could be involved. However, to date, there is no hard evidence to support the presumptions that exercise impairs immune cell function or temporarily increases vulnerability for infection.(20) Whether the open window truly exists, is still the subject of intense debate.(2, 48, 129)

On the other hand, the association between sedentarism, aberrant TLR activation, chronic inflammation and numerous chronic disease states underscores that long-term TLR upregulation and/or activation is likely detrimental.(69) As such, the long-term health benefits of exercise could be mediated (in part) by modulation of TLR responses.

Limitations

Despite the value of translational research in experimental animal models, this systematic review was limited to studies reporting TLR in human subjects. Substantial between-study heterogeneity was observed with regard to the investigated population, the exercise intervention, the TLR evaluation method and the reporting of the outcomes, even when the studies were categorised according to exercise modality and study population. Because of the heterogeneity, it was not practicable to perform a meta-analysis on the published results. Lastly, several studies had small sample size, possibly leaving them underpowered to detect an impact of exercise.

Conclusion and future perspectives

To the best of our knowledge, this is the first systematic review that addresses the effects of acute and chronic exercise on TLRs in humans, without exclusions based on TLR type or health status. According to our findings, the effect of exercise on TLRs is dependent on exercise modality and duration. Acute resistance exercise seems to elicit TLR signalling activation. Acute aerobic exercise does not affect, or especially in healthy participants, even lowers TLR levels. Particularly in intense bouts of longer duration, also increased TLR activation was reported. Exercise training programs generally result in stable or decreased expression levels of TLRs. Within these exercise programs, upregulatory responses were almost exclusively described in aerobic programs. However, the substantial heterogeneity between studies limits the generalisability of these findings. Complementary research, paying attention to careful study design, meticulous phenotyping of the study population, shifts in leukocyte populations, analytical methods (correlating findings at transcript and protein level) and analytical target selection (corroborating TLR findings by evaluation of pathway molecules and functional assays) are required. Future studies comparing the influence of different exercise modalities, durations and intensities could add invaluable information to the field. In addition, the impact of the patient characteristics (age, baseline physical activity, health status) on the TLR response to exercise merits further investigation.

Several features suggest that the anti-inflammatory effects of exercise may be mediated through TLR pathway modulation. However, these effects are most likely not limited to TLR alterations. For instance, in this review, we also described reduced presence of inflammatory cell populations after exercise programs.

Finally, the mechanism(s) underlying the effects of exercise on TLRs remain to be elucidated. Several viable theories have been proposed, but sound evidence is lacking. According to our opinion, several factors are most likely involved simultaneously, with host, exercise and environmental characteristics determining their contribution.

Author contributions

KF and MB were responsible for the article selection process, data extraction and quality and bias assessment. Any disagreements were discussed together with the other authors. KF drafted the manuscript. MB, PD, HF, EVC, JDS, IW, GDM, HH and PJG critically reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

K.F. received personal funding support from the special research fund (BOF) of the University of Antwerp (grant no. FFB190355 and FFB190229). M.B. is a doctoral research fellow of the Research Foundation Flanders (FWO) (grant no. 1S33720N).

Conflict of interest

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

Toll-like receptor (TLR) signalling pathways

TLRs interact with their respective ligand as a homo- or heterodimer along with a co-receptor or accessory molecule.(88) For example, CD14 is a well-known co-receptor for both TLR2 and TLR4.(59) All TLRs share a similar cytoplasmic signalling domain, the Toll/IL-1 receptor (TIR) domain, that initiates the downstream signalling cascade by differentially recruiting members of a set of five TIR domain-containing adaptor proteins.(80, 88, 133, 188) These signalling cascades are discussed in more detail in the next paragraphs and are schematically illustrated in Supplementary Figure 1.

Myeloid differentiation primary response gene 88 (MyD88) is considered the 'universal adaptor protein' as it is used by all TLRs, except for TLR3.(9, 130) The MyD88-dependent pathway sequentially involves IL-1R-associated kinases (IRAKs), TNFR-associated factor (TRAF) 6 and TGF-β-activating kinase (TAK) 1.(9, 88) TAK1 in turn activates two different pathways. In the first pathway, TAK1 activates the IκB kinases (IKK) complex, leading to degradation of the inhibitory protein IκB.(52, 88) Under normal conditions, IκB binds to nuclear factor kappa B (NF-κB) and retains it in the cytosol.(48, 51, 111) The transcription factor NF-κB is a master regulator of the inflammatory response.(155) Degradation of IκB enables translocation of NF-κB into the nucleus with subsequent activation of a wide variety of genes, including genes encoding pro-inflammatory cytokines.(54) The second TAK1-mediated pathway results in activation of mitogen-activated protein kinase (MAPK) family members, such as extracellular signal-regulated protein kinase (ERK) 1/2, p38 and c-Jun N-terminal kinase (JNK). This ultimately leads to activation of the pro-inflammatory transcription factor activator protein-1 (AP-1).(52, 88)

Alternatively, TLR3 and 4 are capable of signalling through a non-MyD88-dependent pathway. The cascade starts with the recruitment of TIR-domain-containing adaptor protein-inducing interferon-β (TRIF). TRIF interacts with TRAF6 and TRAF3. In this pathway, TRAF6 uses the kinase receptor-interacting protein (RIP) 1 to activate the TAK1 complex, leading to induction of inflammatory cytokines through NF-κB and MAPKs as explained above. TRAF3 recruits TANK-binding kinase 1 (TBK1) and inducible IκB kinase IKKi (also known as IKKε) for phosphorylation of interferon regulatory transcription factors (IRFs) 3 and 7, enabling their translocation to the nucleus where they facilitate transcriptional activation of genes responsible for encoding type I interferons (IFNs).(50, 64, 73, 88, 133, 154, 207) IFNs are multifunctional cytokines that play an important role in the first line defence against viral infections.(101)

Besides MyD88 and TRIF, three other TIR domain-containing adaptor proteins exist. MyD88-adaptor-like (MAL) (also termed TIRAP) and TRIF-related adaptor molecule (TRAM) act as bridging adaptors for MyD88 and TRIF respectively. The fifth adaptor protein, sterile α- and armadillo-motif-containing protein (SARM), seems to negatively regulate NF-κB and IRF activation, although more research is required in this regard.(88, 133)

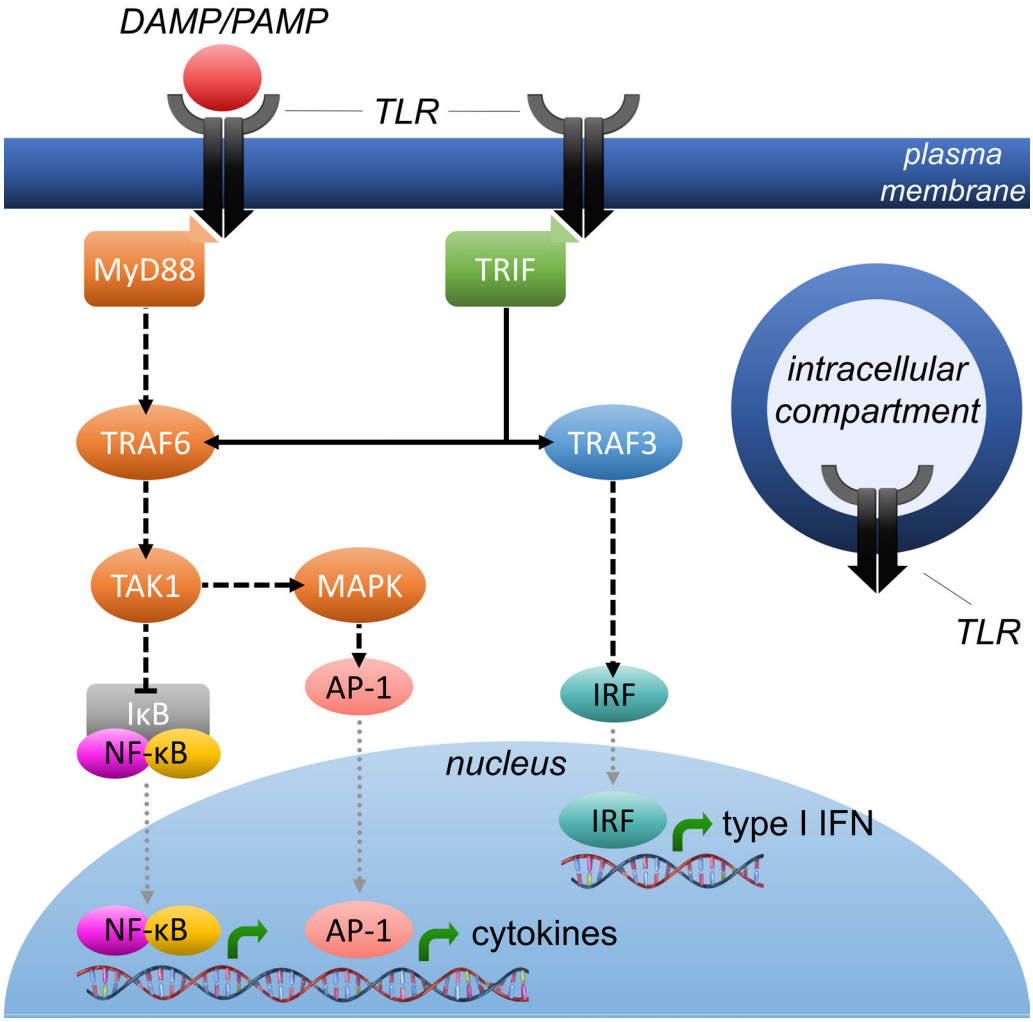
So ultimately, TLR signalling activates transcription factors that promote transcription of a wide variety of genes typically leading to the production of pro-inflammatory cytokines (*i.a.* interleukine (IL) 1, IL-6, tumor necrosis factor alpha (TNF-α), chemokines and type I IFNs).(1, 9, 69) This way, TLRs are pivotal members of the innate immune system. Additionally, TLRs play an important role in priming of adaptive immune responses, including upregulation of antigen-presenting molecules (*e.g.* major histocompatibility complex (MHC) class II), co-stimulatory molecules (*e.g.* CD80/CD86) and specific cytokines (*e.g.* IL-6 and IL-12) in antigen-presenting cells.(9, 69, 96, 136)

Toll-like receptor expression and monocyte and dendritic cell subtypes

TLRs are predominantly expressed in and on immune cells. In exercise literature, most investigators have focused on monocytes and, to a lesser degree, on dendritic cells due to their diverse role in inflammation and immune function.(10, 43)

In monocytes, TLR expression varies according to the subtype. Monocytes are categorised according to the expression of CD-molecules on their cell surface. Classical monocytes express high levels of CD14, but lack expression of the Fc receptor CD16 ($CD14^{++}/CD16^{-}$).(173) Approximately 10-20% of monocytes co-express CD16 and are considered pro-inflammatory. These are further subdivided into an intermediate ($CD14^{++}/CD16^{+}$) subset and a non-classical ($CD14^{+}/CD16^{++}$) subset.(35, 170, 173) Interestingly, pro-inflammatory (CD16-positive) monocytes are characterised by higher surface expression of TLR2 and TLR4.(38, 96, 112, 170, 173, 187) Upon stimulation with lipopolysaccharide (LPS), classical monocytes produce IL-6 (a predominantly pro-inflammatory cytokine) and IL-10 (an anti-inflammatory cytokine). On the other hand, pro-inflammatory monocytes produce IL-6 and the pro-inflammatory TNF-α, but little or no IL-10 following LPS-stimulation.(96, 170, 173) They also express a greater level of HLA-DR (MHC class II) suggesting higher capacity for antigen presentation.(170) It is therefore not surprising that increased circulating levels of pro-inflammatory monocytes are associated with a number of chronic diseases.(38, 112, 187) Dendritic cells (DCs) constitute two major subsets in humans.(42) Myeloid (or conventional) DCs (cDCs) are the largest fraction and use TLRs 1, 2, 4-6 and 8 to detect mainly bacterial pathogen-associated molecular patterns (PAMPs). Upon activation, cDCs secrete pro-inflammatory cytokines and present antigens. Plasmacytoid DCs (pDCs) use TLRs 7 and 9 to detect mainly viral RNA and DNA, respectively.(92) They are less proficient in antigen presentation and react to activation with the production of type I IFNs and other cytokines.(41, 129)

Supplementary Figure 1 | Simplified overview of the MyD88-dependent and –independent signalling pathway in Toll-like receptor activation.



TLR1, TLR2, TLR4-6 and TLR10 localise to the cell surface. In contrast, TLR3 and TLR7-9 are confined to intracellular compartments such as endosomes, (endo)lysosomes and endoplasmic reticulum. Differential adaptor molecules provide specific signalling pathways in different TLR family members. These pathways are described in detail in the text. Full lines indicate direct interaction. Dashed lines indicate indirect linkage. Abbreviations: AP-1, activator protein-1; IC, intracellular; DAMP, damage-associated molecular pattern; IRF, interferon regulatory transcription factor; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary response gene 88; NF-kB, nuclear factor kappa B; PAMP, pathogen-associated molecular pattern; TAK, TGF-β-activating kinase; TLR, toll-like receptor; TRAF, TNFR-associated factor; TRIF, TIR-domain-containing adaptor protein-inducing interferon-β.

Supplementary Table 1 | Overview of the cellular location, PAMPs, DAMPs and synthetic ligands of the different human Toll-like receptors.

Receptor	Cellular location	Ligand: PAMP	PAMP-related pathogens	Ligand: DAMP	Synthetic ligand
TLR1	Cell surface(88, 188)	Peptidoglycan(9) Triacylated lipoproteins (TLR1/TR2 heterodimer)(36, 50, 207)	Bacteria (primarily gram-positive)	β-defensin(63)	Pam_3Cys, Pam_3CSK_4
TLR2	Cell surface(88, 188)	Lipoproteins, peptidoglycan, lipoteichoic acid, zymosan, mannan, hemagglutinin protein, glycosylphosphatidylinositol anchors, Leptospiral LPS, phenol-soluble modulin, porins(13, 15, 21, 69, 74, 88, 113, 200) Diacylated lipoproteins (TLR2/TLR6 heterodimer)(36, 50)	Bacteria (primarily gram-positive, including mycobacteria), fungi, *Measles morbillivirus, Trypanosoma cruzi, Leptospira interrogans*	Free fatty acids, HSP60, HSP70, Gp96, HMGB1, fibronectin, hyaluronan, biglycan, galectin-3, decorin, peroxiredoxins, β-defensin, uric acid, MSU crystal, PAUF, AGE-LDL, versican, EDN(7, 9, 30, 33, 63, 93, 140, 152, 162, 164, 166, 191, 192, 205, 211)	Pam_3Cys, Pam_3CSK_4, MALP-2 (TLR2/TLR6 heterodimer)
TLR3	IC compartments(88, 188)	Double-stranded RNA(69, 88)	Virus (both RNA and DNA)(188)	Self- RNAs (including mRNA and small interfering RNA), angiotensin II(9, 88, 171)	Poly (I:C)
TLR4	Cell surface(88, 188)	LPS, fusion protein, mannan, RSV protein F, MMTV and MMLV envelope proteins, VSV glycoprotein G, DENV NS1, EBOV glycoprotein(6, 69, 134, 148, 207)	Bacteria (gram-negative), virus, fungi	Free fatty acids, HSP22, HSP60, HSP70, HSP72, Gp96, HMGB1, fibronectin, hyaluronan (including lower molecular weight fragments), biglycan, galectin-3, decorin, peroxiredoxins, β-defensin, uric acid, MSU crystal, PAUF, AGE-LDL, heparan sulfate, heme, s-100 proteins (including calprotectin), fibrinogen, tenascin C, α-crystallin, surfactant protein, mmLDL, cardiolipin, serum amyloid A 3, fetuin-A, resistin, granulysin, peroxiredoxin 1, angiotensin II, ceramide, MRP 8/14(7, 9, 14, 29, 30, 33, 44, 55, 70, 72, 79, 93, 122, 138, 140, 143, 151, 157, 162, 164, 166, 171, 184, 185, 191, 192, 195, 211, 215) Oxidised LDL, amyloid-β (TLR4/TLR6 heterodimer)(85, 179)	LPS, lipid derivatives(9) Paclitaxel(196)
TLR5	Cell surface(88, 188)	Flagellin(69)	Bacteria		
TLR6	Cell surface(88, 188)	Lipoteichoic acid	Bacteria (gram-positive)	Versican(9)	MALP-2 (TLR2/TLR6 heterodimer)
TLR7	IC compartments(88, 188)	Single-stranded RNA(69, 130)	Virus, RNA from *Streptococcus* B bacteria(88)	Self-RNA, microRNA(202)	Imidazoquinoline, bropirimine, guanine analogs(9, 102)
TLR8	IC compartments(88, 188)	Single-stranded RNA(69)	Virus	Self-RNA	Imidazoquinoline(9)
TLR9	IC compartments(88, 188)	Unmethylated CpG DNA, hemozoin(60, 69, 88, 137)	Virus, bacteria, *Plasmodium falciparum*	Self-DNA (including mitochondrial DNA), HMGB1, chromatin-IgG complexes, interferon-α (30, 100, 197)	CpG ODN(9)
TLR10	Cell surface(88, 188)	No clearly defined ligand	*Listeria monocytogenes*, influenza virus A, HIV-1(9, 78, 88)		

Abbreviations: AGE-LDL, advanced glycation end-products modified low density lipoprotein; DAMP, damage-associated molecular pattern; DENV, dengue virus; EBOV, Ebola virus; EDN, eosinophil-derived neurotoxin; HMGB1, high mobility group box 1 protein; HSP, heat shock protein; IC compartments, intracellular compartments (endosomes, lysosomes, endoplasmic reticulum); IgG, immunoglobulin G; LPS, lipopolysaccharide; MALP-2, macrophage-activating lipopeptide-2; mmLDL, minimally modified low-density lipoprotein; MMLV, Moloney murine leukemia virus; MMTV, mouse mammary tumour virus; mRNA, messenger RNA; MRP, myeloid-related protein; MSU, monosodium urate; NS1, non-structural protein 1; ODN, oligodeoxynucleotides; PAMP, pathogen-associated molecular pattern; PAUF, pancreatic adenocarcinoma upregulated factor; poly(I:C), polyinosinic:polycytidylic acid; RSV, respiratory syncytial virus; TLR, toll-like receptor; VSV, vesicular stomatitis virus.

Supplementary Table 2 | Summary of the characteristics of the articles investigating the effects of acute resistance exercise.

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Fernandez-Gonzalo, 2012(51)	Moderately active undergraduate male students – n=12 – 22.1 (SE 0.5) years	Moderately active undergraduate male students – n=8 – 22.7 (SE 0.4) years	- Exercise bout (week 0), followed by an eccentric training program and repetition of the exercise bout (week 9) - CG: no training program - Exercise bout: 12 sets of 10 repetitions of the negative phase of the barbell squat at 60% MVIC - Training program: 3 sessions/week for 6 weeks - Session: 3-5 sets of 10 repetitions of eccentric barbell squat at 40-50% MVIC	Venous blood – pre, past and 2h after exercise bout	qPCR (CD14, TLR4, TNF-α) Western blot (TLR4, TRAF6, TNF-α, CD14, p65, pIκB, pIKK, TRIF, MyD88, pERK-1/2)	TLR4 mRNA: ↑ post and 2h after both bouts in both groups TLR4 protein: ↑ post and 2h after first bout in both groups; ↓2h after second bout in trained group (↔ ↑ post and 2h after second bout in CG)	CD14 mRNA: ↑ post and 2h after both bouts in both groups CD14 protein: ↑ post and 2h after first bout in both groups; ↓ 2h after second bout in trained group (↔ ↑ post and 2h after second bout in CG) TNF-α mRNA and protein: ↑ post and 2h after first bout in both groups; = in trained group after second bout (↔ ↑ post and 2h after exercise bout in CG) MyD88, TRAF6, TRIF, pIKK, pIκB, p65 protein and pERK-1/2 protein: ↑ post and 2h after first bout in both groups; = after second bout in trained group (except for ↑ pIKK post exercise and ↑ p65 2h after exercise)(↔ ↑ post and 2h after exercise bout in CG)
Fernandez-Gonzalo, 2014(52)	Healthy active (3-5h of recreational activity per week) undergraduate female students – n=12 – 22.5 (SEM 0.3) years	Healthy active (3-5h of recreational activity per week) undergraduate female students – n=8 – 22.5 (SEM 2.3) years	- Exercise bout (week 0), followed by an eccentric training program and repetition of the exercise bout (week 9) - CG: no training program - Exercise bout: 12 sets of 10 repetitions of the negative phase of the barbell squat at 60% MVIC - Training program: 3 sessions/week for 6 weeks - Session: 3-5 sets of 10 repetitions of eccentric barbell squat at 40-50% MVIC	Venous blood – pre, post and 2h after exercise bout	qPCR of PBMC (CD14, TLR4, TRAF6) Western blot (CD14, TLR4, MyD88, p65, pERK, TBK1, IRF3, TNF-α, TRAF6, TRIF, IKK, pIKK, pIκB, pIRF3, p38, pP38)	TLR4 mRNA: ↑ post and 2h after both bouts in both groups TLR4 protein: ↑ post and 2h after first bout in both groups; = after second bout in trained group (↔ ↑ post and 2h after second bout in CG)	Cell counts: no changes PBMC subpopulations CD14 and TRAF6 mRNA: ↑ post and 2h after both bout in both groups CD14 and TRAF6 protein: ↑ post and 2h after first bout in both groups; = after second bout in trained group (↔ ↑ post and 2h after second bout in CG) MyD88, TRIF, pIκB, IKK, p65 and pERK and TNF-α protein: ↑ post and 2h after first both in both groups; = after second bout in trained group (↔ ↑ post and 2h after second bout in CG) pIRF3 protein: =
Flynn, 2003(58)	Apparently healthy resistive-trained women: - taking traditional HRT – n=9 – 74.02 (SD 5.62) years - non-HRT – n=6 – 74.60 (SD 6.47) years - no hormones but medications known to influence bone – n=7 – 72.01 (SD 7.46) years	Apparently healthy sedentary women not taking hormones – n=6 – 74.20 (SD 6.98) years	- Training program with single exercise bout 1 week after last training session - CG: acclimatisation sessions, followed by normal activities - Week 0: 3 acclimatisation sessions - Week 1-10: 3 sessions/week of three sets of 10 upper and lower body exercises - Exercise bout: 10 min treadmill warm-up, three sets of 10 resistive exercises at 80% 1RM	Venous blood – pre, post and 2h after exercise bout (or after rest for CG)	qPCR (TLR4, CD14, IL-6, TNF-α, IL-1β) Whole blood stimulation with LPS (24h) with ELISA	TLR4 mRNA: collapsed over time ↓ in trained groups compared to CG (no significant time differences) TLR4 mRNA/monocyte: ↑ in CG compared to trained group at all time points	Leukocyte count: ↑ post exercise in trained groups with normalisation at 2h (↔ CG =) Mononuclear cell count: ↑ post exercise in HRT and non-HRT with normalisation at 2h (↔ CG and medication with bone influence =) Monocyte count: ↑ post exercise in trained groups with normalisation at 2h (↔ CG ↓ post exercise with normalisation at 2h) CD14 mRNA: collapsed over time ↓ in trained compared to CG CD14 mRNA/monocyte: ↑ in CG compared to trained groups at all time points IL-6 and IL-1β mRNA: no significant differences TNF-α mRNA: collapsed over time ↑ in CG compared to trained groups LPS-stimulation: ↓IL-6, TNF-α in trained groups; IL-1β =

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
McFarlin, 2004(116)	Elderly (≥ 10 years postmenopausal) trained (≥ 72 exercise sessions in the last 6 months) women – n=10 – 67 (SD 5) years	Elderly (≥ 10 years postmenopausal) untrained women – n=10 – 69 (SD 5) years	- Single exercise bout (exercise & control group) - Upper and lower body exercise: 3 sets, 9 exercises, 10 repetitions at 80% of 1RM	Venous blood – pre, post and 2h, 6h and 24h after exercise	Flow cytometry (CD14, TLR4) qPCR (CD14, TLR4, IL-6, TNF-α, IL-1β) Whole blood stimulation with LPS (24h) with ELISA	$CD14^+$ monocyte TLR4: untrained ↑ compared to trained (group averages), no time factor	Cell count: no group factor Total leukocytes: post-exercise ↑ compared to pre-exercise and 24h; at 6h ↑ compared to pre-exercise $CD14^+$ count: at 6h ↑ compared to 2h and 24h; at post-exercise ↑ compared to 2h LPS-stimulated cytokines: IL-6, IL-1β, TNF-α concentration at 6h ↑ than other time points; IL-6 post-exercise ↑ than at 2h Gene expression: no significant results
Age is given as mean unless otherwise stated. Abbreviations: 1RM, one-repetition maximum; CD, cluster of differentiation; CG, control group; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated protein kinase; h, hour; HRT, hormone replacement therapy; IFN, interferon; IKK, IκB kinases; IL, interleukine; IRF, interferon regulatory transcription factor; LPS, lipopolysaccharide; min, minute; mRNA, messenger RNA; MVIC, maximal voluntary isometric contraction; MyD88, myeloid differentiation primary response gene 88; PBMC, peripheral blood mononuclear cells; qPCR, quantitative polymerase chain reaction; SD, standard deviation; SE, standard error; SEM, standard error of the mean; TBK, TANK-binding kinase; TNF, tumor necrosis factor; TLR, toll-like receptor; TRAF, TNFR-associated factor; TRIF, TIR-domain-containing adaptor protein-inducing interferon-β.							

Supplementary Table 3 | Summary of the characteristics of the articles investigating the effects of acute aerobic exercise.

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Bergman, 2016(12)	- Obese sedentary (<2h physical activity/week) individuals – n=14 (9 ♂, 5 ♀) – 39.7 (1.6) years - T2DM – n=15 (11 ♂, 4 ♀) – 42.5 (1.1) years - Endurance-trained athletes (average training 12.3h/week) – n=15 (11 ♂, 4 ♀) – 41.3 (0.86) years	/	- Single exercise bout - Cycling: 1.5h at 50% VO_2max	Venous blood – pre, during last 30 min of exercise and during last 30 min of recovery Muscle biopsy quadriceps femoris – pre, post and 2h after exercise	NEFA kit (NEFA) Western blot of muscle tissue (TLR4, JNK, MAP4K4, ERK1/2, Akt)	TLR4 = (no differences between groups or after exercise)	Serum TNF-α: ↑ after exercise with normalisation after recovery Baseline serum NEFAs: total concentration = between groups; palmitic acid and stearic acid ↑ in T2DM than athletes or obese; arachidic acid ↑ in T2DM compared to obese; eicosapentaenoic acid ↑ in obese than T2DM or athletes; erucic acid ↑ in athletes than in obese or T2DM Serum NEFAs after exercise: ↑ after exercise with further increase during recovery in all groups $JNK^{Thr138/185}$ phosphorylation/total JNK: ↓ in athletes compared to T2DM and obese at baseline; ↑ after exercise in all groups with normalisation after recovery MAP4K4, ERK1/2 phosphorylation/total ERK1/2: no differences between groups or after exercise Akt^{Ser473} phosphorylation/total Akt: ↑ in athletes compared to T2DM or obese at baseline; no impact of exercise

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Booth, 2010(17)	Trained cyclists – n=8 (5 ♂, 3 ♀) - 32.1 (SD 4.2) years)	/	- Single exercise bout - Cycling: 60 km in fastest time possible (mean completion time 92.1 min, mean HR 83% age-predicted MHR)	Venous blood – pre, post and 1h after exercise	Flow cytometry (CD14, CD16, TLR2, TLR4, HLA-DR) Serum stimulation assay of pre-exercise PMBC with flow cytometry	↑ TLR2, TLR4 post and after 1h TLR2 on monocyte subsets: ↑ TLR2 on $CD14^{++}/CD16^{+}$ after exercise, no other exercise effects TLR4 on monocyte subsets: ↑ TLR4 on $CD14^{++}/CD16^{+}$ after 1h, no other exercise effects Serum exposure: TLR2, TLR4 =	Cell count: ↑ total leukocyte, neutrophil, lymphocyte and monocyte count post exercise; ↑ leukocyte, neutrophil and monocyte count after 1h with normalisation of lymphocyte count Monocyte subsets: ↑ $CD14^{++}/CD16^{-}$, $CD14^{+}/CD16^{++}$, $CD14^{++}/CD16^{+}$ post exercise with normalisation after 1h; ↓ proportion $CD14^{++}/CD16$ and ↑ proportion $CD14^{+}/CD16^{++}$ and $CD14^{++}/CD16^{+}$ with normalisation after 1h Monocyte HLA-DR: ↓ post and after 1h Serum exposure: ↑ HLA-DR on $CD14^{+}/CD16^{++}$ cells with 1h post-exercise serum compared to resting serum
Capó, 2014(22) and Capó, 2016(23)	*Male soccer players from the Real Mallorca B team – n=9 – 20.4 (SEM 0.5) years (not considered in this review)*	Male soccer players from the Real Mallorca B team – n=6 – 19.3 (SEM 0.4) years	- Training program with single exercise bout, with docosahexanoic acid or placebo supplementation (5 days/week) throughout the season *(docosahexanoic acid supplementation group not considered in this review)* - CG: placebo supplementation, training program and exercise bout - Training program: 8 weeks of physical activity related to football training season - Single exercise bout: 2h of habitual physical activity (15 min warm-up, Leger Boucher test, 15 min recovery exercise, small-sided games, 20 min soccer match)	Venous blood – at the beginning and end of the nutritional intervention and 2h after the exercise	Capó, 2014 Flow cytometry (cell counts) Chromatography (fatty acids) PBMC stimulation with LPS (2h) with Biochip array Biochip array (GMCSF, MIP1α, MCP1, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-15, TNF-β, IFN-γ ELISA (IL-8, TNF-α) Western blot of PBMC (TLR4) Capó, 2016 PBMC stimulation with LPS (2h)(37°C and 39°C) with ELISA Randox Biochip Array (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α)	TLR4 = after program; ↑ TLR4 after acute exercise	Capó, 2014 Fatty acid composition of erythrocytes: ↓ proportion C20:2 after program compared to baseline Cell count: ↑ proportion basophils after program; leukocytes =; ↑ proportion monocytes and ↓ proportion lymphocytes and basophils after acute exercise Plasma cytokines and growth factors: = after program; ↑ IL-6 after acute exercise PBMC stimulation: ↑IL-6 and TNF-α after exercise Capó, 2016 LPS-stimulated PBMC: ↑ IL-1β, IL-6, IL-8 in 39°C compared to 37°C; ↓ IL-2 in 39°C compared to 37°C LPS-stimulated PBMC: = (when expressed per blood or per monocyte); ↑ IL-1α, IL-1β, IL-4, IL-6, IL-8, IFN-γ and TNF-α after acute exercise; ↓ IL-2 after acute exercise
Capó, 2016(24)	Young and senior male taekwondo athletes (physical activity of 1-2h daily 5-7 days per week) - young: n=10 – 22.8 (SEM 3.8) years - senior: n=8 – 45.6 (SEM 1.6) years	/	- Single exercise bout before and after 5 weeks of nutritional intervention *(test after intervention was not included in this review)* - Treadmill: incremental maximal test until exhaustion (71.84 min for senior group, 72.02 min for young group)	Venous blood – pre and 1h after exercise	Enzymatic Wako kit (NEFA) ELISA (IL-6, TNF-α, NF-κB p50) qPCR (TLR2, TLR4, NF-κB, 5-LOX, 15-LOX2, IL-1β, IL-8, IL-10, IL-15, TNF-α, HSP70)	TLR2, TLR4 =	Plasma NEFA: ↑ in young and old athletes after exercise Plasma IL-6, NF- κB p50, TNF-α and HSP70: no impact of exercise in young and old group Gene expression: no group differences or impact of exercise
Durrer, 2017(43)	T2DM patients with HbA1c <8% – n=10 (5 ♂, 5 ♀) – 57.9 (SD 5.4) years	Age-matched normoglycemic controls completing 150-300 min of light-moderate physical activity per week (self-reported) – n=9 (4 ♂, 5 ♀) – 55.8 (SD 9.0) years	- Single exercise bout of HIIT (patient & control group) - Cycling: 7 times 1 min at 85% of maximal aerobic power output, separated by 1 min of recovery	Venous blood – pre, post and 1h after exercise	MagPix (plasma TNF-α) Flow cytometry (CD14, CD16, TLR2, TLR4) Whole blood stimulation with LPS (4h) with MagPix assay	TLR2 expression on classical and $CD16^{+}$ monocytes: ↓ post and 1h after exercise TLR2 expression on $CD16^{+}$ neutrophils: ↑ in T2DM compared to CG; no effect of exercise TLR4 expression on classical monocytes: no effect of time or group TLR4 on $CD16^{+}$ monocytes and neutrophils: ↑ in T2DM compared to CG; no effect of exercise	Cell count: ↑ total leukocytes, classical monocytes and $CD16^{+}$ monocytes post exercise with normalisation after 1h; proportion $CD16^{+}$ monocytes = Plasma TNF-α: ↓ at 1h compared to pre exercise in T2DM and CG LPS-stimulated blood culture: absolute TNF-α concentration ↓ at 1h post-exercise compared to pre and post exercise; when corrected for leukocyte number ↓ at 1h compared to pre exercise and ↓ overall in T2DM compared to CG

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Falgiano, 2018(48)	Healthy, recreationally active participants – n=8 (6 ♂, 2 ♀) – 19 (SEM 1) years	/	- Single exercise bout with and without preceding curcumin supplementation (average washout period of 38 days)*(test with preceding supplementation is not considered in this review)* - Running: 60 min at 65% VO_2max in 37°C and 25% RH	Venous blood – pre, post, 1h and 4h after exercise	Western blot of PBMC (TLR4, pAMPKα1, p-HSF1, HSP70, pIκBα, IκBα, MyD88, pNF-κB p65, NF-κB p65, SIRT1)	↓TLR4 1h after exercise with normalisation at 4h	MyD88 protein: = pIκB/IκB and pNF-κB/NF-κB ratio: pIκB/IκB =; ↑ pNF-κB/NF-κB at 1h after exercise p-HSF1 and HSP70: p-HSF1 =; ↓HSP70 at 1h compared to 4h after exercise pAMPK and SIRT1: ↓5 pAMPK and SIRT1 1h after exercise
Fuller, 2018(62)	Healthy male participants – n=12 – 23 (SEM 1) years	/	- Consumption high-fat meal (1000 kcal and 57% fat) with and without single exercise bout on preceding day (separated by 4-7 days) - Cycling: 45 min at 65% VO_2peak	Venous blood – pre, and 0.5, 1, 2, 3 and 4h after the high-fat meal	Enzymatic assay (NEFA) Western blot of PBMC (4h after meal consumption)(TRL4, MyD88)	TLR4: = (no impact of high-fat meal or exercise)	Plasma NEFA: high-fat meal (with or without prior exercise) decreased plasma NEFA MyD88: no impact of high-fat meal or exercise
Gleeson, 2006(69)	Endurance-trained male cyclists – n=11 – 20 (SD 2) years)	/	- Single exercise bout - Cycling: 2.5h at 60% VO_2max in 20°C and 40% RH	Venous blood – pre, post and 1h after exercise	Flow cytometry (TLR1, TLR2, TLR3, TLR4)	TLR on $CD14^+$ monocytes post: TLR1, TLR3, TLR4 =; ↓ TLR2 TLR on $CD14^+$ monocytes 1h after: ↓ TLR1, TLR2, TLR3, TLR4	Core temperature: =
Keech, 2016(89)	CFS patients with 1.2h (SD 1) of at least moderate intensity exercise per week – n=10 (4 ♂, 6 ♀) – 41.4 (SD 8.4) years	Matched healthy control participants with 1.7h (SD 1.4) of at least moderate intensity exercise per week – n=12 (4 ♂, 8 ♀)(one subject did not provide blood) – 34.1 (10.2) years	- Single exercise bout (patient & control group) - Cycling: 25 min at 70% age-predicted MHR	Venous blood – 24h pre, post and 1, 4, 24 and 72h after exercise	qPCR of PBMC (IL-1β, IL-6, IL-10, IFN-γ, CD14, TLR4)	TLR4 =	Gene expression: no significant effect of exercise on any gene
Lackermair, 2017(92)	*Male volunteers with a history of ≥1 finished half marathon – n=42 – 40.8 (SD 9) years (not considered in this review)*	Male volunteers with a history of ≥1 finished half marathon – n=58 – 41 (SD 9.5) years	- Regular training (54 km/week on average) for 4 weeks, accompanied by *nonalcoholic beer (1-1.5L/day)(not considered in this review)* or placebo supplementation, followed by a single exercise bout - CG: placebo supplementation, regular training and marathon race - Single exercise bout: marathon race (227 min on average)	Venous blood – at inclusion, in the week before the marathon (after 3 weeks of beverage consumption and training) and 1h, 24h and 72h after finishing	Flow cytometry (leukocyte subsets) qPCR of PBMC (TLR7)	↑TLR7 after training; ↓ TLR7 1h after marathon with ↑ after 24h and ↓ after 72h	mDC: = after training; ↑ after marathon, with partial normalisation after 24h and complete normalisation after 72h pDC: ↑ after training; ↓ after marathon with recovery after 24h
Lancaster, 2005(96)	Healthy moderate-to-well endurance-trained male subjects – n=11 – 25 (SEM 1) years	/	- Single exercise bout - Cycling: 5 min at 40% Wmax, followed by 1.5h at 55% Wmax (~ 65% VO_2max) in 34°C and 30% RH	Venous blood – pre, post and 2h after exercise	Flow cytometry (CD14, TLR1, TLR2, TLR4, TLR9, HLA-DR, CD80, CD86, IL-6) Whole blood stimulation (6 or 24h) with zymosan (TLR2), LPS (TLR4) and poly(I:C) (TLR3) with flow cytometry	Post exercise and after 2h: ↓ $CD14^+$ monocyte TLR1, TLR2, TLR4; TLR9 =	Unstimulated exercise samples: $CD14^+$ monocyte CD86 ↓ and MHC II ↓ 6h stimulated exercise samples: upregulation $CD14^+$ monocyte CD86 and MHC II ↓ compared to resting samples 24h stimulated exercise samples: upregulation $CD14^+$ monocyte CD80 = between rest and exercise 6h LPS-stimulated sample: $CD14^+$ intracellular IL-6 upregulation ↓ compared to resting samples 6h zymosan-stimulated sample: $CD14^+$ intracellular IL-6 upregulation ↑ compared to resting samples

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Lansford, 2016(99)	Healthy, non-smoking recreationally active subjects: - male – n=16 – 24.5 (SEM 0.8) year - female – n=10 – 22.40 (SEM 0.52) years	/	- Single exercise bout - Cycling: 60-70% VO_2peak until reaching total energy expenditure of 2.5 MJ (43.6 min on average in men, 62.7 min on average in female)	Venous blood – pre and post exercise	FACS qPCR (TLR2, TLR4, IL-6R)(male subjects only)	TLR2 expression: ↑ in $CD62E^+$ PBMC at baseline; ↓ in $CD62E^+$ cells after exercise TLR4 expression: ↓ in $CD31^+$ PBMCs compared with $CD34^+$ and $CD62E^+$; no impact of exercise	Circulating angiogenic cells: ↓ $CD14^+/CD31^+$ cells after exercise (men only); no change $CD34^+/VEGFR2^+$ or $CD3^+/CD31^+$ cells Microparticles: ↑ $CD62E^+$ endothelial microparticles after exercise in men (↔ = in women); no change $CD31^+/CD42b^-$ endothelial microparticles; lower baseline $CD34^+$ microparticles in women with ↑ after exercise (↔ = in men) IL-6R expression: ↓ in $CD31^+$ PBMCs compared with $CD34^+$ and $CD62E^+$; no impact of exercise
Light, 2009(106)	CFS patients – n=19 (4 ♂, 15 ♀) – 42.2 (SE 2.7) years	Control subjects – n=15 (5 ♂, 10 ♀) – 35.6 (SE 3.0) years	- Single exercise bout (patient & control group) - Combined arm-leg ergometer: 25 min at 70% age-predicted MHR	Venous blood – pre and 0.5, 8, 24 and 48h after exercise	qPCR (IL-6, IL-10, TLR4, TNF-α, CD14))	TLR4 mRNA: baseline =; ↑ AUC for all time points post-exercise in patient group (↔ CG =) Post-exercise fitness-matched subgroups: TLR4 mRNA =	BMI: higher in CFS patients Total leukocyte count: ↑ at 8h with normalisation after 24 and 48h, no group differences Baseline: IL-6, IL-10, TNF-α, CD14 = between groups Post-exercise: non-significant higher values for IL-6, TNF-α and CD14 across all time points in the CFS group; significantly elevated AUC for all time points for IL-10
Millard, 2013(121)	Healthy volunteers accustomed to exercise – n=29 (19 ♂, 10 ♀) – age range 25-45 years	/	- Single exercise bout - Stairclimbing: up and down 150 stairsteps (duration on average 68.8 s)	Venous blood – pre and post exercise	Flow cytometry (TLR2)(n ≥5) Stimulation of NK cell culture with recombinant human IL-2, IL-12 or LTA and Pam3CSK4 Stimulation of NK cell culture with IL-2/IL-12 or LTA and Pam3CSK4 with MACS cytokine secretion assay Stimulation of NK cell culture with IL-2 (48h) with ELISA (IFN-γ secretion) Stimulation of NK cell culture for degranulation with IL2/IL-12 (overnight) with flow cytometry ^{51}Cr-release assay of freshly isolated or IL-2 activated NK cells (n=5)	$TLR2$ on $CD56^{pos}$, $CD56b^{right}$ or $CD56^{dim}$ NK cells: =	Cell count: ↑PBMC, NK cells after exercise; ↓ proportion of $CD56^{bright}/CD16^{neg/dim}$ in total $CD56^{pos}/CD3^{neg}$ NK population after exercise NK cell IFN-γ secretion, degranulation and cytotoxicity without exogenous stimulation: slight ↓ degranulating NK cells after exercise, no other effects Cytokine stimulated NK cells: IL-2 stimulation at pre-exercise ↑IFN-γ after 4h and 24h (↔ = after exercise); intracellular IFN-γ production similar before and after exercise; ↓ degranulation after exercise; after 5 days of IL-2 stimulation ↓ cytotoxicity of post-exercise NK cells TLR2-stimulated NK cells (similar TLR2 expression of the samples): ↓ frequency IFN-γ-secreting cells after exercise
Neubauer, 2013(128)	Healthy male endurance athletes – n=8 – 25.0 (SD 4.1) years	/	- Training program (6 weeks of endurance exercise, including cycle to run transition training) followed by a single exercise bout - Single exercise bout: 60 min intense cycling at 105% of power output attained at gas exchange threshold, followed by 60 min of intense running at 10-km time trial pace	Venous blood – 1 week before and 3, 48 and 96h after the exercise bout (prior 48h abstinence from exercise)	ELISA (HSP70, IL-1β, IL-1ra, IL-6, IL-10) Microarray gene expression analysis qPCR (IL-1R1, IL-1ra, IRAK3, TLR4)	↑ TLR4 at 3h with normalisation after 48h	Cell count: ↑ total leukocytes and neutrophils at 3h with normalisation after 48h Plasma HSP70: = Plasma cytokines: ↑ IL-1ra, IL-6, IL-10 at 3h; IL-1β =; all values normalised at 48h Microarray analysis: ↑ KEGG TLR signalling pathway at 3h, with normalisation after 48h qPCR: ↑IL-1R1, IL-1ra, IRAK3 at 3h with normalisation after 48h

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Nickel, 2012(129)	See "Nickel *et al.* 2011" in Supplementary Table 5		- Single exercise bout - Running : marathon race (263 min obese non-elite (ONE), 235 min lean non-elite (LNE), 217 min elite (LE))	Venous blood – 2-5 days pre (no exercise in preceding 2 days), post and 24h after marathon	ELISA (serum IL-6, IL-10, TNF-α) Flow cytometry qPCR of PBMC (TLR2, TLR4, TLR7) Western blot of PBMC (TLR2, TLR4, TLR7)	TLR2 mRNA and protein: = (no differences between groups or after exercise) TLR4 mRNA and protein: ↓ mRNA post marathon in LNE; ↑ mRNA 24h after marathon compared to baseline in all groups; protein = TLR7 mRNA and protein: ↓ mRNA post marathon in all groups; ↑ mRNA 24h after marathon compared to baseline in all groups; protein ↓ 24h after marathon in all groups	Myeloid DCs: baseline =; ↑ post marathon with normalisation after 24h, except for ONE with persistent increase Plasmacytoid DCs: baseline =; ↓ post marathon with normalisation after 24h, except for LNE with24h values lower than baseline Serum IL-6: ↓ in LE at baseline; ↑ post and 24h after marathon in all groups Serum TNF-α: baseline =; ↑24h after marathon in all groups Serum IL-10: ↓ in ONE at baseline; ↑ post marathon with normalisation after 24h in all groups
Oliveira, 2010(136)	Healthy endurance-trained men – n=9 – 25 (SD 5) years	Healthy endurance-trained men – n=6 – 25 (SD 2) years	- Three exercise bouts (2 preliminary and 1 main trial separated by ≥ 1 week) - CG: remain seated during trials - Cycling: 1.5h at 75% VO_2peak	Venous blood – pre, post and 1,4 and 24h after exercise (CG: pre and after 1.5 and 2.5h)	Flow cytometry (TLR2, TLR4, CD14)	Monocyte TLR2: = Monocyte TLR4: ↓ post and 1h after with normalisation after 4h (↔ CG =)	Leukocyte count: ↑ total leukocyte and monocyte count post, 1h and 4h post-exercise with normalisation after 24h
Perandini, 2016(141)	Physically inactive (≥6 months physical inactivity) women with SLE: - patients with active SLE (SLE_{active}) – n=4 – 32.5 (SD 3.4) years - patients with inactive SLE ($SLE_{inactive}$) – n=4 – 34.5 (SD 3.4) years	Age- and BMI-matched healthy control women – n=4 – 29.3 (SD 4.8) years	- Single exercise bout (patient & control group) - Treadmill: 5 min warm-up, followed by 30 min at predetermined intensity (50% of difference between ventilator anaerobic threshold and respiratory compensation point)	Venous blood – pre, post and 3h after exercise	qPCR array of leukocytes	Healthy control: ↓TLR3, TLR7 post exercise with normalisation at 3h; ↑ TLR1, TLR8 at 3h after exercise SLE_{active}: ↓ TLR3, TLR6 post exercise with normalisation at 3h after exercise; ↑ TLR7, TLR9 at 3h; ↓ TLR4, TLR8 at 3h after exercise $SLE_{inactive}$: ↑ TLR3 at 3h after exercise	Healthy control: ↓ MyD88, IRF3, TNF, IL-1α, IL-2,IL-4, IL-6, IL-10, IL-17A, IL-18 and CD80 post exercise with normalisation after recovery; ↓ IL-23A, IL-5 and IL-13 post exercise with increased expression at recovery; no change post exercise but ↑ expression at recovery of CD14, IL-1β, IL-1R1 and MAPK8 SLE_{active}: ↓ IL-5, IL-17A and IL-18 post exercise with normalisation after recovery; ↑ CD14 and IRF7 post exercise with normalisation after recovery; ↓ IL-1α, IL2, IL-4, IL-6, IL-10, IL-13 and IFN-γ post exercise with ↑ expression after recovery $SLE_{inactive}$: ↓ IFN-γ, IL-2, IL-5, IL-10, IL-13, IL-17A and IL-18 post exercise with normalisation after recovery; ↓ CD80, IL-4 and IL-6 post exercise with ↑ expression after recovery; ↑ IRF7 post exercise and after recovery; no change at baseline but ↑ MAPK8 at 3h after exercise
Radom-Aizik, 2014(146)	Healthy young men (no elite athletes or subjects with vigorous participation in competitive sports) – n=12 – 26 (SE 0.6) years	/	- Single (intermittent) exercise bout - Cycling: ten 2-min. bouts at 82% VO_2max, with 1-min rest interval between each bout	Venous blood – pre and post exercise	Flow cytometry (CD14, CD16) Gene expression microarray of monocytes qPCR (TNF, HSPA1A, HSPA8, TLR4)	↓ TLR4	Cell count: ↑ classical and non-classical monocyte number post exercise; ↑ proportion non-classical monocytes post exercise Gene expression microarray: alteration of MAPK signalling pathway after exercise qPCR: ↑ HSPA1A, HSPA8 post exercise; ↓TNF post exercise

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Simpson, 2009(170)	Moderately trained male subjects – n=15 – 26.4 (SD 6.7) years	/	- Single exercise bout - Treadmill: 45 min at 75% VO_2max	Venous blood – pre, post and 1h after exercise	Flow cytometry (CD14, CD16, TLR2, TLR4, HLA-DR)	Post exercise: $CD14^+$ monocyte TLR2 = ; $CD14^+/CD16^+$ monocyte TLR2 ↓ (~ $CD14^{++}/CD16^+$ monocytes); monocyte TLR4 = After 1h: monocyte TLR2 = (compared to baseline; $CD14^+$ monocyte TLR4 ↓ (~ $CD14^+/CD16^-$ monocytes); $CD14^+/CD16^+$ monocyte TLR4 ↑(~ $CD14^+/CD16^{++}$ monocytes)	Difference TLR2, TLR4 and HLA-DR expression between subsets not affected by exercise Post exercise: ↑ proportion $CD14^+/CD16^+$ within $CD14^+$ monocyte population After 1h: ↑ proportion $CD14^+/CD16^+$ monocytes within $CD14^+$ monocyte After 1h: $CD14^+$ monocyte HLA-DR ↓ (~ $CD14^+/CD16^-$ and $CD14^{++}/CD16^+$ monocytes)
Slusher, 2018(173)	Healthy male participants – 24.2 (SD 4.0) years: - aerobically trained(≥150 min of moderate-to-vigorous aerobic exercise/week) – n=12 - aerobically untrained (<150 min of any moderate-to-vigorous physical activity/week – n=13	/	- Single exercise bout - Treadmill: maximal oxygen consumption until voluntary exhaustion (798.24 s on average)	Venous blood – pre and post exercise	Flow cytometry (CD14, CD16, TLR4) PBMC stimulation with LPS (24h) with ELISA	Monocyte TLR4: ↓ on total monocyte population, classical and intermediate monocytes	Cell count: proportion monocytes of PBMC = Monocyte subpopulations: ↓ classical monocytes; ↑ intermediate and non-classical monocytes CD14 expression: ↓ on total monocytes and all subpopulations CD16 expression: ↑ on intermediate monocytes (but still lower than non-classical monocytes) LPS-stimulated cytokines: ↓IL-6 and IL-10 production; ↑ IL-6/IL-10 ratio; ↑ TNF-α
Sureda, 2014(181)	Professional male divers – n=9 – 33.9 (SE 3.8) years	/	- Single exercise bout - Immersion to a depth of 50 m for a total time of 35 min, participants continuously swam while at depth	Venous blood – pre and 0.5 and 3h after exercise	qPCR of neutrophils (TLR2, TLR4, NF-κB, TNF-α, IL-1B IL-6, IL-8, IL-10, HSP72, lipoxygenase and myeloperoxidase) Colorimetric assay (MDA)	TLR2 =; ↑ TLR4 0.5h after exercise with further increase at 3h	Cell count: ↑ neutrophils after 3h compared to baseline and 0.5h after exercise MDA: = NF-κB: ↑ NF-κB 0.5h after exercise with further increase after recovery TNF-α: = IL-1B, IL-6, IL-8 and IL-10: ↑ IL-6 0.5h after exercise with further increase after recovery; ↑ IL-1B, IL-8 and IL-10 after 3h HSP72: ↑ after 3h Myeloperoxidase and lipoxygenase: myeloperoxidase =; ↑ lipoxygenase after 3h
Ulven, 2015(189)	Healthy non-smoking men – n=10 – median 25 years (range 22-28)	/	- Two test days separated by 1 week (data are calculated as average of the 2 test days) - Cycling: 1h at 70% VO_2max	Venous blood – pre and post exercise	ELISA (TNF-α, IL-6, IL-10) qPCR of PBMC (IL-1β, IL-8, IL-18, TNF-α, IFN-γ, TLR2, TLR4, TLR6)	↓TLR2; TLR4, TLR6 =	Serum cytokines: ↑ TNF-α, IL-6, IL-10 Cytokine mRNA: ↑ IL-1β, IL-8; TNF-α, IFN-γ, IL-18 =
VanHaitsma, 2016(194)	Trained cyclists – n=20 (10 ♂, 10 ♀) – 36.1 (SD 9.7) years	/	- Single exercise bout under two different conditions, separated by ≥ 1 week and performed at random order - Cycling: 40 km at race effort at temperate (21°C and 20% RH)(75.2 min) and hot (35°C and 25% RH)(79.0 min) conditions	Venous blood – pre and 0.5, 8, 24 and 48 after exercise	qPCR of whole cell layer (IL-6, IL-10, TLR4)	↓ TLR4 at 0.5 and 8h after exercise in both conditions, levels remained decreased at 24h and 48h after hot conditions, but returned to baseline after temperate conditions	IL-10 mRNA: ↑ at 0.5 and 8h in both groups IL-6 mRNA: only group effect with higher IL-6 levels for exercise in hot conditions across all time points

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
White, 2012(201)	- CFS patients – n=22 (3 ♂, 19 ♀) – 40.8 (SE 2.0) years - RRMS patients with self-reported fatigue and definite MS – n=20 (2 ♂, 18 ♀) – 41.5 (SE 2.0) years	Healthy controls – n=4 (4 ♂, 19 ♀) – 38.7 (SE 2.4) years	- Single exercise bout (patient & control group) - Schwinn Air-Dyne ergometer: 30 min at 70% age-predicted MHR	Venous blood – pre and 0.5, 8, 24 and 48 after exercise	qPCR of leukocytes (CD14, TLR4, IL-6, IL-10, lymphotoxin-α)	TLR4 mRNA: baseline =; ↑ 0.5h after exercise in control; ↓ 0.5h, 8h and 48h after exercise in MS; = after exercise in CFS	CD14 mRNA: baseline =; ↑ 0.5h after exercise in all groups; ↑8h after exercise in CFS and control group; ↑24h after exercise in CFS group only; at 24h normalisation in all groups IL-6 mRNA: baseline =; ↓ 0.5h after exercise and ↑ 48h after exercise in MS IL-10 mRNA: ↓ 0.5h after exercise in control and MS group; ↑ 8h after exercise in control and MS group; ↑ 48h after exercise in CFS group Lymphotoxin-α mRNA: ↓ 0.5h after exercise in control group

Age is given as mean unless otherwise stated. Abbreviations: AMPK, 5'-AMP-actived protein kinase; AUC, area under the curve; CD, cluster of differentiation; CFS, chronic fatigue syndrome; CG, control group; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated protein kinase; FACS, fluorescence-activated cell sorting; GMCSF, granulocyte macrophage colony-stimulating factor; h, hour; HIIT, high-intensity interval training; HR, heart rate; HLA-DR, human leukocyte antigen – DR isotype; HSF, heat shock factor; HSP, heat shock protein; IFN, interferon; IL, interleukine; IRAK, IL-1R-associated kinase; IRF, interferon regulatory transcription factor; JNK, c-Jun N-terminal kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LOX, lipoxygenase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4; MDA, malondialdehyde; MCP, monocyte chemotactic protein; mDC, myeloid dendritic cell; MHC, major histocompatibility complex; MHR, maximal heart rate; min, minute; MIP, macrophage inflammatory protein; mRNA, messenger RNA; MS, multiple sclerosis; MyD88, myeloid differentiation primary response gene 88; NEFA, non-esterified fatty acid; NF-κB, nuclear factor kappa B; NK, natural killer; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid dendritic cell; poly(I:C), polyinosinic:polycytidylic acid; qPCR, quantitative polymerase chain reaction; ra, receptor antagonist; RH, relative humidity; RRMS, relapsing-remitting multiple sclerosis; SD, standard deviation; SE, standard error; SEM, standard error of the mean; SIRT, sirtuin; SLE, systemic lupus erythematosus; T2DM, type 2 diabetes mellitus; TNF, tumor necrosis factor; TLR, toll-like receptor; W, power output.

Supplementary Table 4 | Summary of the characteristics of the articles investigating the effects of resistance exercise programs.

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Alfatlawi, 2019(5)	Women without experience in exercise – n=10 – 64.1 (SEM 1.1) years	Women without experience in exercise – n=10 – 69.0 (SEM 0.9) years	- Training program: 2 sessions/week for 10 weeks - CG: maintained physical activity routines - Upper and lower body exercises: 10 min warm-up, exercises at 60-80% 1RM	Venous blood – pre and post program	qPCR of peripheral blood cells (TLR4) ELISA (IL-6, IL-10)	↓ TLR4 in trained group (↔ CG =)	IL-6 and IL-10: IL-6 =; ↑ IL-10 in trained group (↔ CG =)
Cheng, 2015(34)	Patients with chief complaint of nonspecific low back pain – n=30 (15 ♂, 15 ♀) – 45 (3.25) years	/	- Training program: 3 sessions/week for 4 weeks - Session: 5 min stretching, 10 min back muscle strengthening, 5 min lower limb strengthening	Venous blood – pre and post program	ELISA (plasma IL-1β, IL-6, IL-8, TNF-α, IFN-γ) qPCR (TLR4, SIRT1 Western blot of lymphocytes (NF-κB, IκB) Enzymatic assay (superoxide dismutase, catalase, hydrogen peroxide)	↓ TLR4	SIRT1 mRNA: ↓ Plasma cytokines: ↓ IFN-γ, IL-1β, IL-6, IL-8 and TNF-α Lymphocyte NF-κB and IκB: ↓ NF-κB p65; ↑ IκB Superoxide dismutase and catalase: ↑ Hydrogen peroxide: ↓
Colleluori, 2019(40)	Obese older adults with a sedentary (regular exercise <1h/week) lifestyle and mild-to-moderate frailty: - aerobic exercise (AE) – n=11 (4 ♂, 7 ♀) – 71 (SE 1) years - resistance exercise (RE) –n=12 (6 ♂, 6 ♀) – 72 (SE 2) years - combined exercise (CE) – n=12 (6 ♂, 6 ♀) – 69 (SE 1) years	Obese older adults with a sedentary (regular exercise <1h/week) lifestyle and mild-to-moderate frailty – n=12 (4 ♂, 8 ♀) – 70 (SE 1) years	- Training program: 3 sessions/week for 26 weeks, weight management program and balanced diet with energy deficit of 500-750 kcal/day - CG: educational classes about healthful diet, no participation in exercise programs - AE: 60 min at 65-85% MHR - RE: 60 min upper and lower body exercise at 65-85% 1RM - CE: 75-90 min aerobic and resistance exercise	Vastus lateralis muscle biopsy – pre and after 6 months	Gene expression assay (IL-6, TNF-α, NF- κB, HSP704_9, TLR2)	↑ TLR2 in AE after program compared to the other groups which showed a downregulatory trend	Gene expression assay: ↑ HSP704_9 AE (↔ = in other groups); IL-6, TNF-α, NF- κB = (no difference between groups or after exercise)

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Markofski, 2014(112)	Physically inactive (≤2 days/week moderate activity in the previous 6 months and estimated VO_2peak ≤ 40th percentile for respective age) overweight or obese (BMI 25-39.9 kg/m²) adults – n=26 (9 ♂, 17 ♀) – 58.0 (SD 5.7) years: - resistance exercise (RT) – n=14 - resistance exercise and energy restriction diet (RT-ER) – n=12	Physically active (≥3 days/week moderate and/or vigorous activity in the previous 6 months and estimated VO_2peak ≥ 70th percentile for respective age) lean-to-overweight (BMI 22.4-29.9 kg/m²) adults – n=9 (6 ♂, 3 ♀) – 60.1 (SD 6.1) years	- Training program: 3 sessions/week for 12 weeks - CG: maintained level of physical activity - RT-ER: additional diet 750 kcal below energy need - Upper and lower body exercises: 5 min warm-up, 3 sets at 70-80% 1RM or more	Venous blood – pre and post program	Flow cytometry (CD14, CD16, TLR4) Whole blood stimulation with LPS with or without polymyxin B (24h) with ELISA	TLR4 = (no group differences, no impact of intervention)	Cell count: $CD14^+/CD16^+$ monocyte proportion ↑ in physically inactive (and more specifically obese) subjects at baseline; after intervention ↓ in RT group (more specifically overweight subgroup) and = in RT-ER group LPS-stimulated TNF-α: no group or intervention differences LPS-stimulated IL-6: ↑ unstimulated IL-6 in physically active subjects; ↓ IL-6 production in RT with low dose LPS after program; ↑ unstimulated IL-6 in RT after program
McKenzie, 2017(118)	Community-dwelling older adults recovering from hip fracture (incurred in preceding 2-6 months), and recently (1-12 weeks) discharged from 8-12 weeks of usual-care physical therapy – n=7 (3 ♂, 4 ♀) – 78.4 (SD 13.3) years	Age-, sex-, and BMI-matched controls (tissue from a previous study) – n=8 (4 ♂, 4 ♀) – 76.4 (SD 4.8) years	- Training program: 3 sessions/week for 12 weeks - CG: no training program - Session: 60-80 min with high-intensity exercises and task orientation (protein drink provided after each session)	Vastus lateralis muscle biopsy – pre and post program	qPCR (IL-6, TLR2, TLR4 TAK1, HMGB1, MyD88, TRAF6, NF-κB)	TLR2, TLR4 = (no group differences, no impact exercise)	Gene expression pre-post: ↓ MyD88, TAK1, NF-κB, IL-6 after exercise training Gene expression in comparison with healthy controls: MyD88, TRAF6, TAK1 and HMGB1 were elevated compared to control before and after the program; IL-6 and NF-κB were elevated compared to control before the program, but normalised thereafter
Nader, 2010(126)	Autoimmune inflammatory myopathy (5 dermatomyositis patients and 3 polymyositis patients) – median 51 (range 44-61) years	/	- Training program: 3 sessions/week for 7 weeks - Upper and lower body exercise: 10 voluntary repetition maximum	Vastus lateralis muscle biopsy – 1 week pre and post program	Gene expression microarray qPCR (IRAK3, IL-10Rβ Immunohistochemistry (IL-1α, IL-1β, IL-1Ra, IL-1RI, IL-1RII, HMGB-1)	↓ TLR8	Microarray and qPCR: ↓ IRAK3, IL10-Rβ, HMGB-1 Immunohistochemistry: IL-1α, IL-1β, IL-1Ra, IL-1RI, IL-1RII =
Phillips, 2012(142)	Obese postmenopausal women without regular exercise in the previous 6 months – n=11 – 64.8 (SD 2.4) years	Obese postmenopausal women without regular exercise in the previous 6 months – n=12 – 66.4 (SD 2.8) years	- Exercise bout ≥1 week after the ending of an acclimatisation training, followed by a training program and repetition of the exercise bout ≥1 week after the program - Training program: acclimatisation week (3 sessions/1 week), followed by 3 sessions/weeks for 12 weeks - CG: acclimatisation at start and after 12-week intervention, intervention with control activities and resting control trial - Exercise bout: 3 sets of 10 exercises at 8RM - Upper and lower body exercise: 3 sets of 10 exercises at 8RM	Venous blood – pre, 4 min, 2 and 24h after exercise (or resting) trial Subcutaneous adipose tissue (SCAT) – 400 mg of the abdominal region at baseline and 48h after the last exercise session (or 1 week after 8RM assessment in CG)	Whole blood stimulation with LPS (24h) with ELISA qPCR (blood: TLR4, SCAT: TLR4)(only at pre time points)	TLR4 =	Cell count: ↑ total leukocytes in exercise group post and 2h after exercise with normalisation after 24h; ↑ total leukocytes in CG at 2h with normalisation after 24h Cell counts at baseline: ↓ monocyte number in exercise group after program Plasma cytokines: ↓ resting TNF-α in exercise group after program; ↑ IL-6 post and 2h after both exercise trials in exercise group with normalisation after 24h; ↑ IL-6 at 2h after both control interventions in CG with normalisation after 24h; ↓ mean IL-6 in CG after control intervention LPS-stimulated IL-10: mean ↑ in exercise group compared to CG; ↓ in CG after intervention LPS-stimulated TNF-α: mean ↑ at 2h in both groups, with further ↑ at 24h in exercise group and normalisation in CG
Prestes, 2015(144)	Sedentary non-obese elderly women: - resistance training with linear periodisation (RT/LP) – n=20 – 69.20 (SD 6.05) years - resistance training with undulating periodisation (RT/UP) – n=19 – 65.52 (SD 4.72) years	Sedentary non-obese elderly women – n=10 – 66.90 (SD 7.56) years	- Training program: 2 sessions/week for 16 weeks - CG: no training program - Upper and lower body exercise: 40-50 min - LP: build-up from 12-14 RM to 6-8 RM - UP: training loads varied on daily basis	Venous blood – pre and post program	ELISA (IL-1β, IL-1Ra, IL-10, IL15, irisin, TLR4)	TLR4 =	Cytokines: ↑ baseline irisin in LP compared to control and UP; no other group differences or impact of training

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Rodriguez-Miguelez, 2014(154)	Healthy elderly subjects – n=16 (mixed gender) – 69.1 (SEM 1.1) years	Healthy elderly subjects – n=10 (mixed gender) – 70.0 (SEM 0.9) years	- Training program: 2 sessions/week for 8 weeks - CG: normal daily routines - Upper and lower body exercise: 10 min warm-up, exercise at 60-80% 1RM	Venous blood – pre and post program	ELISA (CRP, TNF-α) Western blot (HSP60, HSP70, TLR2, TLR4, TRIF, IKKi/IKKε, MyD88, p65, IRF, pIRF3, IRF7, pIRF7, ERK1/2, pERK1/2, p38, pP38, TNF-α, IL-10) qPCR of PBMC (IL-10, TNF-α)	↓ TLR2,TLR4 after program (↔ CG =)	MyD88 and p65 protein: ↓ after program (↔ CG =) pP38 and pERK1/2 protein: ↓pP38 and ↑pERK1/2 after program (↔ CG =); total p38 and ERK1/2 = TRIF, IKK, pIRF3 and pIRF7 protein: ↓TRIF, IKK, pIRF3 and pIRF7 after program (↔ CG =) IL-10 and TNF-α mRNA and protein: ↑ IL-10 mRNA and protein after program (↔ CG =); TNF-α mRNA or protein = Plasma CRP and IL-6: ↓ CRP and IL-6 after program (↔ CG =) HSP60 and HSP70: ↑HSP70 after program; ↓HSP60 after program (↔ CG =)

Age is given as mean unless otherwise stated. Abbreviations: 1RM, one-repetition maximum; CD, cluster of differentiation; CG, control group; CRP, c-reactive protein; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated protein kinase; HMGB, high mobility group box; HSP, heat shock protein; IFN, interferon; IKK, IκB kinases; IL, interleukine; IRAK, IL-1R-associated kinase; IRF, interferon regulatory transcription factor; LPS, lipopolysaccharide; MHR, maximal heart rate; min, minute; mRNA, messenger RNA; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa B; PBMC, peripheral blood mononuclear cells; qPCR, quantitative polymerase chain reaction; ra, receptor antagonist; RT; resistance training; SD, standard deviation; SE, standard error; SEM, standard error of the mean; SIRT, sirtuin; TAK, transforming growth factor beta-activated kinase; TNF, tumor necrosis factor; TLR, toll-like receptor; TRAF, TNFR-associated factor; TRIF, TIR-domain-containing adaptor protein-inducing interferon-β.

Supplementary Table 5 | Summary of the characteristics of the articles investigating the effects of aerobic exercise programs.

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Bartlett, 2017(10)	Healthy inactive individuals: - HIIT – n=14 (4 ♂, 10 ♀) – 42 (SD 12) years - MICT – n=13 (5 ♂, 8 ♀) – 45 (SD 10) years	/	- Training program: 3 sessions/week for 10 weeks, for MICT group 2 additional self-administered sessions/week - HIIT: 18-25 min at >90% HR_{max} during sprint intervals - MICT: 30-45 min at 70% HR_{max}	Blood – pre and post program	Phagotest of whole blood with flow cytometry Phagoburst of whole blood with flow cytometry Flow cytometry (CD14, CD16, TLR2, TLR4) Multiplex luminometry (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, GMCSF, TNF-α)	Neutrophil TLR2 and TLR4: = TLR2 expression: $CD14^+/CD16^-$ and $CD14^+/CD16^{bright}$ cells =; ↓ $CD14^+/CD16^{int}$ cells TLR4 expression: $CD14^+/CD16^-$ and $CD14^+/CD16^{int}$ cells =; ↓ $CD14^+/CD16^{bright}$ cells	Phagocytosis and oxidative burst: ↑ neutrophil phagocytosis and oxidative burst in both groups; ↑ monocyte phagocytosis in both groups; monocyte oxidative =, but % of monocytes producing an oxidative burst ↑ in both groups Cell count: leukocytes, monocytes =; ↑$CD14^+/CD16^-$ and ↓$CD14^+/CD16^{int}$ in both groups; ↓$CD14^+/CD16^{bright}$ in HIIT Neutrophils CD16: =
Bartlett, 2018(11)	Physically inactive adults with rheumatoid arthritis – n=12 (1 ♂, 11 ♀) – 64 (SD 7) years	/	- Training program: 3 sessions/week for 10 weeks - High-intensity interval walking: 30 min with ten ≥60 second intervals at 80-90% $VO2_{reserve}$ separated by lower-intensity intervals at 50-60% $VO2_{reserve}$	Venous blood – pre and 16-24h after exercise	Flow cytometry (CD14, CD16, TLR2, TLR4,HLA-DR, phagocytosis by whole blood) Insall chamber (neutrophil migration) Luminol-amplified chemiluminescence (neutrophil ROS) Sandwich immunoassay (IL-1β, IL-6, IL-10, TNF-α) Enzymatic colorimetric assay (NEFA)	Neutrophil TLR4: = Monocyte TLR2 and TLR4: ↓TLR2, TLR4 on intermediate monocytes after exercise; = on classical and non-classical monocytes	Cell count: no difference in total leukocytes or subpopulations Plasma cytokines and NEFA: no impact of exercise training Neutrophil function: ↑chemotactic index; ↑ chemotaxis; chemokinesis =; ↑ phagocytosis; ↑ ROS production after exercise training Neutrophil CD16: = Monocyte subpopulation: ↓$CD16^+$ monocytes, intermediate monocytes and non-classical monocytes after training Monocyte HLA-DR: ↓ on intermediate monocytes after exercise; = on classical and non-classical monocytes Monocyte function: ↑ phagocytosis after training

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Boehler, 2019(16)	Myositis patients – n=3	/	- Training program: 3 sessions/week for 12 weeks - Cycling: 1h at 70% VO_2max	Muscle biopsy – pre and post program	qPCR (TLR7)	↓ TLR7	/
Capó, 2014(22)	See "Capó *et al.* 2014" in Supplementary Table 3						
Child, 2013(35)	Overweight (BMI >25 kg/m²) sedentary (≤2 exercise sessions per week) men – n=11 – 24 (SD 5) years	/	- Training program: 3 sessions per week for 2 weeks - Cycling: ten 4-min intervals at 85% VO_2peak separated by 2-min rest periods	Venous blood – pre and post program	Flow cytometry (CD14, CD16, TLR2, TLR4)	TLR2 =; ↑ TLR4 on $CD14^+$ cells, $CD14^{++}/CD16^-$ and $CD14^+/CD16^{++}$ cells; TLR4 = on $CD14^{++}/CD16^+$ cells	Cell count: total leukocyte count, leukocyte and monocyte subpopulations =
Colleluori, 2019(40)	See "Colleluori *et al.* 2019" in Supplementary Table 4						
Estébanez, 2020(46)	Healthy elderly subjects without participation in aerobic exercise training in the last year – n=9 (2 ♂, 7 ♀) – 68.68 (SD 1.24) years	Healthy elderly subjects without participation in aerobic exercise training in the last year – n=5 (2 ♂, 3 ♀) – 70.78 (SD 1.51) years	- Training program: 2 sessions/week for 8 weeks - CG: maintained regular physical activity - Cycling: 5 min warm-up, 15-20 min at 70-75% MHR with progressive introduction of short periods of high intensity (1 min at 90-95%	Venous blood – pre and post program	Spectrophotometer (Reduced glutathione (GSH), total antioxidant capacity (TEAC)) Dichloro-dihydro-fluorescein diacetate assay (reactive oxygen species/reactive nitrogen species (ROS/RNS)) Western blot of PBMC (TLR4, 4-hydroxynonenal (4-	TLR4 = (no baseline difference, no impact of exercise)	GSH, TEAC and ROS/RNS: no baseline differences, no impact of exercise PC, 4-HNE and 3NT: no baseline difference, no impact of exercise
			MHR), 5 min active recovery		HNE), 3-nitrotyrosine (3NT)) OxyBlot kit (protein carbonyls (PC))		
Ferrari, 2019(53)	Patients with essential hypertension (immediately after diagnosis or in the context of sub-optimal treatment): - prehypertensive – n=14 (9 ♂, 5 ♀) – majority age group 61-70 years - hypertensive – n=30 (24 ♂, 6 ♀) – majority age group 41-60 years	Healthy normotensive subjects – n=24 (17 ♂, 7 ♀) – majority age group 41-60 years	- Training program: ≥4 sessions/week for 12 weeks (patient & control group) - Session: cycling or jogging with ≥30 min reaching heart rate corresponding to anaerobic threshold	Venous blood – pre and post program	PCR pyrosequencing (DNA methylation assay)	TLR2 = (non-significant increase in methylation after exercise)	PCR: TNF = (moderate non-significant increase in methylation)
Ghosh, 2015(65)	Healthy, non-smoking community-dwelling sedentary (≤1 exercise session/week) elderly – n=12 (8 ♂, 4 ♀) – 73.8 (2.1) years (11/12 participated to the exercise program)	Healthy, non-smoking community-dwelling sedentary (≤1 exercise session/week) young participants – n=13 (6 ♂, 7 ♀) – 25.5 (1.0) years	- Training program: 3-4 sessions/week for 16 weeks - CG: continuation habitual behaviour - Cycling: 20-45 min at 65-80% VO_2max (gradual increase in duration and intensity)	Vastus lateralis muscle biopsy – pre and 48-72h after program	ELISA (IL-6, TNF-α, LPS-binding protein) Limulus Amoebocyte Lysate assay (plasma LPS) Western blot (TLR4, NF-κB p50, NF-κB p65, JNK, pJNK, ERK, pERK, p38, pP38) qPCR (TLR4)	At baseline ↑ TLR4 (mRNA and protein) in elderly; no impact of exercise	Plasma cytokines: baseline IL-6 ↑ in elderly compared to young; no difference in TNF-α; no impact of exercise Plasma LPS and LPS-binding protein: baseline LPS and LPS-binding protein ↑ in elderly compared to young and not affected by exercise NF-κB p65 and NF-κB p50: baseline NF-κB p65 and NF-κB p50 ↑ in elderly compared to young; no impact of exercise JNK and pJNK: baseline pJNK/PNK ratio ↑ in elderly compared to young; no impact of exercise ERK, pERK, 38, pP38: no differences between groups or after exercise
Lackermair, 2017(92)	See "Lackermair *et al.* 2017" in Supplementary Table 3						
Mejías-Peña, 2016(119)	Healthy old subjects without experience in aerobic exercise training – n=16 (mixed gender) – 69.6 (SEM 1.0) years	Healthy old subjects without experience in aerobic exercise training – n=13 (mixed gender) – 70.0 (SEM 0.9) years	- Training program: 2 sessions/week for 8 weeks - CG: no intervention - Cycling: 25-30 min at 70-75% MHR (with progressive introduction of short periods of intense activity (90-95% MHR)	Venous blood – 5-6 days pre and post program	Western blot of PBMC (TLR2, TLR4, TRIF, MyD88)	TLR2, TLR4 = (between and within groups)	TRIF and MyD88 protein: no group differences or impact of training

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Nickel, 2011(130)	Male marathon runners: - obese non-elite (ONE) – n=15 – 40 (SDM 6) years - lean non-elite (LNE) – n=16 – 40 (SDM 6) years - elite (LE) – n=16 – (n=16)(40 (SDM 7) years)	/	- Training program: 4 sessions/week for 10 weeks - Running: continuous aerobic exercise and interval training with gradual increase in duration and intensity (average training mileage 35 km/week for ONE, 38 km/week for LNE and 54 km/week for LE)	Venous blood – pre and post program	Flow cytometry (leukocyte subpopulations) qPCR (TLR2, TLR4, TLR7, MyD88, NF-κB) Western blot (TLR2, TLR4, TLR7, MyD88)(8 subjects of LNE only) ELISA (IL-6 and TNF-α)	Baseline: TLR2, TLR4, TLR7 = Post-training TLR2 mRNA: ↑in LNE, similar patterns in other groups without significance Post-training TLR4 and TLR7 mRNA: ↑ in all groups Post-training western blot: TLR2 =; ↑ TLR4, TLR7	Leukocyte and monocyte count: = Dendritic cell subsets: ↓ myeloid DCs and ↑ plasmacytoid DCs in ONE at baseline with normalisation after program Baseline and post-training MyD88 and NF-κB: = Post-training western blot: MyD88 = IL-6: ↓ in LE at baseline with further decrease post program in LE TNF-α: ↓in LE at baseline; no changes after program
Niemiro, 2018(131)	Lean sedentary (≤30 min of moderate/high intensity exercise per week) individuals – n=17 (9 ♂, 8 ♀) – 23.9 (SD 5.4) years	Obese sedentary (≤30 min of moderate/high intensity exercise per week) individuals – n=10 (3 ♂, 7 ♀) – 29.0 (SD 8) years	- Training program: 3 sessions/week for 6 weeks (active & control group) - Cycling or running: 30-60 min at 60-75% MHR	Venous blood – 3-4 days pre and post program	MethoCult (CFU assay) Flow cytometry	Concentration of hematopoietic stem cells expressing TLR4 ↓ after exercise; TLR4 content ↑ after exercise; proportion of hematopoietic stem cells expressing TLR4 =	Cell count: ↓ circulating progenitor cells, ↓ adipose derived mesenchymal stem cells, ↓ common lymphoid progenitor cells in lean participants after exercise (↔ = in obese); ↓ hematopoietic stem cells in both groups after exercise program CFU count: baseline CFU potential of granulocytes and granulocyte/monocyte ↑ in obese participants; total number of CFU and CFU in granulocyte/monocyte ↑ after exercise
Reyna, 2013(150)	Sedentary (≤1 exercise bout/week) obese subjects with stable body weight: - non-diabetic – n=8 – 40 (SE 3) years - T2DM – n=11 – 50 (SE 3) years	Sedentary (≤1 exercise bout/week) subjects with stable body weight: - lean – n=17 – 39 (SE 2) years	- Training program: daily training for 15 days (patient & control groups) - Cycling: 4 identical periods of 8 min at 70% VO_2peak, 2 min at 90% VO_2peak and 2 min complete rest	Venous blood – pre and post program	Western blot mononuclear cells (TLR2, TLR4, ERK, JNK) ELISA (NF-κB p65)	TLR2 =; TLR4 ↑ at baseline in T2DM (significant) and obese (non-significant) compared to lean; no effect of exercise	ERK and JNK: baseline ERK phosphorylation ↑ in T2DM with non-significant ↓ after exercise; no impact of group or training on JNK phosphorylation NF-κB p65 binding: no impact of group or exercise
Robinson, 2015(153)	Subjects with prediabetes, BMI >24kg/m² and inactive lifestyle (<2 30-min bouts of moderate-to-vigorous exercise/week): - HIIT – n=20 (3 ♂, 17 ♀) – 52 (SD 10) years - MICT – n=18 (4 ♂, 14 ♀) – 52 (SD 10) years	/	- Training program: 5 sessions/week for 2 weeks - HIIT: four to ten 1-min intervals at 85-90% Wpeak with 1-min recovery at 20% Wpeak - MICT: 20-50 min at 32.5% Wpeak	Venous blood – pre and post program	ELISA (TNF-α, IL-1β, IL-6, IL-10) Flow cytometry (CD14, TLR2, TLR4) Whole blood stimulation with LPS and PamCSK4 (24h) with ELISA	TLR2: ↓ on lymphocytes; no change on monocytes and neutrophils TLR4: ↓ on lymphocytes and $CD14^+$ monocytes in both groups; ↓ on $CD15^+$ neutrophils in MICT (↔ HIIT =)	Cell count: no effect of training on leukocyte subpopulation concentrations Plasma cytokines: ↓ IL-10 after MICT LPS- and PamCSK4-stimulated blood: no effect of training
Sloan, 2018(172)	Healthy young adults without regular exercise and with VO_2max <43 mL/kg/min for men and <37 mL/kg/min for women – n=60 (28 ♂, 32 ♀) – 31 (SD 6) years	Healthy young adults without regular exercise and with VO_2max <43 mL/kg/min for men and <37 mL/kg/min for women – n=59 (28 ♂, 31 ♀) – 31 (SD 6) years	- Training program: 2 week run-in stretching period followed by 4 sessions/week for 12 weeks and 4 weeks of sedentary deconditioning - CG: 2 week run-in stretching period followed by maintenance of sedentary lifestyle - Session: 10-15 min warm-up, 30-40 min aerobic activity at 55-75% MHR, 10-15 min cool-down	Venous blood – after run-in period (2 weeks), post program (14 weeks) and post deconditioning (18 weeks)	Whole blood stimulation with LPS (4h) with Human Cytokine Array Western blot (TLR4)	TLR4 = (no impact of group, exercise or deconditioning)	Whole blood stimulation: no group differences; ↑ inducible TNF-α, inducible IL-6 post program compared to baseline in the trained group; ↓ inducible TNF-α, inducible IL-6 post deconditioning compared to post program in exercise group

Age is given as mean unless otherwise stated. Abbreviations: 3NT, 3-nitrotyrosine; 4-HNE, 4-hydoxynonenal; CD, cluster of differentiation; CG, control group; CFU, colony-forming unit; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated protein kinase; GMCSF, granulocyte macrophage colony-stimulating factor; GSH, reduced glutathione; h, hour; HIIT, high-intensity interval training; HR, heart rate; HLA-DR, human leukocyte antigen – DR isotype; IL, interleukine; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MHR, maximal heart rate; MICT, moderate-intensity continuous training; min, minute; mRNA, messenger RNA; MyD88, myeloid differentiation primary response gene 88; NEFA, non-esterified fatty acid; NF-κB, nuclear factor kappa B; PBMC, peripheral blood mononuclear cells; PC, protein carbonyls; qPCR, quantitative polymerase chain reaction; RNS, reactive nitrogen species; ROS, reactive oxygen species; SD, standard deviation; SDM, standard-deviation of the mean; SE, standard error; SEM, standard error of the mean; T2DM, type 2 diabetes mellitus; TEAC, total antioxidant capacity; TNF, tumor necrosis factor; TLR, toll-like receptor; TRIF, TIR-domain-containing adaptor protein-inducing interferon-β; W, power output.

Supplementary Table 6 | Summary of the characteristics of the articles investigating the effects of combined exercise programs.

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Colleluori, 2019(40)	See "Colleluori *et al.* 2019" in Supplementary Table 4						
Lambert, 2008(94)	Obese sedentary elderly with evidence of frailty – n=8 (4 ♂, 4 ♀) – 68.5 (SE 1.4) years	Obese sedentary elderly with evidence of frailty – n=8 (4 ♂, 4 ♀) – 69.6 (SE 1.4) years	- Training program: 3 sessions/week for 12 weeks - CG: energy-deficit diet and behaviour therapy - Session: 90 min with endurance exercise at 75-90% MHR and resistance exercise at 65-80% 1RM	Vastus lateralis muscle biopsy – pre and post program	qPCR (TLR4, IL-6, TNF-α) ELISA (serum IL-6 and TNF-α)	↓ TLR4 in exercise group; no difference in weight loss group	IL-6 and TNF-α mRNA: ↓ in exercise group; no change in weight loss group Serum IL-6 and TNF-α: =
Liu, 2015(108)	Non-insulin dependent T2DM without frequent exercise (<2/week for <15 min) – n=42 (12 ♂, 30 ♀) – 52.59 (SD 11.43) years: - conventional therapy (CT) – n=20 - intensive therapy (IT) – n=22	Healthy people – n=20 (7 ♂, 13 ♀) – 51.20 (SD 11.34) years	- Training program: home-based and supervised exercises: 3 aerobic sessions/week and 2-3 resistance sessions/week for 12 weeks - CT: regular drug treatment and diet guidance - IT: regular drug treatment, diet guidance and training program - CG: no intervention - Aerobic session: 40-60 min at 40-60% VO_2max	Venous blood – pre and post program (CG 1 measurement not related to the program)	qPCR of PBMC (TLR4, NF-κB p65) Western blot of PBMC (TLR4, NF-κB p65) ELISA (IL-18, IL-33)	TLR4 mRNA and protein: ↑ in T2DM at baseline compared to CG; after program mRNA ↓to control level in CT but still higher than IT with greater reduction; after program protein = in CT but ↓ to control level in IT	NF-κB p65 mRNA and protein: ↑ in T2DM at baseline compared to control; after program mRNA ↓to control level in CT but still higher than IT with greater reduction; after program protein = in CT but ↓ to control level in IT IL-18: ↑ in T2DM at baseline; after program = in CT but ↓ to control level in IT IL-33: ↓ in T2DM at baseline; after program ↑ in T2DM (more in IT), but still reduced compared to CG
			- Resistance session: 50-60% 1RM				
Munters, 2016(124)	Patients with definite or probable polymyositis or dermatomyositis, exercising ≤ 1/week – n=4 (1 ♂, 3 ♀) – median 66 (IQR 19) years	Patients with definite or probable polymyositis or dermatomyositis, exercising ≤ 1/week – n=4 (1 ♂, 3 ♀) – median 63 (IQR 16) years	- Training program: 3 sessions/week for 12 weeks - CG: maintained stable level of physical activity - Session: 60 min with 30 min cycling at 70% VO_2max and 30 min knee extensor exercises at 30-40% 1RM	Vastus lateralis muscle biopsy – pre and post program	Gene microarray (n=8)	↑ TLR7 after program	Gene microarray: ↑ HSPD1 gene (HSP60) after program in trained group; ↓ HSPA2 (HSP70-2) after program in trained group; ↑ IL28A in CG after program
Shimizu, 2011(168)	Healthy sedentary, independently living elderly – n=12 (3 ♂, 9 ♀) – 67.1 (SE 1) years	Healthy sedentary, independently living elderly – n=12 (4 ♂, 8 ♀) – 67.5 (SE 0.7) years	- Training program: 2 sessions/week with additionally a limited number of body weight resistance exercises for home (≥3 days/week) for 12 weeks - CG: no exercise Session: 10 min stretching, 10 min cycling, resistance training at 20-40% 1RM, 10 min stretching	Venous blood – pre and post program	Flow cytometry (CD14, TLR4, CD80)	$CD14^+/TLR4^+$ cells =	Leukocyte count: no influence of training on leukocyte, lymphocyte and monocyte number CD28: number of $CD28^+/CD8^+$ cells ↑ after training in the exercise group CD80: number of $CD14^+/CD80^+$ cells↑ after training in the exercise group
Soltani, 2020(174)	Female overweight or obese bachelor students with a sedentary lifestyle (≥6 months no participation in any exercise training) – n=13 – 21.3 (SD 1.37) years	Female overweight or obese bachelor students with a sedentary lifestyle (≥6 months no participation in any exercise training) – n= 13 – 20.69 (SD 1.54) years	- Training program: 5 sessions/week for 2 weeks - CG: no exercise intervention - Session: warm-up 10-12 min, upper and lower body cycling at 50-90% MHR and resistance	Venous blood – 5 days pre and 48h post program	qPCR of PBMC (TLR4, IRF3, NF-κB)	↓ TLR4 after exercise (↔ = in control group)	IRF3, NF-κB: = (no group differences or alterations by exercise)
			training at 40-60% 1RM for 25 min, cool-down 8-10 min				

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Stewart, 2005(180)	Subjects with (very) low physical activity score and estimated VO_2max < average: - young – n=14 – 24.9 (SE 4.7) years - older – n=17 – 71.0 (SE 4.3) years	Subjects (very) high physical activity score and estimated VO_2max good to excellent: - young – n=15 – 25.2 (SE 5.0) years - older – n=14 – 71.2 (SE 4.4) years	- Training program: 3 sessions/week for 12 weeks - CG: maintained usual levels of physical activity - Session: warm-up, 20 min endurance training, resistance training, stretching, cool-down	Venous blood – pre and post program	Flow cytometry (CD14, TLR2, TLR4) Whole blood stimulation with LPS or PGN (24h) with ELISA	TLR2 on $CD14^+$ cells: no training-effect; overall post-value ↓ compared to pre-value TLR4 on $CD14^+$ cells: ↓ in trained group; CG =	CD14: no significant differences LPS-stimulated IL-6: ↓ in trained groups; values post program lower in young compared to elderly LPS-stimulated IL-1β and TNF-α: no training effect; IL-1β values post program lower in young compared to elderly PGN-stimulated IL-6: no training effect; post program values lower in young compared to pre; pre values in young higher compared to elderly PGN-stimulated TNF-α: no significant differences PGN-stimulated IL-1β: post values on average lower than pre
Timmerman, 2008(187)	Physically inactive subjects (PI) (no regular physical activity in the last 6 months and VO_2max <26 mL/kg/min for male and <23 mL/kg/min for female) – n=15 (4 ♂, 11 ♀) – 71 (SD 5.74) years	Physically active subjects (PA) (exercising ≥3 days/week in the last 6 months and VO_2max >35 mL/kg/min for male and >28 mL/kg/min for female) – n=15 (8 ♂, 7 ♀) – 70 (SD 4.56) years	- Training program: 3 sessions/week for 12 weeks - CG: maintain physically active lifestyle - Session: treadmill running for 20 min at 60-70% HRR and resistance exercise for 30 min at 70-80% 1RM (2 sets of 8 exercises)	Venous blood – pre and post program	Flow cytometry (CD14, CD16, TLR4) Whole blood stimulation with LPS or LPS + polymyxin B (24h) with ELISA	TLR4: ↑ on classical monocytes from PI than from PA; no baseline difference $CD14^+$TLR4; no post-training differences	BMI: lower in PA at baseline Monocyte counts: ↓ inflammatory monocytes in PA at baseline; after training ↓ in PI Unstimulated whole blood: baseline TNF-α/$CD14^+$ monocyte lower in PA compared to PI; ↓ in PI after training LPS-stimulated whole blood: ↓TNF-α in PI after training; addition of polymyxine B blunted cytokine production

Age is given as mean unless otherwise stated. Abbreviations: 1RM, one-repetition maximum; CD, cluster of differentiation; CG, control group; ELISA, enzyme-linked immunosorbent assay; HHR, heart rate reserve; HSP, heat shock protein; IL, interleukine; IQR, interquartile range; IRF, interferon regulatory transcription factor; LPS, lipopolysaccharide; MHR, maximal heart rate; min, minute; mRNA, messenger RNA; NF-κB, nuclear factor kappa B; PBMC, peripheral blood mononuclear cells; PGN, peptidoglycan; qPCR, quantitative polymerase chain reaction; SD, standard deviation; SE, standard error; T2DM, type 2 diabetes mellitus; TNF, tumor necrosis factor; TLR, toll-like receptor.

Supplementary Table 7 | Summary of the characteristics of the articles investigating the effects of exercise programs which are not otherwise classifiable.

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
Ferrer, 2018(54)	Healthy elderly volunteers (n=116) with later categorisation into active and passive based on terciles of average MET in the last year: - active (1st tercile): male 62.5 (SEM 0.9) years, female 67.4 (SEM 1.0) years - sedentary (3rd tercile): male 64.6 (SEM 1.1) years, female 67.3 (SEM 1.1) years	/	/	Venous blood – after overnight fast	ELISA (IL-6) Guaiacol oxidation (MPO) qPCR of PBMC (TLR4, NF-κB, IL-1β, IL-1Ra, IL-6, IL-10, TNF-α) Western blot of PBMC (TLR2, TLR4)	TLR4 mRNA: ↑ in active males compared to active females TLR2 and TLR4 protein: ↑ TLR2 in active compared to the sedentary; TLR4 = (no impact of sex or activity)	Cell count: ↓ total leukocyte, neutrophil and lymphocyte counts in the active group Plasma IL-6 and MPO: ↓ levels of IL-6 in the active group; MPO = Gene expression: ↑ IL-10 in active males compared to sedentary males; ↑ NF-κB in active participants compared to sedentary; no impact of group or sex on IL-1Ra, IL-1β, IL-6, NF-κB or TNF-α
Flynn, 2003(58)	See "Flynn *et al.* 2003" in Supplementary Table 2						
Lundeland, 2012(110)	Healthy and well-trained male cadets – n=8 – 24.1 (2.5) years	/	- Ranger training course - Semi-continuous physical strain with restriction on sleep and food intake during 7 days	Venous blood – day 0, 3, 5 and 7	Flow cytometry (CD14, TLR4) Whole blood stimulation with saline or LPS (during 6h) with ELISA Gas chromatography (NEFAs)	Monocyte TLR4: = (non-significant ↑ expression over time)	Cell count: total leukocyte count ↑ until day 5 (with a parallel ↑ in granulocytes); monocyte count ↓ until day 3 followed by an increase Cytokine after LPS stimulation: TNF-α, IL-1β and IL-6 (per monocyte) ↑ at day 3 followed by ↓ to below baseline at day 5 and 7 Whole blood stimulation: ↑ TLR expression day 3-7 with saline- and LPS-stimulation
							Gas chromatography: NEFA ↑ on day 3, 5 and 7; ↑ UFA/SFA ratio

First author, year	Study population – n (sex distribution) – age	Control group if applicable – n (sex distribution) - age	Experimental design Exercise intervention	Study specimen – moment of collection	Analytical technique (analyses)	Outcome related to TLR	Other outcomes
McFarlin, 2004(116)	See "McFarlin *et al.* 2004" in Supplementary Table 2						
McFarlin, 2006(115)	Subjects with an active lifestyle and VO_2max > average - young – n=21 – 24 (SD 4.8) years - elderly – n=23 – 72 (SD 5) years	Subjects with an inactive lifestyle and VO_2max < average - young – n=19 – 24 (SD 5.1) years - elderly – n=21 – 69 (SD 4) years	/	Venous blood – after 72h without prior exercise	Flow cytometry (TLR4, CD14) Whole blood stimulation with LPS (24h) with ELISA	↑ TLR4 in inactive compared to active	Leukocyte counts: no differences leukocyte or monocyte count $CD14^+$ count: no significant differences LPS-stimulated IL-6: inactive ↑ compared to active LPS-stimulated IL-1β: young active ↑ compared to old active; old-inactive ↑ compared than young active LPS-stimulated TNF-α: inactive ↑ compared to active
Rodriguez-Miguelez, 2015(155)	Seniors without experience in whole body vibration – n=16 – 71.04 (SEM 1.5) years	Seniors without experience in whole body vibration – n=12 – 70.0 (SEM 0.9) years	- Whole body vibration training program - CG: normal routine - 2 sessions/week for 8 weeks of static and dynamic exercises (training volume and vibration frequency gradually increased)	Venous blood – 5-6 days pre and post program	qPCR of PBMC (TNF-α, IL-10) Western blot of PBMC (HSP60, HSP70, TLR2, TLR4, TRIF, MyD88, TNF-α) ELISA (TNF-α)	↓TLR2, TLR4 in trained group only	TNF-α serum level, mRNA and protein: mRNA =; ↓ plasma and protein in trained group IL-10 mRNA and protein: ↑ mRNA and protein after program in trained group HSP60 and HSP70: ↑HSP60 and ↓HSP70 after program in trained group MyD88, TRIF and p65: ↓MyD88, TRIF and p65 after program in trained group
Shimizu, 2015(167)	Competitive collegiate male kendo athletes: - placebo-supplemented group – n=9 – 19.7 (SE 0.9) years *- coenzyme Q10-supplemented group – n=9 (not considered in this review)*	/	- Kendo training camp - morning (2.5h) and afternoon session (3h) each day during 6 days	Venous blood – 14 days before camp and day 1, 3, and 5 of the camp and 7 days after finishing	Flow cytometry (CD14, TLR4)	$CD14^+/TLR4^+$ cells: ↑ day 3, 5 and post-training compared to baseline	Cell count: ↑ total leukocytes on day 3 with normalisation post-training; monocytes =
Timmerman, 2016(186)	Healthy elderly volunteers – n=26 (9 ♂, 17 ♀) – 68 (SD 4) years	/	/ (self-reported physical activity questionnaire and cardiorespiratory fitness test)	Vastus lateralis muscle biopsy – no physical activity in preceding 48h	Western blot (TLR4, membrane-bound TNF-α, soluble TNF-α)	TLR4 expression: ↑ in women compared to men; negative correlation with self-reported physical activity in men (also after correction for body fat or BMI); positive correlation with self-reported physical activity in women (not significant after controlling for body fat or BMI)	Membrane-bound and soluble TNF-α: no correlation with self-reported physical activity
Zheng, 2015(212)	Students of the university badminton club with 3/week 2h of exercise – n=20 (10 ♂, 10 ♀) – 20.8 (SD 2.1) years	Healthy sedentary students (CG) – n=25 (12 ♂, 13 ♀) – 21.8 (2.1) years	None	Venous blood - after obtaining informed consent	ELISA (IL-6, TNF-α, IFN-γ) Flow cytometry (DC subsets) PBMC or DC stimulation assay with heat-inactivated *S. pyogenes*, hepatitis B core antigen (24h) qPCR of PBMC (TLR2, TLR4 TLR7, MyD88)	Unstimulated, hepatitis core antigen or *S. pyogenes* stimulated mRNA: ↑ TLR2, TLR7 and in exercise group vs. CG except for TLR7 *in S. pyogenes* =; TLR4 =	Plasma cytokines: IL-6, TNF-α, IFN-γ = DC subsets: no differences between both groups Unstimulated PBMC culture: ↑IL-6 and TNF-α in CG vs. exercise group; IFN-γ = PBMC stimulation with hepatitis core antigen or *S. pyogenes*: ↑ IL-6, TNF-α and IFN-γ in exercise group vs. CG DC stimulation with hepatitis core antigen or *S. pyogenes*: ↑ IL-12 and IFN-α in exercise group vs. CG Unstimulated, hepatitis core antigen or *S. pyogenes* stimulated MyD88 mRNA: ↑ MyD88 in exercise group vs. CG

Age is given as mean unless otherwise stated. Abbreviations: CD, cluster of differentiation; CG, control group; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; h, hour; HSP, heat shock protein; IFN, interferon; IL, interleukine; LPS, lipopolysaccharide; MET, metabolic equivalent of task; MPO, myeloperoxidase; mRNA, messenger RNA; MyD88, myeloid differentiation primary response gene 88; NEFA, non-esterified fatty acid; NF-κB, nuclear factor kappa B; PBMC, peripheral blood mononuclear cells; qPCR, quantitative polymerase chain reaction; ra, receptor antagonist; SD, standard deviation; SE, standard error; SEM, standard error of the mean; SFA, saturated fatty acid; TNF, tumor necrosis factor; TLR, toll-like receptor; TRIF, TIR-domain-containing adaptor protein-inducing interferon-β; UFA, unsaturated fatty acid.

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Exercise training effects on natural killer cells: a preliminary proteomics and systems biology approach

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ABSTRACT

Background: Regular exercise, particularly moderate-intensity continuous training (MICT), can improve immune function. Natural killer (NK) cells, a subset of lymphocytes that react to infections, are the most responsive innate immune cells to exercise, but the mechanisms underlying this are poorly understood. A type of exercise training that is gaining popularity in recent years is high-intensity interval training (HIIT), but how it affects NK cells is largely unknown. In fact, intense exercise has been traditionally viewed as a potential stressor to immune homeostasis. The purpose of this study was to determine in healthy, previously untrained adults (N=8 [3 male; 40±6 years]) the effects of an intervention consisting of 4-week MICT followed by 4-week HIIT on NK cells as compared with a pre-training (baseline) state.

Methods: Participants were studied at three time points: baseline, mid-intervention (after MICT), and post-intervention (after HIIT). Main assessments included cytotoxicity assays, flow-cytometry analysis of NK cell surface markers, and interrogation of the cellular proteome using a systems biology approach.

Results: A significant time effect was found for NK cell cytotoxicity ($p<0.001$), which was increased ~10-fold at both mid- and post-intervention versus baseline. No significant intervention effect was found for NK surface receptor expression, except for CXCR3 determined as mean fluorescence intensity ($p=0.044$, although with no significant differences in post hoc pairwise comparisons).

The proteins showing a higher differential expression (Log2 fold-change>10 and false discovery rate [FDR] q-value<0.001) were COP9 signalosome subunit 3 (COPS3), DnaJ heat shock protein family member B11 (DNAJB11), histidyl-TRNA synthetase 1 (HARS), NIMA related kinase 9 (NEK9), nucleoporin 88 (NUP88), phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1), regulator of chromosome condensation 2 (RCC2), TAO kinase 3 (TAOK3), transducin beta like 2 (TBL2), and ring finger protein 40 (RNF40). All were upregulated at mid-intervention compared with baseline, with the exception of HARS, which was downregulated. Four enriched pathways (FDR <25%) were found: two related to transmembrane transport and cellular composition (downregulated at mid-intervention vs baseline), and two related to oxidation-reduction reactions (upregulated at post-intervention versus baseline).

Conclusions: A progressive exercise intervention of MICT followed by HIIT induces a remarkable improvement in NK function compared with the untrained state, although at the mechanistic level the pathways involved seem to differ over time during the intervention.

Keywords: immune system; high interval training; NK cell; cytotoxicity.

INTRODUCTION

Regular exercise, especially (but not only) if performed at low-moderate workloads (termed 'moderate-intensity continuous training' [MICT], typically ≥ three sessions/week of ≥ 30-minute duration at ~60 to 80% of peak oxygen uptake [VO_2peak] during several weeks), induces robust biological adaptations that positively impact human health, as reflected by an improvement in cardiorespiratory (or 'aerobic' fitness) indicators. The benefits of MICT may also extend to the immune system, particularly (but not only) the innate arm (73). Among the innate immune cell subtypes that can be potentially receptive to exercise, the evidence is especially strong for an exer-

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cise-induced mobilization of natural killer (NK) lymphocytes to the circulation, which might be accompanied by an improved cytotoxicity (or 'killing capacity') of these cells (28).

MICT has been shown to increase NK cytotoxic activity in preclinical models (13, 34) and human studies (for a review see Zimmer et al (80)); for instance, in women with mild obesity (45), in patients (52) or survivors of breast cancer (15), in patients with stomach cancer after curative surgery (38), or in children with pediatric cancer undergoing hematopoietic stem cell transplantation (9). Improvements have also been reported after resistance training in older women (35) or in women recovering from breast cancer (22). In addition, some cross-sectional studies have reported that NK cytotoxicity is greater in young/old athletes (36), trained marathon runners (40) and cyclists (40), female elite rowers (44), and endurance-trained older women (67 to 85 years) (42) than in their non-athletic/sedentary peers. And in general, NK cell cytotoxicity has been shown to be higher among older people who are active than in their less active age-matched controls (27, 61, 76). In other studies, however, no improvement was reported in patients with breast cancer after MICT alone (41) or combined with a healthy eating intervention (56), or in older women after 12-week MICT (42). Furthermore, 3 months of training with multicomponent exercises (strength, balance, walking, stretching) of gradually increasing intensities reduced NK cytotoxic activity in frail older adults (54) and one month of heavy exercise training during the pre-competition season had a similar effect in female volleyball players (65).

The underlying mechanisms by which MICT may increase NK number or function remain to be clearly elucidated. One pre-clinical study using tumor-bearing mice found that exercise training (wheel running) for six weeks induced the mobilization of NK cells and increased their infiltration into tumors (51). Mechanistic analyses revealed that NK cell mobilization to the circulation was dependent on epinephrine, the hormonal effector of the 'fight-or-flight' response that is activated during exertion, whereas tumor homing of NK cells was dependent on interleukin (IL)-6, a myokine released from contracting muscles. Although exercise did not enhance NK cell cytotoxicity per se, it 'prepared' the tumor environment for their infiltration by enhancing the expression of ligands for some NK cell-activating receptors (e.g., NKG2D and NKp46). This is consistent with human studies reporting acute transcriptomic (53) or epigenetic modifications (79) in NK cells after a single exercise session in humans, although a recent study found that a 12-week resistance training intervention failed to induce significant changes in the NK cell transcriptome of patients with breast cancer undergoing adjuvant therapy (48).

A type of exercise training that is gaining popularity in Western societies is high-intensity interval training (HIIT), because it is thought to stimulate aerobic fitness and muscle molecular adaptations that are comparable (if not superior) to those elicited by MICT despite a lower time commitment (i.e., lower total exercise volume) (19). The HIIT training model typically involves short, repeated bouts of intense effort (e.g., fast running or intensive bicycling), interspersed with short recovery periods (each lasting a few minutes or less). There is a paucity of data about the effects of HIIT on immune function, especially on NK cells, and the chronic effects of this training modality are unknown. Yet, this is an interesting question in light of the ongoing debate regarding the potential immunosuppressive effects of intense exercise training (of which HIIT is a good example), versus the documented beneficial effect of MICT for host immune defense (61). To the best of our knowledge, only one study found that an acute session of HIIT (performed at the end of a 6-week intervention of this training modality) increased NK cell count and activation (as assessed by the percentage of circulating CD56+CD3−NK cells) compared with pre-session levels in women with overweight/obesity (n=3) (4). It thus remains to be determined whether the effects of MICT on NK function are altered with the addition of subsequent vigorous training sessions (e.g., HIIT). An acute session of MICT (80% of VO_{2peak}) induced a rise in NK cell activity in well-trained young men that was not corroborated at lower intensities (50% of VO_{2peak}) (43). Yet, how this result translates to higher ('HIIT-like') intensities, especially with regard to chronic (i.e., training-induced) rather than acute effects, is unknown. One way to address this is to conduct a study where the same participants are assessed longitudinally along different training states. This might help to draw more conclusions about the effects of exercise training on NK function, particularly in light of the dearth of comparative studies currently available and the heterogeneity among them (particularly with regard to model [preclinical or human], participants' characteristics such as age and training status, or type of training programs). Mechanistically, proteomic analysis might provide important information on causal links as the proteome reflects the interaction of both inherent (genetics) and environmental factors in the responses and adaptations of tissues and cells to different stimuli (3). In addition, systems biology can help to understand the protein networks involved (55).

The purpose of the present study was to determine, in healthy adults, the effects of an exercise training intervention of progressive intensities (MICT followed by HIIT) on NK cells compared to a pre-training (baseline) state, using an integrative proteomics and systems biology approach.

METHODS

Participants and experimental design

The study was approved by the local ethics committee and was performed during 2018 in accordance with the Declaration of Helsinki. Inclusion criteria were as follows: (i) middle-aged (30–50 years) man/woman; (ii) not diagnosed with any major cardiorespiratory or systemic disease contraindicating exercise; (iii) not taking any medication; (iv) not having an infectious condition; and (v) not having performed structured exercise training (< 2 sessions/week of < 30 minutes) or practiced regularly any sport in the last 3 months. Of the 20 potentially eligible subjects originally contacted (University staff at two Faculties of the Universidad Europea de Madrid [UEM]), nine subjects meeting all the aforementioned criteria agreed to participate. One subject declined to continue in the study despite performing all the baseline assessments because he changed jobs and moved to a different city. Thus, eight apparently healthy individuals (3 male, 5 female; mean ± standard deviation [SD] age: 40 ± 6 years [range: 32, 50]; body mass index: 24.0 ± 2.1 $kg \cdot m^{-2}$) were finally enrolled in the study.

All the participants were assessed at three time points: (i) at baseline (untrained); (ii) after a 4-week MICT phase (mid-intervention); and (iii) after a subsequent 4-week HIIT phase (post-intervention). The study design is summarized in **Figure 1**.

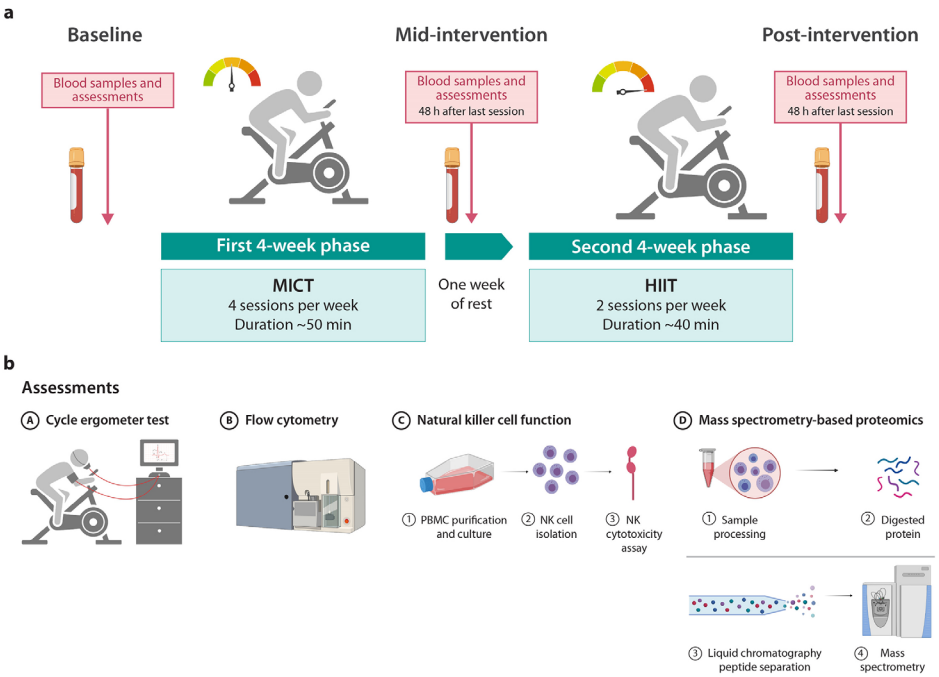

Figure 1. Study design. Protocol and intervention (a) and assessments (b).

Intervention

All exercise-training sessions were individually supervised (I.P.A. and L.B.A.), and were performed indoors with the same bicycle-ergometer (Ergometrics Ergoline 800; Jaeger, Bitz, Germany) that was used during the exercise tests for determination of VO_2peak, ventilatory threshold (VT) and respiratory compensation point (RCP), which are described later.

Moderate-intensity continuous training

This first phase started the week after baseline assessments and lasted four weeks in total, including four sessions per week (Monday, Wednesday, Friday and Sunday). The total duration of all the sessions was the same (~50 minutes), barring gradual build-up to this duration during the first week. The core part of the session lasted 40 minutes and the workload (power output [PO]) was gradually increased as follows: PO corresponding to the VT (first week), 5% above the VT (second week), midpoint between VT and RCP (third week), and 10% below the RCP (fourth week). All the sessions were preceded and followed by a 5–7-minute warmup below the VT and five minutes of cool-down (at the preferred PO), respectively.

The subjects refrained from doing any exercise sessions for one week after the MICT intervention and then started the HIIT program.

High-intensity interval training

This second phase also lasted four weeks in total, and included two sessions per week (Monday and Wednesday). The total duration of all the sessions was ~40 minutes. Each session started with a ~15-minute warmup and ended with a 5–10-minute cool-down period (at the preferred PO). For the Monday sessions, after a ~15-minute warmup slightly below the VT, participants performed three (in the first week) to five (in the remainder of weeks) 3-minute intervals at a PO above the RCP (starting from just above the RCP in the first week and increasing the PO by 2-3% per week thereafter) interspersed with 3-minute rest periods. For the Wednesday sessions, participants performed 10 (in the first week) to 15 (in the last two weeks) 30-second sprints (at maximal or near-maximal intensity) interspersed with 80-second rest periods. Rating of perceived exertion (RPE, on a 0 to 10 scale) were recorded in each session, with subjects instructed to aim at an RPE value ≥ 9 during the intervals versus 4 and 2 in the recovery periods between the 3-minute intervals and 30-second sprints, respectively.

Measurements

Exercise tests

All the participants performed a graded cycle ergometer test until volitional exhaustion, at baseline and at mid- and post-intervention, respectively (after a 48-hour rest period from the last training session). All tests were performed between 9:00 am and 12:00 pm at the exercise physiology laboratory of the UEM. Before the tests (~08:00 am), venous blood samples were collected from the antecubital vein into VacutainerR (sodium-heparin) tubes (BD Biosciences, San José, CA) for performing all the studies on NK cells that are described in the following sections.

Gas exchange data were recorded 'breath-by-breath' with a metabolic cart (Vmax 29C; SensorMedics Corp., Yorba Linda, CA). The test started at 20 watts and the load was increased in a ramp-like fashion (5 watts/12 sec [20 watts/minute on average]) while cadence was kept constant at 60 to 70 rpm. Participants were verbally encouraged to continue pedalling until volitional exhaustion, and were continuously monitored electrocardiographically. The VO_2peak was defined as the highest value (mean of 30 seconds) reached during the test. We also determined the VT and RCP (also termed 'second ventilatory threshold') using the following criteria: VT, the point at which the ventilatory equivalent for oxygen (ventilation [VE]·VO_2^{-1}) starts to increase with no concomitant increase in the ventilatory equivalent for carbon dioxide (VE·VCO_2^{-1}) and with departure from linearity of VE; RCP, the point at which both VE·VO_2^{-1} and VE·VCO_2^{-1} increase together with a decrease in end-tidal pressure of carbon dioxide (31).

Flow cytometry

Flow cytometry analyses (FACSCantoTM II, BD Biosciences) were conducted to assess the presence of various cell surface markers related to cellular activation processes (**Table 1**). The gating strategy was based on dead/live cells and doublets discrimination. When possible, a minimum of 10,000 events of the population of interest was analyzed. FACSDiva™ software (BD Biosciences) was used for analysis.

Natural killer cell function

Peripheral blood mononuclear cell purification and cultures. Heparinized blood was diluted two-fold with phosphate buffered saline (PBS) and layered on top of Ficoll-Paque in a 50 mL conical polypropylene tube (BD Biosciences). After centrifugation, peripheral blood mononuclear cells (PBMC) were recovered and washed twice in calcium- and magnesium-free PBS. Cells were seeded in culture flasks containing Rosewell Park Memorial Institute 1640 medium and 10% fetal bovine serum (FBS) (both from Gibco/Thermo Scientific, Waltham, MA). After 48 hours, cell suspensions were stimulated with 10 µg/mL phytohemagglutinin (PHA) (Sigma Aldrich, St Louis, MO) (18, 49) and functional assays were performed as indicated below.

Natural killer cell isolation. NK cells were purified from PBMC using the NK Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, non-NK cells (T, B, stem, dendritic and erythroid cells, monocytes, and granulocytes) were magnetically labeled using a cocktail of biotin-conjugated antibodies and the NK Cell MicroBead Cocktail (Miltenyi Biotech). Isolation of highly pure NK cells was achieved by depletion of magnetically-labeled cells.

Natural killer cell cytotoxicity assay. K562 cells were used as NK target cells, and were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% FBS. To assess NK cytotoxic capacity, K562 cells were loaded with bis[acetoxymethyl] 2,2′:6′,2′′-terpyridine-6,6′′-dicarboxylate (BATDA), a hydrophobic ligand that penetrates the plasma membrane quickly, according to the DELFIA® EuTDA Cytotoxicity Kit (Perkin Elmer, Waltham, MA). The assay was performed with 5×10^3 BATDA-loaded K562 cells and 100 µL of primary NK cells at various concentrations (2×10^4, 1×10^4, 5×10^3 and 2.5×10^3 cells). After cytolysis, the released ligand (TDA) reacts with a europium solution to constitute a fluorescent chelate. The fluorescent signal correlates directly with the amount of lysed cells:

$$\% \text{ Specific release} = \frac{\text{Experimental release (counts)} - \text{Spontaneous release (counts)}}{\text{Maximum release (counts)} - \text{Spontaneuos release (counts)}} \times 100$$

Mass spectrometry-based proteomics

Sample processing and protein digestion. Frozen sample aliquots of 1–3 × 10^6 NK cells were lysed in 150 µL SDT-lysis buffer (4% SDS and 0.1 M DTT in 0.1 M Tris/HCl, pH 7.6) using 1:10 sample to buffer ratio, at 95°C for 5 minutes. Samples were briefly sonicated to reduce the viscosity of the lysates and then centrifuged at 16,000 × g for 10 minutes. Sample processing was based on the procedure of Wisniewski et al. (75) with minor modifications. Aliquots (100 µL) of lysates were loaded onto spin ultrafiltration units (Amicon Ultra-0.5 Ultracel-30 membrane; Merck, Kenilworth, NJ) and washed with 355 µL of buffer UA (8 M urea in 0.1 M Tris/HCl, pH 8.5). Spin unit were then centrifuged at 14,000 × g for 15 minutes and washed again with 375 µL of buffer UA. Alkylation of cysteine residues in the samples was carried out by adding 100 µL of freshly prepared iodoacetamide solution (50 mM in UA buffer) into the spin filters and incubating the samples in the dark for 20 minutes. Filters were washed three times with 200 µL of UA followed by three washes with 100 µL of 50 mM ammonium bicarbonate. On-filter digestion was performed overnight with trypsin at 37°C. The released peptides were collected by centrifugation at 14,000 × g for 10 minutes followed by an additional wash with 50 µL of 50 mM ammonium bicarbonate. The peptide-containing eluates were finally desalted using C-18 spin columns (C-18 Micro Spin Column; Harvard Apparatus, Holliston, MA) and dried in a speed vacuum (Thermo Scientific). Samples were resuspended in 0.1% formic acid and peptide concentration was measured with the Qubit Protein Assay Kit (Thermo Scientific). Before the liquid chromatography-mass spectrometry (LC-MS/MS) analysis, 200 ng of each biological replicate (baseline, MICT, HIIT) and 0.66 µg sample was analyzed.

Liquid chromatography-tandem mass spectrometry. LC-MS/MS analysis was performed using a Q Exactive mass spectrometer interfaced with an Easy-nLC 1000 nanoUPLC System (both from Thermo Scientific). Digested peptides were loaded onto an Acclaim PepMap100 precolumn (75 µm × 2 cm) connected to an Acclaim PepMap RSLC (50 µm × 15 cm) analytical column (Thermo Scientific). Peptides were eluted with a 180 minute linear gradient of 3–30% acetonitrile in 0.1% formic acid at a flow rate of 300 nL/minute directly onto the nanoES Emitter (Thermo Scientific). The mass spectrometer was operated in a top 10 data-dependent mode. Survey scans were acquired at a resolution of 70,000 (m/z 200) and fragmentation spectra at 17,500 (m/z 200). Peptide selection was done with an isolation window of 2.0 Th and normalized collision energy of 28 was applied for peptide fragmentation. The maximum injection time was 120 milliseconds for survey, and MS/MS scans and automatic gain control target values of 3e6 for survey scans and 5e5 for MS/MS scans were used. Acquired raw data files were processed with MaxQuant (10) software (v1.6.0.16) using the internal search engine Andromeda, and searched against the UniProt database restricted to Homo sapiens entries (release 2018_11). Carbamidomethylation was set as fixed modification, whereas methionine oxidation and protein N-terminal acetylation were defined as variable modifications. Mass tolerance was set to 8 and 20 ppm at the MS and MS/MS level, respectively. Enzyme specificity was set to trypsin, allowing for a maximum of two missed cleavages. The "match between runs" option was enabled with a 1.5-minute time window and a 20-minute alignment window to match identification across samples. The minimum peptide length was set to seven amino acids. The false discovery rate (FDR) for peptides and proteins was set to 1%. Normalized spectral protein label-free quantification intensities were calculated using the MaxLFQ algorithm.

MaxQuant output data was analyzed with the Perseus module (v1.6.5.0) (67). Proteins only identified by site, contaminants and reverse hits were removed. Changes in protein

abundance higher than 4-fold in proteins identified with more than two unique peptides were considered as relevant protein differences.

Statistical analyses

Exercise capacity and natural killer cell numbers/cytotoxicity

Data are presented as mean ± SD. Normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test) of the data were checked before any statistical treatment. A one-way repeated-measures ANOVA was used to examine differences across the three conditions (i.e., baseline, mid-intervention, and post-intervention). Bonferroni's test was performed post hoc when a significant condition (or 'time') effect was present. The Greenhouse-Geisser correction was applied when Mauchly's test of sphericity was violated. Statistical analyses were conducted using a statistical software package (SPSS v23; IBM, Amonk, NY) setting the significance level at α=0.05.

Proteomics

Candidate proteins. To identify proteins in plasma that were differentially expressed across the three conditions (baseline, mid-intervention and post-intervention) the magnitude of the changes was calculated by taking the base 2 logarithm of the mean fold change (log2FC). For proteins with no expression in either of the three samples, log2FC values were adjusted by adding one to each mean and then calculating the ratio. Negative values indicate down-regulation whereas positive values indicate up-regulation. Multi-test correction was performed according to the Benjamini-Hochberg method, that is, p-values were adjusted with FDR correction (5).

Processing of protein expression data. Proteins identified in the plasma profiles were filtered to unique human-reviewed protein entries according to the UniProt Knowledgebase (UniProtKB) (67). To reduce potential interference for high signals, keratin protein entries were excluded from the analysis. In addition, the protein entries that were traced to an unreviewed UniProtKB entry were manually curated to find a valid reviewed entry. When more than one protein entry was traced to the same reviewed UniProtKB entry, the following criteria were applied to prevent duplications: (i) to prioritize protein entries with an identical protein ID to the one noted in UniProtKB; (ii) to prioritize protein entries that were automatically traced to the reviewed UniProtKB entry over those that were first traced to an unreviewed or deleted one and then manually curated; (iii) to prioritize protein entries with valid signals; (iv) to prioritize whole protein entries over those referring to fragments; and (v) to apply an alphabetical criterion when all the criteria above were not applicable.

Contextualization of the differentially expressed proteins within "natural killer cell" protein network. Effector proteins were used to focus the analysis on the biological condition of interest in the human biological network. The relationships between the differentially expressed proteins and the immune system were assessed. Different publicly available databases were consulted for the human protein network generation (Reactome, Molecular INTeraction database (MINT) and Biological General Repository for Interaction Datasets [BioGrid]) (25, 26).

Gene set enrichment analysis. Proteomic data were analyzed using the Gene Set Enrichment Analysis (GSEA) tool (63) to compare the differential pathways and molecular processes between conditions. This approach categorizes genes according to a specific metric, ranking the most statistically significant genes (focusing on log2FC values) at the top end of the list. The FDR cutoff was set at 25% to maximize hypothesis generation. Specifically, the enrichment was run over the following databases: Gene Ontology (GO) terms (Biological Process, Cellular Component, Molecular Function) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Visualization as a network of the relationship between enriched pathways. Cytoscape 3.5.1. software was used to represent the relationship between the enriched pathways identified by GSEA (based on the number of common annotated genes between datasets).

RESULTS

No adverse effect was noted during the exercise sessions and no participant reported any medical condition (e.g., infection/s) during the study that could have altered the results.

Exercise capacity

No significant time effect ($p = 0.455$) was found for participants' body mass (baseline: 69.5 ± 9.5 kg; mid-intervention: 69.1 ± 9.0 kg; and post-intervention: 69.4 ± 9.1 kg). A significant time effect ($p = 0.016$) was found for VO_2peak, which showed an increasing trend during the study (baseline: 34.9 ± 4.8 $mL \cdot kg^{-1} \cdot min^{-1}$; mid-intervention: 38.7 ± 7.6 $mL \cdot kg^{-1} \cdot min^{-1}$; and post-intervention: 39.5 ± 7.5 $mL \cdot kg^{-1} \cdot min^{-1}$). In post hoc pairwise comparisons, a quasi-significant difference was found for post-intervention versus baseline ($p = 0.071$). The peak PO (PPO) attained during the tests ($p = 0.004$ for time effect; baseline: 2.4 ± 0.2 $watts \cdot kg^{-1}$; mid-intervention: 2.8 ± 0.4 $watts \cdot kg^{-1}$; and post-intervention: 2.9 ± 0.4 $watts \cdot kg^{-1}$), as well as the PO eliciting the RCP ($p = 0.006$ for time effect; baseline: 2.1 ± 0.2 $watts \cdot kg^{-1}$; mid-intervention: 2.4 ± 0.4 $watts \cdot kg^{-1}$; and post-intervention: 2.5 ± 0.3 $watts \cdot kg^{-1}$) also showed an increasing trend during the study, with significant post hoc differences for post-intervention versus baseline ($p = 0.028$ for PPO and 0.044 for RCP). No significant time effect ($p = 0.154$) was, however, found for the PO eliciting the VT (baseline: 1.1 ± 0.1 $watts \cdot kg^{-1}$; mid-intervention: 1.1 ± 024 $watts \cdot kg^{-1}$; and post-intervention: 1.2 ± 1.2 $watts \cdot kg^{-1}$).

NK number and cytotoxicity

A significant time effect was found for PBMC ($p < 0.001$, **Figure 2A**) and NK ($p < 0.001$, Figure 2B) cell counts, with both cell counts considerably increasing at both mid- and post-intervention compared with baseline, and with a clear training time-dependent effect (i.e., a further, significant increase was found at post-intervention as compared with mid-intervention).

A significant time effect was also found for NK cell cytotoxicity ($p < 0.001$, **Figure 2C**); which significantly increased at both mid- and post-intervention compared with baseline; however, no training time-dependent effect was found (i.e., no significant differences were found for mid- versus post-intervention).

Flow cytometry

No significant condition effect was found except for the surface expression of the chemokine receptor CXCR3 expressed as mean fluorescence intensity ($p = 0.044$, with a trend towards a decrease at mid- and post-intervention compared with baseline), although no significant differences were found in post hoc pair-

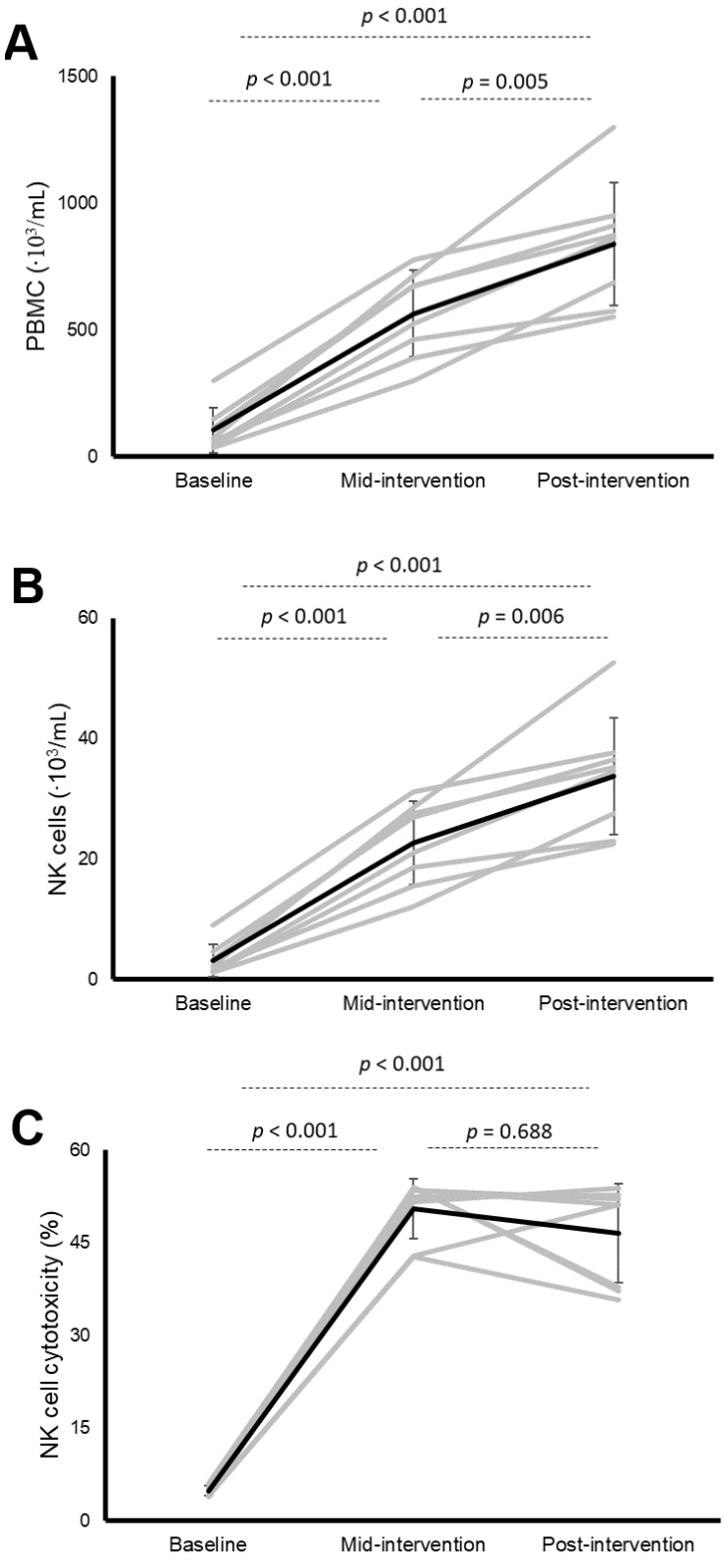

Figure 2. Within-subject comparison of the number of peripheral blood mononuclear cells ([PBMC], **panel A**), and of the number (**panel B**) and cytotoxicity (**panel C**, N=8) of natural killer (NK) cells. Cytotoxicity was expressed as % specific fluorescence release (see text for more details) at a ratio of effector (NK) to target (K562) cells of 1:1, with the results replicated (i.e., same p-values) at different E:T ratios (1:2, 2:1 and 4:1).

wise comparisons (p = 0.244 and 0.093 for baseline versus mid- and post-intervention, respectively; and p = 1.000 for mid- versus post-intervention) (**Table 2**).

Proteomics

A total of 2538 human proteins were identified by LC-MS/MS, of which 2448 valid protein entries were used in the next steps of the study and 90 were filtered out (i.e., potential contaminants, reverse and only identified by site).

Protein candidates

Mid-intervention versus baseline. The expression of 74 plasma proteins was significantly different between mid-intervention and baseline (FDR q-value < 0.01), of which 49 and 25 were upregulated and downregulated, respectively, at mid-intervention (**Table 3**).

Moreover, one and nine unique proteins were identified (FDR q-value < 0.01) at baseline and at mid-intervention, respectively. Among the candidate proteins, 17 are known to be potentially related to immune function (**Figure 3**):

Proteins upregulated at mid-intervention (versus baseline): N-acylsphingosine amidohydrolase 1 (ASAH1); V-type proton

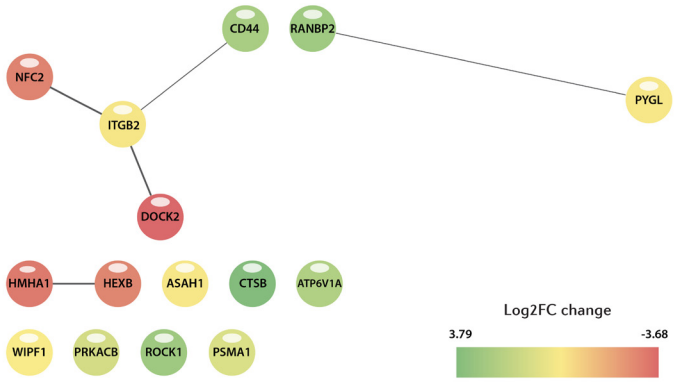

Figure 3. Visual protein interaction network of the proteins related to the immune system that were differentially expressed (upregulated or downregulated) at mid-intervention compared to baseline.

ATPase catalytic subunit A (ATP6V1A); CD44; cathepsin B (CTSB); integrin beta chain-2 (ITGB2, also known as 'CD18'); nucleoporin 88 (NUP88); phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1); cAMP-dependent protein kinase catalytic subunit beta (PRKACB); proteasome (prosome, macropain) subunit, alpha 1 (PSMA1); glycogen phosphorylase, liver isoform (PYGL); RAN binding protein 2 (RANBP2, also known as 'nucleoporin 358'); Rho-associated coiled-coil containing protein kinase 1 (ROCK1); and WAS/WASL interacting protein family member 1 (WIPF1).

Proteins downregulated at mid-intervention (versus baseline): dedicator of cytokinesis 2 (DOCK2); hexosaminidase subunit beta (HEXB); minor histocompatibility protein HA-1 (HMA1, also known as 'Rho GTPase activating protein 45'); and neutrophil cytosolic factor 2 (NCF2).

Post-intervention versus baseline. The expression of 76 plasma proteins was significantly different between post-intervention and baseline, of which 22 and 54 were upregulated and downregulated, respectively, at post-intervention (**Table 4**). No unique proteins were identified (FDR q-value < 0.01) at post-intervention compared with baseline.

Table 1. Antibodies per tube and per peripheral blood sample used for flow cytometry analysis.

	AC	APC	APC-Cy7	FITC	PB	PE	PE-Cy7	PerCP/Cy5.5
Tube 1	CD45	IgG	IgG	IgG	CD3	IgG	CD56	IgG
Tube 2	CD45	NKp46	NKG2D	CD94	CD3	NKp30	CD56	NKp44
Tube 3	CD45	CXCR3	CD69	CD25	CD3	CD57	CD56	CXCR4

Antibody	Reference	Brand
AC CD45 V500-C	655873	BD Horizon
APC IgG Fc	409306	BioLegend
APC CD335 (NKp46)	331918	BioLegend
APC CD183 (CXCR3)	353708	BioLegend
APC/Cy7 CD314 (NKG2D)	320824	BioLegend
APC/Cy7 CD69	310914	BioLegend
APC/Cy7 IgG Fc	409314	BioLegend
FITC IgG Fc	409310	BioLegend
FITC CD25	555431	BD Pharmingen
FITC CD94	305504	BioLegend
PB V405 CD3	560365	BD Horizon
PE IgG Fc	409304	BioLegend
PE CD337 (NKp30)	325208	BioLegend
PE CD57	322312	BioLegend
PE-Cy7 CD56	335826	BD Biosciences
PerCP/Cy5.5 IgG Fc	409312	BioLegend
PerCP/Cy5.5 CD184 (CXCR4)	306516	BioLegend
PerCP/Cy5.5 CD336 (NKp44)	325114	BioLegend

Abbreviations: AC, AmCyan; APC, Allophycocyanin; APC-Cy7, Allophycocyanin cyanin-7; Ig, Immunoglobulin; FITC, Fluorescein isothiocyanate; PB, Pacific blue; PE, Phycoerythrin; PE-Cy7, Phycoerythrin cyanin-7; PerCP-Cy7, Peridinin chlorophyll protein.

Table 2. Flow cytometry results in NK cells.

A

Variable	Baseline	Mid-intervention	Post-intervention	p-value
CD94	5.5 ± 1.6	6.2 ± 2.0	5.4 ± 2.2	0.305
NKp30	190 ± 149	53 ± 52	25 ± 21	0.367
NKp44	0.10 ± 0.06	0.09 ± 0.1	0.13 ± 0.08	0.595
NKp46	619 ± 449	214 ± 324	246 ± 379	0.057
NKG2D	123 ± 149	146 ± 172	172 ± 127	0.676
CD25	0.79 ± 0.09	0.75 ± 0.09	0.74 ± 0.11	0.527
CD57	11.2 ± 24.8	3.1 ± 4.1	2.1 ± 2.8	0.318
CXCR4	0.16 ± 0.13	0.15 ± 0.20	0.18 ± 0.12	0.708
CXCR3	436 ± 302	171 ± 274	138 ± 166	**0.044**
CD69	13.9 ± 17.1	11.2 ± 11.7	13.4 ± 18.1	0.946

B

Variable	Baseline	Mid-intervention	Post-intervention	p-value
CD94	61.3 ± 13.9	58.6 ± 14.9	59.6 ± 16.2	0.574
NKp30	66.6 ± 20.3	62.4 ± 16.3	65.3 ± 21.2	0.697
NKp44	0.06 ± 0.09	0.08 ± 0.12	0.06 ± 0.05	0.950
NKp46	51.0 ± 17.0	50.8 ± 13.2	54.8 ± 18.2	0.660
NKG2D	31.0 ± 13.9	17.3 ± 10.6	17.7 ± 9.8	0.078
CD25	0.21 ± 0.20	0.24 ± 0.24	0.18 ± 0.13	0.818
CD57	5.9 ± 5.0	6.0 ± 4.9	6.9 ± 6.1	0.246
CXCR4	0.19 ± 0.25	0.16 ± 0.09	0.08 ± 0.07	0.393
CXCR3	30.0 ± 11.5	26.6 ± 7.1	30.6 ± 9.2	0.513
CD69	1.8 ± 0.9	1.4 ± 0.7	1.5 ± 1.4	0.611

Table footnote. Values are mean ± SD of fluorescence intensity (**A**) or of percent positive (**B**) for each NK cell receptor. The only significant p-value for the time (condition) effect is in bold. Abbreviations: CXCR3, C-X-C chemokine receptor type 3; CXCR4, C-X-C chemokine receptor type 4 (also known as fusin or CD184 [cluster of differentiation 184]).

Table 3. List of proteins differentially expressed at mid-intervention versus baseline.

UniProt	Gene	Number of unique peptides	Log2FC change	FDR (q-value)	Related to immune system	Expression at mid-intervention compared to baseline
Q9UBS4	DNAJB11	3	27.921	<.001	No	↑
P27986	PIK3R1	3	27.050	<.001	Yes	↑
Q9UNS2	COPS3	3	27.023	<.001	No	↑
Q9P258	RCC2	3	26.768	<.001	No	↑
Q99567	NUP88	3	26.728	<.001	Yes	↑
Q9H2K8	TAOK3	3	26.724	<.001	No	↑
O75150	RNF40	3	26.628	<.001	No	↑
Q8TD19	NEK9	3	26.468	<.001	No	↑
Q9Y4P3	TBL2	3	26.246	<.001	No	↑
Q9UHD8	SEPT9	8	3.957	<.001	No	↑
P07858	CTSB	3	3.788	<.001	Yes	↑
O75439	PMPCB	5	3.500	<.001	No	↑
Q13464	ROCK1	8	3.386	<.001	Yes	↑
P49792	RANBP2	5	3.379	<.001	Yes	↑
P46060	RANGAP1	4	3.319	<.001	No	↑
P10412	HIST1H1E	8	3.319	<.001	No	↑
P16070	CD44	4	3.250	<.001	Yes	↑
Q14683	SMC1A	8	3.157	<.001	No	↑
O15372	EIF3H	5	3.146	<.001	No	↑
P38606	ATP6V1A	5	3.136	<.001	Yes	↑
Q9H4G4	GLIPR2	3	3.006	<.001	No	↑
P34897	SHMT2	3	2.963	<.001	No	↑
O94776	MTA2	3	2.849	<.001	No	↑
Q9UQ35	SRRM2	7	2.698	<.001	No	↑
Q15424	SAFB	4	2.678	<.001	No	↑
P22694	PRKACB	4	2.671	<.001	Yes	↑
Q92878	RAD50	5	2.670	<.001	No	↑
Q9H583	HEATR1	3	2.668	<.001	No	↑
P55769	NHP2L1	3	2.609	<.001	No	↑
P25786	PSMA1	3	2.568	<.001	Yes	↑
Q92888	ARHGEF1	6	2.554	<.001	No	↑
P49588	AARS	6	2.498	<.001	No	↑
O00410	IPO5	3	2.467	<.001	No	↑
Q13045	FLII	11	2.424	<.001	No	↑
P05198	EIF2S1	5	2.375	<.001	No	↑
Q12907	LMAN2	4	2.306	<.001	No	↑
Q14980	NUMA1	19	2.237	<.001	No	↑
O43516	WIPF1	6	2.203	<.001	Yes	↑
P00390	GSR	3	2.202	<.001	No	↑
Q9NTI5	PDS5B	9	2.195	<.001	No	↑
Q3KQU3	MAP7D1	3	2.162	<.001	No	↑
Q13510	ASAH1	5	2.103	<.001	Yes	↑
Q96HE7	ERO1L	3	2.098	<.001	No	↑
Q5JSL3	DOCK11	4	2.079	<.001	No	↑
Q99497	PARK7	5	2.054	<.001	No	↑
P05107	ITGB2	11	2.052	<.001	Yes	↑
P06737	PYGL	4	2.050	<.001	Yes	↑
P23634	ATP2B4	3	2.045	<.001	No	↑
Q15942	ZYX	9	2.040	<.001	No	↑
Q9UBQ7	GRHPR	4	-2.188	<.001	No	↓
O75964	ATP5L	3	-2.214	<.001	No	↓
P84243	H3F3A	3	-2.231	<.001	No	↓
P07686	HEXB	4	-2.272	<.001	Yes	↓
Q7Z2W4	ZC3HAV1	6	-2.366	<.001	No	↓
P19878	NCF2	5	-2.386	<.001	Yes	↓
Q6P2Q9	PRPF8	11	-2.478	<.001	No	↓
P16403	HIST1H1D	5	-2.487	<.001	No	↓
P17844	DDX5	4	-2.547	<.001	No	↓
Q92896	GLG1	8	-2.552	<.001	No	↓
P45954	ACADSB	4	-2.656	<.001	No	↓
Q92619	HMHA1	7	-2.817	<.001	Yes	↓
P62081	RPS7	3	-2.874	<.001	No	↓
Q9H0U4	RAB1B	3	-2.945	<.001	No	↓
P57737	CORO7	5	-3.053	<.001	No	↓
O94919	ENDOD1	3	-3.131	<.001	No	↓
Q00325	SLC25A3	5	-3.370	<.001	No	↓
O94906	PRPF6	5	-3.448	<.001	No	↓
P52209	PGD	5	-3.634	<.001	No	↓
Q92608	DOCK2	10	-3.683	<.001	Yes	↓
P00505	GOT2	7	-4.115	<.001	No	↓
P46781	RPS9	4	-4.234	<.001	No	↓
P47985	UQCRFS1	4	-4.541	<.001	No	↓
Q9H299	SH3BGRL3	3	-4.923	<.001	No	↓
P12081	HARS	3	-26.319	<.001	No	↓

Table footnote. Symbols: ↑ upregulation at mid-intervention compared to baseline; ↓ downregulation at mid-intervention compared to baseline. Abbreviations (other than protein names): FDR, false discovery rate; Log2FC, log 2 fold change. Abbreviations (proteins): AARS, alanyl-tRNA synthetase 1; ACADSB, acyl-coA dehydrogenase short/branched chain; ARHGEF1, Rho guanine nucleotide exchange factor 1; ASAH1, N-acylsphingosine amidohydrolase 1; ATP2B4, ATPase plasma membrane Ca^{2+} transporting 4; ATP5L, ATP synthase membrane subunit G; ATP6V1A, ATPase H^{+} transporting V1 subunit A (also known as V-type proton ATPase catalytic subunit A); CD44, CD44 molecule; COPS3, COP9 signalosome subunit 3; CORO7, CORO7-PAM16 readthrough; CTSB, cathepsin B; DDX5, DEAD-box helicase 5; DNAJB11, DnaJ heat shock protein family (Hsp40) member B11; DOCK11, dedicator of cytokinesis 11; DOCK2, dedicator of cytokinesis 2; EIF2S1, eukaryotic translation initiation factor 2 subunit alpha; EIF3H, eukaryotic translation initiation factor 3 subunit H; ENDOD1, endonuclease domain containing 1; ERO1L, endoplasmic reticulum oxidoreductase 1 alpha; FLII, FLII actin remodeling protein; GLG1, Golgi glycoprotein 1; GLIPR2, GLI pathogenesis related 2; GOT2, glutamic-oxaloacetic transaminase 2; GRHPR, glyoxylate and hydroxypyruvate reductase; GSR, glutathione-disulfide reductase; H3F3A, H3.3 histone A; HARS, histidyl-TRNA synthetase 1; HEATR1, hEAT repeat containing 1; HEXB, hexosaminidase subunit beta; HIST1H1D, H1.3 linker histone, cluster member; HIST1H1E, H1.4 linker histone, cluster member; HMHA1, minor histocompatibility protein HA-1 (also known as Rho GTPase activating protein 45); IPO5, importin 5; ITGB2, integrin subunit beta 2 (also known as 'CD18'); LMAN2, lectin, mannose binding 2; MAP7D1, MAP7 domain containing 1; MTA2, metastasis associated 1 family member 2; NCF2, neutrophil cytosolic factor 2; NEK9, NIMA related kinase 9; NHP2L1, NHP2-like protein 1 (also known as 'small nuclear ribonucleoprotein 13) [SNU13]); NUMA1, nuclear mitotic apparatus protein 1; NUP88, nucleoporin 88; PARK7, parkinsonism associated deglycase; PDS5B, PDS5 cohesin associated factor B; PGD, Phosphogluconate dehydrogenase; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PMPCB, mitochondrial-processing peptidase subunit beta; PRKACB, protein kinase CAMP-activated catalytic subunit beta; PRPF6, pre-MRNA processing factor 6; PRPF8, pre-MRNA processing factor 8; PSMA1, proteasome (prosome, macropain) subunit alpha 1; PYGL, glycogen phosphorylase L; RAB1B, RAB1B, member RAS oncogene family; RAD50, RAD50 double strand break repair protein; RANBP2, RAN binding protein 2; RANGAP1, Ran GTPase activating protein 1; RCC2, regulator of chromosome condensation 2; RNF40, ring finger protein 40; ROCK1, Rho associated coiled-coil containing protein kinase 1; RPS7, ribosomal protein S7; RPS9, ribosomal protein S9; SAFB, scaffold attachment factor B; SEPT9, septin 9; SH3BGRL3, SH3 domain binding glutamate rich protein like 3; SHMT2, serine hydroxymethyltransferase 2; SLC25A3, solute carrier family 25 member 3; SMC1A, structural maintenance of chromosomes 1A; SRRM2, serine/arginine repetitive matrix 2; TAOK3, TAO kinase 3; TBL2, transducin beta like 2; UQCRFS1, ubiquinol-cytochrome C reductase, Rieske iron-sulfur polypeptide 1; VIP36, lectin, mannose binding 2; WIPF1, WAS/WASL interacting protein family member 1; ZC3HAV1, zinc finger CCCH-type containing, antiviral 1; ZYX, zyxin.

Table 4. List of proteins differentially expressed at post-intervention versus baseline.

UniProt	Gene	Number of unique peptides	Log2FC change	FDR (q-value)	Related to immune system	Expression at post-intervention compared to baseline
P10412	HIST1H1E	8	5.613	<.001	No	↑
P20701	ITGAL	5	4.200	.009	Yes	↑
Q92522	H1FX	5	3.784	<.001	No	↑
Q16777	HIST2H2AC	3	3.588	.008	No	↑
Q92878	RAD50	5	3.531	.008	No	↑
P16402	HIST1H1D	5	3.213	<.001	No	↑
P55809	OXCT1	3	3.107	<.001	No	↑
P06454	PTMA	3	2.710	<.001	No	↑
P31943	HNRNPH1	3	2.667	<.001	No	↑
P34897	SHMT2	3	2.666	.008	No	↑
P13073	COX4I1	5	2.621	<.001	No	↑
P62244	RPS15A	4	2.594	<.001	No	↑
Q9UH99	SUN2	6	2.568	<.001	No	↑
Q92888	ARHGEF1	6	2.397	<.001	No	↑
P30273	FCER1G	3	2.338	.008	Yes	↑
P22307	SCP2	3	2.301	<.001	No	↑
P62805	HIST1H4A	9	2.265	<.001	No	↑
Q9BUJ2	HNRNPUL1	7	2.142	<.001	No	↑
P16104	H2AFX	4	2.080	<.001	No	↑
Q9UKM9	RALY	8	2.069	<.001	No	↑
P23634	ATP2B4	3	2.045	<.001	No	↑
P00505	GOT2	7	2.022	<.001	No	↑
Q96KP4	CNDP2	3	-2.103	<.001	No	↓
O15400	STX7	5	-2.110	<.001	No	↓
P60174	TPI1	6	-2.137	<.001	No	↓
O43290	SART1	3	-2.161	<.001	No	↓
Q01813	PFKP	3	-2.225	<.001	No	↓
Q15007	WTAP	3	-2.311	<.001	No	↓
P18669	PGAM1	3	-2.332	<.001	Yes	↓
Q9NTJ5	SACM1L	9	-2.348	<.001	No	↓
P12931	SRC	7	-2.352	<.001	Yes	↓
O15511	ARPC5	5	-2.388	<.001	Yes	↓
Q0VD83	APOBR	6	-2.389	<.001	No	↓
Q9HBI1	PARVB	4	-2.402	<.001	No	↓
P24557	TBXAS1	5	-2.407	<.001	No	↓
Q13418	ILK	9	-2.409	<.001	No	↓
P78417	GSTO1	4	-2.457	<.001	Yes	↓
P18754	RCC1	3	-2.470	<.001	No	↓
P09960	LTA4H	10	-2.531	<.001	Yes	↓
Q86UE4	MTDH	7	-2.536	<.001	No	↓
Q9H4B7	TUBB1	5	-2.565	<.001	Yes	↓
P12814	ACTN1	9	-2.596	<.001	No	↓
O60763	USO1	7	-2.658	<.001	No	↓
P32455	GBP1	3	-2.689	<.001	Yes	↓
Q5JTV8	TOR1AIP1	7	-2.754	<.001	No	↓
Q14766	LTBP1	17	-2.812	<.001	No	↓
P24534	EEF1B2	3	-2.930	<.001	No	↓
P16615	ATP2A2	5	-2.934	<.001	No	↓
P54577	YARS	10	-2.975	<.001	No	↓
P19878	NCF2	5	-2.977	<.001	Yes	↓
P09326	CD48	4	-3.020	<.001	No	↓
Q02218	OGDH	8	-3.049	<.001	No	↓
Q9NYU2	UGGT1	10	-3.206	<.001	No	↓
Q7Z2W4	ZC3HAV1	6	-3.252	<.001	No	↓
P33176	KIF5B	5	-3.257	<.001	Yes	↓
O60488	ACSL4	5	-3.288	<.001	No	↓
P57737	CORO7	5	-3.296	<.001	No	↓
O60264	SMARCA5	10	-3.373	<.001	No	↓
Q13464	ROCK1	8	-3.419	<.001	Yes	↓
P30153	PPP2R1A	4	-3.456	<.001	Yes	↓
P07814	EPRS	7	-3.531	<.001	No	↓
P31948	STIP1	7	-3.587	<.001	No	↓
Q9H299	SH3BGRL3	3	-3.613	<.001	No	↓
P17301	ITGA2	8	-3.676	<.001	No	↓
Q14764	MVP	10	-3.697	<.001	Yes	↓
Q04917	YWHAH	6	-3.953	<.001	No	↓
P31150	GDI1	5	-4.063	<.001	No	↓
P04275	VWF	14	-4.066	<.001	Yes	↓
P11413	G6PD	9	-4.159	<.001	No	↓
P51858	HDGF	8	-4.321	<.001	No	↓
Q9Y6C2	EMILIN1	9	-4.942	<.001	No	↓
P30041	PRDX6	5	-5.199	<.001	Yes	↓
P40925	MDH1	4	-5.888	<.001	No	↓
Q13043	STK4	3	-6.160	<.001	No	↓
P52209	PGD	5	-6.619	<.001	No	↓
P12081	HARS	3	-26.319	<.001	No	↓

Table footnote. Symbols: ↑ upregulation at post-intervention compared to baseline; ↓ downregulation at post-intervention compared to baseline. Abbreviations (other than protein names): FDR, false discovery rate; Log2FC, log 2 fold change. Abbreviations (proteins): ACSL4, acyl-CoA synthetase long chain family member 4; ACTN1, actinin alpha 1; APOBR, apolipoprotein B Receptor; ARHGEF1, Rho guanine nucleotide exchange factor 1; ARPC5, actin related protein 2/3 complex subunit 5; ATP2A2, ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} Transporting 2; ATP2B4, ATPase plasma membrane Ca^{2+} transporting 4; CD48, CD48 molecule; CNDP2, carnosine dipeptidase 2; CORO7, CORO7-PAM16 readthrough; COX4I1, cytochrome C oxidase subunit 4I1; EEF1B2, eukaryotic translation elongation factor 1 beta 2; EMILIN1, elastin microfibril interfacer 1; EPRS, glutamyl-prolyl-tRNA synthase 1; FCER1G, Fc fragment of IgE receptor Ig; G6PD, glucose-6-phosphate dehydrogenase; GBP1, guanylate binding protein 1; GDI1, GDP dissociation inhibitor 1; GOT2, glutamic-oxaloacetic transaminase 2; GSTO1, glutathione S-transferase omega 1; H1FX, H1.10 linker histone; H2AFX, H2A.X variant histone; HARS, histidyl-tRNA synthetase 1; HDGF, heparin binding growth factor; HIST1H1D, histone cluster 1 H1 family member D; HIST1H1E, histone cluster 1 H1 family member E; HIST1H4A, H4 clustered histone 1; HIST2H2AC, H2A clustered histone 20; HNRNPH1, heterogeneous nuclear ribonucleoprotein H1; HNRNPUL1, heterogeneous nuclear tibonucleoprotein U Like 1; ILK, integrin linked kinase; ITGA2, integrin subunit alpha 2; ITGAL, integrin subunit alpha L; KIF5B, kinesin family member 5B; LTA4H, leukotriene A4 hydrolase; LTBP1, latent transforming growth factor beta binding protein 1; MDH1, malate dehydrogenase 1; MTDH, metadherin; MVP, major vault protein; NCF2, neutrophil cytosolic factor 2; OGDH, oxoglutarate dehydrogenase; OXCT1, 3-oxoacid CoA-transferase 1; PARVB, parvin beta; PFKP, phosphofructokinase, platelet; PGAM1, phosphoglycerate mutase 1; PGD, phosphogluconate dehydrogenase; PPP2R1A, protein phosphatase 2 scaffold subunit alpha; PRDX6, peroxiredoxin 6; PTMA, prothymosin alpha; RAD50, RAD50 double strand break repair protein; RALY, RALY heterogeneous nuclear ribonucleoprotein; RCC1, regulator of chromosome condensation 1; ROCK1, Rho associated coiled-coil containing protein kinase 1; RPS15A, ribosomal protein S15a; SACM1L, SAC1 like phosphatidylinositide phosphatase; SART1, spliceosome associated factor 1, recruiter of U4/U6.U5 tri-SnRNP; SCP2, sterol carrier protein 2; SH3BGRL3, SH3 domain binding glutamate rich protein like 3; SHMT2, serine hydroxymethyltransferase 2; SMARCA5, SWI/SNF related, matrix associated, actin dependent regulator of Chromatin, subfamily A, member 5; SRC, SRC proto-oncogene, non-receptor tyrosine kinase; STIP1, stress induced phosphoprotein 1; STK4; serine/threonine kinase 4; STX7, syntaxin 7; SUN2, Sad1 and UNC84 domain containing 2; TBXAS1, thromboxane A synthase 1; TOR1AIP1, torsin 1A interacting protein 1; TPI1, triosephosphate isomerase 1; TUBB1, tubulin beta 1 class VI; UGGT1, UDP-glucose glycoprotein glucosyltransferase 1; USO1, USO1 vesicle transport factor; VWF, Von Willebrand factor; WTAP, WT1 associated protein; YARS, tyrosyl-tRNA synthetase 1; YWHAH, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein Eta; ZC3HAV1, zinc finger CCCH-type containing, antiviral 1

Among the candidate proteins, 16 proteins are known to be potentially related to immune function (**Figure 4**):

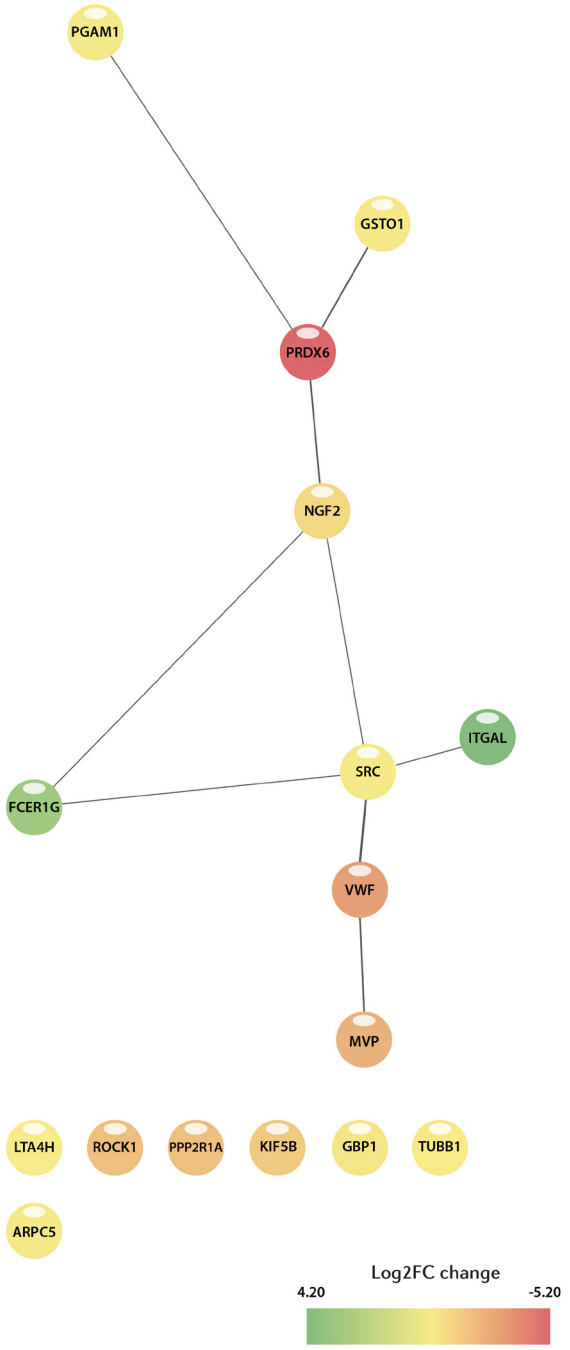

Figure 4. Visual protein interaction network of the proteins related to the immune system that were differentially expressed (upregulated or downregulated) at post-intervention compared to baseline.

Proteins upregulated at post-intervention (versus baseline): integrin subunit alpha L (ITGAL); and Fc fragment of IgE receptor Ig (FCER1G).

Proteins downregulated at post-intervention (versus baseline): ARPC5 actin related protein 2/3 complex subunit 5 (ARPC5); guanylate binding protein 1 (GBP1); glutathione S-transferase omega 1 (GSTO1); kinesin family member 5B (KIF5B); leukotriene A4 hydrolase (LTA4H); major vault protein (MVP); neutrophil cytosolic factor 2 (NCF2); phosphoglycerate mutase 1 (PGAM1); protein phosphatase 2 scaffold subunit alpha (PPP2R1A); peroxiredoxin 6 (PRDX6); Rho associated coiled-coil containing protein kinase 1 (ROCK1); SRC proto-oncogene, non-receptor tyrosine kinase (SRC); tubulin beta 1 class VI (TUBB1); and Von Willebrand factor (VWF).

Post-intervention versus mid-intervention. The expression of 106 plasma proteins was significantly different between mid- and post-intervention (FDR q-value < 0.01) (**Table 5**), with 26 and 80 upregulated and downregulated, respectively, at post-intervention. Among the candidate proteins, 24 proteins are known to be potentially related to immune function (**Figure 5**):

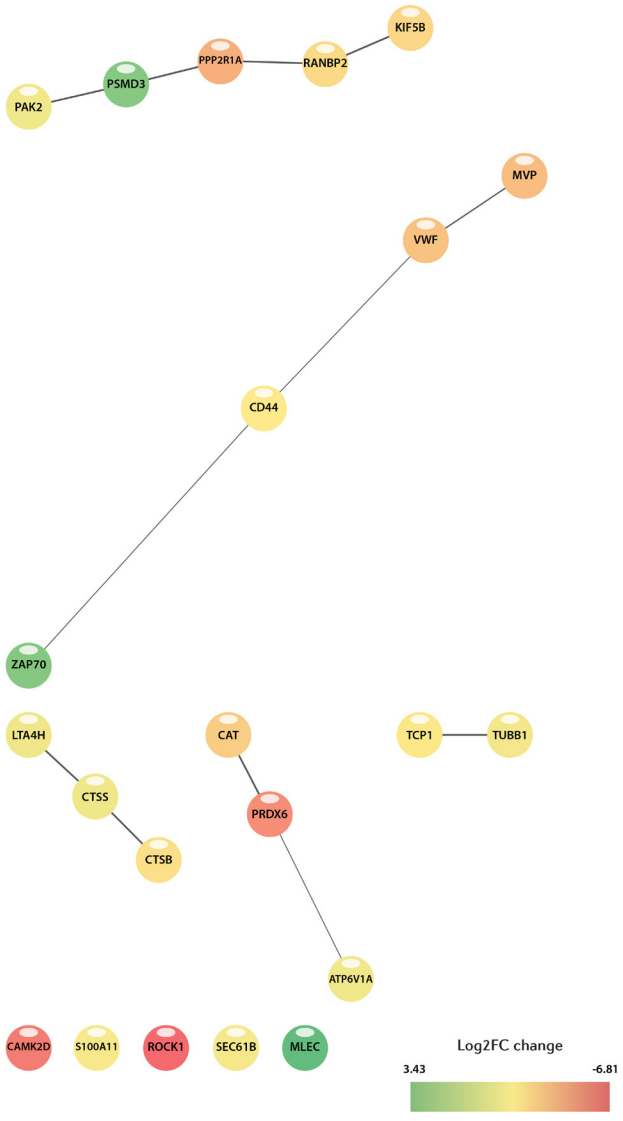

Figure 5. Visual protein interaction network of the proteins related to the immune system that were differentially expressed (upregulated or downregulated) at post-intervention compared to mid-intervention.

Proteins upregulated at post-intervention (versus mid-intervention): malectin (MLEC); proteasome 26S subunit (PSMD3); and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

Proteins downregulated at post-intervention (versus mid-intervention: ATPase H^+ transporting V1 subunit A (ATP6V1A); calcium/calmodulin dependent protein kinase II delta (CAMK2D); catalase (CAT); CD44 molecule (CD44); cathepsin B (CTSB); cathepsin S (CTSS); kinesin family member 5B (KIF5B); leukotriene A4 hydrolase (LTA4H); major vault protein (MVP); nucleoporin 88 (NUP88); P21 (RAC1) acti-

Table 5. List of proteins differentially expressed at post-intervention versus mid-intervention.

UniProt	Gene	Number of unique peptides	Log2FC change	FDR (q-value)	Related to immune system	Expression at post-intervention compared to mid-intervention
P00505	GOT2	7	6.137	<.001	No	↑
P16402	HIST1H1D	5	5.700	<.001	No	↑
Q00325	SLC25A3	5	4.547	<.001	No	↑
P06454	PTMA	3	4.112	<.001	No	↑
Q16777	HIST2H2AC	3	3.769	<.001	No	↑
O00479	HMGN4	3	3.695	<.001	No	↑
Q92522	H1FX	5	3.669	<.001	No	↑
P84243	H3F3A	3	3.615	<.001	No	↑
Q6DD88	ATL3	4	3.512	<.001	No	↑
Q14165	MLEC	3	3.434	<.001	Yes	↑
Q9Y2W1	THRAP3	7	3.380	<.001	No	↑
P22307	SCP2	3	3.163	.008	No	↑
P46781	RPS9	4	3.066	<.001	No	↑
P13073	COX4I1	5	3.030	<.001	No	↑
P17844	DDX5	4	2.634	<.001	No	↑
Q9H9B4	SFXN1	4	2.408	<.001	No	↑
Q13151	HNRNPA0	4	2.396	<.001	No	↑
P62805	HIST1H4A	9	2.356	<.001	No	↑
Q99623	PHB2	9	2.334	.008	No	↑
P10412	HIST1H1E	8	2.294	<.001	No	↑
P46063	RECQL	6	2.281	<.001	No	↑
P08758	ANXA5	3	2.151	<.001	No	↑
P49368	CCT3	6	2.123	<.001	No	↑
P43403	ZAP70	3	2.070	<.001	Yes	↑
P16104	H2AFX	4	2.069	<.001	No	↑
O43242	PSMD3	4	2.061	<.001	Yes	↑
Q9NTI5	PDS5B	9	-2.006	<.001	No	↓
P61981	YWHAG	5	-2.011	<.001	No	↓
P09960	LTA4H	10	-2.025	<.001	Yes	↓
P51659	HSD17B4	6	-2.037	<.001	No	↓
Q15404	RSU1	3	-2.041	<.001	No	↓
Q13177	PAK2	6	-2.071	<.001	Yes	↓
P25774	CTSS	4	-2.074	<.001	Yes	↓
P38606	ATP6V1A	5	-2.102	<.001	Yes	↓
Q15717	ELAVL1	3	-2.103	<.001	No	↓
Q8TCU6	PREX1	3	-2.143	<.001	No	↓
Q13428	TCOF1	17	-2.147	<.001	No	↓
Q9H4B7	TUBB1	5	-2.172	<.001	Yes	↓
Q86UE4	MTDH	7	-2.197	<.001	No	↓
P60468	SEC61B	3	-2.201	<.001	Yes	↓
Q9UGI8	TES	10	-2.224	<.001	No	↓
P09326	CD48	4	-2.271	<.001	No	↓
P05387	RPLP2	3	-2.280	<.001	No	↓
P31949	S100A11	3	-2.287	<.001	Yes	↓
P46782	RPS5	4	-2.307	<.001	No	↓
Q12906	ILF3	7	-2.340	<.001	No	↓
O95831	AIFM1	6	-2.366	<.001	No	↓
O60832	DKC1	3	-2.377	<.001	No	↓
O75533	SF3B1	9	-2.387	<.001	No	↓
P60174	TPI1	6	-2.396	<.001	No	↓
P13489	RNH1	10	-2.397	<.001	No	↓
Q8TC12	RDH11	3	-2.421	<.001	No	↓
O60763	USO1	7	-2.424	<.001	No	↓
P16070	CD44	4	-2.489	<.001	Yes	↓
P17987	TCP1	6	-2.498	<.001	Yes	↓
Q01813	PFKP	3	-2.537	<.001	No	↓
Q13620	CUL4B	3	-2.590	<.001	No	↓
Q9NTJ5	SACM1L	9	-2.625	<.001	No	↓
P16615	ATP2A2	5	-2.628	<.001	No	↓
Q02218	OGDH	8	-2.669	<.001	No	↓
Q0VD83	APOBR	6	-2.677	<.001	No	↓
Q96T37	RBM15	3	-2.754	<.001	No	↓
P07858	CTSB	3	-2.804	<.001	Yes	↓
Q9NZB2	FAM120A	3	-2.845	<.001	No	↓
P05198	EIF2S1	5	-2.889	<.001	No	↓
Q13098	GPS1	4	-2.896	<.001	No	↓
P80723	BASP1	7	-2.906	<.001	No	↓
Q9NYU2	UGGT1	10	-2.922	<.001	No	↓
P52209	PGD	5	-2.985	<.001	No	↓
P07814	EPRS	7	-3.029	<.001	No	↓
Q9UHD8	SEPTIN9	8	-3.030	<.001	No	↓
P49792	RANBP2	5	-3.056	<.001	Yes	↓
Q8WYJ6	SEPTIN5	4	-3.101	<.001	No	↓
P33176	KIF5B	5	-3.108	<.001	Yes	↓
Q9H3N1	TMX1	4	-3.139	<.001	No	↓
96KP4	CNDP2	3	-3.145	<.001	No	↓
Q14766	LTBP1	17	-3.164	<.001	No	↓
Q15942	ZYX	9	-3.236	<.001	No	↓
Q3KQU3	MAP7D1	3	-3.254	<.001	No	↓
O60488	ACSL4	5	-3.298	<.001	No	↓
P11413	G6PD	9	-3.355	<.001	No	↓
P04040	CAT	7	-3.486	<.001	Yes	↓
P04275	VWF	14	-3.802	<.001	Yes	↓
P31948	STIP1	7	-3.819	<.001	No	↓
Q14764	MVP	10	-3.957	<.001	Yes	↓
P24534	EEF1B2	3	-4.280	<.001	No	↓
P31150	GDI1	5	-4.288	<.001	No	↓
P30153	PPP2R1A	4	-4.464	<.001	Yes	↓
P17301	ITGA2	8	-4.588	<.001	No	↓
Q04917	YWHAH	6	-4.940	<.001	No	↓
Q9Y6C2	EMILIN1	9	-5.342	<.001	No	↓
P51858	HDGF	8	-5.433	<.001	No	↓
P30041	PRDX6	5	-5.545	<.001	Yes	↓
P40925	MDH1	4	-5.759	<.001	No	↓
Q13557	CAMK2D	3	-6.154	<.001	Yes	↓
Q13464	ROCK1	8	-6.805	<.001	Yes	↓
Q13043	STK4	3	-7.982	<.001	No	↓
Q9UBS4	DNAJB11	3	-27.921	<.001	No	↓
P27986	PIK3R1	3	-27.050	<.001	Yes	↓
Q9UNS2	COPS3	3	-27.023	<.001	No	↓
Q9P258	RCC2	3	-26.768	<.001	No	↓
Q99567	NUP88	3	-26.728	<.001	Yes	↓
Q9H2K8	TAOK3	3	-26.724	<.001	No	↓
O75150	RNF40	3	-26.628	<.001	No	↓
Q8TD19	NEK9	3	-26.468	<.001	No	↓
Q9Y4P3	TBL2	3	-26.246	<.001	No	↓

Table footnote. Symbols: ↑ upregulation at post-intervention compared to mid-intervention; ↓ downregulation at post-intervention compared to mid-intervention. Abbreviations (other than protein names): FDR, false discovery rate; Log2FC, log 2-fold change. Abbreviations (proteins): ACSL4, acyl-CoA synthetase long chain Family member 4; AIFM1, apoptosis inducing factor mitochondria associated 1; ANXA5, annexin A5; APOBR, apolipoprotein B receptor; ATL3, atlastin GTPase 3; ATP2A2, ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2; ATP6V1A, ATPase H^{+} transporting V1 subunit A; BASP1, brain abundant membrane attached signal protein 1; CAMK2D, calcium/calmodulin dependent protein kinase II delta; CAT, catalase; CCT3, chaperonin containing TCP1 subunit 3; CD44, CD44 molecule; CD48, CD48 molecule; CNDP2, carnosine dipeptidase 2; COPS3, COP9 signalosome subunit 3; COX4I1, cytochrome C oxidase subunit 4I1; CTSB, cathepsin B; CTSS, cathepsin S; CUL4B, cullin 4B; DDX5, DEAD-box helicase 5; DKC1, dyskerin pseudouridine synthase 1; DNAJB11, DnaJ heat shock protein family (Hsp40) member B11; EEF1B2, eukaryotic translation elongation factor 1 beta 2; EIF2S1, eukaryotic translation initiation factor 2 subunit alpha; ELAVL1, ELAV like RNA binding protein 1; EMILIN1, elastin microfibril interfacer 1; EPRS, glutamyl-prolyl-tRNA synthetase 1; FAM120A, family with sequence similarity 120A; G6PD, glucose-6-phosphate dehydrogenase; GDI1, GDP dissociation inhibitor 1; GOT2, glutamic-oxaloacetic transaminase 2; GPS1, G protein pathway suppressor 1; H1FX, H1.10 linker histone; H2AFX, H2A.X variant histone; H3F3A, H3.3 histone A; HDGF, heparin binding growth factor; HIST1H1D, H1.3 linker histone, cluster member; HIST1H1E, H1.4 linker histone, cluster member; HIST1H4A, H4 clustered histone 1; HIST2H2AC, H2A clustered histone 20; HMGN4, high mobility group nucleosomal binding domain 4; HNRNPA0, heterogeneous nuclear ribonucleoprotein A0; HSD17B4, hydroxysteroid 17-beta dehydrogenase 4; ILF3, interleukin enhancer binding factor 3; ITGA2, integrin subunit alpha 2; KIF5B, kinesin family member 5B; LTA4H, leukotriene A4 hydrolase; LTBP1, latent transforming growth factor beta binding protein 1; MAP7D1, MAP7 domain containing 1; MDH1, malate dehydrogenase 1; MLEC, malectin; MTDH, metadherin; MVP, major vault protein; NEK9, NIMA related kinase 9; NUP88, nucleoporin 88; OGDH, oxoglutarate dehydrogenase; PAK2, P21 (RAC1) activated kinase 2; PDS5B, PDS5 cohesin associated factor B; PFKP, phosphofructokinase, platelet; PGD, phosphogluconate dehydrogenase; PHB2, prohibitin 2; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PPP2R1A, protein phosphatase 2 scaffold subunit alpha; PRDX6, peroxiredoxin 6; PREX1, phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1; PSMD3, proteasome 26S subunit, non-ATPase 3; PTMA, prothymosin alpha; RANBP2, RAN binding protein 2; RBM15, RNA binding motif protein 15; RCC2, regulator of chromosome condensation 2; RDH11, retinol dehydrogenase 11; RECQL, RecQ like helicase; RNF40, ring finger protein 40; RNH1, ribonuclease/angiogenin inhibitor 1; ROCK1, Rho associated coiled-coil containing protein kinase 1; RPLP2, ribosomal protein lateral stalk subunit P2; RPS5, ribosomal protein S5; RPS9, ribosomal protein S9; RSU1, Ras suppressor protein 1; S100A11, S100 calcium binding protein A11; SACM1L, SAC1 like phosphatidylinositide phosphatase; SCP2, sterol carrier protein 2; SEC61B, SEC61 translocon beta subunit; SEPTIN5, septin 5; SEPTIN9, septin 9; SF3B1, splicing factor 3b subunit 1; SFXN1, sideroflexin 1; SLC25A3, solute carrier family 25 member 3; STIP1, stress induced phosphoprotein 1; STK4, serine/threonine kinase 4; TAOK3, TAO kinase 3; TBL2, transducin beta like 2; TCOF1, treacle ribosome biogenesis factor 1; TCP1, T-complex 1; TES, testin LIM domain protein; THRAP3, thyroid hormone receptor associated protein 3; TMX1, thioredoxin related transmembrane protein 1; TPI1, triosephosphate isomerase 1; TUBB1, tubulin beta 1 class VI; UGGT1, UDP-glucose glycoprotein glucosyltransferase 1; USO1, USO1 vesicle transport factor; VWF, Von Willebrand factor; YWHAG, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma; YWHAH, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein Eta; ZAP70, zeta chain of T cell receptor associated protein kinase 70; ZYX, zyxin.

vated kinase 2 (PAK2); phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1); protein phosphatase 2 scaffold subunit alpha (PPP2R1A); peroxiredoxin 6 (PRDX6); Ran binding protein 2 (RANBP2); Rho associated coiled-coil containing protein kinase 1 (ROCK1); S100 calcium binding protein A11 (S100A11); SEC61 translocon beta subunit (SEC61B); T-complex 1 (TCP1); tubulin beta 1 class VI (TUBB1); and Von Villebrand Factor (VWF).

Selection of the ten best candidate proteins. The proteins that showed a higher differential expression (all with a Log-2FC > 10 and FDR q-value < 0.001) were as follows: COP9 signalosome subunit 3 (COPS3); DnaJ heat shock protein family (Hsp40) member B11 (DNAJB11); histidyl-TRNA synthetase 1 (HARS); NIMA related kinase 9 (NEK9); NUP88; PIK3R1, regulator of chromosome condensation 2 (RCC2); TAO kinase 3 (TAOK3); transducin beta like 2 (TBL2); and ring finger protein 40 (RNF40). All of them were upregulated with MICT versus baseline, except HARS, which was downregulated with MICT versus baseline. Of these ten candidate proteins only two, PIK3R1 and NUP88, are currently known to play a documented role in immune function.

Gene set enrichment analysis. A total of four enriched pathways (FDR < 25%) were found, of which two related to transmembrane transport and cellular composition were downregulated at mid-intervention versus baseline, and two related to oxidation-reduction reactions were upregulated at post-intervention vs baseline (**Table 6**).

DISCUSSION

Our study reports several novel findings. We found that the first 4-week phase of the program (MICT) increased the number of PBMC and NK cells in blood over baseline, and this was further increased by the second 4-week phase (HIIT). However, the second part of the intervention did not lead to additional improvements over the first one for NK cell function with respect to cytotoxicity (or 'killing capacity'). In fact, a ceiling effect was observed, where both training phases induced a comparable and remarkable (~10-fold) improvement in NK cell cytotoxicity over the untrained, baseline state. Of note, such a noticeable benefit on NK function was observed after only four weeks of regular exercise (MICT in this case). Importantly, because immune function was assessed after a 48-hour rest upon termination of both MICT and HIIT phases, controlling for an acute exercise effect, our results point to a chronic benefit of regular exercise training on NK function.

Flow cytometry analysis showed that exercise training benefits do not necessarily involve upregulation of NK activating receptors. A trend towards a decrease in CXCR3 expression (determined as mean fluorescence intensity) was found at both mid- and post-intervention compared with baseline, yet there were no significant differences in post hoc pairwise comparisons. Using proteomics coupled to systems biology, we identified some candidate proteins and pathways (or 'protein networks') enriched by the training intervention. Thus, compared with baseline, the MICT phase downregulated the expression of proteins related to transmembrane transport and cellular composition, whereas the expression of proteins related to redox reactions was upregulated after the subsequent HIIT phase. Another interesting finding was that while the increment in NK cytotoxicity was similar at both mid- and post-intervention, the candidate proteins and pathways involved were different between the two time points. Exercise studies like the one described here might contribute to identify new proteins with a previously unreported role on NK function.

Given the increasingly important role of NK cells, it is fundamental to unravel the effects of regular exercise on NK cell function and the potential mechanisms involved. Indeed, NK cells, which share features of both innate and adaptive immunity, have a powerful killing function against diseased target cells that can compromise organismal homeostasis. NK cells are constantly on high alert for malignant cell-transformation and bacterial/viral infection, and monitor target cells for surface expression of stress-regulated self-molecules that are ligands for NK cell activating receptors (37). Engagement of these receptors triggers the exocytic release by NK cells of granules with cytotoxic mediators – granzymes and perforins – to destroy such abnormal cells. A high presence of NK cells in tumor microenvironments has been reported to be a positive prognostic factor for patients with a variety of malignancies, including during metastatic disease (30). However, despite the rapid and efficient capacity of NK cells to recognize and kill

Table 6. Processed differentialy expressed after endurance exercise training (moderate-intensity continuous training followed by high-intensity interval training) compared with baseline.

Gene set	FDR (q-value)	Nominal p-value	Related process	Expression
GO_OXIDOREDUCTASE_ACTIVITY	.017	<.001	Catalysis of oxidation-reduction reaction	↑ at post-intervention *vs* baseline
GO_OXIDATION_REDUCTION_PROCESS	.060	<.001	Oxidation-reduction reaction	↑ at post-intervention *vs* baseline
GO_TRANSMEMBRANE_TRANSPORT	.091	<.001	Transmembrane transport	↓ at mid-intervention *vs* baseline
GO_CYTOPLASMIC_REGION	.198	.006	Cellular composition	↓ at mid-intervention *vs* baseline

tumor cells, their function is impaired by the tumor microenvironment, even leading to their dysfunction or exhaustion (78). Thus, numerous strategies to improve NK cell cytotoxic capacity have been attempted, including activation with cytokines or analogs (78). In this context, our results showing an increase in NK cytotoxicity of one order of magnitude with both training programs provide support to the notion that a non-pharmacological intervention – regular exercise – could be considered as a coadjuvant to cancer immunotherapies and cancer treatment in general (6, 7, 60).

Our findings that MICT enhances NK cell cytotoxicity in healthy adults is in agreement with several prior studies in adults (15, 38, 45, 52), although others did not report an improvement (41, 42, 56). In this regard, a novelty of our study was that, at least in healthy non-immunocompromised adults, the addition of heavy exercise (HIIT) following MICT is essentially as beneficial as prior MICT in terms of improving NK function. In fact, a further increase in the release of NK cells to the bloodstream was observed at post-intervention compared with mid-intervention. Whether this result is due to an additional benefit of HIIT per se (i.e., higher work intensities) or to the accumulation of more exercise bouts in general (i.e., longer duration of the intervention) remains to be determined. That addition of HIIT did not negatively affect NK cell function and might actually considerably improve the number and function of these cells is an important finding because intense exercise has been traditionally viewed as a stressor to immune function that could potentially lead to a certain state of immunosuppression with subsequent increase in the risk of infections, at least of the upper respiratory tract (8). In fact, one month of heavy exercise training during the pre-competition season was reported to reduce NK cytotoxicity in female volleyball players (65), and another study found reductions in resting NK cell numbers and proportions after a 7-month training season in elite swimmers (20). It remains to be determined, however, whether a HIIT intervention with no previous MICT in untrained subjects elicits similar adaptations to those observed here.

The mechanisms by which exercise might increase NK number or function remain to be clearly elucidated. A preclinical murine study by Pedersen et al. found that exercise training did not enhance NK cell cytotoxicity per se, but rather 'prepared' the tumor microenvironment for their infiltration by enhancing the expression of ligands for NK cell-activating receptors such as NKG2D and NKp46 (51). Of note, no training effects were noted in our study for surface expression of NKG2D or NKp46 in flow cytometry analyses. NK cells – especially $CD56^{dim}$ cells, which make up 90% of the total cell population and are classified as cytotoxic (68) – can respond to exercise-induced epinephrine (51). In this context, the Pedersen et al. study showed that NK cells were mobilized by exercise-induced epinephrine and were redistributed to tumors in an IL6-dependent manner (51). More recently, another mechanistic study showed that the acute mobilization of NK cells in response to a 30-minute exercise bout at an intensity above the lactate threshold – and thus also above a typical MICT session – in cyclists was largely dependent on epinephrine signaling through β_2-adrenergic receptors (21). In this regard, our proteome analyses failed to find significant changes in NK b_2-adrenergic or IL-6 receptors at mid- or post-intervention, suggesting that, if exercise were able to change the expression levels of these receptors in humans, it would be more an acute than a chronic effect. Indeed, increases in the secretion of epinephrine and of the myokine IL-6 occur during (and in the hours following) an acute exercise bout whereas in the present study we aimed at assessing the chronic effects of regular exercise bouts, and as such we studied the participants' NK cells under resting conditions (after a previous 48-hour rest).

Transcriptomic (53) and epigenetic (79) modifications have been reported in NK cells after an acute exercise session. Just two-minute bouts of MICT (cycle ergometer exercise at 77% of VO_2peak) interspersed with one-minute rest result in the upregulation of genes related to pathways involved in cancer and cell communication (p53 signaling pathway, melanoma, glioma, prostate cancer, adherens junction, and focal adhesion) in healthy young men (53). Also, running a half marathon was found to induce global histone modifications in NK cells and a subsequent increase in the expression of the activating NK cell receptor NKG2D in patients with cancer and their controls (80). Another study found that acute exercise can provoke epigenetic modifications in NK cells, in this case affecting the balance between the activating immunoglobulin-like receptor KIR2DS4 and the inhibiting KIR3DL1 receptor, with potential benefits on NK cell function (57). Finally, acute exercise could also have a stimulating effect on NK cell cytotoxicity through intracellular signaling, for instance by mediating an increase in perforin levels inside these cells (23), with perforin-mediated cytolysis playing a key role in the control of acute viral infections by NK cells (64).

The effects of chronic exercise on NK cells at the molecular level are much less clear. Dias et al. (12) found that 18 weeks of aerobic endurance training changed the expression of 211 gene transcripts involved in cell cycle regulation, proliferation, and development of immune cells in PBMC. Similarly, a study comparing young endurance athletes and non-athlete controls identified 72 candidate transcripts in PBMC involved in encoding ribosomal proteins and oxidative phosphorylation (29). It should be borne in mind, however, that PBMC are a heterogeneous mix of immune cells, and changes in gene expression over time may be driven by alterations in immune cell proportions and not necessarily in NK cells per se. In contrast to the aforementioned studies in PBMC, a recent study showed that a 12-week resistance training intervention had negligible effects on the NK cell transcriptome (48). Slight increases were found for some candidate gene transcripts, of which only one of them, ten-eleven translocation methylcytosine dioxygenase 1 (TET1, involved in DNA demethylation) is actually known to play a relevant role with regard to NK cell function.

The ten candidate proteins that showed a higher differential expression in the present study were COPS3, DNAJB11, HARS, NEK9, NUP88, PIK3R1, RCC2, TAOK3, TBL2 and RNF40 (all FDR values < 0.001). All of them were upregulated at mid-intervention versus baseline, except HARS, which was downregulated at mid-intervention versus baseline. Of the ten candidate proteins two – PIK3R1(p85a) and NUP88 – have bona fide roles in immune function. The PI3K signaling pathway is involved in a broad range of cellular processes, including growth, metabolism, differentiation, proliferation,

motility, and survival (46). The PI3Kδ enzyme complex is primarily present in the immune system and comprises a catalytic (p110δ) and regulatory (PIK3R1/p85α) subunit. Dynamic regulation of PI3Kδ activity is required to ensure normal function and differentiation of immune cells, including NK cells. PI3K signaling plays indeed an important role in multiple key aspects of NK cell biology, including development/maturation, homing, priming, and function of these cells (33). PI3K activation leads to mobilization of intracellular calcium stores, which is required for NK cell migration and granule exocytosis (33). In addition to activating cytotoxicity, PI3K plays a pivotal role in signaling downstream (through PI3K-mammalian target of rapamycin [mTOR] pathway) of cytokine activation, particularly of IL-15 – the critical NK cell development and survival cytokine (32) – thereby coupling the metabolic sensor mTOR to NK cell anti-viral responses (39). Importantly, inhibition of the PI3K-signaling pathway blocks this priming effect and attenuates the antitumor response of these cells (39, 72).The other protein related to immune function, NUP88, is a structural constituent of the nuclear pore complex (nucleoporins), which are large protein complexes residing in the nuclear envelope. Nucleoporin 88 kDa (Nup88) selectively mediates the nucleocytoplasmic transport of NF-kappaB, an ubiquitous transcription factor involved in immune responses, apoptosis, and cancer (66).

The candidate proteins COPS3, DNAJB11, HARS, NEK9, RCC2, TAOK3, TBL2 and RNF40, have not yet been assigned a role related to NK function per se. COPS3 is a subunit of the COP9 signalosome (CSN), which regulates protein degradation (de-ubiquitination) and protein kinase activities in a variety of processes (59, 74). DNAJB11 is a member of the heat shock protein (HSP) family, whose main function is to facilitate folding of other proteins. HSPs are generally stress-inducible as they play a particularly important cytoprotective role in cells exposed to stress conditions (81). They are generally regarded as danger signaling biomarkers that prompt the immune system to react to prevailing adverse cellular conditions. Accordingly, DNAJB11 might also stimulate immune responses, for instance through activation of bone-marrow derived dendritic cells (77). In turn, HARS can mediate deleterious adaptive immune responses, contributing to the disease phenotype of the anti-synthetase syndrome (1). TAOK3 may be involved in T cell receptor signaling as well as in the regulation of early signaling from receptors that utilize Src kinases in cells other than T lymphocytes (47). NEK9 might play an important role in cell cycle control, contributing to the establishment of the microtubule-based mitotic spindle (17). RCC2 belongs to the so-called 'interactome', connecting integrins with the cell-migration machinery (11). TBL2 is an endoplasmic reticulum-localized transmembrane protein that is involved in cell survival and in translation of activating transcription factor 4 through its association with mRNA (69). Finally, RNF40 is a RING finger protein that is known to be involved in protein-protein and protein-DNA interactions, and has been documented to exert pro-tumorigenic functions in colorectal cancer in vitro by increasing clonogenic potential as well as by suppressing apoptosis (58).

Systems biology analysis highlighted four enriched pathways (FDR <25%), of which two related to transmembrane transport and cellular composition were downregulated at mid-intervention (after MICT) compared with baseline. It is possible that MICT could temporarily block endocytic signaling of NK cell membrane surface receptors, thereby keeping activating receptors – with a preserved cytotoxic capacity – at the membrane for a longer time with no need for receptor upregulation. This would explain the flow cytometry results (Table 2), where no major changes were found in activating NK surface receptors compared to baseline, perhaps reflecting a longer duration of biological signals due to the aforementioned decrease in endocytosis. Further knowledge of the role that transmembrane transport and cellular composition have with regard to activating/inhibitory membrane surface receptors in NK cells and other lymphocytes will be important to understand how regulation of receptor function within the endocytic compartments relates to the functional status of these cells. The same analysis showed that redox reactions were upregulated at post-intervention compared with baseline. In this regard, oxidants play a role in the regulation of NK cell function. Of note, Evans et al. (14) reported a crucial role of oxidant production in cancer cell killing or sensitization by NK cells. A possible mechanism underlying the sensitizing effect of oxidants to NK-mediated killing of cancer cell may be the upregulation of NK cell activating molecules on the surface of cancer cells (22).

We are aware of several limitations in our study. In addition to the relatively small sample size, we did not use a counterbalanced design with regard to the two training modalities, with the MICT program preceding the HIIT phase for all the participants. However, we felt it more appropriate for individuals who were untrained at baseline and thus not familiarized with exercise training programs to start with the less stressful/demanding MICT intervention. Furthermore, owing to the short resting period between MICT and HIIT, we could not discern whether the results found at post-intervention are attributable to HIIT alone and/or to a potential carry-over effect of the previous MICT phase. Implementation of a more controlled design would have strengthened our study (i.e., with a longer 'washout' period between MICT and HICT and also using a quasi-experimental design, with all the participants assessed at an additional 'control' time point, that is, after a long training cessation period following the intervention). In turn, we believe there are major strengths in our study, notably the use of a proteomics approach, which is a more rational strategy than transcriptomic approaches. Indeed, while the cellular concentration of proteins correlates with the abundance of their corresponding mRNAs, the correlation is not strong and, as such, relative abundances of proteins may not occur in proportion to their relative mRNA levels (71). The proteome is the final product of genome expression and comprises all the proteins present in a cell at a particular time. It supplements the other "omics" technologies to explain the identity of proteins in an organism, tissue or cell and to study the structure and functions of a particular protein (2). Further, proteomics analyses, especially if complemented with systems biology as here, allow to study the role of functional protein pathways in different conditions (2).

In conclusion, the training program induced a very remarkable improvement in NK function compared with the

untrained state although at the mechanistic level the pathways involved seem to differ over the intervention (i.e., between mid-intervention, which allowed to assess MICT-only effects, and post-intervention, which reflected HIIT effects as well as potential carry-over effects of previous MICT). The main findings of our study are summarized in **Figure 6**.

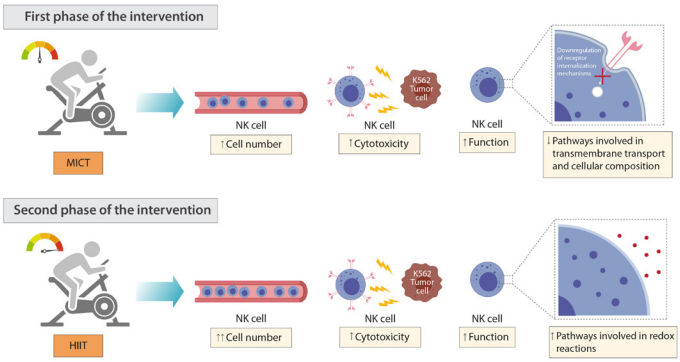

Figure 6. Summary of the main study results. Abbreviations: HIIT, high-intensity interval training; MICT, moderate-intensity continuous training.

Acknowledgements

AL, CFL, and JLZ are supported by grants from the Spanish Ministry of Science and Innovation (Fondo de Investigaciones Sanitarias [FIS]), Fondos FEDER (grant numbers PI18/00139, PI20/00645, and PI18/00207). JLZ is also supported by a grant from the University of the Basque Country (UPV/EHU, # US 19/04). CFL is supported by the Spanish Ministry of Science and Innovation (Instituto de Salud Carlos III, post-doctoral contract Miguel Servet, # CP18/00034) and by a grant from the National Strength and Conditioning Association (NCSA) Foundation. We thank Dr. K. McCreath for his editorial assistance.

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The effect of exercise on regulatory T cells: A systematic review of human and animal studies with future perspectives and methodological recommendations

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ABSTRACT

Many of the exercise-related health-promoting effects are attributed to beneficial immunomodulation. The restoration of immune homeostasis is context-dependent, meaning either to increase anti-inflammatory signaling to counteract disease progression of non-communicable (auto)inflammatory diseases or to enhance (local) activity of proinflammatory immune cells to slow down or inhibit cancer progression. Regulatory CD4$^+$ T cells (T_{regs}) represent the main regulatory component of the adaptive immune system that fine-tunes inflammatory responses, keeps them in check and prevents long-lasting autoimmunity. Because often dysregulated in the context of various diseases, emerging treatment approaches aim to modulate their number or inherent anti-inflammatory and immunosuppressive function in a highly disease-specific way. Exercise represents a non-pharmacologic strategy in disease prevention and rehabilitation and may be an effective treatment with few to no side effects to counteract dysregulation of T_{regs}. To date, several studies have evaluated the effect of exercise on T_{reg}-related outcomes. This review aims at providing a comprehensive overview on alterations of blood- or tissue-derived T_{reg} counts, proportion and functionality following acute and chronic exercise in humans and animal models. From the 60 reviewed studies, an overall disease-specific beneficial effect of chronic exercise on T_{reg} levels in animal models can be stated, while both acute and chronic effects in human studies are less definite. However, T_{reg} phenotyping is less sufficient in the animal studies compared to human studies. Only a limited number of studies investigated T_{reg} functionality. There is a large heterogeneity concerning study design, human population or animal model, exercise protocol, and T_{reg} outcome measure specification which makes it difficult to compare results and draw clear conclusions. Study results are discussed in the context of current concepts in exercise immunology. Finally, future perspectives and methodological recommendations are provided to promote research in this field.

Keywords
Exercise, Physical activity, Immune homeostasis, T_{regs}, Exercise immunology

INTRODUCTION

The proper functioning of immune cells with regulatory function is indispensable for a balanced and fine-tuned immune system in mammals. Within the CD4$^+$ T cell compartment, specialised regulatory cells (T_{regs}) exist that are characterized by CD25 upregulation, CD127 downregulation and expression of the transcription factor forkhead box P3 (Foxp3) as the master regulator in T_{reg} development and function. They mature in the thymus as a small subset of positively selected thymocytes that receive signals slightly weaker than those which are negatively selected by clonal deletion (51). Consequently, their T cell receptors possess a high affinity for MHC:self peptide complexes. However, activation of T_{regs} leads to contact-dependent (e.g. via CTLA4-B7 interaction or gap junction formation) and contact-independent (mainly via cytokines) suppression of effector immune cells from the innate and adaptive immune system (12, 83), which favors immune homeostasis and self-tolerance, and prevents excessive inflammation. This becomes apparent in cases of genetic ablation or mutations in the Foxp3 gene which lead to severe disease phenotypes in mice (14) and humans (8). T_{regs} can also be derived from naïve CD4$^+$ T cells in the periphery by the orchestrated action of certain cytokines, however, there is no clear method to distinguish between the thymus-derived and peripherally induced subsets (52, 131). Due to their "immune harmony" role, T_{regs} have been investigated thoroughly in the context of several disease states where immune dysregulation plays a key role in pathogenesis and disease progression (see table 1), but also in transplantation and aging (55, 122). Thus, treatment approaches that target T_{reg} metabolism, and therefore functionality, are a hot topic in autoimmunity, cancer, and organ transplantation (113). Although it is proposed to stain at least for CD4, CD25, and Foxp3 to identify human and animal T_{regs} (112), species-specific differences in both the T_{reg} phenotype and the overall immune system makeup in general need to be considered (81, 86, 114). Other commonly used markers are those for defining the maturation status with CD45RA$^+$CD45RO$^-$CD95$^-$ T_{regs} being naïve and CD45RA$^-$CD45RO$^+$CD95$^{+/high}$ T_{regs} representing a highly differentiated or memory phenotype (93).

Regular physical activity and exercise training are widely accepted determinants of a healthy lifestyle with implications for disease prevention and partial rehabilitation. In contrast to medical treatments, exercise does not cause any side-effects which makes it an attractive supportive therapy option

in many disease conditions. There are several existing concepts addressing why exercise exerts those effects. However, mainly due to complex inter-organ crosstalk, the underlying tissue-specific cellular and molecular mechanisms in response to exercise are unclear, and the focus of ongoing research (95, 145). Accumulating evidence in the field of exercise immunology revealed that the immunomodulatory characteristics of exercise may account for some health-promoting effects (73, 138). The positive impact of exercise-induced immunomodulation is attributable to the long-term establishment of a more anti-inflammatory state on the systemic level since most of the Western diseases arise from a phenotype of chronic silent inflammation as a consequence of metaflammation and inflammaging (50). Other existing concepts refer to senescence and redistribution of immune cells within body compartments. The former concept describes the idea of an exercise-induced decline in senescent or exhausted T cells, e.g. via apoptosis, to "make space" for newly built, naïve T cells, which ultimately results in improved immunocompetence (123). The latter concept describes the (re)entry of lymphocytes from margination pools into the circulation in response to acute exercise, reflected by an increased number of cells, that is followed by the migration of lymphocytes back to tissues that generate strong chemotactic signals.

Most of the research has been conducted in effector $CD8^+$ T cells and Natural Killer cells. However, there is a lack of knowledge on the effects of acute and chronic exercise interventions on the anti-inflammatory arm of $CD4^+$ T cells. Since T_{regs} represent the main and best studied regulatory part within the adaptive immune system, they may play a major role in exercise-induced health promotion by the restoration of local and systemic immune homeostasis. Therefore, this review should inform about all human and animal research that focused on the impact of acute and chronic exercise interventions and the physical activity level on T_{reg} cell numbers, proportions, functionality and T_{reg}-related outcomes (i.e. Foxp3 gene expression). Limitations concerning study design, choice of outcome measures, and heterogeneity between studies are discussed. To promote and improve further research, methodological recommendations and promising research questions in the context of current concepts in exercise immunology are provided.

Table 1: Regulatory T cell characteristics in major disease states.

DISEASE STATE	T_{REG} NUMBER/FUNCTIONALITY	CONSEQUENCE	REF
Autoimmunity	*Decreased/Dysfunctional*	*Increased activity of autoreactive immune cells*	(28)
Cancer	*Increased*	*Decreased activity of cytotoxic immune cells in the tumor microenvironment*	(103)
CNS pathologies	*Decreased/Dysfunctional*	*Increased neuroinflammatory signaling in several brain and spinal cord regions*	(34)
Cardiovascular diseases	*Decreased/Dysfunctional*	*Decreased anti-inflammatory counter-regulation in the vasculature*	(85)

METHOD

This study was conducted in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) (90). Since research on $CD4^+CD25^+$ regulatory T cells started after the publication of the seminal paper of Sakaguchi et al. in 1995 (118), the literature search considered a period of the last 25 years (1995 to April 1st, 2020). To not miss any relevant published study in this field, the references of included studies were screened.

Search strategy

The literature search was conducted using MEDLINE (via PubMed), Web of Science, and SportDiscus for human studies and MEDLINE (via PubMed) and Web of Science for animal studies. Search strategies involved Medical Subject Headings (MeSH) and text words combined through Boolean operators (''AND'', ''OR'').

For human studies, the search string included common synonyms for the concepts of [1] physical exercise, [2] human population, and [3] T_{reg} characteristics: *(exercise OR "physical activity" OR sport* OR training OR HIIT OR HIT OR cycling OR running OR walking OR swimming) AND (human OR men OR women OR athlete* OR runner* OR cyclist*) AND ("regulatory T cells" OR Treg* OR "Forkhead-Box Protein P3" OR foxp3 OR "immune homeostasis" OR immunsuppress*)*. For animal studies, the search string included synonyms for [1] physical exercise, [2] animal (i.e. rodent) population, and [3] T_{reg} characteristics: *(exercise OR fitness OR sport* OR training OR "wheel running" OR HIIT OR HIT OR cycling OR walking OR swimming OR running) AND (murine OR mouse OR mice OR rat OR rats OR rodent OR animal) AND ("regulatory T cells" OR Treg* OR "Forkhead-Box Protein P3" OR foxp3 OR CD25 OR "immune homeostasis" OR immunsuppress*)*.

For both search strategies, the search string was adjusted according to the formal requirements of each database and the scope of the search was restricted to peer-reviewed articles published in English. After deduplication and screening (title/abstract) by two reviewers (SP and MW), 33 human studies (34 animal studies) were independently assessed for full-text analysis. Through cross-referencing, ten additional human studies (one animal study) were included in the review process. Finally, 37 human studies (23 animal studies) were included in the systematic review (see figure 1 and figure 2).

Eligibility criteria

All types of acute or chronic exercise interventions, regardless of additional interventions (e.g. supplementation, medication), were considered eligible. Studies that did not report on group effects (animals) or group and/or time effects (humans) were excluded. For both the human and animal category, studies that examined the T_{reg} cell counts, proportion, and suppressive function in any tissue or body compartment were included. Further, studies that measured Foxp3 gene expres-

sion, which was linked to T_{reg} cells by the authors, were included as well. Studies with insufficient or unclear reporting on T_{reg} outcome measures were excluded. Human studies that used a randomized, non-randomized controlled, non-controlled, or cross-sectional design were eligible for inclusion. For animal studies, only randomized and non-randomized controlled trials were included. Case-studies, editorials, reviews, and conference abstracts were excluded.

Data extraction

The following data of included studies were extracted and summarized in table 2 and 3: first author and year of publication; sample size; detailed description of the intervention group, exercise protocol and control group; study design; time point of blood sampling (table 2 only); type of tissue used for analysis and time point of tissue sampling (table 3 only); characterization/phenotyping of T_{reg} population; T_{reg}-related outcome measures; T_{reg}-related results.

Quality assessment and Risk of Bias

Quality assessment of internal validity and risk of bias within included human studies were assessed with the Tool for the assEssment of Study qualiTy and reporting in EXercise (TESTEX) (127). The TESTEX scale contains 12 items and has been designed to assess the quality of reporting of exercise training studies. It covers eligibility criteria, random allocation, concealment of allocation, comparability of groups at baseline, blinding of assessors, reporting of study adherence/exercise attendance/adverse events, analysis by intention to treat, between-group statistical comparisons, point measures and variability data, activity monitoring in control group, constant relative exercise intensity, and reporting of exercise volume and energy expenditure. The TESTEX is not adequate for assessing the study quality of acute exercise interventions and there is no other study quality and reporting assessment tool for acute exercise studies. Therefore, acute studies were not rated.

Quality assessment of internal validity and risk of bias within included animal studies were assessed with the "Office of health assessment and translation risk of bias rating tool" – for Human and Animal Trials (OHAT) (157). The tool contains 11 Risk-of-bias questions that cover 6 different fields of biases (selection, confounding, performance, attrition/exclusion, detection, selective reporting). Eight of the eleven questions can be answered using one of four predefined answer choices that enable the researcher to categorize and quantify the outcome. The remaining three questions are closed-ended questions ("yes" or "no").

Both the quality and the risk of bias of each study were independently assessed by two reviewers (MW and NJ) with an intraclass inter-rater correlation coefficient of 94,56% (TESTEX) and 92,17% (OHAT), respectively. In case of disagreement between the two reviewers, a third reviewer (SP) was consulted.

Differences in T_{reg} phenotyping between studies represent an additional risk of bias. As it is recommended to stain at least for CD4, CD25 and Foxp3/CD127 to identify human and animal T_{regs} (112), considering that CD127 expression inversely correlates with FoxP3 expression (78), phenotyping of T_{regs} is categorized (see table 2 and 3) as sufficient (green color: at least CD4, CD25, Foxp3/CD127), insufficient (light red color: only CD4, CD25 or CD4, Foxp3) or highly insufficient (dark red color: Foxp3 gene expression in leukocytes or PBMCs).

Results

The search strategy for human studies led to 1610 results. Ten additional studies were identified via cross-referencing. After removing duplicates, the titles and abstracts of 1460 studies were screened for eligibility. The remaining 43 studies were assessed for a full-text screen. After applying the selection criteria, six studies were excluded (no/unclear reporting on Treg outcome measures, study protocol, review). Finally, 37 studies were included in the systematic review. The PRISMA flow diagram is provided in figure 1.

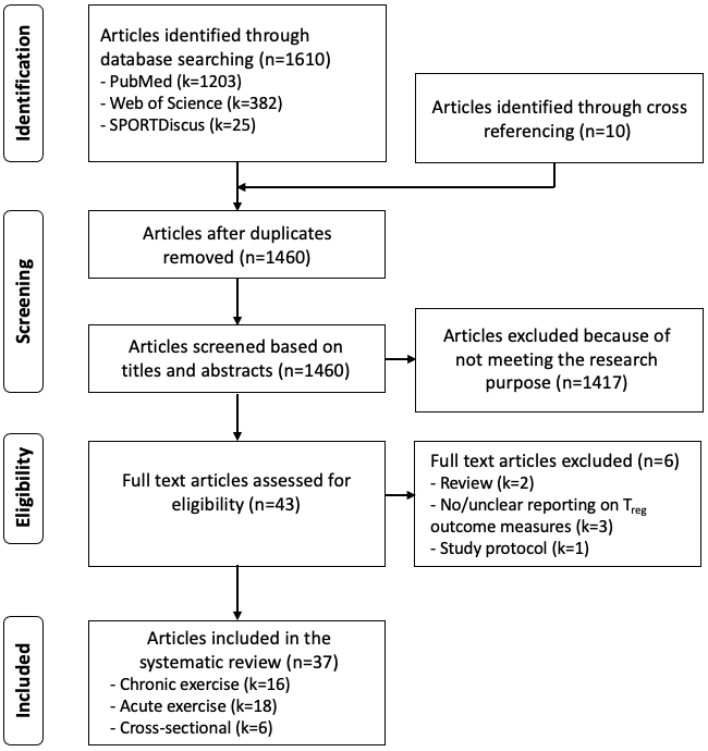

Figure 1: Literature search and results of human studies. Three studies (29, 48, 143) fit into the acute and chronic exercise category.

The search strategy for animal studies led to 1642 results. One additional study was identified via cross-referencing. After removing duplicates, the titles and abstracts of 1267 studies were screened for eligibility. The remaining 34 studies were assessed for a full-text screen. After applying the selection criteria, eleven studies were excluded (no exercise intervention, no/unclear reporting on T_{reg} outcome measures, T_{reg} knockout). Finally, 23 studies were included in the systematic review. The PRISMA flow diagram is provided in figure 2.

Characteristics of included studies

There is a tendency for an increased inclusion of T_{reg} outcome measures into human and animal studies in recent years. The overall sample size of included participants in the human chronic exercise studies is 619, with a mean intervention duration of 70.25 days (ranging from four days to six months). In the acute studies, a total of 398 participants performed either an "exercise-to-exhaustion" protocol [6], another intense [9] or a moderate training protocol [4]. The six cross-sectional

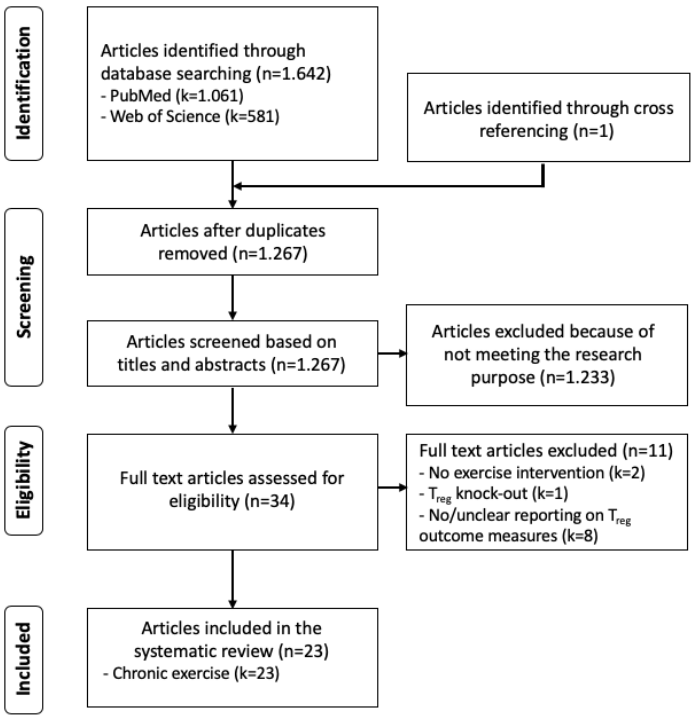

Figure 2: Literature search and results of animal studies.

studies comprise 745 predominantly healthy participants, although the participant characteristics strongly differ. There are only three studies that measured the single or combined effect of strength training on T_{regs}. Seventeen human studies collected data on cell counts, 31 studies on cell proportions, while only ten studies measured both markers. Three human studies measured cellular suppressive capacity either directly, in T_{regs} and peripheral blood mononuclear cells (PBMCs), or indirectly via Interleukin (IL)-10 production of whole blood. Another three studies solely identified Foxp3 gene expression in immune cells that the authors related to T_{reg} cells.

The overall sample size of included animals in the 23 animal studies (only chronic exercise interventions) is 713, with a mean intervention duration of 44.7 days (ranging from four days to twelve weeks). There is only one study that measured the single effect of strength training on T_{regs}. Concerning T_{reg} outcome measures, four animal studies collected data on cell counts, 18 on cell proportions, while two studies measured the suppressive capacity of tissue-specific T_{regs}. Two studies only identified Foxp3 gene expression in tissue homogenates that the authors related to T_{reg} cells.

Rating of bias

None of the included human studies fulfilled all the TESTEX quality criteria. No studies met item 6.2 (reporting of adverse events). Three items were fulfilled only by one study (intention-to-treat analysis (77), activity controlling in control group (77), exercise volume and energy expenditure (38)). On average, each study fulfilled 4,44 criteria representing 29,6% of all items. Figure S1 (supplement material) shows detailed information on each trial rating.

None of the included animal studies fulfilled all OHAT quality criteria. No study considered adequate concealment to study groups (increased selection bias) and outcome data completion without attrition or exclusion from analysis (increased attrition/exclusion bias). Regarding performance bias, only two studies adequately blinded research personnel to the study group (129, 133). One study did not fulfill exposure characterization (107), two studies did not fulfill outcome assessment (84, 107) (increased detection bias). All researchers adhered to the study protocol, but only 26,1% of all studies used appropriate statistical methods. Figure S2 (supplement material) shows detailed information on each trial rating.

Effects of exercise on T_{reg} cells in healthy people

Studies that examined the effect of acute and chronic exercise on T_{reg} measures in the healthy population focus on active/physically fit and inactive/physically unfit adults, active elderly adults (master athletes and octogenarians), and (elite) athletes. Of note, the majority (82%) of the acute studies included healthy people, whereas only 25% of the chronic studies tested healthy people. In the following sections, cross-sectional studies [5] are presented first, followed by acute [14] and chronic [4] studies.

Handzlik et al. (48) showed that a higher physical activity level correlated with an increased T_{reg} proportion and whole blood in vitro IL-10 production when comparing sedentary, recreationally active, sprint- and endurance-trained males. Although the activity level did not differ between the endurance- and sprint-trained groups, the IL-10 production was significantly higher in the endurance group. Dorneles et al. (29) recently showed that obese men with high cardiorespiratory fitness (CRF), characterized as peak oxygen uptake (VO_{2peak}), had higher proportions of $CD4^+CD25^{high}CD127^{low}$ T_{regs} and $CD4^+CD25^+CD39^+$ memory T_{regs} than lean men with low CRF. Within the lean and obese cohorts, the high CRF groups consistently showed higher T_{reg} proportions compared to the low CRF groups. In another cross-sectional study, Weinhold et al. (143) found an almost linear relationship between the relative VO_{2peak} level and the T_{reg} proportion in a heterogeneous cohort of 245 elite athletes. Moreover, both the T_{reg} proportion and suppressive function of T_{regs} from athletes were significantly higher compared to an age- and sex-matched healthy control group. Rehm et al. (110) showed that the T_{reg} proportion of marathon-trained runners near peak in training volume was decreased compared to demographically matched controls. This group difference disappeared when the body mass index was considered as a covariate. Another cross-sectional study of master cyclists (55-79 years) revealed no differences in the T_{reg} proportion compared to younger controls, while both groups showed lower Treg levels than the elderly age-matched control group (36).

Concerning the acute effects of exercise on T_{reg} measures, the aforementioned study of Handzlik et al. (48) further examined the influence of cycling for 60 minutes at 70% VO_{2max} on T_{reg} counts and the T_{reg} proportion within the endurance-trained subgroup. However, no alterations were observed. Three separate studies measured the effect of a single strenuous endurance exercise (marathon, half-ironman triathlon) on T_{reg} counts and proportions. Perry et al. (102) observed a significant decrease in both measures immediately and ten days after a half-ironman triathlon or a marathon race. A subsequent in vitro assay revealed an increased suppressive effect of the athletes´ post-exercise serum on T_{reg} proliferation compared to athletes´ pre-exercise serum. In response to a marathon race, similar changes in T_{reg} kinetics have been observed by Clifford et al. (21). However, the decrease of T_{reg} counts and propor-

tion one hour after the marathon raised above baseline levels after one day. The same post-exercise kinetic accounted for naïve CD45RA$^+$ T_{regs}, whereas the proportion of terminally differentiated HLA-DR$^+$ T_{regs} only increased one day after the marathon. Another study in marathoners revealed no changes in the T_{reg} proportion on day seven post-marathon compared to pre-race levels (111). In elite soccer players, treadmill running until exhaustion induced an increase in the T_{reg} proportion at 17 hours post-exercise, whereas no differences were observed immediately after exercise (66). In a parallel-group study with elite soccer players, the effect of two different exhaustive exercise protocols (YO-YO test vs. Beep test) on CD4$^+$ Foxp3$^+$ T_{regs} was examined (67). Following the YO-YO test, the T_{reg} proportion declined at 17 hours post-exercise, while there was a significant increase in T_{regs} immediately and 17 hours after the Beep test. Minuzzi et al. (88) tested the effect of cycling exercise until exhaustion on the proportions and cell counts of several CD4$^+$CD25^{++}CD127$^{-/low}$ T_{reg} populations in active master athletes (53.2 ± 9.08 years) and age-matched controls. An increase in T_{reg} counts ten minutes post-exercise and a concomitant return to baseline levels after one hour was observed in both groups. No changes in IL-10$^+$-, TGF-β$^+$-, Foxp3$^+$-expressing T_{regs}, naïve (CD45RA$^+$), memory (CD45RA$^-$), and terminally differentiated (KLRG1$^+$) T_{regs} were shown. Dorneles et al. (30) revealed an increase in the CD4$^+$CD25$^+$CD39$^+$ memory T_{reg} proportion in healthy men with low and high CRF immediately and one hour after a single high-intensity interval training (HIIT). Of note, T_{reg} proportions were significantly greater in high CRF compared to low CRF men at baseline. In a study on middle-aged women (64), no change in the T_{reg} proportion in response to cycling until exhaustion was shown. However, the participants´ CRF correlated positively with baseline T_{reg} levels. By using a randomized cross-over design of acute moderate resistance and endurance exercise, the group of Zimmer et al. (57, 121) showed no changes in CD4$^+$CD25$^+$CD127^{dim} T_{reg} counts and proportions in response to resistance exercise, but significant increases in T_{reg} counts immediately after the endurance exercise that was accompanied by a decrease to baseline levels one hour after exercise cessation. Another study conducted by Krüger et al. (69) compared the effect of acute moderate continuous exercise to HIIT in untrained healthy men by using a cross-over design as well. There was an increase in circulating T_{regs} only following HIIT, lasting for three hours, whereas a higher proportion of apoptotic Annexin V$^+$ T_{regs} was shown only in response to the moderate continuous exercise with a significant group difference three hours post exercise.

With respect to chronic effects of exercise on T_{reg} measures in healthy subjects, the aforementioned study of Weinhold et al. (143) conducted a one-week intense training intervention (German Olympic Hockey Team) and revealed a mean increase of 12,9% in the T_{reg} proportion. In two different studies, Yeh et al. (149, 152) focused on the effect of a long-term moderate exercise intervention on the T_{reg} compartment in middle-aged healthy adults. Both the twelve-week Tai chi chuan (149) and the gymnastic exercise intervention (152) led to an increase in T_{reg} counts. However, when compared to the passive control group, the results did not reach significance (152). Another study by Van Geest et al. (135) found that a strenuous endurance exercise (4-day walk, 30 km each day) in octogenarians (81.3 ± 1.9 years) increased naïve CD45RA$^+$ T_{reg} counts, whereas no change was observed in memory CD45RA$^-$ T_{regs}.

Effects of exercise on T_{reg} cells in pathological conditions

The included human studies comprise a wide spectrum of diseased population. Most of them are complemented by studies of animal disease models. In the following sections, studies on MS and cancer, as being the most investigated disease categories, are presented separately, followed by a section that comprises other disease categories. For each category, human studies are described first, followed by related animal studies.

In a cross-sectional study, Waschbisch et al. (141) found no difference in circulating CD4$^+$ CD25$^+$Foxp3$^+$ T_{regs} between physically active and inactive patients with relapsing-remitting MS. A subsequent incremental test to exhaustion revealed no association between VO_{2max} and the baseline T_{reg} proportion. The study of Deckx et al. (25) showed that twelve weeks of combined endurance and resistance exercise did not alter the T_{reg} proportion. In line with these results, a parallel-group study from Mähler et al. (80) observed no changes in the CD4$^+$CD25$^+$CD127$^-$ T_{reg} proportion in relapsing-remitting MS patients after 4 weeks of either normoxic or hypoxic endurance exercise. However, normoxic training led to a shift towards an increased proportion of CD39$^+$ T_{regs}. At the same time, a reduced CD31 expression, a marker for recent thymic emigrants, was observed. Hypoxic training led to the opposite effect with increased CD31 and reduced CD39 expression, respectively.

By using mice with induced experimental autoimmune encephalomyelitis (EAE) as the main MS model, Souza et al. (129) found a significant higher splenic CD4$^+$CD25$^+$ T_{reg} proportion at day 14 (strength and endurance training) and day 30 (strength training only) after EAE induction compared to EAE passive controls. Xie et al. (147) revealed that eight weeks of high-intensity swimming significantly increased CD4$^+$CD25$^+$Foxp3$^+$ T_{regs} in the central nervous system (CNS)- and lymph node compared to the moderate training and control groups. Results from Bernardes et al. (9) did not confirm these findings in EAE mice, as no group difference in T_{reg} counts or proportion in the spinal cord was observed after six weeks of swimming exercise.

In the context of cancer disease, Ligibel et al. (77) showed that, on average, four weeks (ranging from 10 to 70 days) of combined strength and aerobic exercise in invasive breast cancer patients did not change counts of tumor tissue-infiltrated Foxp3$^+$ leukocytes.

In two mammary tumor mouse models of 4T1 cell line injection, Hagar et al. (47) and Bianco et al. (10) performed analyses of T_{reg} measures in dissected tumor tissues. Hagar et al. used an immunohistochemical approach and showed significant reductions in CD4$^+$Foxp3$^+$ T_{regs} and an increase in the CD8/T_{reg} ratio compared to a passive control group. Bianco et al. identified no changes in mean fluorescent intensity of tumor-infiltrating CD4$^+$Foxp3$^+$ T_{regs} between tumor-trained and untrained mice. However, splenic CD4$^+$CD25$^+$ T_{regs} were decreased, while Foxp3$^+$ gene expression in splenocytes was increased in the exercise group. In a 7,12-dimethylbenzanthracene-induced mammary tumor model (1), an eight-week exercise intervention before tumor induction lowered the splenic T_{reg} proportion in tumor-trained mice compared to passive controls. When three weeks of moderate swimming exercise before and six weeks after liver tumor induction was applied,

reductions of the T_{reg} proportion in several tissues, i.e. tumor tissue, spleen, and blood, were observed. In a genetic model of intestinal tumorigenesis, McClellan et al. (84) revealed a decrease in Foxp3 gene expression in mucosal scrapings after a twelve-week running intervention.

In adolescent elite swimmers with and without allergic asthma, Wilson et al. (146) found that acute intense swimming exercise increased $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T_{regs}, respectively, in both groups. However, no group difference for any cell population was reported. In response to 12 weeks of chronic moderate exercise, Liao et al. (76) revealed increases in the T_{reg} proportion in asthmatic children, but again, without a difference compared to passive controls. In three mouse models of allergic asthma, using an ovalbumin sensitization protocol beginning three weeks before exercise commencement, Fernandes et al. (40) found that five weeks of moderate exercise increased counts of $IL\text{-}10^+$ and LAP^+ (an important marker of TGF-β excretion in T_{regs}) T_{regs} in lung tissue of asthmatic but not healthy mice. Similar findings were shown for the T_{reg} proportion and suppressive capacity in lungs and lung-draining lymph nodes of asthmatic mice compared to passive controls after four weeks of exercise (79). Although no changes in the T_{reg} proportion was observed, a significant group difference was evident for T_{reg} suppressive function. Dugger et al. (37) adoptively transferred either wild type β2 adrenergic receptor (β2AR) T_{regs} or β2AR knockout T_{regs} into T_{reg}-depleted asthmatic mice. Results show that an adoptive transfer of wild-type $\beta2AR^+T_{regs}$ into mice prior to asthma induction increased cyclic AMP (cAMP) levels in splenic and lymph node T_{regs} in response to chronic exercise compared to mice with $\beta2AR^{-/-}$ T_{regs}. This increase in intracellular cAMP was associated with an enhanced cell-dependent suppressive function. The catecholamine-dependent increase in T_{reg} functionality was confirmed by elevations in the cAMP concentration due to β2AR agonist application.

Six months of intradialytic cycling did not change $CD4^+CD25^+CD127^{low/-}$ T_{reg} counts in haemodialysis patients (38). In the usual care control group, however, T_{regs} decreased below baseline levels which resulted in a significant group difference after the intervention period. In a mouse model of cisplatin-induced acute kidney injury, Miyagi et al. (117) found that five weeks of aerobic running exercise before disease induction did not affect $CD4^+Foxp3^+$ T_{reg} proportion in spleen and kidney lymph nodes, respectively, compared to passive controls. However, significantly lower levels in $CD4^+CD25^+$ (spleen and lymph nodes) were identified in response to exercise.

Yeh et al. showed that twelve weeks of Tai Chi Chuan increased both $CD4^+CD25^+$ T_{reg} counts and proportions in a non-controlled trial with diabetic adults (151). The same exercise protocol was used in a controlled trial which revealed an increased Foxp3 gene expression in leukocytes only in the diabetic cohort, however, without a group effect (150). Another study did not find any changes in the T_{reg} proportion in response to twelve weeks of moderate cycling (144). In a genetic mouse model of diabetes type II, six weeks of whole-body vibration exercise resulted in an increased proportion of circulating T_{regs} compared to passive controls (153).

Dorneles et al. (29) showed that one week of HIIT in obese men with low CRF increased both $CD4^+CD25^{high}CD127^{low}$ T_{reg} and $CD39^+$ memory T_{reg} proportions, correlating positively with exercise-induced increases in VO_{2peak}. However, in a study that tested eight weeks of running exercise on the T_{reg} proportion in diet-induced obese mice, no change was observed (74).

Barhoumi et al. (6) examined the effect of an eight-week swimming intervention on Foxp3 gene expression in renal cortex cells in a mouse model of hypertension and found that the exercise-induced increased expression was associated with lower erythropoietin-induced adverse vascular effects such as oxidative stress, inflammation, and immune activation. In a study using an atherosclerosis-prone mouse model, five weeks of running did not change the proportion of lymph node $CD4^+CD25^+Foxp3^+$ T_{regs} (56).

In a murine heart transplantation model (133), running exercise for one week after transplantation had the most beneficial effect on transplantation success compared to other exercise regimens and led to increases in the splenic T_{reg} proportion. When injecting whole splenocytes or $CD4^+$ splenocytes from this exercise group to heart-transplanted mice that did not exercise, significantly longer graft survival rates were observed compared to untreated mice. In line with these findings, Rael et al. (107) observed a beneficial effect of combined preceding (four weeks) and succeeding (one week) voluntary exercise on skin transplantation success. However, they did not find any changes in the $CD4^+Foxp3^+$ T_{reg} proportion in the skin allograft and even a decline in skin-draining lymph nodes.

Table 2: Human studies on the effect of exercise and physical activity on regulatory T cells. Phenotyping of T_{regs} is categorized as sufficient (green: at least CD4, CD25, CD127 or CD4, CD25, Foxp3), insufficient (light red: only CD4, CD25 or CD4, Foxp3) and highly insufficient (dark red: Foxp3 gene expression in leukocytes or PBMCs). Each section (chronic, acute, cross-sectional) is sorted by T_{reg} outcome measure (first proportion & counts, then proportion, then counts, then Foxp3 gene expression).

AUTHOR, YEAR	N	POPULATION, INTERVENTION GROUP	EXERCISE PROTOCOL	STUDY DESIGN, CG SPECIFICATION	TIMEPOINT OF SAMPLING	T_{REG} PHENO-TYPING	T_{REG} OUTCOME MEASURES	RESULTS
CHRONIC INTERVENTION STUDIES								
DUNGEY 2017 (38)	31 f+m	Haemodialysis patients; n=16	6 months of progressive intradialytic exercise on recumbent cycle at RPE 12–14 (offered thrice weekly)	CT / CG: same population; passive (usual care); n=15	Before and after intervention (at least 48h after last exercise)	$CD4^+ CD25^+ CD127^{low/-}$	Proportion (%$CD4^+$ cells), Counts (cells/µL)	↔ T_{reg} count post vs. baseline in exercise group; ↓ T_{reg} count post vs. baseline in control (p=.003); ↑ T_{reg} count in exercise vs. control group (p=.02); ↔ T_{reg} proportion in either group after intervention; No difference between groups
YEH 2007 (151)	32 f+m	Type 2 diabetic patients	12 weeks of Tai Chi Chuan exercise (classified as moderate exercise), 3 sessions/week à 60 min	NCT	Before and 3 days after intervention	$CD4^+ CD25^+$	Proportion (%$CD4^+$ cells), Counts (cells/mm^3)	↑ T_{reg} count after exercise (p=.029) ↑ T_{reg} proportion after exercise (p=.001)
DECKX 2016 (25)	45 f+m	MS patients (all types, EDSS ≤ 6); n=29	12 weeks of combined endurance/resistance exercise (5 sessions in 2 weeks, continuous progression of duration [1×6min/session to 3×10min/session] and intensity [1×10 rep. to 4×15 rep.])	RCT / CG: same population; passive; n=16	Before & within the week after intervention (overnight fast at 8 a.m.)	$CD4^+ CD25^{high} Foxp3^+$	Proportion (%$CD4^+$ cells)	↔ T_{reg} proportion in exercise and control group; No differences between groups
MÄHLER 2018 (80)	30 f+m	Relapsing-remitting MS patients (EDSS ≤ 4)	4 weeks of either normoxic (NO, n=16) or hypoxic (HP, corresponding to 2.500m altitude, n=14) treadmill exercise (3 sessions/week à 1h)	Pilot parallel-group RT	Before and after intervention (12h overnight fast)	$CD4^+ CD25^+ CD127^-$ (T_{regs}) + $Foxp3^+$, CD31, CD39, CD45RA on T_{regs}	Proportions of T_{regs} and all subpopulations (%T_{regs})	↔ T_{reg} proportion and any T_{reg} subpopulation in the HO group; ↑ $CD39^+ CD31^-$ T_{reg} proportion in the NO group; ↑ $CD39^+$ T_{reg} proportion in the NO group; ↓ $CD39^- CD31^+$ T_{reg} proportion in the NO group; ↑ $CD39^+$ $Foxp3^+ CD45RA^+$ T_{reg} proportion in the NO group; Significant group differences for $CD39^+ CD31^-$ T_{reg} / $CD39^+ Foxp3^+ CD45RA^+$ T_{reg} (higher in NO) and $CD39^- CD31^+$ T_{reg} (higher in HO) after exercise
DORNELES 2019 (29)	7 m	Low CRF obese men (VO_{2peak} of 34.54 ± 3.27 mL/kg/min)	1 week of HIIT-Training on treadmill (3 sessions à 10 bouts of 60 sec (85-90% HR_{max}), 75 sec recovery (50% HR_{max}) between bouts)	NCT	Immediately before, 24h after last session	$CD4^+ CD25^{high} CD127^{low}$ / $CD4^+ CD25^+ CD39^+$ (mT_{reg})	Proportion (%lymphocytes)	↑ T_{reg} proportion (p=.04) ↑ mT_{reg} proportion (p=.042)
WEINHOLD 2016 (143)	19 m	Olympic hockey team	Single 1-week intense training phase	NCT	Immediately before + after intervention (not specified)	$CD4^+ CD25^{high} CD127^{low}$	Proportion (%$CD4^+$ cells)	↑ T_{reg} proportion after intervention (p=.021)
DOS SANTOS 2015 (91)	60 f	Overweight (n=15) and eutrophic (n=15) pubescent children	18 weeks of circus activities in a circus school (2 sessions/ week à 60 min)	RCT / CG: same population; passive; n=2x15	No pre assessment; at least 48h after last exercise session	$CD4^+ CD25^+ Foxp3^+$	Proportion (% n.a.)	Lower T_{reg} proportion in overweight exercise vs. eutrophic exercise group (p<.05) and overweight control vs. eutrophic control group (p<.05); No difference between overweight exercise vs. control group
WENNING 2013 (144)	14 m	Type 2 diabetic patients	12 weeks of moderate aerobic cycling (2 sessions/week, progressively increasing from 15 to 45 min/session)	NCT	Before and after intervention (not specified)	$CD4^+ CD25^{++} CD127^{low}$	Proportion (%$CD4^+$ cells)	↔ T_{regs} proportion after intervention
LIAO 2019 (76)	40 f+m	Children (6-12y) with mild to severe persistent asthma; n=25	12 weeks of Tai Chi Chuan exercise (1 session/week à 60 min); specifically designed for asthmatic children	CT / CG: same population; passive; n=15	Before and after intervention (not specified)	$CD4^+ CD25^+ Foxp3^+$	Proportion (%$CD4^+$ cells)	↑ T_{reg} proportion in exercise group (p=.008); No differences between groups
WANG 2016 (140)	87 m	Heroin users; n=30	12 weeks of Baduanjin qigong training (5 sessions/week à 60min)	CT / CG: 2 groups; 1st group: same population, passive; n=30 2nd group: healthy men; n=27	Before and after intervention (not specified)	$CD4^+ CD25^+ Foxp3^+$	Proportion (%n.a.), Suppressive function of PBMCs (Thymidine H^3 in T_{Eff})	↓ T_{reg} proportion in heroin exercise group after vs. before intervention (p<.001); ↓ T_{reg} proportion in heroin exercise vs. heroin control group after intervention (p<.001); ↓ suppressive function in heroin exercise group (time and group effect, both p<.001))
YEH 2014 (152)	44 f	Inactive middle-aged women (Ø age: 47); n=22	12 weeks of regular music aerobic exercise (classified as moderate exercise), 3 sessions/ week à 60 min	CT / CG: same population; passive; n=22	Before and 30 min after last exercise session	$CD4^+ CD25^+$ / Foxp3 gene expression in leukocytes	Counts (n.a.)	↑ T_{regs} in exercise group (p<.01) ↑ Foxp3 gene expression in exercise group (p<.01); No differences between groups
YEH 2006 (149)	37 f+m	Middle-aged healthy adults (Ø age: 55)	12 weeks of Tai Chi Chuan exercise (classified as moderate exercise), 3 sessions/week à 60 min	NCT	Before and 3 days after intervention	$CD4^+ CD25^+$	Counts (cells/mm^3)	↑ T_{reg} count after intervention (p=.015)
VAN DER GEEST 2017 (135)	20 f+m	Octogenarian walkers (Ø age: 81)	Nijmegen Four Days Marches (30 km a day, for 4 consecutive days at self-selected pace, (4.0±0.7 km/h ≙ moderate intensity))	NCT	Before, after (within 10 min after exercise termination)	$CD4^+ CD25^+ Foxp3^{high} CD45RA^-$, $CD4^+ CD25^+ Foxp3^{low} CD45RA^+$	Counts (x10^9/L)	↔ $CD4^+ CD25^+ CD45RA^- Foxp3^{high}$ count after the march ↑ $CD4^+ CD25^+ Foxp3^{low} CD45RA^+$ count after the march (p<.001)
YEH 2009 (150)	60 f+m	Type 2 diabetic patients; n=30	12 weeks of Tai Chi Chuan exercise (classified as moderate exercise), 3 sessions/week à 60 min	CT / CG: age-matched healthy adults; active (same protocol); n=30	Before and 3 days after intervention period	Foxp3 gene expression (in leukocytes)	Fold change (%) in mRNA expression	↑ Foxp3 gene expression in diabetic patients after exercise (p=.026), % change not reported; No differences between groups
RAYGAN 2017 (108)	44 m	Asymptomatic ischemic heart	12 weeks of submaximal aerobic exercise (3 sessions/week à 40	RCT / CG: same population; active	Before (24h) and after (24h)	Foxp3 gene expression (in	Fold change (%) in mRNA	↑ Foxp3 gene expression after exercise intervention (p<.02)

		disease patients	min)	(routine regular physical activities); n=23	intervention period	PBMCs)	expression	↑ Foxp3 gene expression in exercise vs. control group (p=.012)
LIGIBEL 2019 (77)	49 f	Invasive breast cancer patients (no metastases); n=27	Multi-week (Ø 4 weeks, range 10-70 days) combined strength and moderate-intense aerobic exercise of 220 min/week	RCT / CG: same population; passive (mind-body exercise); n=22	Tumor tissue collection at baseline and surgical tumor excision	Foxp3 gene expression (in tumor sample infiltrated leukocytes)	Fold change (%) in mRNA expression	↓ $Foxp3^+$ gene expression in exercise group (x0.76) ↑ $Foxp3^+$ gene expression in control group (x2.86) No differences between groups (p=.08)
ACUTE INTERVENTION STUDIES								
MINUZZI 2017 (88)	29 f+m	Active master athletes (different disciplines, Ø age: 53); n=19	CPET to exhaustion on cycle ergometer (25 watts increase every 3 min)	CT / CG: healthy inactive body weight- and age-matched controls; active (same protocol); n=10	Before, 10 min post, 1h post	$CD4^+CD25^{++}$ $CD127^{-low}$ (T_{regs}) $CD45RA^{+/-}$ T_{regs}, $KLRG1^+$ T_{regs}, IL-10^+ T_{regs}, $TGF\beta^+$ T_{regs}, $Foxp3^+$ T_{regs}	Proportion (%lymphocytes, $CD4^+$, T_{regs}), Counts ($x10^3/\mu l$), Foxp3, TGF-β and IL-10 gene expression in T_{regs}	Time effects for both groups: ↑ T_{reg} count at 10min post exercise (p<.05) ↔ for T_{regs} proportion (%$CD4^+$ & lymphocytes) ↔ $CD45RA^+$- and $CD45RA^-$ T_{regs} ↔ KLRG1 expression on T_{regs} ↔ Foxp3, TGF-β and IL-10 gene expression in any group No differences between groups for any outcome measure
CLIFFORD 2017 (21)	17 f+m	Experienced runners	Marathon race	NCT	Before (in the week leading up to the marathon), 1h post, 1-day post	$CD4^+CD25^{++}$ $CD127^-$ $Foxp3^+$ (T_{regs}), $CD45RA^+$ T_{regs}, $HLA\text{-}DR^+$ T_{regs}	Proportion (%$CD4^+$ & %lymphocytes), Counts (cells/μL)	↓ T_{reg} proportion (% $CD4^+$) at 1h post vs. before; ↑ T_{reg} proportion (% lymphocytes) at 1-day post vs. before; ↓ T_{reg} count at 1h post and ↑ at 1-day post (both vs. before); ↓ $CD45RA^+$ T_{reg} proportion (both %$CD4^+$ cells and lymphocytes) and count 1h post vs. before; ↑ $CD45RA^+$ T_{reg} count and $HLA\text{-}DR^+$ T_{reg} proportion (both %$CD4^+$ cells & %lymphocytes) 1-day post vs. before; all outcomes p<.05
PERRY 2013 (102)	38 f+m	Trained triathletes/ marathoners	Marathon race (n=16) and half-ironman triathlon (n=22)	NCT	4 days before race, immediately after race, 10 days after race	$CD4^+CD25^+$ $Foxp3^+$	Proportion (%$CD4^+$), Counts ($x10^4$/ml)	↓ T_{reg} count immediately and 10 days after race vs. before (each p=.002) ↓ T_{reg} proportion immediately and 10 days after race vs. before (each p<.001)
HANDZLIK 2013 (48)	10 m	Recreationally active healthy university students	60min on cycle ergometer at 70% of VO_{2max}	NCT	Before, immediately post, 1h post	$CD4^+CD25^+$ $CD127^{low/-}$	Proportion (%lymphocytes, %$CD4^+$), Counts($x10^9$/ml)	↔ T_{reg} count and proportion at any timepoint
GUSTAFSON 2017 (45)	15 m	Healthy active (n=10) & in-active (n=5) men	45min on cycle ergometer at 60% of PPO	NCT	Before, 2-5min post	$CD4^+CD25^+$ $CD127^{low}$	Proportion (%$CD4^+$)	↓ T_{reg} proportion 2-5 min post exercise (p=.006) in whole population; no post-exercise subgroup difference reported
SCHLAGHECK 2020 (121)	24 m	Healthy, physically fit men	Acute bout of endurance (45 min at 60% of PPO) and resistance exercise (4 sets at 5 resistance machines; 8-10 reps at 70% of 1RM)	RCT (crossover design)	Immediately before, immediately post, 1h post	$CD4^+CD25^+$ $CD127^{dim}$	Proportion (%$CD3^+$), Counts (10^3/μL)	↔ T_{reg} count after resistance exercise at any timepoint, ↑ T_{reg} count immediately post endurance exercise vs. before (p<.001); ↓ T_{reg} count 1h post endurance exercise vs. immediately post (p<.009), ↔ T_{reg} proportion in any group at any timepoint
KRÜGER 2016 (69)	23 m	Untrained healthy males	Single HIIT (5 bouts, 3 min each at 90% of PPO; 3 min active break with no resistance) and continuous exercise session (70% of VO_{2max} for 30 min) on cycle ergometer	CT (crossover design)	Before, immediately post, 3h post, 24h post	$CD4^+CD25^+$ $CD127^-$ (T_{regs}), Annexin V^+ T_{regs}	Proportion of Annexin V^+ T_{regs}, Counts ($x10^9$/l) of T_{regs}	↑ T_{reg} count immediately and 3h after HIIT exercise (each p<.05), sig. group difference immediately post vs. continuous exercise, ↔ in continuous exercise group; ↑ proportion of Annexin V^+ T_{regs} at 3h after continuous exercise, sig. group difference at 3h post vs. HIIT, ↔ in HIIT group
HARBAUM 2016 (49)	26 f+m	Idiopathic Pulmonary Arterial Hypertension patients; n=16	CPET on cycle ergometer (10 watts every min until exhaustion)	CT / CG: healthy age- and BMI-matched controls; active (same protocol); n=10	Before, immediately post, 1h post	$CD4^+CD25^{high}$ $CD127^{low}$	Proportion (% n.a.), Counts ($x10^6$/l)	↔ T_{reg} proportion and count in either group after exercise; No difference between groups IPAH patients with a higher baseline T_{reg} proportion than control (p<.05)
PERRY 2012 (101)	8 f+m	Chronic lympho-cytic leukemia patients; n=4	CCL patients: 45-60min walking/running on treadmill; Athletes: 60-120min running; both with HF at 70% of HRR	CT; CG: healthy athletes; n=4	Before, immediately post, 1h post	$CD4^+CD25^{high}$ $Foxp3^+$	Proportion (% n.a.)	↔ T_{reg} proportion in either group immediately after exercise; ↓ T_{reg} proportion 1h post exercise in the CLL group (p=.017)
KOLIAMITRA 2019 (64)	19 f	Healthy, physically fit women, age > 50	CPET on cycle ergometer (25 watts increase every 2 min until exhaustion)	NCT	Immediately before, 1 min post	$CD4^+CD25^+$ $CD127^{dim}$	Proportion (%$CD4^+$)	↔ T_{regs} proportion after exercise
REHM 2013 (111)	19 f+m	Recreational marathoners	Marathon race	NCT	4 weeks before (baseline), 24-48h before (pre race), 1 week after (recovery)	$CD4^+CD25^{high}$ $Foxp3^+$	Proportion (%$CD4^+$)	↔ T_{reg} proportion at any timepoint
KOSTRZEWA-NOWAK 2018 (66)	14 m	Elite soccer players	CPET on treadmill until exhaustion (speed increase of 2 km/h every 3 min) each in autumn and spring under same conditions	NCT (longitu-dinal design)	Before, max. 5 min post, 17h post	$CD4^+$ $Foxp3^+$	Proportion (%$CD4^+$)	↑ T_{reg} proportion at 17h post vs. before and 5 min post exercise (each p<.001) in autumn ↑ T_{reg} proportion at 17h post vs. before and 5 min post exercise (each p=.001) in spring
KOSTRZEWA-NOWAK 2020 (67)	62 m	Elite soccer players	Progressive exercise until exhaustion (YO-YO test [YYRL1 protocol, n=31] vs. Beep test [max. multistage 20 m shuttle run], n=31)	Parallel-group NCT	Before, max. 5 min post, 17h post	$CD4^+$ $Foxp3^+$	Proportion (%lymphocytes)	↓ T_{reg} proportion at 17h post vs. before (p<.01) and 5 min post (p<.001) in the YO-YO group ↑ T_{reg} proportion at 5 min post and 17h post vs. before (both p<.001) in the Beep group
KOSTRZEWA-NOWAK 2019 (65)	3 m	Elite karate athletes	CPET on treadmill until exhaustion (speed increase of 2 km/h every 3 min)	NCT	Before, max. 5 min post, 17h post	$CD4^+$ $Foxp3^+$	Proportion (%$CD4^+$)	↑ T_{reg} proportion at 17h post vs. before exercise (p<.05)
DORNELES 2019 (30)	30 m	Healthy men with high (n=15) and low (n=15) physical fitness	Single HIIT session on treadmill (10 bouts of 60 sec (85-90% HR_{max}) with 75 sec of recovery (50% HR_{max}) between bouts)	NCT	Before, immediately post, 1h post	$CD4^+CD25^+$ $CD39^+$ (mT_{reg})	Proportion (%$CD4^+$)	↑ mT_{reg} proportion in both groups immedi-ately and 1h post vs. baseline; ↑ mT_{reg} proportion in high vs. low physical fitness group at all timepoints (including baseline)
JUSZKIEWICZ 2018 (59)	19 m	Professional rowers; n=10	Controlled 2000-m time trial on rowing ergometer (before and after a 6-week training camp with Spirulina extract or placebo supplementation)	RCT / CG: same population; active (same protocol); n=9	Immediately before, 1 min post, 24h post	$CD4^+CD25^+$ $CD127^-$	Counts (pg/ml)	↔ T_{regs} count in either group after 1st trial (before training camp) ↑ T_{reg} count at 1min vs. baseline and ↓ T_{reg} count at 24h vs. 1min after 2nd trial (after training camp) in placebo group

JUSZKIEWICZ 2019 (60)	20 m	Professional rowers; n=10	controlled 2000-m time trial on rowing ergometer before and after a 6-week training camp (with L-Theanine or placebo supplementation)	RCT / CG: same population; active (same protocol, placebo); n=10	Immediately before, 1 min post, 24h post	$CD4^+ CD25^+ CD127^-$	Counts (pg/ml)	↔ T_{reg} count at any timepoint in any group after 1st trial (before training camp); ↑ T_{reg} count at 24h post vs. baseline in both placebo and supplementation group after 2nd trial (after training camp)
WILSON 2009 (146)	22 f+m	Elite adolescent swimmers with allergic rhinitis or asthma	7 min "race-swimming" exercise	CT / CG: swimmers without allergic rhinitis or asthma; active (same protocol); n=10	Before and post-race swimming exercise (not specified)	$CD4^+ CD25^+$, $CD4^+ Foxp3^+$	Counts (cells/µl)	↑ $CD4^+ CD25^+$ count after exercise in both groups (p<.001); ↑ $CD4^+ Foxp3^+$ cell count after exercise in both groups (p<.001); No differences between groups for any outcome
CROSS-SECTIONAL STUDIES								
HANDZLIK 2013 (48)	40 m	Active and inactive healthy university students	n.a.	4 groups (each n=10): sprint, endurance, recreational, sedentary	Once in resting condition	$CD4^+ CD25^+ CD127^{low/-}$	Proportion (%lymphocytes, $CD4^+$),Counts ($x10^9$/ml), in vitro IL-10 production of whole blood culture	↓ T_{reg} proportion (%$CD4^+$) in sedentary compared with all other groups (p<.001); ↑ proportion (%lymphocytes) in endurance compared to sedentary (p<.05); No group differences in T_{reg} cell count; ↑ IL-10 production in endurance athletes compared to all other groups
WEINHOLD 2016 (143)	280 f+m	Elite athletes with differing $relVO_{2peak}$ values (low vs. intermediate [int.] vs. high); n=245	n.a.	Compared to age- and sex-matched healthy persons (n=35)	Once in resting condition	$CD4^+ CD25^{high} CD127^{low}$	Proportion (%$CD4^+$), Counts (cells/µl), Suppressive function of T_{regs} (proliferation of T_{Eff}(CFSE labeling))	↑ T_{reg} count in high VO_{2peak} athletes vs. int. (p=.04) and low (p<.0001), ↑ T_{reg} proportion in high VO_{2peak} athletes vs. int. (p<.0001) and low (p<.0001) athletes; ↑ T_{reg} proportion int. vs. low (p=.038), ↑T_{reg} proportion in athletes vs. control (p=.0001), ↑T_{reg} proportion in male athletes vs. female athletes (p=.002), ↑ suppressive function of athlete T_{regs} vs. control (p=.04)
DORNELES 2019 (29)	90 m	Healthy obese (n=45) and lean (n=45) men with VO_{2peak} between 25.30 and 58.8 mL/kg/min	n.a.	6 groups (each n=15): lean (low, moderate high CRF), obese (low, moderate high CRF)	Once in resting condition	$CD4^+ CD25^{high} CD127^{low}$ (T_{reg}), $CD4^+ CD25^+ CD39^+$ (mT_{reg})	Proportion (%lymphocytes)	↑ T_{reg} proportion in lean high CRF vs. lean low and lean mod. CRF; ↓ T_{reg} in obese low CRF vs. lean low CRF; ↑ T_{reg} in obese high CRF vs. obese and lean low CRF; ↑ mT_{reg} in lean high CRF vs. lean low and lean mod. CRF; ↑ mT_{reg} in lean mod. CRF vs. lean low CRF; ↑ mT_{reg} in obese high CRF vs. obese low CRF; ↑ mT_{reg} in obese high and obese mod. CRF vs. lean low CRF
DUGGAL 2018 (36)	255 f+m	Active master athletes (non-elite cyclists, age 55-79); n=125	n.a.	Compared to healthy inactive age-matched (n=75) & young (n=55) adults	Once in resting condition	$CD4^+ CD25^{high} Foxp3^+$	Proportion (%$CD4^+$)	↑ T_{reg} proportion in inactive old vs. master cyclists and inactive young (both p=.001), no difference between master cyclists vs. inactive young
REHM 2015 (110)	38 f+m	Healthy recreational marathoners; n=19	n.a.	Compared to demographically matched controls (n=19)	Once in resting condition	$CD4^+ CD25^{high} Foxp3^+$	Proportion (%PBMCs)	↓ T_{reg} proportion in marathoners vs. controls (p=.044)
WASCHBISCH 2012 (141)	42 f+m	Relapsing-remitting MS patients (EDSS ≤ 3.5)	n.a.	Physically active (n=21) vs. inactive (n=21) patients	Once in resting condition	$CD4^+ CD25^+ Foxp3^+$	Proportion (%$CD4^+$)	No difference between groups

Abbreviations: BMI: body mass index; CCL: Chronic lymphocytic leukemia; CFSE: Carboxyfluorescein succinimidyl ester; CG: control group; CPET: cardiopulmonary exercise testing; CRF: cardiorespiratory fitness; CT: controlled trial; EDSS: Expanded Disability Status Scale; f: female; HIIT: high intensity interval training; HR_{max}: maximal heart rate; HRR: heart rate reserve; IPAH: Idiopathic Pulmonary Arterial Hypertension; m: male; mRNA: messenger RNA; MS: multiple sclerosis; mT_{regs}: memory T_{regs}; n.a.: not available; NCT: non-controlled trial; nT_{regs}: naive T_{regs}; PBMCs: Peripheral blood mononuclear cells; PPO: peak power output; RCT: randomized controlled trial; RPE: Rate of Perceived Exertion; RT: randomized trial; T_{Eff}: $CD4^+$ $CD25^-$ effector T cells; TGF-β: Transforming growth factor β; T_{regs}: regulatory T cells; $VO_{2max/peak}$: maximal/peak oxygen consumption; ↑: significant increase/higher; ↓: significant decrease/lower; ↔: no significant change.

Table 3: Animal studies on the effect of exercise on regulatory T cells. Phenotyping of T_{regs} is categorized as sufficient (green: at least CD4, CD25, CD127 or CD4, CD25, Foxp3), insufficient (light red: only CD4, CD25 or CD4, Foxp3) and highly insufficient (dark red: Foxp3 gene expression in tissue homogenate). The table is sorted by T_{reg} outcome measure (first proportion & counts, then proportion, then counts, then Foxp3 gene expression).

AUTHOR YEAR	*N*	*ANIMAL, MODEL ORGANISM*	*EXERCISE PROTOCOL*	*STUDY DESIGN, CG SPECIFICATION*	*TISSUE SAMPLE*	*TIMEPOINT OF SAMPLING*	*T_{REG} PHENOTYPING, METHODS*	*T_{REG} OUTCOME MEASURES*	*RESULTS*
BER-NARDES 2016 (9)	47 f	C57BL/6 mice with induction of EAE (multiple sclerosis model); n=23	6 weeks of swimming exercise (30 min/day, 5 day/week with 7% of body weight); EAE induction at the beginning of the 5th week	RCT / CG: passive; n=24	Spinal cord	42 days after EAE induction (=32 days after intervention)	$CD4^+$ $CD25^+$ $Foxp3^+$ / FC	Proportion (% n.a.), Counts (n.a.)	No difference in T_{reg} count or proportion between exercise vs. control group
XIE 2019 (147)	72 f	C57BL/6 mice with induction of EAE (multiple sclerosis model); 2 groups, each n=24	8 weeks of moderate-intensity swimming (=MIT) (50 min/day, 5 day/week with 0% of body weight) or high-intensity swimming (=HIT) (50 min/day, 5 day/week with 4% of body weight); EAE induction after the completion of the 6th week	RCT / CG: passive; n=24	CNS (brain, spinal cord), Lymph nodes	14 days after EAE induction	$CD4^+$ $CD25^+$ $Foxp3^+$ / FC	Proportion (%$CD4^+$)	↑ CNS T_{reg} proportion in HIT vs. MIT ($p<.05$) and control group ($p<.05$); ↑ Lymph node T_{reg} proportion in HIT vs. MIT ($p<.05$) and control group ($p<.01$); No difference of CNS or lymph node T_{reg} proportion between MT and control group
SOUZA 2017 (129)	12 f	C57BL/6 mice with induction of EAE (multiple sclerosis model); 2 groups, each n=6	4 weeks of strength training (=ST) (5 sessions/week à 30 min; climbing a ladder, carrying load that increases from 25-75% of body weight) or endurance training on a treadmill (=ET) (5 sessions/week à 30min; intensity of 13–17 m/min); EAE induction after completion of the 2nd week	RCT / CG: 2 groups; passive; 1st group: naive 2nd group: EAE each n=6	Spleen	7, 14 and 30 days after EAE induction	$CD4^+CD25^+$ / FC	Proportion (%spleno-cytes)	↑ T_{reg} proportion in ST vs. EAE control group at day 14 ($p<.001$) and 30 ($p<.05$); ↑ T_{reg} proportion in ET vs. naive control group at day 14 ($p<.001$) No difference of T_{reg} proportion between ST and ET at any timepoint
BIANCO 2017 (10)	40 f	BALB/c mice with/without mammary tumor induction (injection of 4T1 cell line); 2 groups, each n=10	4 weeks of swimming exercise after tumor induction (5 sessions/week; progression from 15 min in 1st week to 30 min in 2nd week to 45 min in weeks 3+4)	CT / CG: 2 groups; passive; each n=10	Spleen, Tumor tissue	On last exercise day (not specified)	$CD4^+$ $CD25^+$ / FC $Foxp3^+$ gene expression / PCR	Proportion (%$CD4^+$), Fold change of $Foxp3^+$ splenocytes, MFI of tumor infiltrating lymphocytes	↓ splenic T_{reg} ($p<.005$) proportion in tumor trained vs. control group; no differences between healthy groups; ↑ splenic $Foxp3^+$ gene expression in tumor trained vs. control group ($p<.03$); no differences in MFI of tumor infiltrating $CD4^+$ $Foxp3^+$ cells
ZHANG 2016 (154)	12 m	C57BL/6 mice with liver tumor induction (injection of Hepa 1-6 cell line); n=6	9 weeks of moderate swimming exercise (5 sessions/week à 5 min in 1st week, 6 min in 2nd week and 8 min in weeks 3-9); liver tumor induction at the end of week 3	CT / CG: passive; n=6	Whole blood, Spleen, Tumor tissue	Day 42 after tumor cell inoculation	$CD4^+$ $CD25^+$ $Foxp3^+$ / FC	Proportion (%$CD4^+$)	↓ T_{reg} proportion in blood, spleen and tumor tissue in exercise vs. control group (all $p<.05$)
ABDALLA 2013 (1)	56 f	BALB/c mice with/ without mammary tumor induction (via oral 7,12-DMBA); each n=14	8 weeks of swimming exercise after tumor induction (5 sessions/week à 45 min)	CT / CG: 2 groups; passive; each n=14	Spleen	After the intervention period; no specification	$CD4^+$ $CD25^+$ / FC	Proportion (%$CD4^+$)	↓ $CD4^+$ $CD25^+$ proportion in tumor trained vs. control group ($p<.05$); no differences between healthy groups
DOS SANTOS 2019 (32)	66 f	Normolipidemic/diet-induced hyper-lipidemic C57BL/6 mice with/without tumor induction (injection of B16F10 melanoma cells); 4 groups, each n=8	10 weeks of moderate training on treadmill (5 sessions/week à 1h at 45-55% of maximal speed, 5° incline); melanoma induction in 8th week	RCT / CG: 4 groups; passive; n=8/9/8/9	Mesenteric lymph nodes	21 d after melanoma cell or PBS injection (=shortly after intervention)	$CD4^+$ $CD25^+$ $Foxp3^+$ / FC	Proportion (%$CD4^+$)	No difference of T_{reg} proportion between any group ↓ T_{reg} proportion in hyperlipidemic control vs. normolipidemic control mice
MIYAGI 2018 (117)	38 m	C57BL/6 mice with (n=11)/without (n=10) cisplatin-induced nephrotoxicity	5 weeks of aerobic treadmill exercise before AKI induction (5 sessions/ week at 10/15/20 m/min; progression from 30 to 60 min in 1st week; 60minin weeks 2-5)	CT / CG: 2 groups; passive; n=8/9	Spleen, Kidney lymph node	5 days after AKI induction (=3 days after intervention period)	$CD4^+Foxp3^+$, $CD4^+$ $CD25^+$ / FC	Proportion (%$CD4^+$)	↓ splenic ($p<.01$) and lymph node ($p<.05$) $CD4^+$ $CD25^+$ cell proportion in AKI exercise group vs. control group; no difference in $CD4^+$ $Foxp3^+$ proportion in exercise AKI vs. control group
CHEN 2018 (18)	60 m	Sprague Dawley rats with induced ischemic cardiomyopathy; n=20	12 weeks of treadmill exercise after induction of ischemic cardiomyopathy (5 sessions/week à 30 min at 12m /min, 0° incline)	RCT / CG: 2 groups; 1st group: passive; 2nd group: sham passive;each n=20	PBMCs	post intervention (after overnight fast)	$CD4^+$ $CD25^+$ $Foxp3^+$ / FC	Proportion (%$CD4^+$)	↑ T_{reg} proportion in exercise vs. passive control group ($p<.05$) ↓ T_{reg} proportion in exercise and passive control group vs. sham passive control group ($p<.01$)
JAKIC 2019 (56)	48 f	Young/old C57BL/6 mice with/without high cholesterol diet; 4 groups, each n=6	5 weeks of treadmill exercise (5 sessions/week à 1h at 16 m/min, 5° incline)	CT / CG: 4 groups; passive; each n=6	Lymph nodes - inguinal - brachial - axillary	2 d after last exercise session	$CD4^+$ $CD25^+$ $Foxp3^+$ / FC	Proportion (%lympho-cytes)	No effect of exercise on lymph node T_{reg} proportion in any group; No difference in T_{reg} proportion between the groups
JAKIC 2019 (56)	48 f	Young/old C57BL/6 $ApoE^{-/-}$ mice (prone to atherosclerosis) with/without high cholesterol diet; 4 groups, each n=6	5 weeks of treadmill exercise (5 sessions/week à 1h at 16 m/min, 5° incline)	CT / CG: 4 groups; passive; each n=6	Lymph nodes - inguinal - brachial - axillary	2 d after last exercise session	$CD4^+$ $CD25^+$ $Foxp3^+$ / FC	Proportion (%lympho-cytes)	No effect of exercise on lymph node T_{reg} proportion in any group; No difference in T_{reg} proportion between the groups
LE GARF 2019 (74)	24 f	Diet-induced obese C57Bl/6J mice with/ without PPAR-β/δ agonist; each n=6	8 weeks of treadmill exercise (3 sessions/week; progression from 20 min (20 cm/s) in 1st week to 45min (30 cm/s) in week 8); begin of intervention: change to normal diet	CT / CG: passive; each n=6	Lymph nodes - inguinal - brachial - cervical	After intervention period (not specified)	$CD4^+$ $Foxp3^+$ / FC	Proportion (% n.a.)	↑ T_{reg} proportion in exercise + PPAR-β/δ agonist vs. control group; No difference between only exercise vs. control group
YIN 2015 (153)	12 m	Diabetic db/db and Non-diabetic db/m mice; 2 groups, each n=6	6 weeks of whole-body vibration exercise (5 sessions/week à 20 min at 30Hz (intensity not specified))	CT / CG: 2 groups; passive; each n=6	Whole blood	After intervention (not specified)	$CD4^+$ $Foxp3^+$ / FC	Proportion (%$CD4^+$)	↑ $Foxp3^+$ cell proportion in diabetic exercise vs. control group ($p<.05$); No group difference of $Foxp3^+$ cell proportions in non-diabetic mice
SONG 2019 (128)	n.a. n.a.	Healthy C57BL/6 mice; n=n.a.	30 consecutive days of whole-body vibration exercise (30 min/day: 10 min at 13Hz (moderate), 20 min at 17Hz (high intensity))	CT / CG: passive; n=n.a.	Spleen	After intervention period (not specified)	$CD4^+$ $CD25^+$ $Foxp3^+$ / FC	Proportion (%spleno-cytes)	↑ T_{reg} proportion in exercise vs. control group ($p<.01$)
RAEL 2019 (107)	20 f+m	C57BL/6 B6 mice (tail skin transplant, male B6 donors & female B6 recipients); n=10	4 weeks of voluntary wheel-running prior to skin transplantation with continuation of exercising thereafter for 10 days	CT / CG: same population; passive; n=10	Skin-draining lymph nodes, Skin allograft	10 days after transplan-tation	$CD4^+$ $Foxp3^+$ / FC	Proportion (%$CD4^+$)	↓ T_{reg} proportion in lymph nodes in exercise vs. control group ($p<.01$) No group difference of T_{reg} proportion in skin allograft

UCHIYAMA 2015 (133)	15 m	Heart transplanted CBA mice; n=5	7 days of endurance training on a treadmill after transplantation (1h/day at 9.6 m/min on 1st day and 12.8 m/min on days 2-7, 5° incline).	RCT / CG: 2 groups; 1st group passive; 2nd group: no operation (nOP); each n=5	Spleen, cardiac allograft tissue	1, 2 and 4 weeks after transplantation (spleen), 4 weeks after transplantation (cardiac allograft)	$CD4^+$ $CD25^+$ $Foxp3^+$ (T_{regs}) / FC $CD4^+$ $Foxp3^+$ / IHC	Proportion (%$CD4^+$)	↑ splenic $CD4^+$ $CD25^+$ $Foxp3^+$ proportion after week 1, 2 and 4 in exercise vs. passive and nOP group (all p<.001) except for proportion in exercise vs. passive group after 1 week; ↑ allograft infiltration of $CD4^+$ $Foxp3^+$ cells after 4 weeks in exercise vs. passive control group
WALKER 2018 (137)	12 n.a.	C57BL/6 Foxp3 reporter mice; n=6	4 days of voluntary wheel running	CT / CG: same population; passive; n=6	Spleen, Bone marrow, lymph nodes	n.a.; presumably 1 day after intervention period	$CD4^+$ $Foxp3^+$ (T_{reg}), $CD44^{+/-}$ $CD62L^{+/-}$ T_{reg}, $CXCR4^{+/-}$ T_{reg}, $CCR9^{+/-}$ T_{reg} FC	Proportion (%$CD4^+$, $Foxp3^+$ T_{regs})	↑ in splenic $CD4^+$ $Foxp3^+$ $CD44^+$ $CD62L^-$ proportion in exercise vs. control group (p<.05); No group differences of any cell proportion in lymph nodes or bone marrow; No group differences in T_{reg} chemokine receptor expression
WANG 2012 (139)	40 f	Healthy C57BL/6 mice; 2 groups, each n=8	6 weeks of moderate training (VO_{2max}~70%) or HIIT (VO_{2max}~91%) on a treadmill (5 sessions/week; moderate: week 1: 30 min at 8–18 m/min, week 2-6: 60 min at 18 m/min; HIIT: week 1: 30 min at 5m/min, week 2: 60 min at 15-26.8 m/min, week 3-6: 60 min at 26.8 m/min, 5-10° incline)	RCT / CG: 2 groups; 1st group: moderately active (5 m/min, 5 min/ day, 5 days/ week); n=8 2nd group group: "naïve": free exercise; n=8	Spleen	16-20h after intervention period	$CD4^+$ $CD25^+$, $CD4^+$ $Foxp3^+$ / FC	Proportion (%$CD4^+$)	↑ proportions of both cell types ($CD4^+$ $CD25^+$, $CD4^+$ $Foxp3^+$) in HIIT vs. moderate exercise, control and naïve group (all p<.05), no difference between the other groups; no difference between the other groups
LOWDER 2010 (79)	n.a. f	OVA-sensitized BALB/cj (asthma model) and healthy Foxp3 reporter mice; 2 groups, n=n.a.	4 weeks of moderate training on treadmill (3 sessions/week; 10.0 m/min for 30min to 13.5 m/min for 45min in 1st week, 13.5 m/min for 45min in weeks 2-4, 0° incline); begin of OVA-sensitization 3 weeks before intervention period	RCT / CG: 2 groups; passive; n=n.a.	Lung, Mediastinal lymph nodes, Spleen	24h after last exercise bout	$CD4^+$ $CD25^+$ $Foxp3^+$ / suppression assay $CD4^+$ $Foxp3^+$ / FC	Proportion (% n.a.), Suppressive function of $CD4^+$ $CD25^+$ $Foxp3^+$ (Thymidine H^3 in T_{Eff})	↑ $CD4^+$ $Foxp3^+$ proportion in lung & lymph node in OVA-exercise vs. control group (p=.01); no differences between healthy exercise vs. control group; ↑ suppression capacity of splenic, lung and lymph node $CD4^+$ $CD25^+$ $Foxp3^+$ in OVA-exercise vs. control group (p<.01); same results for healthy exercise vs. control group
FERNANDES 2019 (40)	40 m	OVA-sensitized (asthma model) and healthy BALB/c mice; 2 groups, each n=10	5 weeks of moderate training on treadmill (5 sessions/week à 1h at 50% of maximal speed); begin of OVA-sensitization 3 weeks before intervention period	CT / CG: 2 groups; passive; each n=10	Lung	1 d after intervention	$CD4^+$ $CD25^+$ $Foxp3^+$ LAP^+, $CD4^+$ $CD25^+$ $Foxp3^+$ $IL\text{-}10^+$ / FC	Counts (x10^6/n.a.)	↑ of both LAP^+ and $IL\text{-}10^+$ T_{reg} count in OVA-exercise vs. control group (p<.001); No group effect in either LAP^+ or $IL\text{-}10^+$ T_{reg} count in healthy mice; No difference in T_{reg} count between OVA and healthy mice
HAGAR 2019 (47)	16 f	BALB/c mice with mammary tumor induction (injection of 4T1 cell line); n=7	8 weeks of wheel-running before tumor induction (5 sessions/week; acclimatization in weeks 1+2 with zero to low intensity; 8 min/session in 2nd week to 26 min in week 8)	Pilot RCT / CG: passive; n=9	Tumor tissue	Euthanasia after tumor volume exceeded 1000mm^3	$CD4^+$ $Foxp3^+$ / IHC	Counts (per mm^2)	↓ T_{reg} count in exercise vs. control group ↓ T_{reg}/$CD8^+$ ratio in exercise vs. control group (p=.024)
BARHOUMI 2014 (6)	16 m	Wild-type and transgenic eET-1 overexpressing mice (hypertension model) with/ without EPO; n=7	8 weeks of swimming (6 sessions/ week à 60min with continuous progression in 1st week from 10 to 60 min [+10 min each day])	CT / CG: passive; n=9	Renal cortex	Immediately after the last exercise session	$Foxp3^+$ renal cortex cells/ Immunofluorescence microscopy	Counts (cells/mm^2)	↑ $Foxp3^+$ cell count in $eET\text{-}1^+$ exercise vs. control group (p<.05); ↑ $Foxp3^+$ cell count in $eET\text{-}1^+$ EPO exercise vs. control group (p<.05)
MCCLELLAN 2014 (84)	15 m	$Apc^{Min/+}$ mice (model of intestinal tumorigenesis); n=6	12 weeks of treadmill exercise (6 sessions/week à 1h at 15 m/min)	RCT / CG: passive; n=9	Mucosal tissue of small/large intestine	After intervention period (not specified)	$Foxp3$ gene expression in mucosal scrapings/ PCR	Fold change $Foxp3$ expression (in %)	↓ $Foxp3$ expression in exercise vs. control group (p<.05)

Abbreviations: AKI: acute kidney injury; CG: control group; cpm: counts per minute; CNS: Central nervous system; CT: controlled trial; DMBA: dimethylbenzanthracene; EAE: experimental autoimmune encephalomyelitis; eET-1: endothelial-specific endothelin-1; EPO: Erythropoietin; f: female; FC: Flow cytometry; HIIT: high intensity interval training; IHC: Immunohistochemistry; m: male; MFI: mean fluorescence intensity; n.a.: not available; OVA: Ovalbumin; PBMCs: Peripheral blood mononuclear cells; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; RCT: Randomized controlled trial; T_{Eff}: $CD4^+$ $CD25^-$ effector T cells; T_{reg}: regulatory T cell; VO_{2max}: maximal oxygen consumption; ↑: higher values; ↓: lower values.

Discussion of results and future perspectives

This is the first systematic review that examined the effect of exercise-induced changes in T_{reg} outcome measures in human and animal studies, respectively. Taken together, higher CRF levels and a non-obese state are associated with an increased peripheral T_{reg} proportion in both species (29, 32, 64, 74, 91, 143). As T_{regs} are implicated in tumor progression, it needs to be elucidated whether these observations are also true for cancer patients. Acute bouts of exercise tend to either increase or to not change circulating T_{regs} post-exercise in humans (30, 48, 49, 59, 60, 64, 67, 69, 88, 121, 146), while strenuous endurance exercise such as a marathon race reduces T_{reg} levels (21, 102). With respect to T_{reg} kinetics during the recovery phase, levels tend to increase or remain constant rather than being decreased (30, 48, 49, 60, 65–67, 69, 88). However, this might not be the case in patients that suffer from cancer where a decline in T_{reg} levels has been observed (101). It is important to note that there are no acute exercise studies in animals. Only three long- (twelve weeks) (149, 151, 152) and two short-term (one week) (30, 143) chronic exercise interventions in humans showed increases of T_{reg} counts or proportion, whereas the remaining eight studies did not observe any changes in the peripheral T_{reg} compartment.

It is important to note that these three long-term studies (149, 151, 152) were conducted by the same research group that used the same moderate training protocol. With respect to animal studies, only three studies did not observe any difference in T_{reg}-related outcome measures (9, 32, 56), while seven studies reported reduced T_{reg} levels (1, 10, 47, 84, 107, 117, 154). Five out of the seven studies were mouse tumor models, thus a decline in T_{reg} counts or proportion is associated with a better clinical outcome. The remaining twelve studies revealed an increase in tissue-specific or circulating T_{regs}.

In the following sections, Treg assessment and study heterogeneity is discussed first, followed by discussions of study results in the context of current concepts in exercise immunology, i.e. T_{reg} differentiation, redistribution/apoptosis, and immunosenescence.

T_{reg} assessment

T_{reg} outcome measures often vary in the way how counts (e.g. 10^9/L, 10^4/ml, 10^3µl, cells/mm^3) and proportions (% of $CD4^+$/ $CD3^+$/ lymphocytes/ leukocytes) are presented. Moreover, T_{reg} phenotyping ($CD4^+CD25^+$, $CD4^+Foxp3^+$, $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^+CD127^-$, Foxp3 gene expression) often differs between studies. Recently, a consortium of leading experts discussed existing human T_{reg} markers and, after validating marker combinations, agreed on a rationally composed ranking list of markers (119). Therefore, it is highly recommended to adhere to the following panel for proper (basal) T_{reg} characterization: CD4+, CD25+, and Foxp3/$CD127^{low}$. When further phenotyping of T_{reg} subsets was done in the included studies, $CD39^+$, $CD45RA^-$, $CD45RO^+$, $CD28^+CD57^-$, or $CD62L^-CD44^+$ T_{regs} were categorized as memory T_{regs}, while $CD45RA^+$, $CD45RO^-$, or $CD62L^+CD44^-$ T_{regs} were used to specify naïve/low differentiated T_{regs}. Recent thymic emigrants (RTEs) were identified by being either $PTK7^+$ or $CD31^+$, while $CD28^-CD57^+$ or $KLRG1^+$ T_{regs} were characterized as senescent or highly/terminally differentiated T_{regs}. The high variation in the used subset markers makes it difficult to adequately compare study results.

Some studies only measured T_{reg} counts without assessing the T_{reg} $CD4^+$ proportion which may be of limited significance when similar changes occurred in the overall $CD4^+$ T cell population after acute or chronic exercise. For studies that only measured the T_{reg} proportion relative to lymphocyte or leukocyte counts, interpretation of changes in the T_{reg} proportion can be misleading if there are concomitant exercise-induced increases or decreases in the overall lymphocyte or leukocyte compartment. Exercise-induced mobilization differs within the major leukocyte subtypes and is characterized by a more profound mobilization of $CD8^+$ T-cells, Natural Killer (NK) cells, γδ T-cells, and neutrophils (126). In contrast, the $CD4^+$ compartment shows a less intense response. Therefore, it should be of interest to consider both measures, T_{reg} counts and the T_{reg} proportion relative to $CD4^+$ cells to guarantee an appropriate interpretation of results. A further assessment of T_{reg} functionality, either by staining for functionality-associated cytokines (e.g. IL-10, TGF-β), suppressive markers (CTLA-4), or by conducting functional assays to determine the contact-dependent/independent suppressive capacity (54), gives a more reliable indication of clinically important improvements. It should be taken into account that an increase in cell counts does not necessarily reflect an increase in the overall functional capacity of the cell population. For example, a current meta-analysis from our research group on the effect of acute exercise on the NK cell cytotoxic activity in healthy adults revealed a significant and intensity-dependent increase, but the improved function was not associated with an increased number of circulating NK cells (116).

Heterogeneity

There is a large heterogeneity regarding population, exercise protocols, and either blood sampling time points in human studies or time point of disease induction in animal models (see table 2 and 3). Therefore, our initial aim to conduct a meta-analysis was discarded. For acute studies, blood was not taken immediately, namely within the first minute after exercise cessation, in seven out of the 18 studies (21, 45, 65–67, 88, 111), but at five, ten, or 60 minutes after exercise. There is even more variability within recovery measurement time points, as blood samples were taken one hour (21, 30, 48, 49, 88, 101, 121), three hours (69), 17 hours (65–67), 24 hours (21, 69), seven days (111), and ten days (102) post-exercise. Although three studies tested a relatively homogeneous population with a similar exercise protocol (21, 102, 111), measurement time points at baseline, post and recovery were highly heterogeneous which impedes comparison of results.

Similar heterogeneity was found for chronic human studies. The post-intervention time point of blood sampling differed between 10 minutes (135), 30 minutes (152), 24 hours (29, 108), at least two days (38, 91), and three days (149–151). It is not recommended to take blood samples shortly after the final training session since cytokines and cellular immune components need approximately 24 hours to return to baseline levels. Of note, the remaining seven chronic studies did not report on the time point of sampling. This inconsistency further impedes a comparison between study results and hampers research reproducibility. Thus, more research with varying exercise modalities and intensities in different populations is needed to gain knowledge on modality- and intensity-depen-

dent chronic adaptions in the T_{reg} compartment.

With respect to animal studies, relevant differences between study designs need to be taken into account when interpreting results. In the studies of EAE mouse models (9, 129, 147), disease induction was consistently done two weeks before exercise cessation. However, Xie et al. (147) applied six weeks of exercise before EAE induction, while Bernardes et al. (9) applied four weeks and Souza et al. (129) two weeks of exercise. Concerning cancer studies, Hagar et al. (47) trained mice eight weeks before breast tumor induction, whereas Bianco et al. (10) applied four weeks of exercise training before induction in the same mouse model. Zhang et al. (154) tested the effect of three weeks of moderate swimming exercise before liver tumor induction, while McClellan et al. (84) used a genetic model of intestinal tumorigenesis so that no exercise was possible before disease onset. Of note, studies that investigated the effects of exercise in asthmatic mouse models (37, 40, 79), only small variations were evident regarding exercise protocol and disease induction. This homogeneity was also seen in the results, as all studies showed improved function and increased T_{reg} counts or proportion in asthmatic mice.

Exercise-induced T_{reg} differentiation

Currently there are two different concepts for exercise-induced T_{reg} differentiation. First, we highlight the kynurenine (KYN)-/kynurenic acid (KYNA)-dependent T_{reg} differentiation hypothesis that is supplemented by an intertwined TGF-β signalling mechanism. Then we discuss another, mainly cell metabolism-focused T_{reg} differentiation hypothesis.

The involvement of KYN- and KYNA dependent pathways in T_{reg} differentiation was delineated in the last issue of this journal (57). The interconnection between the tryptophan catabolism and T_{reg} generation via the rate-limiting enzyme, indolamin-2,3-dioxygenase (IDO) 1, has been confirmed. There is now strong evidence that KYN and KYNA or other aryl hydrocarbon receptor (AhR) agonists can induce T_{reg} differentiation (87, 148). Schlagheck et al. found an increase in circulating T_{regs} immediately after moderate endurance exercise that was followed by a decline to baseline levels after one hour (121). This was in line with the KYNA kinetics after endurance exercise. However, neither KYN levels nor the KYN-to-tryptophan ratio increased in response to the endurance exercise. Interestingly, the T_{reg} proportion significantly correlated with IDO1 implying an enhanced conversion from tryptophan to KYNA via KYN (57). In contrast, exhaustive exercise provoked the opposite response by increasing the KYN levels and KYN-to-tryptophan ratio, whereas no changes in T_{reg} levels immediately after exercise were observed (64). According to these results, it was suggested that repeating acute exercise-induced increases in KYN or KYNA might lead to a long-term increase in T_{regs}. However, no study so far examined the influence of exercise on cytoplasmatic or nucleic acid level (trans)differentiation pathways, e.g. KYN-AhR binding and translocation to the nucleus, within the $CD4^+$ T cell population. Since plasmacytoid dendritic cells play an important role in the KYN/KYNA-to-T_{reg} axis (17, 104), it may be interesting to include analyses of these cells into future studies. Deckx et al. (25) measured both T_{regs} and dendritic cells after twelve weeks of combined exercise in MS patients and found no changes in T_{regs} while circulating activated $CD80^+CD62L^+$ plasmacytoid dendritic cells increased. Similar results were found in the lymph nodes of asthmatic mice in response to chronic exercise (40). Future research should focus on cell culture approaches of combined cell lines in regards to tryptophan degradation, KYN/KYNA production and transport kinetics, SLC7A5 expression (KYN transporter) on naïve $CD4^+$ T_{reg} precursor cells and T_{regs}, or AhR translocation to the nucleus. While an increased (trans)differentiation is desirable in metabolic and autoimmune disease, the opposite would be beneficial in cancer diseases. As cell differentiation processes take some time, it may be reasonable to choose longer-term recovery measurement time points for blood or tissue sampling. Increases in T_{reg} counts immediately after exercise may result from effects other than true differentiation events of naïve $CD4^+$ T cells (e.g. demargination or transmigration).

As noted by Pallotta (98), TGF-β is critical in establishing a regulatory phenotype in plasmacytoid dendritic cells which in turn maintains the function of T_{regs} through constant KYN production. Thus, T_{regs} may sustain their activity by a self-reinforcing process through TGF-β production and signaling to dendritic cells. In this regard, exercise-induced increases in serum/tissue TGF-β or $CD4^+TGF\text{-}\beta^+$ regulatory T helper 3 cells (Th3) may represent important additional contributors to this signaling cascade. However, Rehm et al. (111) found an increase in $TGF\text{-}\beta^+$ producing Th3 cells, but no changes in T_{regs} one week after a marathon. Moreover, resting levels of Th3 are increased in marathoners compared to healthy controls, while T_{regs} are decreased (110). Therefore, future in vitro approaches that ensure cell-dependent or cell-independent interactions between T_{regs}, plasmacytoid dendritic cells, and Th3 cells may give insights into possible mechanistic interrelationships.

The other mechanism for T_{reg} induction that has not been addressed in original research yet, but was already discussed elsewhere (31), is the role of exercise-induced alterations in cellular metabolic pathways that may be important for T_{reg} functioning. The metabolic machinery in T_{regs} differs significantly from conventional effector T cells (42) in relying mainly on oxidative phosphorylation. It was recently shown that the mitochondrial complex III is necessary to maintain suppressive function in T_{regs} (142). It is suggested that exercise activates the histone deacetylase SIRT1 and SIRT3, which in turn promotes mitochondrial biogenesis and increases mitochondrial oxidative capacity (136). SIRT1 partly contributes to mitochondriogenesis through PGC-1α-dependent mechanisms. Further, the deletion of SIRT3 and PGC-1α leads to the loss of T_{reg} suppressive function both in vitro and in vivo (7). Interestingly, KYNA enhances PGC-1α expression and cellular respiration in adipocytes (3). These findings point to exercise-induced immunometabolic adaptations in T_{regs}. Thus, it remains to be elucidated if exercise can modulate T_{reg} differentiation and functioning through these pathways.

Tissue-specificity

In the human studies only circulating T_{regs} have been investigated in response to exercise interventions. However, T_{reg} cells are known to display tissue-specific heterogeneity. According to recent single-cell RNA and genome-wide sequencing studies, significant tissue-specific T_{reg} signatures were identified as "tissular programs" and suggest continuum-like differentiation states, adding even more complexity to T_{reg} phenotyping (26, 89). Although these findings are relatively new, unique T_{reg} phenotypes in tumor tissue (75) have been identified ear-

lier. A recent meta-analysis suggested that regular exercise may slow down tumor growth in rodents (39). Targeting T_{reg} functionality or eradicating T_{reg} subpopulation, i.e. terminally differentiated effector T_{regs}, is a hot topic in current cancer immunotherapy (130). Of the reviewed studies, Hagar et al. (47), Zhang et al. (154), and McClellan et al. (84) revealed a reduction in tumor growth and a decrease in T_{regs} or T_{reg}-related outcomes in tumor tissue. Identifying molecular pathways that may underly the proposed exercise-induced anti-tumor effects of T_{regs} should be the focus of future preclinical models. Due to heterogeneity in the validity of different tumor mouse models (44), subsequent validation in human cancer cohorts is of high clinical importance. Further, it would be interesting to understand how exercise influences the metabolism in the tumor microenvironment, as the tumor cell-induced microenvironment is implicated in T_{reg} differentiation and, therefore, increased immunosuppression (23).

It widely accepted that accumulation of excessive adipose tissue significantly contributes to disturbed immune homeostasis and is associated with decreased local T_{reg} counts and function (13, 50). Despite the known role of exercise-induced improvements in murine adipose tissue inflammation due to a suggested macrophage class switch from inflammatory M1 to anti-inflammatory M2 (61) and a proposed increase in local T_{regs} (68), no study to date investigated alterations in T_{reg} number/proportion or function in adipose tissue. Given the results of Dorneles et al. (29) and dos Santos et al. (32, 91) regarding reduced circulating T_{regs} in obesity and the positive association between CRF and circulating T_{regs} (29, 30, 64, 143), it remains elusive why no research made use of already existing improvements in techniques to gather human abdominal subcutaneous adipose tissue (5) to close this knowledge gap. From a mechanistic point of view, KYNA could be a molecule of interest being upregulated in response to exercise. It is involved in the expression of M2 macrophage and T_{reg} genes in murine adipose tissue (3). The adipokine leptin, which is mainly produced by adipocytes, may represent another target molecule as it can decrease T_{reg} differentiation while simultaneously increasing T helper 1 cell responses (24, 82).

Post-exercise T_{reg} kinetics – apoptosis and redistribution

Some studies explain exercise-induced reductions in circulating T_{regs} (21, 59) with increased apoptosis. This mechanism has been observed in other human and animal immune cells. It is likely mediated by the training status as well as exercise intensity (71). However, despite remarkable similarities of expressed genes responsible for apoptosis between mice and humans, the human regulatory machine is more complex making a flawless translation of findings in animals difficult (109). Krüger et al. (69) found that acute continuous moderate exercise, but not HIIT, provoked a significant increase in apoptotic T_{regs} three hours post-exercise compared to baseline levels. This finding was explained by an association between circulating markers of oxidative stress and apoptotic T_{regs}. However, this association is partly contradictory to the concept of an intensity-dependent increase of oxidative stress (106) which would suggest a higher number of apoptotic T_{regs} in response to HIIT. Interestingly, no significant group difference in oxidative stress markers between HIIT and moderate training groups were observed. A hypothesis attributes the beneficial hormetic-like effect of regular exercise to oxidative stress-associated damage by upregulation of cellular antioxidants and damage repair enzymes after acute exercise (105). This suggests that the antioxidant capacity from the untrained participants of Krüger et al.´s study (69) may be lower compared to that of the trained marathoners and rowers in the studies of Clifford et al. (21) and Juszkiewicz et al. (59). Thus, further complexity is added to an adequate comparison of study results. In regards to apoptosis sensitivity, it has been shown earlier that highly differentiated T cells are less sensitive to oxidative stress-induced apoptosis compared to low differentiated T cells (120, 134). However, this may not account for T_{regs}. When blood-isolated naïve $CD45RA^+$ and $CD45RA^-$ memory T_{regs} from healthy humans were exposed to pro-oxidants, naïve T_{regs} showed higher resistance to both cell death and suppressive decline compared to memory T_{regs} (92). Therefore, further research is warranted to test exercise-induced changes in apoptosis sensitivity in T_{reg} subpopulations as well as intracellular levels of antioxidants and damage repair enzymes.

Clifford et al. (21) and Juszkiewicz et al. (59) also discussed T_{reg} redistribution to peripheral tissues as an underlying mechanism for decreased circulating concentrations following exercise. The exercise-induced mobilization and subsequent migration of leucocytes (2), i.e. in T cells (16, 70, 125) and NK cells (100), is an established concept in exercise immunology. Krüger et al. (69) found an association between norepinephrine plasma levels and mobilized T_{regs} which partly confirms the catecholamine-dependent mechanism of T cell mobilization (27, 70).

Mobilized leukocytes show higher expression of integrins, adhesion molecules, and a range of chemokine receptors necessary for effective cell wall attachment and tissue transmigration. Only one of the reviewed studies considered phenotyping of $CD4^+Foxp3^+$ T_{regs} for adhesion molecules (CD44, CD62L) and chemokine receptors (CXCR4, CCR9) (137). A current review highlights the fundamental migratory role of T_{regs} for controlling local inflammation at the "sites of need" (20). Therefore, the precise characterization of human and animal T_{regs} by staining for selected integrins, adhesion molecules, and/or chemokine receptors is mandatory in future exercise immunology research. Reoccurring evidence from various disciplines reveals different expression patterns between animal and human immune cell homing factors. This fact needs to be carefully considered when translating results from the preclinical into the clinical setting (20, 33, 86) and when planning future research.

Taken together, it cannot be ruled out that both T_{reg} apoptosis and T_{reg} redistribution may occur at the same time. As with other immune cells, it is possible that unfavorable T_{reg} subsets, depending on the (disease) context, are deleted by apoptosis, while favorable T_{regs} migrate into target tissues to balance inflammation and to guarantee immune homeostasis (see figure 3). Despite species-specific differences in the $CD4^+$ T cell compartment regarding longevity and expression of adhesion molecules and chemokine receptors, measuring acute exercise effects in animal models will be mandatory to elucidate migration pattern of T_{regs} to different tissues.

T_{reg} functionality and infection susceptibility

Referring to the aforementioned role of catecholaminergic signaling in T cell mobilization after exercise, the results of Dugger et al. (37) may give mechanistic insights into exercise-induced catecholamine-dependent changes in T_{reg} functionality. By using a mouse model of allergic asthma and $\beta2AR^{-/-}$ knockout T_{regs}, Dugger et al. (37) found that reduced intracellular cAMP lev-

els in the T_{reg} $\beta2AR^{-/-}$ variants were associated with a decreased contact-dependent suppressive function after chronic exercise when compared to exercise-trained mice that harbor wild type $\beta2AR^{+/+}$ T_{regs}. By using this approach, they translated similar findings from Bopp et al. (12) into the exercise immunology context. The catecholamine-dependent increase in T_{reg} functionality was confirmed by increased cAMP levels after pharmacological β2AR agonist application. As all these observations were made in both healthy and asthmatic mice, a universal catecholamine-dependent mechanism can be assumed. More importantly, the cAMP-dependent functionality was confirmed in human T_{regs} when their function was assessed in vitro and in a humanized mouse model in vivo (62). For future research, it would be interesting to examine how T_{reg} phenotypes, ranging from naïve to terminally differentiated, differ in β2AR expression, catecholamine responsiveness, and intracellular cAMP levels when analyzed in physically active/inactive humans or in response to exercise.

Some studies propose a higher susceptibility to upper respiratory tract infection and recurrent infections in athletes due to exercise-related increases in regulatory T cell counts or function (45, 98, 126). This could at least partly be explained by the catecholaminergic mechanism delineated by Dugger et al. (37), as the increase in catecholamine levels during exercise seems to be higher in in highly trained compared to untrained subjects, also known as the "sports adrenal medulla" (156). Factors such as age, exercise type, duration, and intensity contribute to the catecholamine response (156). These factors should be taken into account when designing studies, conducting statistical analyses, or interpreting results. Moreover, some studies found positive correlations between CRF and T_{reg} counts or proportions (29, 30, 64, 143). In contrast, the serum of athletes taken immediately after a marathon was shown to exert an increased suppressive effect on T_{reg} proliferation (102). In this regard, it would be interesting to compare cAMP levels and catecholamine-responsiveness in athletes and non-athletes depending on their CRF and physical activity levels.

However, the immunosuppressive effect of both acute and long-lasting intense exercise is still a matter of debate and was discussed extensively in the last issue of this journal (124). There is a clear indication to conduct further studies that focus on exercise-induced changes in T_{reg} function in conjunction with clinical outcomes of immunosuppression. Lowder et al. (79) and Fernandes et al. (40) found increased T_{reg} counts and proportions in lung tissue from asthmatic mice after chronic exercise. Interestingly, T_{reg} suppressive capacity in lung tissue after exercise was elevated in both asthmatic and healthy mice, although no changes in T_{reg} counts and proportions were observed in healthy mice. Whether the higher anti-inflammatory or the immunosuppressive capacity has context-dependent advantageous or disadvantageous implications remains to be elucidated. However, as mice harbor significant bronchus-associated lymphoid tissue, whereas humans do not (97), the transferability of results may be difficult when appropriate mouse models, e.g. mice with humanized-lungs, are not applied. Whether such models are compatible with studies in the sports medicine field, without exerting significant confounding effects on exercise physiology, remains to be answered.

Role of exercise on T_{regs} in the elderly

There is an increasing interest in the role of exercise in immunosenescence (35, 132). An existing concept describes an exercise-induced decline in senescent or exhausted T cells, e.g. via apoptosis, to "make space" for newly built, naïve T cells, thereby boosting immunocompetence (123). However, most of the research focused on $CD8^+$ T cells. Concerning T_{regs}, mainly apoptosis-resistant T_{reg} subsets accumulate in the elderly which is associated with a higher risk of infections through increased immunosuppression (55, 72). This may be due to an age-dependent decrease in the expression of the pro-apoptotic molecule Bim in T_{regs} which was found in mice (19). Although it is known that the proportion of naïve $CD45RA^+$ T_{regs} decreases, while memory $CD45RO^+$ T_{regs} increase with age (11), there is evidence that $CD45RA^+$ T_{regs} are highly apoptosis-resistant compared to their memory counterpart (92). This may challenge the idea of replacing memory T cells by naïve cells to counteract immunosenescence in the context of T_{regs}. Increases in $CD25^+Foxp3^{dim}CD45RA^+$ naïve T_{reg} counts and decreases in $CD25^+Foxp3^{high}CD45RA^-$ memory T_{reg} counts after a strenuous multi-day march in octogenarians (81.3 ± 1.9 years) (135) indicates the mobilization of more apoptosis-resistant cells. As shown by Duggal et al. (36), a large cohort of master cyclists (55–79 years) has decreased proportions of $CD4^+CD25^{high}Foxp3^+$ T_{regs} compared to inactive age-matched controls, whereas these levels do not differ from inactive young adults. That may support an exercise-induced delay of immunosenescence. However, since only T_{reg} proportions were considered without measuring cell counts, some of the differences between the young inactive and old active subjects may be misleading as lymphocyte numbers fall with age. Further, low and highly differentiated T_{regs} were not specified. In response to acute exhaustive exercise (88), master athletes of a younger age (53.2 ± 9.08 years) showed no alterations in counts and proportions of T_{reg} subsets (low/highly differentiated $CD45RA^{+/-}$, terminally differentiated $KLRG1^+$). However, no study assessed T_{reg} functionality. As this parameter is the most reliable to verify changes in (age-related) immunosuppression, contact-dependent and/or contact-independent suppressive capacity should be addressed in future exercise studies. Additionally, exercise-induced effects on apoptosis sensitivity and antioxidant capacity in high and low differentiated T_{reg} subsets should be measured in elderly people.

In the literature, it is suggested that the ratio of peripherally induced T_{regs} (pT_{regs}) to thymus-derived T_{regs} (tT_{regs}) decreases with age due to thymic involution and a concomitant decline in RTEs as the main precursors of pT_{regs} (22). This is a proposed mechanism for the increased immunosuppressive state in the elderly. Interestingly, the cell counts of $CD31^+$ RTEs increased significantly in octogenarians after a strenuous multi-day march (135). Moreover, higher proportions of $PTK7^+CD45RA^+$ RTEs were observed in master cyclists compared to age-matched inactive controls with the same levels seen in the younger group (36). However, there is no valid method to date to truly differentiate between pT_{regs} and tT_{regs}, since the best-known proposed marker of tT_{regs}, the transcription factor Helios, is still controversial (43, 131). Research during the last decade revealed important epigenetic regulatory mechanisms within the Foxp3 locus, i.e. the promotor and three conserved non-coding sequences, that are necessary for stable Foxp3 expression. The first sequence is needed for pT_{reg} generation, but is dispensable for tT_{reg} generation (52). Therefore, elucidating what determinants of exercise (modality, duration, frequency) induce histone modification and change gene expression of transcription factors and their binding to the first non-coding sequence of the Foxp3 locus could give future implications for increasing peripherally induced T_{regs} while simultaneously decreasing thymus-derived T_{regs} in the elderly.

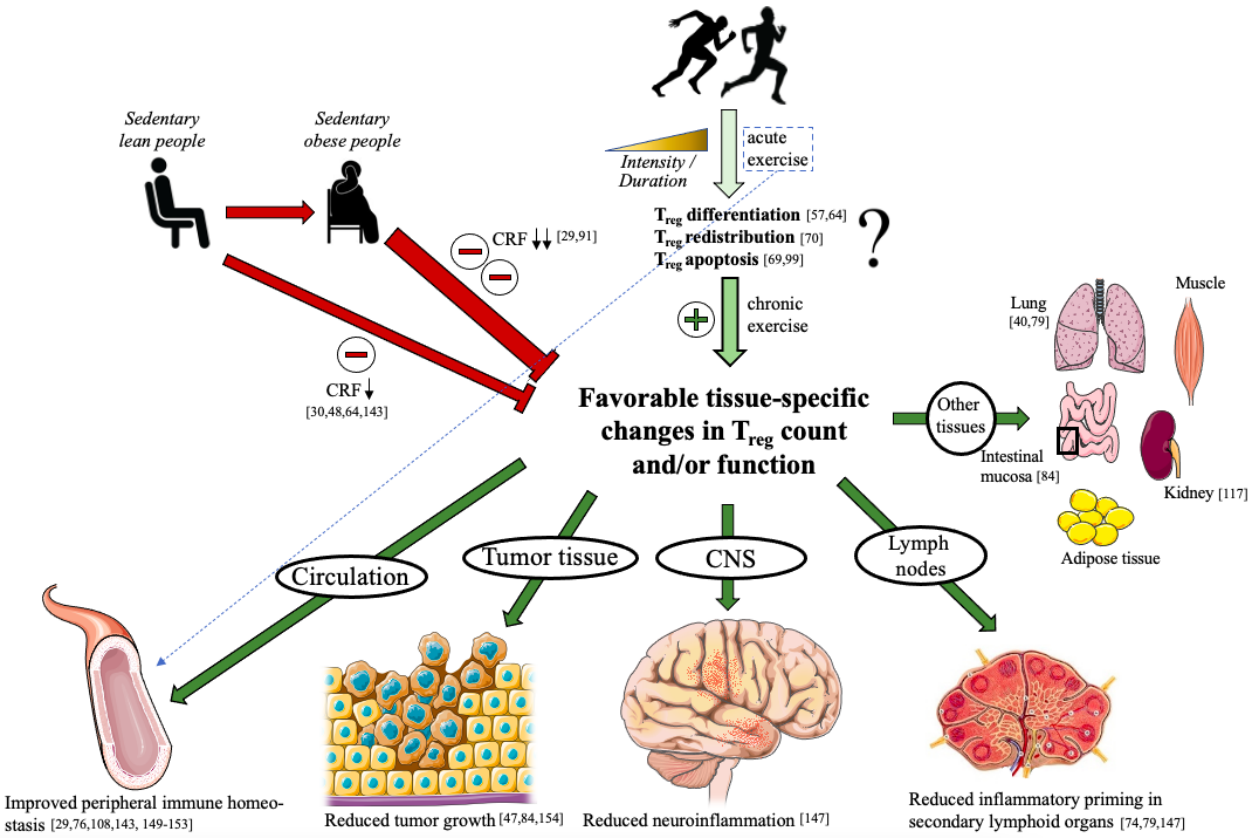

Figure 3: Schematic illustration of proposed exercise-induced effects on regulatory T cells and clinical implications. Acute exercise may lead to T_{reg} differentiation or redistribution and apoptosis of T_{reg} subsets in an intensity- and duration-dependent manner. Repetitive acute bouts over several months, namely chronic exercise, may consequently lead to favorable tissue-specific changes in T_{reg} count and/or function, depending on the (disease) context. This results in reduced tumor growth and metastatic spread, decreased neuroinflammation and loss of neural tissue, reduced inflammatory priming and generation of inflammatory (autoreactive) T cells, and improved peripheral immune homeostasis. T_{reg} count and functionality in other tissues like the lung, muscle, kidney, intestinal mucosa, and adipose tissue may also be beneficially influenced by chronic exercise. A decline in cardiorespiratory fitness due to inactivity has detrimental effects on immunity and favor T_{reg} dysregulation. The establishment of an obese state with concomitant decreases in CRF further diminishes immune regulation. Of note, the effects of acute exercise on T_{regs} have only been examined in the human blood compartment (blue arrow). Corresponding references are provided in brackets.
CNS: central nervous system; CRF: cardiorespiratory fitness; T_{reg}: regulatory T cell

Recommendations for future research

The single sections within the discussion integrated findings of exercise-induced changes in T_{reg} measures into current frameworks of exercise immunology and emphasized existing knowledge gaps. Several key elements should be considered to improve quality, validity, and interpretation of results while simultaneously reducing heterogeneity. Thus, to promote future research in this field, it is recommended that researchers should

- consider T_{reg} measures as primary outcomes with powered sample sizes to draw clearer conclusions on exercise-induced effects. To date, T_{reg} measures often represent exploratory endpoints.
- adhere to the panel proposed by Santegoets et al. (119) for proper T_{reg} characterization in humans. Basal phenotyping should include CD4, CD25, and CD127 to isolate T_{regs} for functional assays, and CD4, CD25, and Foxp3 to measure T_{reg} counts or calculate T_{reg} proportions. The same strategy accounts for rodent T_{regs} (112). It is further suggested to use CD45RA, CD45RO, and CD95 for discriminating between human naïve and highly differentiated/memory T_{regs} (93). For defining mouse memory T_{regs}, high expression of CD27 and CD44 is proposed (114). Surface CD39 and intracellular CTLA-4 expression can be used to identify highly suppressive T_{regs} in humans, while in mice, CD39 and CD73 should be used (63). CD31 measurement is proposed to characterize human RTE T_{regs} (46).
- stain T_{regs} for integrins, adhesion molecules and/or chemokine receptors. Naïve T_{regs} express CD62L (for rolling) and both CCR7 and LFA-1 (for adhesion), while PSGL-1 and CD44 are rather expressed on activated T_{regs} (20). The expression of these molecules highly depends on the disease context and target tissue of which the T_{regs} are attracted by. For example, chemokine receptors that are expressed on T_{regs} and are implicated in migration to tumor sites are CCR4, CCR7, CCR8, CXCR4, and CXCR5 (53, 155). In this context, cell migration and invasion assays of T_{regs} represent promising approaches to reveal tissue-specific properties of adhesion and migration (58).
- calculate and report the delta of pre and post values regarding T_{reg} viability when an acute exercise protocol was applied. This may be valuable to gain insights into exercise-induced T_{reg} fate and to not misinterpret an increase in cell counts. T_{regs} should be further stained for apoptosis markers (e.g. Annexin V, caspase 3 or 9, CD95) to identi-

fy viable but non-functional cells (99).

- use functional assays to quantify T_{reg} suppressive capacity as a main outcome. To avoid heterogeneity, researchers are referred to a current review that addresses important issues (e.g. choice of responder T cells, monitoring suppression of proliferation) when conducting an ex vivo approach (54). It is recommended to do functional testing with freshly isolated T_{regs}. When using cryopreserved T_{regs}, appropriate cryopreservation-thawing techniques should be applied to prevent decreases in viable T_{regs} (4).
- calculate T_{reg} proportions relative to the CD4 population instead of the lymphocyte or leukocyte compartment. To overcome the wide heterogeneity regarding T_{reg} count units, it may be reasonable to find a consensus on one unit for flow cytometry and one unit for immunohistochemical studies that should be used throughout future studies. Increasing homogeneity in presentation of T_{reg} proportion and counts will allow meta-analysis approaches.
- conduct more acute exercise studies in humans that include blood sampling at several measurement time points, i.e. immediately (within one minute), ten minutes, one hour, three hours, and 24 hours post-exercise. This will give more detailed information on exercise-induced alterations of circulating T_{regs}.
- conduct acute exercise studies in animals to examine acute effects on tissue-specific or tissue-infiltrated T_{regs}.
- conduct more chronic studies in humans, especially in diseased populations, with at least ten weeks of exercise to guarantee chronic adaptions in the T_{reg} compartment. Further, varying exercise modalities (upper body vs. lower body or combined, resistance vs. endurance or combined exercise) and intensities (moderate vs. intense or alternating exercise) should be applied to increase knowledge of intensity- and modality-specific effects on T_{reg} measures.
- should wait at least 24 hours to take blood samples after chronic interventions, since acute effects on cytokines and cellular immune components need approximately 24 hours to disappear. Further, reporting on measurement time points should be as detailed as possible. Reporting like "blood samples were collected at least 24/48/72 hours after the last training session" hampers interpretation and reproducibility.
- do consequent and precise reporting on methods and study design, e.g. according to the TESTEX (127) and OHAT (157) scales, to reduce the risk of bias and improve study quality. In this context, reporting of the exercise prescription components of frequency, intensity, time, and type (FITT) in chronic human studies is of special interest as they are crucial for optimizing exercise interventions and are suspected to influence exercise adherence (15, 94).
- make use of existing methods in molecular immunology for in-depth analysis (e.g. chromatin immunoprecipitation, single cell sequencing, -omics approaches) to examine exercise-induced changes in T_{reg} cellular metabolism or epigenetic alterations. This accounts especially for animal models when analyses are done in tissue-specific T_{regs} such as CNS, peripheral organs, and tumor microenvironment.

Limitations

Due to the holistic approach of this review covering all research in the exercise-T_{reg} context, some limitations should be mentioned. The review considered the inclusion of all T_{reg} phenotypes, i.e. those $CD4^+$ T cells only stained for $CD25^+$ or $Foxp3^+$ that is today known as being insufficient for T_{reg} characterization (112). Further, studies that link gene expression of Foxp3 in any immune cell compartment to T_{regs} were included, while only Foxp3 gene expression in the $CD4^+CD25^+(CD127^-)$ should be considered appropriate. Another limitation may be the inclusion of studies with low quality or a high risk of bias confining the validity of results. In this regard, no assessment of acute studies was done, as there is no valid tool to be used to calculate study quality or risk of bias.

CONCLUSION

Regulatory $CD4^+$ T cells represent the main and best studied regulatory component within the adaptive immune system and are increasingly appreciated as a cornerstone for immune therapy approaches in cancer and autoimmune diseases. From the included studies, only a limited number examined exercise-induced effects on T_{reg} functionality which, however, is of high clinical significance and should therefore be addressed by future studies. A disease-specific beneficial effect of chronic exercise on T_{reg} measures can be stated from animal models, that is an increase in T_{reg} levels during autoimmune conditions or chronic inflammation and a decrease of T_{reg} levels in cancer. As T_{reg} phenotyping in the included animal studies is largely insufficient, these findings should be interpreted with caution and be validated with appropriate staining procedures. Due to the relatively high risk of bias, drawbacks in study design, and the large heterogeneity in human and animal studies (i.e. population, outcome measure specification, exercise protocol), no clear overall conclusions can be drawn. Unraveling the impact of exercise on systemic and tissue-specific T_{reg} functionality and phenotypic changes in future studies is highly warranted. It should further be taken into account that regulatory $CD4^+$ T cells and their subpopulations are not the only cells within the immune system that possess immunosuppressive functions. Therefore, it might be reasonable to include other cell types with suppressive characteristics when analyzing exercise-induced effects on the immune system to view the whole (complex) picture instead of considering only one piece of the puzzle. Emerging cell types of interest are the Myeloid-Derived Suppressor Cells (96), different subtypes of $CD8^+$ regulatory T cells (41) and regulatory B cells (115). Methodological recommendations and research questions that are outlined in this review should be considered in the future when studying healthy and diseased humans or respective animal models. However, the results of animal studies should always be interpreted with caution as "mice are not men" in regards to immunology.

Funding

This research was conducted in the absence of any external funding.

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SUPPLEMENTARY MATERIAL

Table S1: Rating of human chronic exercise studies according to (127).

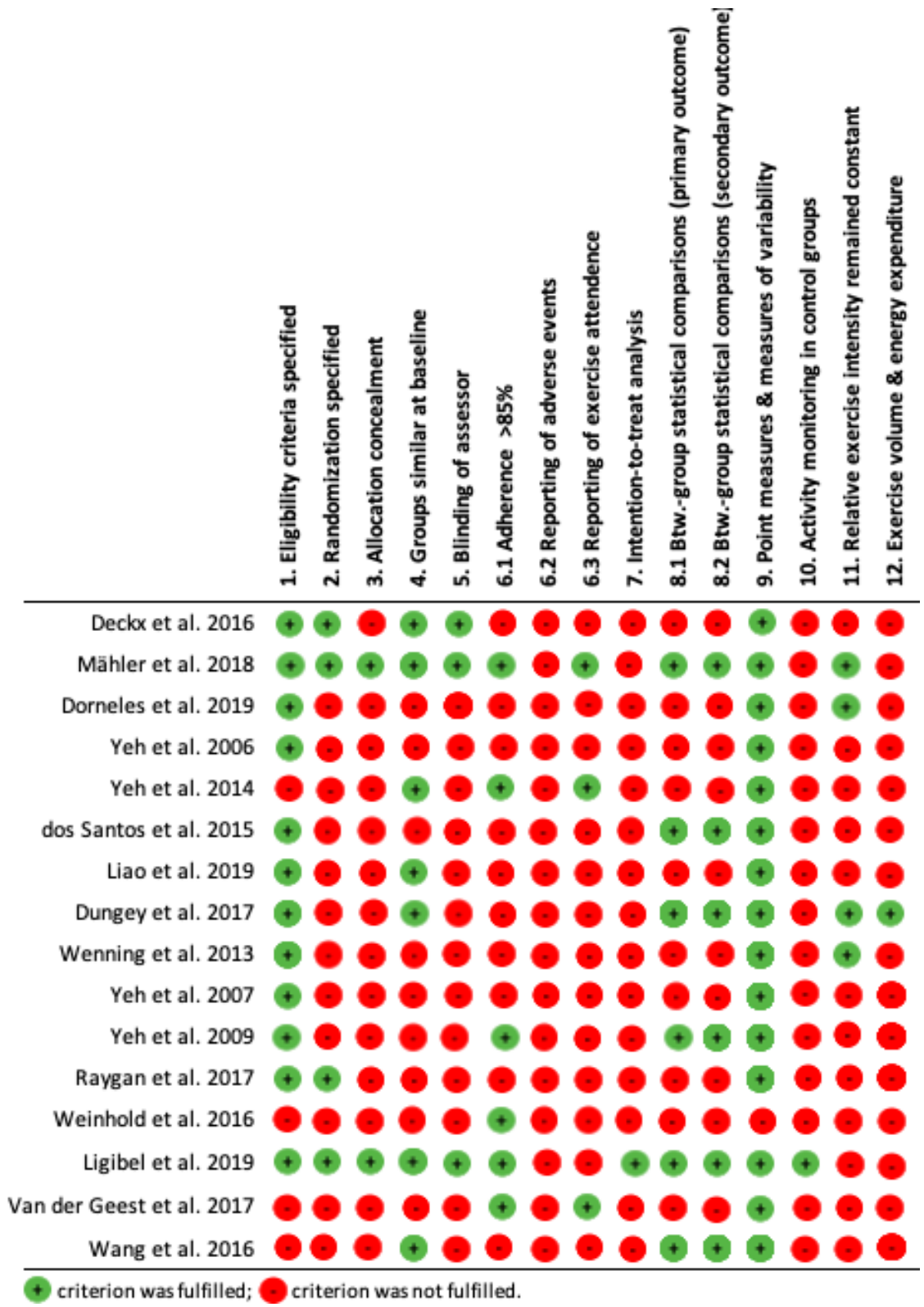

Table S2: Rating of animal studies according to (157).

		Zhang et al. 2016	Bianco et al. 2019	dos Santos et al. 2019	Hagar et al. 2019	Abdalla et al. 2013	McClellan et al. 2014	Miyagi et al. 2018	Chen et al. 2018	Jakic et al. 2019	Barhoumi et al. 2014	Le Garf et al. 2019	Yin et al. 2015	Song et al. 2018	Lowder et al. 2010	Dugger et al. 2018	Fernandes et al. 2019	Rael et al. 2019	Uchiyama et al. 2014	Souza et al. 2017	Xie et al. 2019	Bernardes et al. 2016	Walker et al. 2018	Wang et al. 2011
Selection bias?	1 Was the administered dose of exposure level adequately randomized?	-	-	+	+	-	+	-	+	-	-	+	-	-	+	+	-	-	+	+	+	+	-	+
	2 Was allocation to study groups adequately concealed?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Performance bias?	3 Were experimental conditions identical across study groups?	-	+	+	+	+	+	+	-	+	-	+	-	-	-	+	-	-	-	+	-	-	-	-
	4 Were the research personnelblinded to the study group?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Attrition/exclusion bias?	5 Were outcome data complete without attrition or exclusion from analysis?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Detection bias?	6 Can we be confident in the exposure characterization?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
	7 Can we be confident in the outcome assessment?	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Selective reporting bias?	8 Were all measured outcomes reported?	++	+	++	++	++	+	+	+	++	++	++	+	+	+	++	++	+	+	+	+	++	++	+
Other bias	9 Were statistical methods appropriate?	Y	Y	Y	N	Y	N	N	Y	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N
	10 Did researchers adhere to the study protocol?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	11 Did the study design or analysis account for important confounding variables in experimental studies?	Y	Y	Y	Y	Y	*NA*	*NA*	*NA*	*NA*	*NA*	Y	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*

++ definitely low risk of bias (dark green); + probably low risk of bias (light green); – probably high risk of bias (light red); – – definitely high risk of bias (dark red).

NA: Not Applied

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