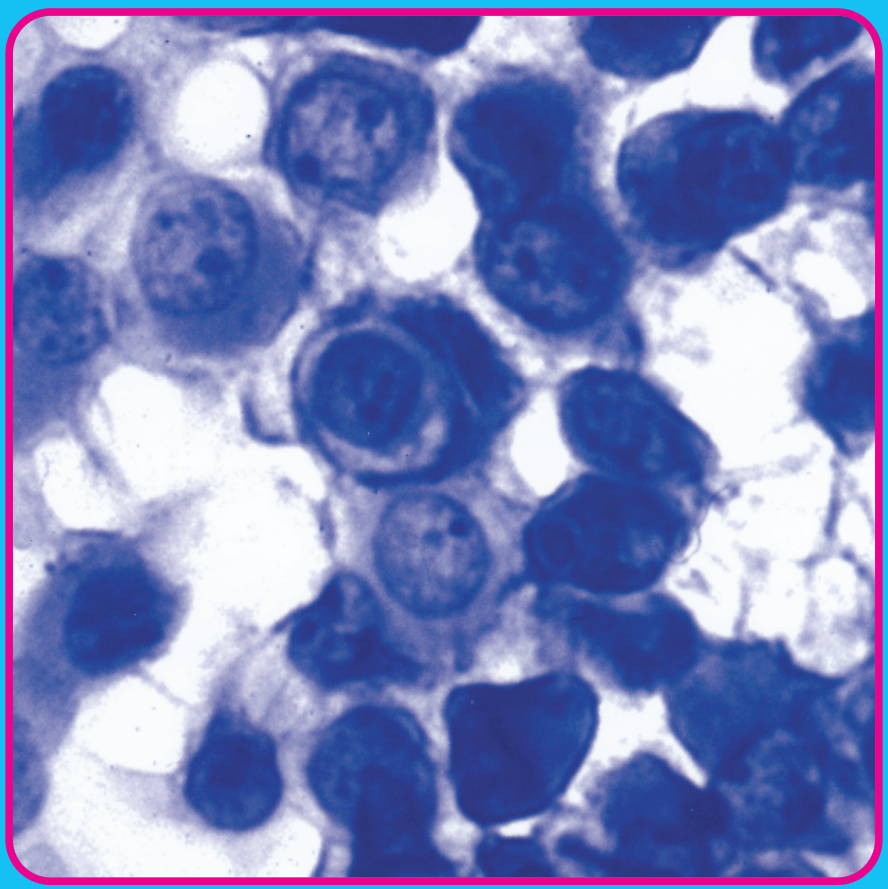
EXERCISE IMMUNOLOGY REVIEW



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EXERCISE IMMUNOLOGY REVIEW

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Hinnak Northoff
for the editorial team and all our readers

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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.

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From the editors

This year's issue of EIR marks 20 proud years as official organ of the International Society of Exercise and Immunology (ISEI) which was founded less than 5 years before EIR was born. For many years it has been supported and co-published by the Deutsche Gesellschaft für Sportmedizin und Prävention (DGSP) which I hereby would personally like to thank most sincerely for its engagement. To shorten the traditional self praise let me just mention that we enjoy our present impact factor of around 7 and hope that we will be able to keep it up in that range for the foreseeable future.

EIR 20 contains 3 studies, 3 reviews, one introduction of a new method and one review with inclusion of own data . The first two articles have a new and interesting common subject- the reaction of oral-respiratory mucosal antimicrobial proteins (AMPs) to exercise. While He et al. report higher secretion rates of AMPs in males than females in a study with a remarkable number (210) of subjects, the second, smaller study by Gillum et al. confirms these findings and also controls for menstrual phase. The third by Kanda et al. examines an array of potential novel markers for damage, inflammation or oxidative stress in calf raise –type exercise. Although positive results are usually appreciated best, it is nevertheless valuable information that none of these could replace CK, ALD or LDH as markers for damage.

The next article by Sako et al. introduces a newly developed genome wide analysis of the translational response. In many cases, the latter can bridge the rift between transcriptomics and proteomics and the authors explore the potential of this novel method as a tool in exercise immunology by demonstrating its robustness in a model with LPS stimulated macrophages.

In the following article, Senchina et al. review what is known about the effects of caffeine and other alkaloids on athlete immune function. It is refreshing to hear that, in spite of the omnipresence of these substances in beverages, there is no scientific basis for any beneficial effect in this context. Nijs et al. then present a systematic review about exercise and chronic fatigue patients. It finds level B evidence that the immune response to exercise is altered in these patients - with oxidative metabolism as a hot spot. The following review by Krüger and Mooren summarizes our present knowledge about exercise and leukocyte apoptosis, discussing possible mediators and the potential significance of apoptosis within the process of adaptation to exercise. Finally, Makarova et al. present a review on the role of miRNAs in the reaction to exercise.The authors hypothesize that miRNAs may play a key role in governing the immunological response to exercise at a very early stage. They also expect that this area of research will rapidly expand in the years to come.

This year's issue of EIR is also the last one to appear under my (Hinnak Northoff) editorship. Karsten Krüger will be the next editor, assisted by Mike Gleeson and Jonathan Peake, and I am very satisfied to see a smooth and efficient transition going on. I know they will assure a brilliant future for our journal and I will enjoy watching the further developments from the perspective of a retired old man. Cheers! – and many many thanks to all my friends and supporters of the exercise immunology community who have given me so much.

For the editors

Hinnak Northoff

Sex differences in upper respiratory symptoms prevalence and oral-respiratory mucosal immunity in endurance athletes

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ABSTRACT

The purpose of this study was to examine sex differences in oral-respiratory mucosal immunity and the incidence, severity and duration of upper respiratory symptoms (URS) episodes in endurance athletes during a 16-week winter training period. Blood was collected from 210 subjects (147 men and 63 women) at the start and end of the study for determination of differential leukocyte counts. Timed collections of unstimulated saliva were obtained at the start and at 4-week intervals during the study period. Saliva samples were analysed for salivary antimicrobial peptides and proteins (AMPs). Weekly training and daily illness logs were kept using validated questionnaires. Training loads averaged 11 h/week of moderate-vigorous physical activity and were not different for males and females. The salivary concentration of lysozyme and lactoferrin (both $P<0.04$) but not salivary immunoglobulin A (SIgA) or amylase were higher in males than females. Saliva flow rates were significantly higher in males than females ($P < 0.03$) and consequently so were the salivary secretion rates of lysozyme, lactoferrin and amylase (all $P<0.01$) but not SIgA ($P = 0.097$). Total blood leukocyte, monocyte and lymphocyte counts were not different between the sexes but females had higher numbers of circulating neutrophils ($P = 0.040$). The average number of URS episodes was 0.6 ± 0.8 (mean ± SD) in males and 0.8 ± 1.0 in females ($P = 0.103$) and the number of URS days was higher in females (4.7 vs 6.8 days, $P < 0.02$). The duration of URS episodes was longer in females (11.6 vs 15.5 days, $P < 0.03$). The findings of this study concur with recent reports of illness incidence at major competitive games indicating that female athletes may be more susceptible than their male counterparts to URS and that lower oral-respiratory mucosal immunity may, in part, account for this.

Keywords: exercise training, leukocytes, immunoglobulins, antimicrobial proteins, illness

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INTRODUCTION

Resistance to infection is strongly influenced by the effectiveness of the immune system in protecting the host against pathogenic micro-organisms. Within the general healthy human population there is a range of immuno-competency due to genetic differences, age, nutritional deficiencies and lifestyle habits. The sex of the individual also affects immune function. In females, oestrogens and progesterone modulate immune function (41) and thus immunity is influenced by the menstrual cycle and pregnancy (29). Consequently, sex-based differences in responses to infection, trauma and sepsis are evident (6). Within the general population women are generally more resistant to viral infections and tend to have more autoimmune diseases than men (6). In a review of the literature on respiratory tract infections (RTIs) (20) in which data from 84 studies was extracted it was concluded that males are more susceptible than females to most types of RTIs in all age groups (children, adolescents, adults and the elderly). Anatomic, lifestyle, behavioural and socioeconomic differences between males and females may explain the observed findings and the involvement of sex hormones in the regulation of immune function may also contribute to the reported sex differences in the incidence and severity of the various types of RTIs, especially in adults and adolescents (20). Oestrogens are generally immune enhancing, whereas androgens, including testosterone, exert suppressive effects on both humoral and cellular immune responses. Females have higher levels of plasma immunoglobulin M (IgM) than men and exhibit more vigorous responses to exogenous antigens, indicating a higher level of humoral immunity in females than in males (8). In females, there is increased expression of some cytokines in peripheral blood and vaginal fluids during the follicular phase of the menstrual cycle and with use of hormonal contraceptives (9). In the luteal phase of the menstrual cycle, blood leukocyte counts are higher than in the follicular phase (18), mononuclear cell expression of the heterodimeric transcription factor 1 (a key regulator of the innate immune response) is lower (47), and the immune response is shifted towards a T helper (Th) 2-type response (18). Thus, in the general population, there are differences in some aspects of immune function between men and women that appear to result in women getting fewer viral infections, including those affecting the respiratory tract.

In addition to these sex differences in resting conditions, several studies have documented sex differences in some aspects of the immunological response to exercise (22, 49, 50) including larger post-exercise increases in circulating lymphocytes and natural killer cells in females. The expression of pro-inflammatory and anti-inflammatory genes in response to exercise is also influenced by the menstrual cycle and there are distinct differences in gene expression between women in the luteal phase and men (40). Prolonged strenuous exercise has been associated with a transient depression of immune function (23, 24, 53) and a heavy schedule of training and competition can lead to immune impairment in both male and female athletes (53). This is associated with an increased susceptibility to upper respiratory symptoms (URS) (7, 19, 27, 30, 39, 43, 53) and several studies suggest that reduced secretion of salivary immunoglobulin A (SIgA) and possibly other mucosal antimicrobial proteins may be an important causal factor

(19, 24, 26, 27, 37, 53). However, it is not clear whether any substantial sex differences exist in any aspect of oral-respiratory mucosal immune function in an athletic population or whether any such differences affect URS risk. In contrast to what has been reported for the general population, some recent reports of illness rates among athletes attending large competitive events (e.g. winter and summer Olympic games, athletic and aquatic sport world championships) suggest that URS episodes may actually be more prevalent in the women than the men (4, 5, 14, 16, 17, 36, 46).

The aims of the present study were to determine if sex differences exist in resting immune variables including saliva antimicrobial proteins (AMPs) including SIgA, lysozyme, lactoferrin and amylase secretion and the numbers of circulating leukocyte numbers in an athletic population. We also wished to determine if the prevalence of URS episodes was different in male and female athletes during a period of winter training and competition. Our hypothesis was that saliva AMP secretion rates would be lower in females and that this might be associated with a higher prevalence of URS episodes.

METHODS

Subjects

Two hundred and sixty seven subjects (83 females, 184 males) aged 21 ± 3 years who were engaged in regular sports training (predominantly endurance-based activities such as running, cycling, swimming, triathlon, team games and racquet sports) from Loughborough University, UK volunteered to participate in the study during November 2011. Since our study population was a group of university athletes on a single campus site it is likely that environment and pathogen exposure were similar for all subjects. Subjects ranged from recreationally active to Olympic triathletes and their self-reported training loads (determined by a prescreening questionnaire) averaged 10 ± 3 h/week (mean ± SD). Two hundred and ten subjects (63 females, 147 males) completed the study and provided sufficient blood for routine haematology on 2 occasions and sufficient saliva for analysis of AMPs on all 5 occasions. Their baseline characteristics as shown in Table 1. Among the females 96% reported having regular periods and 40% were taking oral contraceptives. Reasons for dropout were given as foreign travel, injury or persistent non-respiratory illness (preventing subjects from performing training) or due to undisclosed reasons.

Subjects were required to complete a comprehensive health-screening questionnaire prior to starting the study and had not taken any regular medication or antibiotics in the 3 months prior to the study. All subjects were fully informed about the rationale for the study and of all experimental procedures to be undertaken. Subjects provided written consent to participate in the study, which had earlier received the approval of Loughborough University ethical advisory committee. Subjects were enrolled after having fulfilled all inclusion criteria, and presenting none of the exclusion criteria (determined by both questionnaire and interview). Subjects could be included if they were currently healthy, had been

involved in endurance training for at least 2 years, engaged in at least 3 sessions and at least 3 h of total moderate/high-intensity training time per week and were between 18-40 years of age. Subjects representing one or more of the following criteria were excluded from participation: smoking or use of any medication, suffering from or had a history of cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal, haematological or psychiatric illness.

Study protocol

For the first visit to the laboratory, subjects arrived in the morning at 08:30-10:30 following an overnight fast of approximately 12 h and no strenuous exercise in the previous 24 h and their body mass and height were recorded. Information about the study was given to them and they then signed an informed consent form. Subjects then sat quietly for 10 min and completed a health-screening questionnaire and inclusion/exclusion criteria questionnaire before being asked to swallow to empty their mouth of any residual saliva before providing an unstimulated saliva sample by passive dribble into a pre-weighed sterile collection tube for a timed period (usually 2 min; longer was allowed if the volume of saliva collected after 2 min was insufficient). After centrifugation for 2 min at 5000 g to remove cells and insoluble matter, saliva samples were stored frozen at -80ºC prior to analysis. Subsequently, a resting venous blood sample (5 ml) was obtained by venepuncture from an antecubital forearm vein into a vacutainer tube (Becton Dickinson, Oxford, UK) containing K_3EDTA for immediate haematological analysis (including haemoglobin, haematocrit and total and differential leukocyte counts) using an automated cell-counter (A^c.T™5diff haematology analyser, Beckman Coulter, High Wycombe, UK). The intra-assay coefficient of variation for all measured blood variables was less than 3.0%. Subjects had to have all haematological values within the normal healthy range to be included in the study.

During the 4-month study period subjects were requested to continue with their normal training programs. Subjects completed a validated self-report health (URS) questionnaire (29) on a daily basis. Subjects were not required to abstain from medication when they were suffering from illness symptoms but they were required, on a weekly basis, to report any unprescribed medications taken, visits to the doctor or any prescribed medications. The illness symptoms listed on the questionnaire were: sneezing, headache, malaise, nasal discharge, nasal obstruction, sore throat, cough, ear ache, hoarseness, fever, chilliness and joint aches and pains. The non-numerical severity ratings of mild, moderate and severe of severity of symptoms were scored as 1, 2 or 3, respectively to provide a quantitative means of data analysis and the total symptom score for every subject each day was calculated as a sum of multiplied numbers of symptoms experienced by the numerical severity ratings. A URS was deemed present when (i) total symptom score was ≥15 on any two consecutive days and (ii) when a subject positively indicated suffering a common cold on ≥ 3 days according to Jackson et al. (31). Subjects were also asked to rate the impact of illness symptoms on their ability to train (above normal, at the same level, below normal or training stopped). The total number of URS days was also determined as the number of days with a symptom score of ≥5 according to Predy et al. (44).

Subjects were also asked to fill in a standard short form of the International Physical Activity Questionnaire (IPAQ; http://www.ipaq.ki.se/downloads.htm) at weekly intervals, thus providing a quantitative information on training loads in metabolic equivalents (MET)-h/week (13). Subjects attended the laboratory every 4 weeks following an overnight fast. Subjects were required to abstain from any strenuous physical activity for 24 h before coming to the laboratory. During these visits body mass was recorded and an unstimulated saliva sample was collected. Venous blood samples were collected only at the start and end of the study period and the mean values for these haematological values are reported in the Results.

Sample size estimation (21) of 41 subjects per gender group was based on an expected rate of 2.0 ± 1.0 URS episodes (mean ± SD) during the winter months (37), a target difference of 30% in number of episodes (effect size 0.6), statistical power of 80% and a type I error of 5%.

Saliva analysis

The saliva volume collected was estimated by weighing and the saliva flow rate was calculated. Saliva samples were analysed for secretory immunoglobulin A (SIgA) using an ELISA kit (Salimetrics, Philadelphia, USA) and α-amylase activity was measured as previously described (35). Salivary lactoferrin and lysozyme were analysed using commercially available ELISA kits (Calbiochem, USA and Biomedical Technologies, USA, respectively). Secretion rates for each of the salivary AMPs were calculated as the multiple of the saliva flow rate and the AMP concentration. Values obtained from the 5 visits at 4-week intervals were averaged for each subject. All saliva assays were carried out in duplicate. Coefficients of variation (CVs) for the assays were <5% for all salivary AMPs.

Statistical Analysis

The Shapiro-Wilk test was used to determine if data sets were normally distributed. The difference in proportion of subjects who presented with symptoms of infection during the trial between the males and females was assessed by a Chi-squared test. Self-reported training load (h/week), average IPAQ scores (MET-h/week), anthropometric and haematological variables (including blood leukocyte, neutrophil, monocyte and lymphocyte counts) were compared between males and females using unpaired t tests for normally distributed data. Changes in training load over time for both sexes were evaluated by 2-way ANOVA with post hoc Bonferroni t tests to locate differences from week 1. The salivary AMP concentrations and secretion rates were compared between males and females using nonparametric Mann-Whitney tests for data that were not normally distributed. The changes in the secretion rates of salivary AMPs over the 16 weeks of the study in males and females were assessed by non-parametric Friedman tests with post hoc Dunn's tests to compare values at weeks 4, 8, 12 and 16 with baseline within sex. Differences between sexes at specific sampling timepoints were examined using Mann Whitney tests. Statistical significance was accepted at $P < 0.05$. Data are expressed as mean ± SD or median and interquartile range as appropriate.

RESULTS

Anthropometric and haematological variables

There was no significant difference in age between males and females (Table 1) but males were taller, heavier and had higher BMI than females (all $P < 0.01$). Males had higher RBC count, haematocrit and haemoglobin concentration than females (all $P < 0.001$). Total blood leukocyte, monocyte and lymphocyte counts were not different between the sexes but females had higher circulating numbers of neutrophils than males ($P = 0.040$).

Table 1. Anthropometric, training and haematological variables in male and female athletes

	Males (n=147)	Females (n=63)	P
Age (years)	20.4 ± 1.9	20.5 ± 3.1	0.943
Height (cm)	1.80 ± 0.06	1.66 ± 0.06	<0.001
Body mass (kg)	77.7 ± 10.1	63.3 ± 7.6	<0.001
BMI (kg/m^2)	23.8 ± 2.3	22.9 ± 2.3	0.007
Previous training load (h/week)	10 ± 3	10 ± 3	0.857
IPAQ (MET-h/week)	65.2 ± 28.2	66.7 ± 31.4	0.746
RBC count (x10^{12}/L)	4.86 ± 0.55	4.33 ± 0.50	<0.001
Haematocrit (%)	46.8 ± 4.9	41.6 ± 4.6	<0.001
Haemoglobin (g/L)	148 ± 16	130 ± 15	<0.001
Leukocyte count (x10^9/L)	5.85 ± 1.30	6.16 ± 1.51	0.128
Neutrophil count (x10^9/L)	2.96 ± 0.97	3.28 ± 1.15	0.040
Lymphocyte count (x10^9/L)	2.06 ± 0.52	2.05 ± 0.50	0.931
Monocyte count (x10^9/L)	0.58 ± 0.15	0.59 ± 0.22	0.774

Values are expressed as mean ± SD.

Training loads

Self-reported weekly training loads (mean ± SD) based on information gathered in the pre-screening questionnaire were similar in males and females (both 10 ± 3 h/week, $P = 0.857$).Analysis of the IPAQ questionnaires indicated that the weekly training loads were relatively stable within both sexes over the 16 weeks of the study (Figure 1) although there was a significant main effect of time ($P<0.001$) with training load falling below week 1 values in weeks 6-11. There was no significant effect of sex and no significant sex x time interaction. Training loads were, on average, about 66 MET-h/week which is equivalent to about 11 h of moderate-vigorous activity per week. The training loads averaged over the whole

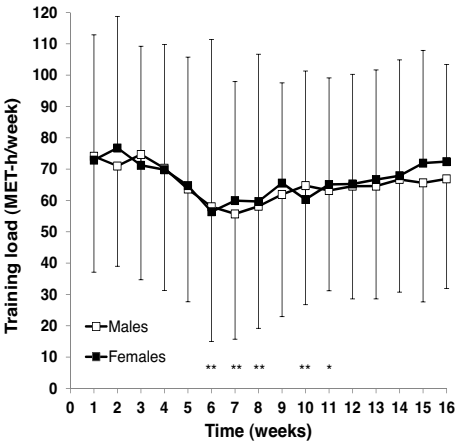

Figure 1. Training loads in MET-h/week over the 16-week study period for men (n=147) and women (n=63) who completed the study. Data are mean ± SD. There was a main effect of time (P<0.001) and the location of significant differences from week 1 are indicated as follows: * P<0.05, ** P<0.01. There was no significant effect of sex and no significant sex x time interaction.

16-week study period were not significantly different between males and females (Table 1).

Salivary variables

When averaged over the 5 sampling occasions, saliva flow rates ($P < 0.03$) and the secretion rates of lactoferrin, lysozyme and amylase (Table 2) were significantly higher in males than females (all $P < 0.01$). Concentrations of SIgA and amylase were not different between the sexes whereas lactoferrin ($P = 0.021$) and lysozyme ($P = 0.033$) concentrations were significantly higher in males. While there were significant effects of time (Friedman, $P < 0.05$) for the secretion rates of lactoferrin, lysozyme, amylase and SIgA in males, there were significant effects of time only for amylase and SIgA secretion rates in females (Figure 2). The changes over time followed the same pattern for both sexes.

Table 2. Salivary variables in male and female athletes

	Males (n=147)	Females (n=63)	P
Saliva flow rate (μL/min)	340 (263-446)	308 (220-404)	0.025
SIgA concentration (mg/L)	68.5 (49.3-97.8)	70.6 (44.8-107.9)	0.775
SIgA secretion rate (μg/min)	23.1 (13.9-34.4)	19.5 (12.2-28.8)	0.097
Lysozyme concentration (μg/L)	2000 (1112-3452)	1426 (886-2649)	0.033
Lysozyme secretion rate (ng/min)	629 (384-1033)	373 (229-685)	0.001
Lactoferrin concentration (μg/L)	2438 (1819-3596)	1930 (1183-3365)	0.021
Lactoferrin secretion rate (ng/min)	805 (561-1238)	554 (329-862)	0.001
Amylase activity (U/mL)	142 (76-243)	134 (75-173)	0.126
Amylase secretion rate (U/min)	51.6 (24.0-85.3)	37.8 (25.5-50.6)	0.009

Values are expressed as median and interquartile range. P value is from Mann-Whitney U test.

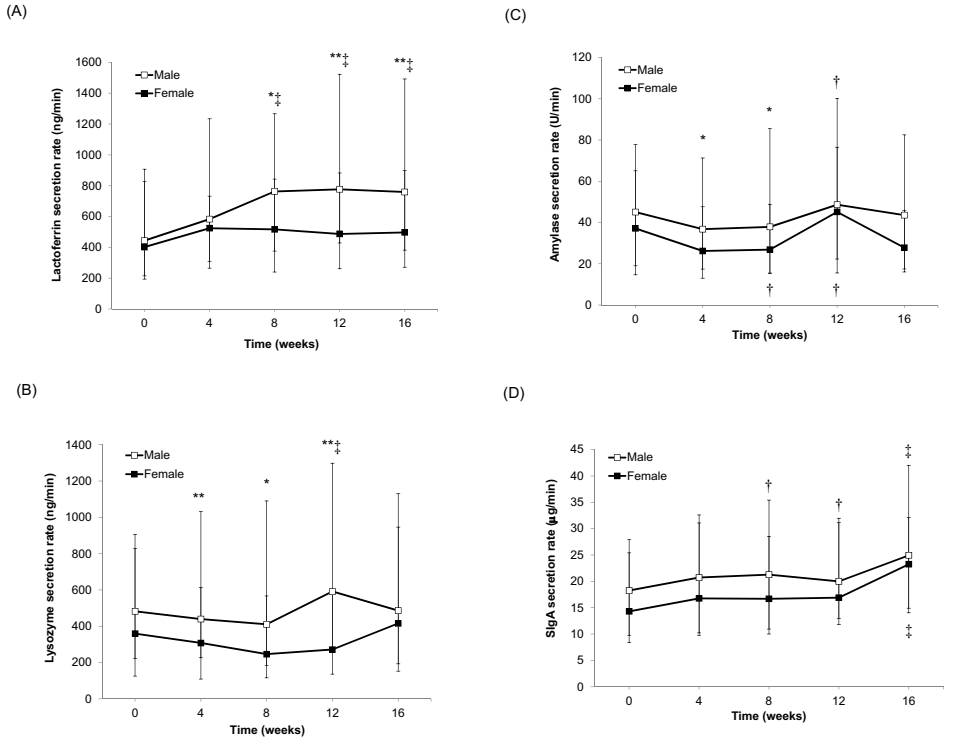

Figure 2. Changes in salivary secretion rates over time for (A) Lactoferrin, (B) Lysozyme, (C) Amylase and (D) SIgA. Data are median and interquartile range. Significantly different from baseline sample within sex: † P<0.05, ‡ P<0.01 (Dunn's post hoc test applied when Friedman test P<0.05). Significant difference between males and females at specific timepoint: * P<0.05, ** P<0.01 (Mann Whitney test).

URS incidence and the severity and duration of URS

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

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

Table 3. Illness incidence among athletes at major competitive events lasting 2-3 weeks.

Games (reference)	Season	Athletes (n)	Males (n)	Females (n)	Illness in all athletes (%)	Illness in males (%)	Illness in females (%)	Respiratory (% of total)
Olympics 2012 (16)	Summer	10568	5892	4676	7.2	5.3	8.6	41
Youth Olympics 2012 (46)	Winter	1021	562	459	8.4	6.0	11.0	61
IAAF 2011 (4)	Summer	1851	971	880	6.8	7.1	7.7	39
Olympics 2010 (17)	Winter	2567	1522	1045	7.2	5.2	8.7	63
IAAF 2009 (5)	Summer	1979	1082	897	6.8	5.6	8.4	36
FINA 2009 (36)	Summer	2318	1306	1012	6.6	5.1	7.9	50
All		20304	11335	8969	7.2 ± 0.6	5.7 ± 0.8	8.7 ± 1.2*	48 ± 12

IAAF: International Association of Athletics Federations; FINA: Federation Internationale de Natation; n = number of registered athletes.
* Significant difference in mean illness rates between male and female athletes ($P < 0.05$).

DISCUSSION

The main findings of the present study were that although URS incidence was not significantly different between male and female athletes, the women had more URS days and their URS episodes lasted several days longer than the men. Salivary SIgA concentration and amylase activity were not significantly affected by

sex but both lactoferrin and lysozyme concentrations were found to be lower in the females. Saliva flow rate was ~17% lower in females and the secretion rates of all the AMPS apart from SIgA were significantly lower in females. In both sexes saliva AMP secretion rates increased over time which could be due to the significant fall in the training loads of the athletes that was observed in the middle part of the study period.

Low SIgA concentration or secretion rate has been identified as a risk factor for development of URS in physically active individuals (19, 26-28, 37). In the present study, we found that female athletes tended to have lower SIgA secretion rates than male athletes during a 16-week winter training period although this difference was not statistically significant. It has been suggested that SIgA levels are a surrogate marker of host protection and the suppression of SIgA after prolonged exercise or heavy training is itself a probable consequence of altered T lymphocyte function (12). Females generally have lower unstimulated saliva flow rates than males (42), whereas SIgA concentration in unstimulated saliva has been reported to be unaffected by sex among relatively large cohorts of healthy young adults (33, 51, 52). A previous smaller scale study reported lower SIgA concentration and secretion rate in females (n=34) than in males (n=46) among a cohort of student athletes (25). Other small scale studies on elite swimmers have also reported lower SIgA concentrations in females compared with males (n= 11 females, n = 15 males (26); n = 5 females, n= 7 males (1)), as has a small scale study of recreational cyclists (n= 8 females, n = 8 males (2)); but, to our knowledge, our investigation is the first large scale study to report a sex difference in salivary AMP secretion rates in athletes from a range of endurance-based sports.

In the present study, it was observed that the female athletes had lower saliva amylase secretion rate than male athletes. Amylase, an enzyme that breaks down starch into maltose, is important to host defence in oral-respiratory mucosal immunity by inhibiting the adherence and growth of certain bacteria (3). The lower amylase secretion rate in the saliva of females could be due to lower circulating adrenaline levels that are observed both at rest (54) and after exercise (45) in females when oestrogen levels are high due to exogenous ovarian hormone administration. Both increased sympathetic nervous activity (48), and elevated plasma adrenaline and noradrenaline (11) are known to increase salivary amylase secretion. In addition, we also found that the secretion rates of lysozyme and lactoferrin were significantly lower in female athletes than male athletes. Lysozyme and lactoferrin play important roles in oral-respiratory mucosal immunity against pathogen infection: Lactoferrin possesses the ability to sequester iron, bind to bacteria, and has antimicrobial activities that act in synergy with SIgA and lysozyme (15, 34). Therefore, it is possible that the lower secretion rates of amylase, lysozyme and lactoferrin might leave female athletes at greater risk of contracting RTIs during winter training periods.

Of course, other factors could also account for differences in infection risk between the sexes. For example, in the general population, women have been reported to have fewer blood monocytes and NK cells, but more CD4+ cells and more neutrophils than men (8, 55) and women appear to suffer from fewer viral

infections including RTIs than men (6). The present study also found athletic females to have higher blood neutrophil counts than their male counterparts but it was the females who appeared to be more susceptible to URS than men. It is possible that the same training load could have a greater depressive effect on humoral and systemic immunity (e.g. lower secretion rates of mucosal AMPs and fewer numbers of circulating B cells and NK cells) for women (25) than for men (that is not evident in the normal, more sedentary population) but this possibility needs to be resolved by future research. Such an effect may be responsible for the reversal of the usual situation of more effective immune function in females into the opposite situation in athletes. Our data support the notion that URS are more prevalent in female athletes than their male counterparts when they engage in similarly high training loads. In recent years medical and sport science support personnel have collected data on rates of injuries and illnesses in large cohorts of athletes attending (and intending to compete in) large competitive events lasting 2-3 weeks (e.g. winter and summer Olympic games, athletic and aquatic sport world championships). The findings from these studies are summarised in Table 3 and suggest that illness episodes actually are more prevalent in the women than the men (4, 5, 14, 16, 17, 36, 46). About half of the illnesses reported at these events were URS episodes. Other epidemiological studies on physically active (38) and athletic (30, 32) men and women also suggest that URS are more prevalent in the females.

A limitation of the present study is that the phase of the menstrual cycle (when blood and saliva samples were taken) was not determined but we did establish that 40% of the females were taking oral contraceptives. It is possible that the high training loads of some of the female endurance athletes in our study could have caused them to be amenorrhoeic and one would expect that this would make their immune variables more similar to that of men. However, according to the health screen questionnaire used at the start of the study 96% of the females reported that they had regular periods so it seems likely that during the study period very few of the females were amenorrhoeic. This aside, menstrual cycle phase was not found to affect resting saliva SIgA responses in endurance trained female athletes (10). Another limitation is that we did not attempt to distinguish between symptoms of an infectious/illness nature vs inflammation throughout the 16 weeks of the study design. However, nearly all studies to date have used self-reported symptoms rather than pathogen identification in studies involving athletes and URS incidence. We used the validated Jackson score questionnaire which is a conservative instrument requiring a substantial symptom score criterion threshold to define a RTI episode (31). Given that the average duration of URS episodes in our study was 13 days, it is very likely that the vast majority of episodes were caused by infections as typically inflammation/allergy symptom episodes generally only last 1-3 days according to Walsh et al. (53).

The major strengths of our study are that we used a validated questionnaire (31) to determine URS episodes and measured saliva AMPs on 5 occasions at monthly intervals to better establish the average values for each individual. The average training loads of the athlete cohort were generally high and were not different between males and females. Our study population was a group of university athletes on a single campus site so that environment and pathogen exposure were likely to have been similar for all subjects.

In conclusion, the findings of this study concur with recent reports of illness incidence at major competitive games indicating that female athletes may be more susceptible than their male counterparts to URS and that lower oral-respiratory mucosal immunity (i.e. lower secretion rates of mucosal AMPs), may account for this in part.

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REFERENCES

1. Allgrove JE. Factors influencing the mucosal immune response to exercise. PhD thesis, Loughborough University 102-121, 2007.
2. Allgrove JE, Geneen L, Latif S, and Gleeson M. Influence of a fed or fasted state on the s-IgA response to prolonged cycling in active men and women. Int J Sport Nutr Exerc Metab 19: 209-221, 2009.
3. Allgrove JE, Gomes E, Hough J, and Gleeson M. Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. J Sports Sci 26: 653-661, 2008.
4. Alonso JM, Edouard P, Fischetto G, Adams B, Depiesse F, and Mountjoy M. Determination of future prevention strategies in elite track and field: analysis of Daegu 2011 IAAF Championships injuries and illnesses surveillance. Br J Sports Med 46: 505-514, 2012.
5. Alonso JM, Tscholl PM, Engebretsen L, Mountjoy M, Dvorak J, and Junge A. Occurrence of injuries and illnesses during the 2009 IAAF World Athletics Championships. Br J Sports Med 44: 1100-1105, 2010.
6. Beery TA. Sex differences in infection and sepsis. Crit Care Nurs Clin North Am 15: 55-62, 2003.
7. Bishop NC. The influence of exercise on infection risk. In: Exercise Immunology, edited by Gleeson M, Bishop NC, and Walsh NP. Abingdon: Routledge, 2013, p. 1-20.
8. Bouman A, Schipper M, Heineman MJ, and Faas MM. Gender difference in the non-specific and specific immune response in humans. Am J Reprod Immunol 52: 19-26, 2004.
9. Brabin L. Interactions of the female hormone environment, susceptibility to viral infections, and disease progression. AIDS Patient Care STDS 16: 211-221, 2002.
10. Burrows M, Bird SR, and Bishop NC. The menstrual cycle and its effect on the immune status of female endurance runners. J Sports Sci 20: 339-344, 2002.
11. Chatterton RT Jr, Vogelsong KM, Lu YC, Ellman AB, and Hudgens GA. Salivary alpha-amylase as a measure of endogenous adrenergic activity. Clin Physiol 16: 433-448, 1996.
12. Clancy RL, Gleeson M, Cox A, Callister R, Dorrington M, D'Este C, Pang G, Pyne D, Fricker P, and Henriksson A. Reversal in fatigued athletes of a defect in interferon gamma secretion after administration of Lactobacillus acidophilus. Br J Sports Med 40: 351-354, 2006.

13. Craig CL, Marshall AL, Sjostrom M, Bauman AE, Booth ML, Ainsworth BE, Pratt M, Ekelund U, Yngve A, Sallis JF, and Oja P. International physical activity questionnaire: 12-country reliability and validity. Med Sci Sports Exerc 35: 1381-1395, 2003.
14. Edouard P, Depiesse F, Hertert P, Branco P, and Alonso JM. Injuries and illnesses during the 2011 Paris European Athletics Indoor Championships. Scand J Med Sci Sports 2012 Nov 28. doi: 10.1111/sms.12027. [Epub ahead of print].
15. Ellison RT, Giehl TJ, and LaForce FM. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect Immun 56: 2774-2781, 1988.
16. Engebretsen L, Soligard T, Steffen K, Alonso JM, Aubry M, Budgett R, Dvorak J, Jegathesan M, Meeuwisse WH, Mountjoy M, Palmer-Green D, Vanhegan I, and Renström PA. Sports injuries and illnesses during the London Summer Olympic Games 2012. Br J Sports Med 47: 407-414, 2013.
17. Engebretsen L, Steffen K, Alonso JM, Aubry M, Dvorak J, Junge A, Meeuwisse W, Mountjoy M, Renström P, and Wilkinson M. Sports injuries and illnesses during the Winter Olympic Games 2010. Br J Sports Med 44: 772-780, 2010.
18. Faas M, Bouman A, Moesa H, Heineman MJ, de Leij L, and Schuiling G. The immune response during the luteal phase of the ovarian cycle: a TH2-type response? Fertil Steril 74: 1008-1013, 2000.
19. Fahlman MM, and Engels HJ. Mucosal IgA and URTI in American college football players: A year longitudinal study. Med Sci Sports Exerc 37: 374-380, 2005.
20. Falagas ME, Mourtzoukou EG, and Vardakas KZ. Sex differences in the incidence and severity of respiratory tract infections. Resp Med 101: 1845-1863, 2007.
21. Faul F, Erdfelder E, Lang A-G, and Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioural and biomedical sciences. Behav Res Methods 39: 175-191, 2007.
22. Gillum TL, Kuennen MR, Schneider S, and Moseley P. A review of sex differences in immune function after aerobic exercise. Exerc Immunol Rev 17: 104-121, 2011.
23. Gleeson M. Exercise and immune function. J Appl Physiol 103: 693-699, 2007.
24. Gleeson M, Bishop NC, and Walsh NP (editors). Exercise Immunology. Abingdon: Routledge, 2013.
25. Gleeson M, Bishop NC, Oliveira M, McCauley T, and Tauler P. Sex differences in immune variables and respiratory infection incidence in an athletic population. Exerc Immunol Rev 17: 122-135, 2011.
26. Gleeson M, Bishop NC, Oliveira M, McCauley T, Tauler P, and Muhamad AS. Respiratory infection risk in athletes: association with antigen-stimulated IL-10 production and salivary IgA secretion. Scand J Med Sci Sports 22: 410-417, 2012.
27. Gleeson M, McDonald WA, Pyne DB, Cripps AW, Francis JL, Fricker PA, and Clancy RL. Salivary IgA levels and infection risk in elite swimmers. Med Sci Sports Exerc 31: 67-73, 1999.
28. Gleeson M, Pyne DB, Austin JP, Francis JL, Clancy RL, McDonald WA, and Fricker PA. Epstein-Barr virus reactivation and upper respiratory illness in elite swimmers. Med Sci Sports Exerc 34: 411-417, 2002.
29. Haus E, and Smolensky MH. Biologic rhythms in the immune system. Chronobiol Int 16: 581-622, 1999.
30. Heath GW, Ford ES, Craven TE, Macera CA, Jackson KL, and Pate RR. Exercise and the incidence of upper respiratory tract infections. Med Sci Sports Exerc 23: 152-157, 1991.

31. Jackson GG, Dowling HF, Spiesman IG, and Boand AV. Transmission of the common cold to volunteers under controlled conditions: the common cold as a clinical entity. Arch Intern Med 101: 267–278, 1958.
32. König D, Grathwohl D, Weinstock C, Northoff H, and Berg A. Upper respiratory tract infection in athletes: influence of lifestyle, type of sport, training effort, and immunostimulant intake. Exerc Immunol Rev 6:102-120, 2000.
33. Kugler J, Hess M, and Haake D. Secretion of salivary immunoglobulin A in relation to age, saliva flow, mood states, secretion of albumin, cortisol, and catecholamines in saliva. J Clin Immunol 12: 45-49, 1992.
34. Legrand D, Elass E, Pierce A, and Mazurier J. Lactoferrin and host defence: an overview of its immuno-modulating and anti-inflammatory properties. Biometals 17: 225-229, 2004.
35. Li T-L, and Gleeson M. The effect of single and repeated bouts of prolonged cycling and circadian variation on saliva flow rate, immunoglobulin A and alpha-amylase responses. J Sports Sci 22: 1015-1024, 2004.
36. Mountjoy M, Junge A, Alonso JM, Engebretsen L, Dragan I, Gerrard D, Kouidri M, Luebs E, Shahpar FM, and Dvorak J. Sports injuries and illnesses in the 2009 FINA World Championships (Aquatics). Br J Sports Med 44: 522-527, 2010.
37. Neville V, Gleeson M, and Folland JP. Salivary IgA as a risk factor for upper respiratory infections in elite professional athletes. Med Sci Sports Exerc 40: 1228-1236, 2008.
38. Nieman DC, Henson DA, Austin MD, and Sha W. Upper respiratory tract infection is reduced in physically fit and active adults. Br J Sports Med 45: 987-992, 2011.
39. Nieman DC, Johanssen LM, Lee IW, and Arabatzis K. Infectious episodes in runners before and after the Los Angeles Marathon. J Sports Med Phys Fitness 30: 316-328, 1990.
40. Northoff H, Symons S, Zicker D, Schaible EV, Schafer K, Thoma S, Loffler M, Abbasi A, Simon P, Niess AM, and Fehrenbach E. Gender- and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise. Exerc Immunol Rev 14: 86-103, 2008.
41. Paavonen T. Hormonal regulation of immune responses. Ann Med 26: 255-258, 1994.
42. Percival RS, Challacombe SJ, and Marsh PD. Flow rates of resting whole and stimulated parotid saliva in relation to age and gender. J Dent Res 73: 1416-1420, 1994.
43. Peters EM, and Bateman ED. Ultramarathon running and URTI: an epidemiological survey. S Afr Med J 64: 582-584, 1983.
44. Predy GN, Goel V, Lovlin R, Donner A, Stitt L, and Basu TK. Efficacy of an extract of North American ginseng containing poly-furanosyl-pyranosyl-saccharides for preventing upper respiratory tract infections: a randomized controlled trial. Can Med Assoc J 173: 1043-1048, 2005.
45. Ruby BC, Robergs RA, Waters DL, Burge M, Mermier C, and Stolarczyk L. Effects of estradiol on substrate turnover during exercise in amenorrheic females. Med Sci Sports Exerc 29: 1160-1169, 1997.
46. Ruedl G, Schobersberger W, Pocecco E, Blank C, Engebretsen L, Soligard T, Steffen K, Kopp M, and Burtscher M. Sport injuries and illnesses during the first Winter Youth Olympic Games 2012 in Innsbruck, Austria. Br J Sports Med 46: 1030-1037, 2012.

47. Schaible E, Boehringer A, Callau D, Niess AM, and Simon P. Exercise and menstrual cycle dependent expression of a truncated alternative splice variant of HIF1α in leukocytes. Exerc Immunol Rev 15: 145-156, 2009.
48. Thoma MV, Kirschbaum C, Wolf JM, and Rohleder N Acute stress responses in salivary alpha-amylase predict increases of plasma norepinephrine. Biol Psychol 91: 342-348, 2012.
49. Timmons BW, Hamadeh MJ, Devries MC, and Tarnoplosky MA. Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling. J Appl Physiol 99: 979-985, 2005.
50. Timmons BW, Tarnoplosky MA, Snider DP, and Bar-Or O. Immunological changes in response to exercise: Influence of age, puberty, and gender. Med Sci Sports Exerc 38: 293-304, 2006.
51. Van Anders SM. Chewing gum has large effects on salivary testosterone, estradiol, and secretory immunoglobulin A assays in women and men. Psychoneuroendocrinol 35: 305-309, 2010.
52. Van Anders SM. Gonadal steroids and salivary IgA in healthy young women and men. Am J Human Biol 22: 348-352, 2010.
53. Walsh NP, Gleeson M, Shephard RJ, Gleeson M , Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, and Simon P. Position Statement Part One: Immune function and exercise. Exerc Immunol Rev 17: 6-63, 2011.
54. Weitz G, Elam M, Born J, Fehm HL, and Dodt C. Postmenopausal estrogen administration suppresses muscle sympathetic nerve activity. J Clin Endocrinol Metab 86: 344-348, 2001.
55. Willemsen G, Carroll D, Ring C, and Drayson M. Cellular and mucosal immune reactions to mental and cold stress: associations with gender and cardiovascular reactivity. Psychophysiol 39: 222-228, 2002.

The effects of exercise, sex, and menstrual phase on salivary antimicrobial proteins

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ABSTRACT

*Salivary antimicrobial protein (AMP) expression is a primary determinant of mucosal immunity. This expression can be altered by exercise. While salivary IgA has been extensively studied, less is known about Lysozyme (Lys) and Lactoferrin (Lac). Knowledge on how sex and menstrual phase affect mucosal immunity is also limited. The purpose of this study was to examine how sex, menstrual phase, and exercise impact IgA, Lys, and Lac expression. Men (n=9) and women (n=9) ran for 45 min at 75% VO_{2peak}. Women were tested in the follicular and luteal phase. Saliva was collected pre-exercise, immediately post-exercise and 1 h post-exercise. Pre-exercise, women had higher secretion rates of IgA compared to men (154±106 vs 85±44 µg/min) ($p<0.05$). Lac secretion rate increased with exercise in both sexes and remained above baseline 1 h after exercise in men (7460±4839 ng/min), but had returned to pre-exercise levels at 1 h post-exercise in women (5720±4661 ng/min) (time*sex interaction, $p<0.05$). Men had higher secretion rates of Lys ($p<0.05$) at each time point compared to women (Men pre-exercise: 31042±23132, post-exercise: 29521±13205, 1 h post-exercise: 41229±31270 ng/min vs Women pre-exercise: 11585±10367, post-exercise: 22719±19452, 1 h post-exercise: 17303±11419 ng/min). Both sexes increased the secretion rate of Lys and Lac with exercise, whereas IgA was unchanged. Menstrual phase did not affect IgA, Lys, or Lac and men and women did not differ in saliva flow rates. In conclusion, regularly menstruating women who are not taking hormonal contraceptives differently express AMPs compared to men.*

Keywords: Lactoferrin; Lysozyme; IgA; upper respiratory symptoms; oestrogen

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Conflict of Interest

The authors report no conflict of interest with the present study. The authors alone are responsible for the writing and content of this paper.

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INTRODUCTION

The mucosal immune system, comprised primarily of salivary IgA, lactoferrin (Lac), and lysozyme (Lys), serves as the first line of defence against invading pathogens at the mucosal surface (21, 42). IgA is the most abundant antibody at the mucosal surface and is the most commonly researched biomarker for innate mucosal immunity during exercise (29). Although IgA has been well studied, it is necessary to assess other constituents of the mucosal immune system that are important to immune protection. The two most abundant antimicrobial proteins (AMPs) are Lys and Lac. These AMPs are produced by salivary glands and epithelial cells, and are located in neutrophils (14). Lys provides protection against gram-positive bacteria (28) while Lac confers protection by inhibiting iron uptake to reduce bacterial growth (43). Lys and Lac function synergistically to augment immunity (12) and Lac may improve Lys ability to remove gram–positive bacteria (28). In short, Lac and Lys play an important role in host defence at the mucosal barrier.

The potential for IgA, Lys, and Lac to be influenced by sex is appropriate because they arise from mucous membranes and mononucleated cells (25, 35), both of which have oestrogen receptors (13). In addition, salivary glands and the oral epithelium express both oestrogen receptor β (41) and progesterone receptors (34), which have been shown to affect saliva composition and flow rate.

Epidemiologic data suggests that active (30) or athletic (24, 27) women experience more upper respiratory symptoms (URS) than men. URS are partially linked to mucosal immunity (16, 23, 26, 31, 36). Salivary IgA concentrations and secretion rates are higher in athletic men compared to athletic women at rest (20) and immediately prior to both prolonged cycling (2) and maximal exercise (38).

Importantly, previous work to quantify sex differences in IgA after exercise has failed to control for both hormonal contraceptive use and menstrual phase and/or status. This lack of control may confound the prior work that has been done in this area. For example, eumenorrhoeic runners demonstrated significantly higher salivary IgA secretion rates and fewer URS than amenorrhoeic runners (39). Similarly, higher salivary IgA concentration and relative IgA (IgA:100 mg protein) was found in resting women compared to men when menstrual phase and contraceptive use was controlled (22). Leukocytes, neutrophils, and monocytes were higher in women who took oral contraceptives compared to both men and women who were not taking oral contraceptives after cycling for 90 min at 65% VO_{2pk} (40). Furthermore, the mRNA of both pro- and anti-inflammatory cytokines were different between sexes and menstrual phase after running for 60 min at a moderate intensity (33). After prolonged, exhaustive running, IL-1ra, IL-6, and IL-10 were different between men and regularly menstruating women not taking hormonal contraceptives (1). When taken together, these studies imply that circulating concentrations of ovarian hormones differ between phases (or occurrence) of the menstrual cycle and according to hormonal contraceptive use. These fluctuations may alter immune parameters. Therefore, in order to accurately assess the impact of sex, both menstrual phase and hormonal contraceptive use need to be controlled.

There are limited data detailing how physiologic fluctuations of ovarian hormones across the menstrual cycle impact IgA, Lac, or Lys expression in response to exercise. Furthermore, potential sex differences in these saliva parameters are needed if relevant clinical or practical recommendations are to be made. Given that menstrual phase and hormonal contraceptive use have the potential to impact immune parameters after exercise, our purpose was to determine sex and menstrual phase differences in IgA, Lac, and Lys in response to acute treadmill running.

METHODS

Participants. 9 men and 9 women volunteered to participate in this study. All procedures were approved by the Institutional Review Board at California Baptist University and subjects gave their informed, written consent prior to participation. All subjects were negative for cardiovascular, pulmonary, and metabolic disease. For at least 3 months prior, subjects had not been ill and were recreationally active, meeting the American College of Sports Medicine's weekly physical activity recommendations (18). Female subjects were eumenorrhoeic. They had maintained a regular menstrual cycle (28-32 days) and had not used hormonal contraceptives for the previous 6 months.

Preliminary Assessment. Male and female subjects were matched according to age and aerobic capacity. Three site skinfold (Lange, Beta Technology, Santa Cruz, CA) measurements (Men: chest, abdomen, thigh; Women: triceps, suprailiac, thigh) were used to determine percent body fat. Each site was measured in duplicate and the mean value was used to calculate percent body fat (5). A continuous graded treadmill test was used to determine VO_{2pea} . This treadmill test started at 6.4 km/h for women and 8 km/h for men with 1% grade. The speed increased 1.6 km/h every minute while the grade remained constant. Subjects ran until volitional fatigue. VO_{2peak} was assessed through open circuit spirometry (Viasys, San Diego CA) and defined as the highest 30-s value when 2 of the following criteria were met: 1) a plateau in VO_2 (change in VO_2 <150 mL/min) with increased workload, 2) a maximal respiratory exchange ratio greater than 1.1, and 3) heart rate greater than 90% of the age predicted maximum (220-age). From the VO_{2peak} testing procedure, a speed that would elicit 75% VO_{2peak} was selected and used for the experimental trial. This workload was shown to optimally increase AMP expression (3). VO_{2peak} was expressed per mL of fat free mass (FFM) so that the aerobic fitness of sexes could be compared. Sex based variations in salivary immune markers may reflect differences in fitness level as elite athletes exhibit higher IgA concentration compared to active or sedentary individuals (17). However, subjects in the current study had similar VO_{2peak} values (when expressed as mL/kg FFM/min) in an effort to minimize the impact of fitness on mucosal markers. Subject characteristics are listed in Table 1.

Experimental Design. Subjects were asked to refrain from exercise and alcohol for 24 h and from caffeine for 12 h prior to testing. They were also instructed to perform an overnight fast (10 h), after which they arrived at our laboratory at

Table 1. Baseline Subject Characteristics.

	Height (cm)	Weight (kg)	Age	% BF	VO_{2pk} (mL/kg FFM/min)
Men (*n* = 9)	182.1 ± 7.9*	79.7 ± 5.7*	21.1 ± 1.1	11.5 ± 4.2*	63.0 ± 5.4
Women (*n* = 9)	167.7 ± 7.1	61.4 ± 8.7	22.4 ± 2.4	18.7 ± 3.8	62.6 ± 9.4

Data are mean ± SD. *Between group difference $p<0.05$

08:00. Subjects were instructed to consume 500 mL of water 1 h before testing to control for hydration. They voided their bowel and bladder before nude body weight was assessed. Subjects ran on a motorized treadmill (Trackmaster, Newton, KS) in a 20°C, 10-15% relative humidity environment for 45 min at a predetermined speed that elicited 75% of VO_{2peak}. Expired gases were collected every 15 min during exercise and heart rate was recorded every 5 min to ensure appropriate exercise intensity. Nude body weight was assessed after the post exercise saliva collection.

All subjects completed 2 exercise trials. Females completed trials in the follicular (Fol) and luteal (Lut) phase using a counterbalanced design (Fol trial 1: *n=4*; Lut trial 1: *n=5*). Men were matched with women to ensure equal time between trials (14.9±0.9 days for women; 17.0±1.7 days for men). Exercise trials for an individual subject were identical in speed, grade, duration, and time of day. Subjects were asked to continue their habitual exercise between trials.

Saliva Collection. During each collection, subjects were seated and asked to swallow to cleanse their mouth prior to un-stimulated collection via passive drool into pre-weighed tubes. Subjects sat with their head tilted forward and were asked to maintain minimal oro-facial movement during collection. Saliva volumes were estimated by weighing to the nearest mg. Density of saliva was assumed to be 1.00 g/mL (8). Flow rate was calculated as the volume of saliva collected divided by the collection time. Secretion rate was calculated as the product of the flow rate and concentration of salivary protein. Saliva was collected at 3 time points: pre-exercise, immediately post-exercise, and 1 h after exercise, as done previously (3). Participants were given 250 mL of water after the post exercise saliva collection in accordance with previous work (3). No other food or drink was consumed until after the 1 h post-exercise saliva collection.

Saliva Analysis. After collection and before storage, saliva was mixed and osmolality was assessed using a freeze point depression osmometer (Advanced Instruments, Norwood, MA, USA) that had been calibrated using 50 mOsm/kg NaCl and 850 mOsm/kg NaCl controls and checked with 290 mOsm/kg NaCl solution according to manufacturer's instruction. Remaining saliva was stored at -80°C for subsequent batch analysis of salivary AMP, with minimal freeze-thaw cycles. These samples were later thawed and analyzed with ELISA according to manufacturer's instruction. IgA (Salimetrics, State College, PA, USA) was detectable at 2.5 µg/mL with an intra-assay coefficient of 4.5% and an inter-assay coefficient of 8.7%. Lac (AssayPro, St. Charles, MO, USA) was detectable at 0.1 ng/mL with an intra-assay coefficient of 4.1% and an inter-assay coefficient of 7.1%. Lys (AssayPro, St. Charles, MO, USA) was detectable at 0.3 ng/mL with

an intra-assay coefficient of 4.3% and an inter-assay coefficient of 7.3%. Data were generated using Gen5 software (BioTek Instruments, Inc, Winooski, VT, USA). All samples for individual subjects were analyzed on the same plate.

Menstrual Phase Analysis. With day 1 as the onset of bleeding, female subjects were tested in the Fol (day 4.1±0.3) and Lut (day 20.4±0.2) phase of their menstrual cycle. Progesterone concentration was used to validate the presence of the Lut phase. 17-β estradiol (Salimetrics, State College, PA) was detectable at 0.1 pg/mL with an intra-assay coefficient of 8.1% and an inter-assay coefficient of 8.9%. Progesterone (Salimetrics, State College, PA) was detectable at 5 pg/mL with an intra-assay coefficient of 8.4% and an inter-assay coefficient of 9.6%. Ovarian hormone concentrations are listed in Table 2.

Table 2. Baseline Ovarian Hormones

	Oestradiol (pg/mL)	Progesterone (pg/mL)
Follicular (*n*=9)	5.4 ± 1.2*	98.1 ± 58.6*
Luteal (*n*=9)	6.6 ± 1.1	207.2 ± 146.9

Data are mean ± SD. *Between group difference $p<0.05$

Statistical Analysis. Data in text and in tables are expressed as mean±SD. For clarity, data on Figures are expressed as mean±SEM. Trial 1 was statistically similar to trial 2 for both men and women. Thus, for simplicity, Figures and Tables that compare sexes combined both trials. A 3 way mixed-design ANOVA (exercise time x trial x sex), using Statistica version 8 (Tulsa, OK, USA), was used to determine the effect of exercise on dependent variables. A 2-way repeated measures ANOVA (exercise time x trial) was used to assess differences in women across the menstrual cycle. Significant differences were further evaluated using Student's paired t test with Holm-Bonferroni adjustments for multiple comparisons. Independent t-tests were used to determine differences between sexes in descriptive data. Pearson's product moment correlation was used to find the association between oestrogen, progesterone, flow rate, IgA, Lac, and Lys. Statistical significance was set at $p<0.05$. All salivary analytes were log transformed to correct for violations of normality. When appropriate, adjustments were made using Greenhouse-Geisser to account for violations against sphericity.

While we could not find published work regarding sex differences in mucosal immunity (specifically Lac and Lys) after exercise, the estimated sample size was 9 subjects, using an alpha level of 0.05 and a beta level of 0.80, to find differences in mucosal immunity after exercise (3).

RESULTS

This study was designed to answer 2 specific questions. First, does sex affect IgA, Lac, Lys at rest or after 45 min of treadmill running? Second, does menstrual phase affect IgA, Lac, Lys at rest or after 45 min of treadmill running? To aid in reader interpretation, the answers for these two questions will each be discussed

for individual dependent variables starting with sex differences before discussing the effect of menstrual phase.

Sex Differences

Exercise Challenge. Metabolic work was not different between men (72.5±3.5% VO_{2peak}) and women (73.6±4.7% VO_{2peak}). Dehydration was similar between sexes as body weight reductions of men (0.8±0.3%) and women (0.6±0.3%) were not significantly different.

Saliva Analysis. Saliva flow rate and osmolality did not differ between sexes. Saliva flow rate increased from pre-exercise to 1 h after exercise ($p<.00$), while saliva osmolality increased from pre-exercise to immediately post-exercise ($p<0.05$) (Table 3).

Table 3. Saliva Analysis

	Pre-Exercise		Immediately Post-Exercise		1 Hour Post-Exercise	
	Osm (mOsm/kg)	Flow Rate (mL/min)	Osm (mOsm/kg)	Flow Rate (mL/min)	Osm (mOsm/kg)	Flow Rate (mL/min)
Men	69.4±15.3	0.53±0.2	96.6±30.4*	0.48±0.2	70.6±15.6	0.71±0.4*
Follicular	63.2±18.3	0.67±0.5	85.3±10.1*	0.67±0.59	68.2±16.1	0.87±0.63*
Lutueal	67.3±18.4	0.90±0.6	91.9±13.3*	0.73±0.56	77.7±14.3	0.93±0.61*

Changes in saliva pre-exercise, post-exercise, and 1 h after 45 min of treadmill running at 75% VO_{2pk}. Data are mean ± SD. Data from exercise Trial 1 and exercise Trial 2 were combined for men. * main effect of time, $p<0.05$ compared to pre-exercise.

IgA Analysis. Women had higher secretion rates ($p<0.05$) and g:Osm ($p<0.05$) of IgA than men pre-exercise, but IgA secretion rates were similar between men and women immediately after and 1 h after exercise. There was a main effect of exercise time for IgA secretion rate ($p<0.05$) and g:Osm ($p<0.001$). The secretion rate was higher 1 h after exercise than it was before or immediately post-exercise, while post-exercise g:Osm values were lower than pre-exercise and 1 h after exercise (Figure 1 A-C).

Lac Analysis. There was an exercise time x sex interaction ($p<0.05$) for Lac secretion rate and µg:mOsm ($p<0.05$). Both sexes' post-exercise value increased from pre-exercise. However, the Lac secretion rate and µg:mOsm in women had returned to pre-exercise values 1 h after exercise while men remained elevated at this time point. There was a main effect of exercise time as Lac concentration increased from pre-exercise to post-exercise ($p<0.05$)(Figure 2 A-C).

Lys Analysis. Men expressed higher Lys concentration ($p<0.001$), secretion rates ($p<0.001$), and µg:mOsm values ($p<0.001$) than women at all time points. There was a main effect of exercise time for Lys concentration ($p<0.05$) and secretion rate ($p<0.05$) as both values increased from pre-exercise to post-exercise. Lys concentration returned to baseline 1 h after exercise while Lys secretion rate remained elevated 1 h after exercise compared to pre-exercise (Figure 3 A-C).

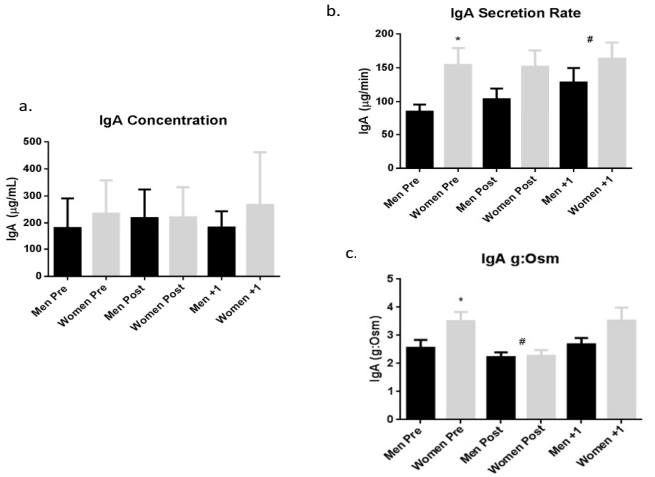

Fig 1. Saliva IgA analysis after 45 min of treadmill running at 75% VO_{2pk}. Trials 1and 2 were combined. Data represented as mean ± SEM. A) IgA concentration. B) IgA secretion rate: *p<0.05 Pre-exercise women compared to pre-exercise men. #p<0.05 main effect of time, 1 h post-exercise greater than pre-exercise and post-exercise samples. C) IgA g:Osm: *p<0.05 pre-exercise women compared to pre-exercise men, #p<0.05 main effect of time, post-exercise compared to pre-exercise and 1 h post-exercise samples.

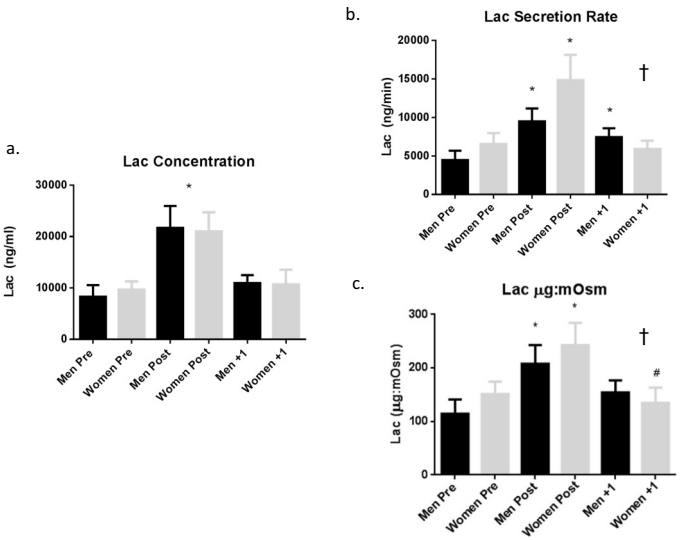

Fig 2. Saliva Lac analysis after 45 min of treadmill running at 75% VO_{2pk}. Trials 1and 2 were combined. Data represented as mean ± SEM. A) Lac concentration: *p<0.05 main effect of time, post-exercise compared to pre-exercise and 1 h post-exercise. B) Lac secretion rate: *p<0.05 from pre-exercise, † time*sex interaction. C) Lac µg:mOsm: *p<0.05 from pre-exercise. #p<0.05 from post-exercise, † time*sex interaction.

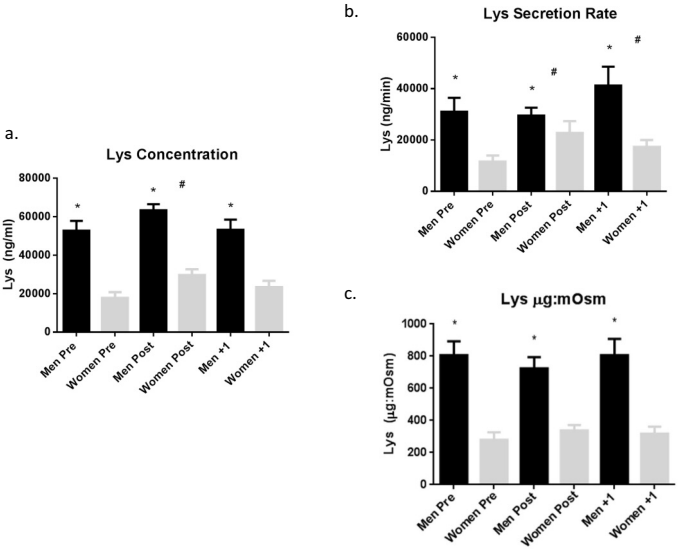

Fig 3. Saliva Lys analysis after 45 min of treadmill running at 75% VO_{2pk}. Trials 1and 2 were combined. Data represented as mean ± SEM. A) Lys concentration: *p<0.05 men compared to women, #p<.05 main effect of time compared to pre-exercise and 1 h after exercise. B) Lys secretion rate: *p<0.05 men compared to women, #p<0.05 main effect of time from pre-exercise. C) Lys µg:mOsm: *p<0.05 men compared to women.

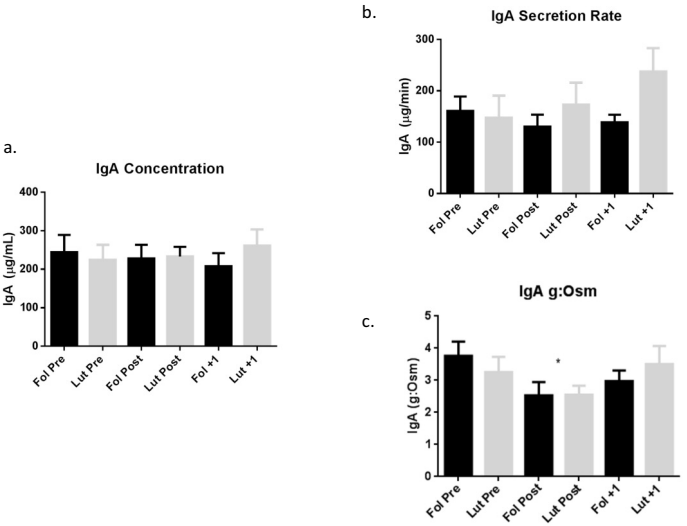

Fig 4. Menstrual phase analysis of salivary IgA after 45 min of treadmill running at 75% VO_{2pk}. Data represented as mean ± SEM. A) IgA concentration. B) IgA secretion rate. C) IgA g:Osm: *p<0.05 compared to pre-exercise.

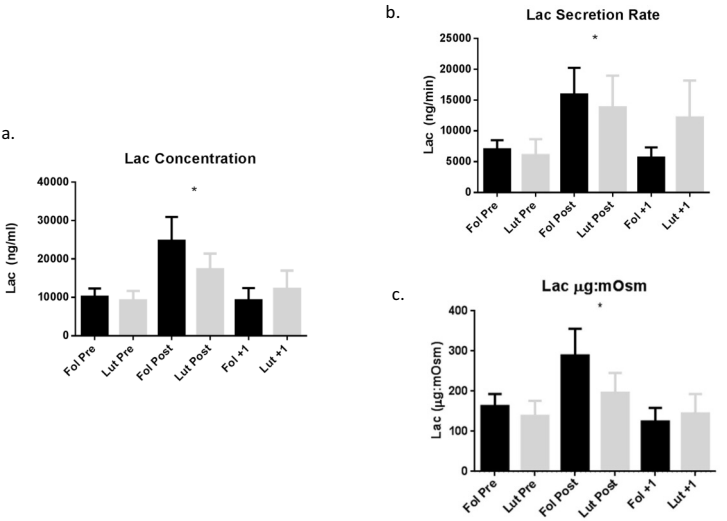

Fig 5. Menstrual phase analysis of salivary Lac after 45 min of treadmill running at 75% VO_{2pk}. Data represented as mean ± SEM. A) Lac concentration: *p<0.05 compared to pre-exercise. B) Lac secretion rate: *p<0.05 compared to pre-exercise. C) Lac µg:mOsm: *p<0.05 compared to 1 h after exercise.

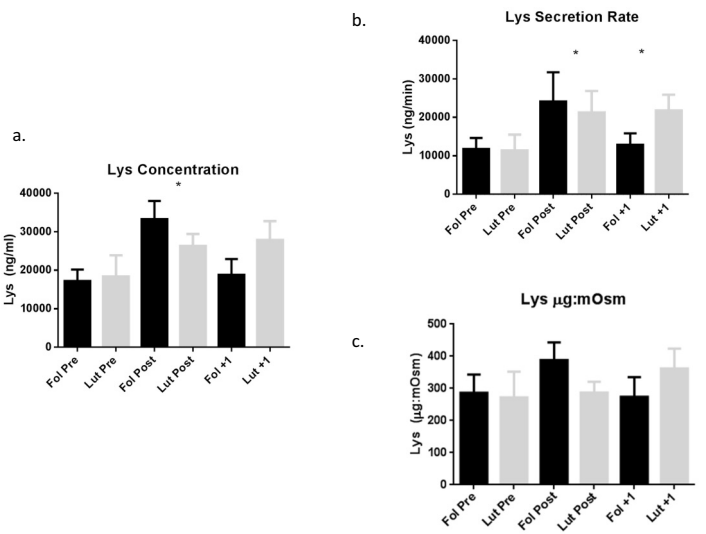

Fig 6. Menstrual phase analysis of salivary Lys after 45 min of treadmill running at 75% VO_{2pk}. Data represented as mean ± SEM. A) Lys concentration: *p<0.05 compared to pre-exercise. B) Lys secretion rate: *p<0.05 compared to pre-exercise. C) Lys µg:mOsm.

Menstrual Phase

Ovarian Hormones. Salivary 17-β estradiol concentration was lower in Fol compared to Lut phase ($p<0.01$), while progesterone values were higher ($p<0.05$) in the Lut phase (Table 2). Ovarian concentrations were similar to prior work (4) and confirmed that women were tested in the appropriate menstrual phase.

Exercise Challenge. Metabolic work was not different between Fol (74.2±4.4% VO_{2peak}) and Lut (73.0±5.2% VO_{2peak}). Body weight reduction was also not different between Fol (0.6±0.3%) compared to Lut (0.7±0.3%).

Saliva Analysis. Saliva flow rate and osmolality were unaffected by menstrual phase. Exercise increased osmolality from pre-exercise to immediately post-exercise ($p<0.05$) and flow rate increased from pre-exercise to 1 h after exercise ($p<0.05$) (Table 3).

IgA Analysis. IgA concentration, secretion rate, and Osm:µg were unaffected by menstrual phase. There was a main effect of exercise time on g:Osm ($p<0.05$). More specifically, pre-exercise values were higher than post-exercise values (Figure 4 A-C).

Lac Analysis. Menstrual phase did not affect Lac concentration, secretion rate, or Osm:ng. Exercise increased both Lac concentration ($p<0.001$) and secretion rate ($p<0.05$) from pre-exercise to post-exercise. Post-exercise µg:mOsm values were higher than those measured 1 h after exercise (Figure 5 A-C).

Lys Analysis. Lys concentration, secretion rate, and µg:mOsm was not affected by menstrual phase. Exercise increased Lys concentration ($p<0.05$) and secretion rate ($p<0.05$) from pre-exercise to post-exercise and the secretion rate remained elevated 1 h after exercise (Figure 6 A-C).

Correlation. Neither progesterone nor oestrogen was correlated with IgA, Lac, or Lys. Further, neither oestrogen or progesterone was associated with flow rate.

DISCUSSION

The main finding of this study was that men and women differ in AMP expression. Specifically, IgA was higher pre-exercise in women while men's Lys values were higher than women's at all time points. Lac values increased after exercise in both sexes, but remained elevated in men. These changes occurred despite men and women having similar levels of exercise-induced dehydration and similar saliva flow rates. We did not detect any changes in AMP expression in Fol compared to Lut phase. Finally, treadmill running at 75% VO_{2peak} increased the secretion rate of Lac and Lys for both sexes.

Sex Differences

Previous work suggests that men have higher IgA levels than women at baseline (2,38) and higher resting IgA secretion rates than women who trained a similar number of hours per week (20). However, this prior work was confounded by the fact that neither hormonal contraceptive use nor menstrual phase was controlled. This is a concern because mucosal immunity in athletic populations has been shown to vary according to the ovarian hormone profile. Indeed, eumenorrhoeic runners demonstrated significantly higher salivary IgA secretion rates than amenorrhoeic runners (26). The authors were only able to find one published report that examined sex differences in salivary IgA and controlled for menstrual phase and hormonal contraceptive use (22). That study reported higher IgA concentration and relative IgA (IgA:100 mg protein) in women compared to men and is consistent with the present analysis. More specifically, our data reflect higher secretion rates and g:Osm of IgA prior to, but not after, treadmill running. As such, these data support the prior observations that sex differences of IgA are only evident at rest and that exercise, *per se,* does not distinctly alter IgA expression between sexes (2, 32).

Since the presence of a menstrual cycle (or ovarian hormone levels) were not accounted for in previous studies that suggest an increase in IgA in men compared to women, the inclusion of amenorrhoeic subjects may partially account for the finding of men possessing higher IgA levels than women. Similarly, higher IgA in men could be due to a blunting of adrenaline (epinephrine) that is seen both at rest (44) and during exercise (37) in the presence of high oestrogen that results during exogenous ovarian hormone intake. Indeed, sympathetic nerve stimulation and adrenaline increase the transport of IgA into the saliva in an animal model (7). Taken together, women consuming exogenous oestrogen may have a blunted adrenaline profile, both at rest and during exercise, and this could lead to lower IgA levels compared to men. The present study suggests that regularly menstruating women who are not taking hormonal contraceptives exhibit higher values compared to men, yet the mechanism responsible for the potential sex difference is unknown. It should be noted that there is high intra- and inter-subject variability in IgA secretion rates and further research is warranted before clinical applications are made.

We are not aware of previously published reports detailing the impact of sex on Lac or Lys in response to exercise. Unlike Lys and IgA, our data suggests that Lac secretion rate and µg:mOsm between sexes is similar at rest. This is supported by previous epidemiologic work (15). However, also unlike Lys and IgA, exercise affected men and women differently. Both groups increased Lac post-exercise, but men were still above baseline values 1 h after exercise while women had returned to pre-exercise values. Lys, however, was higher in men both at rest and in response to exercise. Epidemiologic data regarding resting Lys concentration and secretion rate are equivocal as stimulated submandibular saliva contained greater concentration in men compared to women (46), while stimulated parotid saliva contained a higher secretion rate of Lys in women compared to men (15). The secretion rate of Lys from unstimulated parotid saliva was congruent

for men and women, and across ages (15). While the clinical significance of sex differences is unknown, our data may have practical applications, and as such, should be the focus of further study.

Menstrual Phase

The present data suggest that physiologic variations of ovarian hormones across the menstrual cycle have a limited effect on AMP expression. Similar data were found for non-trained (22) and trained endurance athletes (6). Specifically, there was no difference in saliva flow rate, IgA concentration, or IgA secretion rate in Fol versus the Lut phase (6, 22). Furthermore, the present data show no correlation between oestrogen or progesterone and IgA or flow rate, which is consistent with others (6). Also, Lys is reported to be consistent throughout the menstrual cycle (1) with no published reports for the menstrual phase effect of Lac. Taken together, while ovarian hormones may be partially responsible for sex differences, the physiologic variation across the menstrual cycle did not significantly alter AMP expression.

Exercise Stress

There was a statistically significant increase in the secretion rate of IgA from pre-exercise to 1 h after treadmill running at 75% VO_{2peak}. However, IgA concentration and g:Osm did not increase from pre-exercise values. The increase in the secretion rate likely resulted from the increased flow rate. The flow rate was highest 1 h post-exercise. In support of the current data, post-exercise IgA secretion rates have been similar to pre exercise values after 2 h of running at 75% VO_{2peak} (9), 2 h of cycling at 65% VO_{2peak} (2), and cycling at 75% VO_{2peak} for 22 min (3).

Exercise increased the secretion rate of Lac and Lys for both sexes. This is consistent with previous reports of intense, but not sub-maximal, cycling increasing Lys secretion rate and concentration (3). Similarly, vigorous rowing (45) and sprinting (10) increased Lac and Lys concentration immediately post-exercise. Moderate cycling for 2 h, however, decreased Lys secretion rate and concentration immediately post-exercise, but returned to pre-exercise levels within 1 h post-exercise (11). Prolonged, low intensity running did not alter Lac or Lys secretion rates immediately after or 1.5 h post-exercise (19). Thus, Lys and Lac expression may be altered by exercise; however, this expression is dependent on exercise duration and intensity.

Taken together, the maintenance of IgA in combination with an increase Lac and Lys suggest that exercise of this intensity and duration may serve to enhance the immune system and increase protection against URS in both sexes. While clinical markers were not addressed, our data along with Allgrove and colleagues (3) provides guidelines for the intensity of exercise necessary to increase AMP expression.

Limitations

A limitation to the current study, which analyzed the salivary antimicrobial responses of nine men and nine women at rest and during exercise, is the small sample size. This likely contributed to the large variability we reported with salivary AMPs, particularly in women. While this potential limitation is noteworthy, such variability has also been shown to persist in studies that have used much larger n sizes. For example, Gleeson and colleagues examined sex differences in IgA in an athletic population over four months of training. In that study, the coefficient of variation (CV) for resting IgA concentration and secretion rate for 46 men was 64% and 67%, respectively. The CV for resting IgA concentration and secretion rate for 34 women was 43% and 67%, respectively (20). Similar variation was reported for IgA concentration and secretion rate for 50 men and women runners prior to a marathon. In that study, the CV for IgA concentration and secretion rate was 36% and 50%, respectively (data were not analyzed according to sex) (32). As such, while the CVs for pre-exercise IgA concentration (men = 59% and women = 52%) and secretion rate (men = 52% and women = 68%) are certainly higher than desired, they appear to be in line with prior research.

CONCLUSION

AMP expression differs between sexes. Specifically, regularly menstruating women who are not taking hormonal contraceptives express greater IgA while men demonstrate an increase in Lys. Both sexes increased Lac, but this elevation remained longer in men compared to women. Physiologic variations of ovarian hormones across the menstrual cycle do not affect AMP expression. Both men and women experience an increase in Lac and Lys expression in response to acute treadmill running at 75% VO_{2peak}. Finally, these data highlight the need for greater control in exercise immunology studies that assess the impact of sex.

REFERENCES

1. Abbasi A, Fehrenbach E, Hauth M, Walter M, Hudemann J, Wank V, Niess A, and Northoff H. Changes in spontaneous and LPS-induced ex vivo cytokine production and mRNA expression in male and female athletes following prolonged exhaustive exercise. Exer Immunol Rev 19: 8-28, 2013.
2. Allgrove J.E., Geneen L., Latif S., and Gleeson M. Influence of a fed or fasted state on the s-IgA repsone to prolonged cycling in active men and women. Int J Sport Nutr Exerc Metab 19: 209-221, 2009.
3. Allgrove J.E., Gomes E., Hough J., and Gleeson M. Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. J Sports Sci 26: 653-661, 2008.
4. Andreano J.M., and Cahill L. Menstrual cycle modulation of limbic activity during emotional encoding. Neuroimage 53: 1286-1293, 2010.
5. Brozek J., Grade R., and Anderson J. Densitometric analysis of body composition: revision and some quantitative assumptions. Ann NY Acad Sci 110: 113-140, 1963.

6. Burrows M., Bird S.R., and Bishop NC. The menstrual cycle and its effect on the immune status of female distance runners. J Sports Sci 20: 339-344, 2002.
7. Carpenter G.H., Proctor G.B., Ebersole L.E., and Garrett J.R. Secretion of IgA by rat parotid and submandibular cells in response to autonomimetic stimulation in vitro. Int Immunopharmocol 4: 2004.
8. Cole A, and Eastoe J. Biochemistry and oral biology. London: 1988.
9. Costa R, Fortes M, Richardson K, Bilzon J, and Walsh N. The effect of postexercise feeding on saliva antimicrobial proteins. Int J Sport Nutr Exerc Metab 22: 184-191, 2012.
10. Davison G. Innate immune responses to a single session of sprint interval training. Appl Physiol Nutr Metab 36: 395-404, 2011.
11. Davison G, and Diment BC. Bovine colostrum supplementation attenuates the decrease of salivary lysozyme and enhances the recovery of neutrophil function after prolonged exercise. Br J Sport Nut 103: 1425-1432, 2010.
12. Davison G., Allgrove J., and Gleeson M. Salivary antimicrobial peptides (LL-37 and alpha-defensins HNP1-3), antimicrobial and IgA responses to prolonged exercise. Eur J Appl Physiol 106: 277-284, 2009.
13. Dimitropoulou C, Drakopanagiotakis F, and Catravas J. Estrogen as a new therapeutic target for asthma and chronic obstructive pulmonary disease. Drug News Perspect 20: 241-252, 2007.
14. Dubin RF, Robinson SK, and Widdicombe JH. Secretion of lactoferrin and lysozyme by cultures of human airway epithelium Am J Physiol Lung Cell Mol Physiol 286: 750-755, 2004.
15. Fox PC, Heft MW, Herrera M, Bowers M, Mandel I, and Baum B. Secretion of antimicrobial proteins from the parotid glands of different aged healthy persons. J Gerontol 42: 466-469, 1987.
16. Fox PC, van der Ven PF, Sonies BC, Weiffenbach JM, and Baum BJ. Xerostomia: evaluation of a symptom with increasing significance. J Am Dent Assoc 110: 519-525, 1985.
17. Francis JL, Gleeson M, Pyne DB, Callilster R, and Clancy R. Variation of salivary immunoglobulins in exercising and sedentary populations. Med Sci Sports Exerc 37: 571-578, 2005.
18. Garber C.E., Blissmer B., Deschenes M.R., Franklin B., Lamonte M., Lee I.M., Nieman D.C., and Swain D. Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: Guidance for prescribing exercise. Med Sci Sports Exerc 43: 1334-1359, 2011.
19. Gillum TL, Kuennen MR, Gourley C, Schneider S, Dokladny K, and Moseley P. Salivary antimicrobial protein response to prolonged running. Biol Sport 30: 3-8, 2013.
20. Gleeson M., Bishop NC, Oliveira M., McCauley T., and Tauler P. Sex differences in immune variables and respiratory infection incidence in an athletic population. Exerc Immunol Rev 17: 122-134, 2011.
21. Gleeson M., and Pyne D.B. Exercise effects on mucosal immunity. Immunol Cell Biol 78: 536-544, 2000.
22. Gomez E., Ortiz V., Saint-Martin B., Boeck L., Diaz-Sanchez V., and Bourges H. Hormonal regulation of the secretory IgA (sIgA) system: Estradiol- and Progesterone- induced changes in sIgA in parotid saliva along the menstrual cycle. AJRI 29: 219-223, 1993.

23. Hanson LA, Bjorkander J, and Oxelius VA. Selective IgA Deficiencies. In: Primary and secondary immunodeficiency disorders. Edinburgh: Churchill Livingstone, 1983, p. 62-64.
24. Heath G.W., Macera C.A., and Nieman D.C. Exercise and upper respiratory tract infections. Is there a relationship? Sports Med 14: 353-365, 1992.
25. Jolles P, and Jolles J. What is new in lysozyme research? Mol Cell Biochem 63: 165-189, 1984.
26. Kazuhiro S., Natsumi S., Nakamura M., Katsuju A., Imai T., Suzuki S., Eda N., Hanaoka Y., Naota S., Mesaki N., Kono I., and Akama T. Mucosal immune function comparison between amenorrheic and eumenorrheic distance runners. J Strength Cond Res 26: 1402-1406, 2012.
27. Konig D., Grathwohl D., Weinstock C., Northoff H., and Berg A. Upper respiratory tract infection in athletes: influence of lifestyle, type of sport, training effort, and immunostimulant intake. Exerc Immunol Rev 6: 102-120, 2000.
28. Leitch EC, and Willcox MD. Elucidation of the antistaphylococcal action of lactoferrin and lysozyme. J Med Microbiol 48: 867-871, 1999.
29. Neville V, Gleeson M, and Folland JP. Salivary IgA as a risk factor for upper respiratory infection in elite professional athletes. Med Sci Sports Exerc 40: 1228-1236, 2008.
30. Nieman D.C., Henson D.A., Austin M.D., and Sha W. Upper respiratory infection is reduced in physically fit and active adults. Br J Sport Med 45: 987-992, 2010.
31. Nieman DC. Immune function responses to ultramarathon race competition. Medicina Sportiva 13: 189-196, 2009.
32. Nieman DC, Henson DA, Fagoaga OR, Utter AC, Vinci DM, Davis JM, and Nehlsen-Cannarella SL. Change in salivary IgA following a competitive marathon race. Int J Sports Med 23: 69-75, 2002.
33. Northoff H, Symons S, Zieker D, Schaible E, Schafer K, Thoma S, Loffler M, Abbasi A, Simon P, Niess A, and Fehrenbach E. Gender-and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise. Exerc Immunol Rev 14: 86-103, 2008.
34. Ozono S, Onozuka M, Sato K, and Ito Y. Immunohistochemical localization of estradiol, progesterone, and progesterone receptor in human salivary glands and salivary adenoid cystic carcinomas. Cell Struct Funct 17: 169-175, 1992.
35. Perera S, Sabin E, Nelson P, and Lowe D. Increase in salivary lysozyme and IgA concentrations and secretory rates independent of salivary flow rates following viewing of a humorous videotape. Int J Behav Med 5: 118-128, 1998.
36. Rossen R.D., Butler W.T., Walsman R.H., Alford R.H., Hornick R.B., Togo Y., and Kasel J.A. The proteins in nasal secretions. II. A longitudianl study of IgA and neutralizing anitbody levels in nasal washings from men infected with influenza virus. JAMA 211: 1157-1161, 1970.
37. Ruby B.C., Robergs R.A., Waters D.L., Burge M., Mermier C., and Stolarczyk L. Effects of estradiol on substrate turnover during exercise in amenorrheic females. Med Sci Sports Exerc 29: 1160-1169, 1997.
38. Schouten WJ, Verschuur R, and Kemper HC. Habitual physical activity, strenuous exercise, and salivary immunoglobulin A levels in young adults; the Amsterdam Growth and Health Study. Int J Sport Med 9: 289-293, 1988.

39. Shimizu K, Suzuki K, Nakamura M, Katsuji A, Imai T, Suzuki S, Nobuhiko E, Hanaoka Y, Nakao K, Suzuki N, Mesaki N, Kono I, and Akama T. Mucosal immune function comparison between amenorrheic and eumenorrheic distance runners. J Strength Cond Res 26: 1402-1406, 2012.
40. Timmons B, Hamadeh M, Devries M, and Tarnopolsky M. Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling. J Appl Physiol 99: 979-985, 2005.
41. Valimaa H, Savolainen S, Soukka T, Silvoneimi P, Makela S, Kujari H, and Laine M. Estrogen receptor-B is the predominant receptor subtype in human oral epithelium and salivary glands. J Endocrinol 180: 55-62, 2004.
42. Walsh N, Glesson M., Shephard R., Gleeson M., Woods J., Bishop NC, Fleshner M., Green C., Pedersen B., Hoffman-Goetz L., Rogers C., Northoff H., Abbasi A., and Simon P. Position statement part one: Immune function and exercise. Exerc Immunol Rev 17: 6-63, 2011.
43. Weinberg ED. Iron depletion: a defense against intracellular infection and neoplasia Life Sci 1289-1297, 1992.
44. Weitz G., Elam M., Born J., Fehm H.L., and Dodt C. Postmenopausal estrogen administration supresses muscle sympathetic nerve activity. J Clin Endocrinol Metab 86: 344-348, 2001.
45. West NP, Pyne DB, Kyd JM, Renshaw GM, Fricker PA, and Cripps AW. The effect of exercise on innate mucosal immunity. Bri J Sports Med 44: 227-231, 2010.
46. Yeh C. K, Dodds M. W, Zuo P., and Johnson D. A. A population based study of salivary lysozyme concentrations and canidal counts. Arch Oral Biol 42: 25-31, 1997.

Evaluation of serum leaking enzymes and investigation into new biomarkers for exercise-induced muscle damage

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ABSTRACT

This investigation determined whether existing muscle damage markers and organ damage markers respond to an acute eccentric exercise protocol and are associated with affected muscle symptoms. Nine healthy-young men completed one-leg calf-raise exercise with their right leg on a force plate. They performed 10 sets of 40 repetitions of exercise at 0.5 Hz with a load corresponding to half of their body weight, with 3 min rest between sets. The tenderness of medial gastrocnemius, lateral gastrocnemius and soleus, and the ankle active range of motion (ROM) were assessed before, immediately after, 24 h and 48 h, 72 h, 96 h and 168 h after exercise. Blood and urine were collected pre-exercise and 2 h, 4 h, 24 h, 48 h, 72 h and 96 h post-exercise. Serum was analyzed for creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and aldolase (ALD) activities. We also determined heart-type fatty acid-binding protein (H-FABP), intestinal-type fatty acid-binding pro-

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tein (I-FABP) and liver-type fatty acid-binding protein (L-FABP), neutrophil gelatinase-associated lipocalin (NGAL), interleukin (IL)-17A, IL-23, nerve growth factor (NGF), soluble-Endothelial (sE)-selectin, s-Leukocyte (L)-selectin, s-Platelets (P)-selectin, and 8-isoprostane in plasma and urine. The tenderness of proximal and middle gastrocnemius increased significantly 72 h ($p<0.05$, $p<0.01$) after exercise. Ankle active ROM in dorsal flexion decreased significantly 48 h ($p<0.05$) and 72 h ($p<0.01$) after exercise. CK and ALD activities significantly increased at 72 h ($p<0.05$) and remained elevated at 96 h ($p<0.01$) post-exercise compared to pre-exercise values. Also, ALD which showed relatively lower interindividual variability was significantly correlated with tenderness of middle gastrocnemius at 72 h. LDH activity significantly increased 96 h post-exercise ($p<0.01$), whereas the increase in AST and ALT activities 96 h post-exercise was not significantly different from pre-exercise values. There were no significant changes in FABPs, NGAL, IL-17A, IL-23, NGF, selectins and 8-isoprostanes in plasma and urine. In conclusion, calf-raise exercise induced severe local muscle damage symptoms which were accompanied by increases in both serum CK and ALD activities, but we could not detect any changes in examined markers of organ damage, inflammation and oxidative stress. Further research is needed to determine other more sensitive biomarkers and the underlying mechanisms of exercise-induced muscle damage.

Key words: exercise-induced muscle damage, organ damage markers, acute eccentric exercise, creatine kinase, aldolase

INTRODUCTION

Exercise-induced muscle damage has been one of the most important targets in sport science research. Direct assessment of muscle damage involves histological examination of muscle tissue via biopsy. However, there is difficulty gathering tissue samples with biopsy technique and demonstrating disruption of muscle cells (1, 3, 48), in combination with the process being an invasive experience for research participants. Therefore, the less invasive measures for the accurate assessment of exercise-induced muscle damage are needed. As indirect indicator of muscle damage, delayed-onset muscle soreness (DOMS) is a poor reflector of eccentric exercise-induced muscle damage, and changes in other indicators of muscle damage are not necessarily accompanied by DOMS (27). On the other hand, the changes of range of motion (ROM) have been used as an indirect variable of DOMS (8, 9). Although a large number of studies have reported the effects of exercise on muscle damage, DOMS and inflammatory responses in humans (26, 30, 31, 34, 41, 44, 46), the mechanisms of exercise-induced muscle damage are not fully understood at present. DOMS is characterized by tenderness and movement-related pain, that is, mechanical hyperalgesia in the exercised muscle (22), and ROM might be affected by muscle soreness (33). Therefore, it is necessary to assess the muscle symptoms in a multifaceted manner, and investigate the associations with other muscle damage markers carefully.

Serum enzyme activities such as creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase

(LDH), aldolase (ALD) and myoglobin which leak into circulation from damaged muscle have been used as indirect markers of exercised-induced muscle damage (5, 28). Serum levels of these damage indicators depend on gender, muscle mass, exercise intensity and duration in addition to the individual training state, and there is a remarkable interindividual variability in the degree to which serum enzyme activities increase with exercise (30). Instead of the existing leaking enzymes, fatty acid-binding protein (FABP) and neutrophil gelatinase-associated lipocalin (NGAL) have been introduced as organ damage markers. FABPs are present in almost all tissues, and were named after the tissue in which they were discovered or are prominently expressed: L-FABP, liver-type fatty acid-binding protein; I-FABP, intestinal-type fatty acid-binding protein; H-FABP, heart-type fatty acid-binding protein (50). H-FABP is mainly expressed in the heart, but to a lesser extent also in the skeletal muscle (47). NGAL was identified as a 25 kDa protein secreted by neutrophils (45), and is expressed in human tissues, including kidneys, lungs, stomach, and colon (10). NGAL is focused in recent years as a biomarker in several benign and malignant diseases, especially as biomarker in acute kidney injuries (11), and may have the potential to protect against cellular injury mediated by reactive oxygen species (ROS) (36, 37). Although we have observed that neutrophils produce ROS following exercise (14, 42-44), NGAL might become a good alternative variable which is easier to measure simply in plasma and urine by conventional enzyme-linked immunosorbent assays (ELISA) than the existing neutrophil functional analyses that contain rather complex procedures and need to be determined *ex vivo* soon after blood sampling.

Exercise-induced muscle damage causes local inflammation which degenerates and regenerates muscle and surrounding connective tissue (30). Briefly, neutrophils are mobilized into the circulation after exercise, and soon infiltrate into the damaged tissue (2). Neutrophils are primed by the chemoattractants such as complement 5a (C5a) and interleukin (IL)-8 from the exercise-induced damaged muscle, causing rolling, arrest and transmigration which is mediated by intercellular adhesion molecules, selectins (16), initially. Neutrophils are present in muscle within a day after exercise (3, 17, 18, 19, 34, 40), and after neutrophils' infiltration, macrophages are replaced and present in muscle from 1 to 14 days after exercise (2, 3, 12, 13, 19, 32, 38). Neutrophils and macrophages produce ROS to degrade the damaged muscle tissue (23, 24), and may produce pro-inflammatory cytokines (6). The pro-inflammatory cytokines such as IL-1β, tumor necrosis factor (TNF)-α, and IL-6 are expressed in skeletal muscle following eccentric exercise (30), but we could not detect any changes of these cytokines in plasma and urine in our previous study (14). On the other hand, Sugama et al. reported that IL-17 induced by IL-6 and activated by IL-23 might promote neutrophil activation and muscle damage in a different way from the classical pro-inflammatory cytokines IL-1β and TNF-α following prolonged endurance exercise (41). Furthermore, nerve growth factor (NGF) which is involved in pathological pain conditions (15, 49) increased in the muscle after lengthening contraction in rats (22). 8-isoprostane is a prototypical biomarker of oxidative stress by exercise-induced free radical production (21). Fluctuations of these substances may be detected following exercise-induced muscle damage and inflammation, and can be one of the underlying mechanisms of it.

We have shown in a previous study that increased myoglobin concentration at 72 h after exercise was correlated with the appearance of DOMS at 72 h ($r=0.73$, $p<0.05$), and there was a positive correlation between exercise-induced increases in neutrophil migratory activity at 4 h and increases in myoglobin at 48 h ($r=0.67$, $p<0.05$) following one-leg calf-raise exercise (14), suggesting that neutrophils may be involved in muscle damage and that myoglobin is a very sensitive muscle damage marker. Based on these findings, the aims of the present study were to examine 1) changes in serum leaking enzyme activities and their correlation with DOMS, 2) concentrations of novel organ damage markers, novel pro-inflammatory cytokines and an oxidative stress marker and their relationship to muscle damage, and 3) urinary excretion rates of these markers as potential surrogate non-invasive indicators of muscle damage, using a local muscle damage model of one-leg calf-raise exercise.

METHODS

Subjects

Nine untrained healthy males (age 24.8±1.3 (mean±SD) yrs, body mass 62.3±6.3 kg and height 1.72±0.05 m) volunteered for this study. At the time of the study, the subjects had not been involved in any hard exercise or resistance training for at least two weeks before the exercise bout, and were not taking any supplements, or participating in recovery strategies such as massage, stretching or cryotherapy. The subjects were instructed to maintain their usual daily schedule during the experiment. All subjects completed a medical questionnaire and gave written informed consent. The experimental procedure was approved by the ethics committee of Waseda University.

Experimental design

Subjects performed a calf-raise exercise, including repetitive eccentric muscle contractions with their right leg on a force plate. The range of motion of the ankle joint during the exercise was regulated from -20° (dorsiflexion position) to 15° (plantar flexion position) using a goniometer (SG 110/A, Biometrics, Newport, UK) with its ends attached onto the skin over the tibia and calcaneus. They performed 10 sets of 40 repetitions of exercise at 0.5 Hz with a load corresponding to the half of their body weight, with 3 min rest between sets. The tenderness of the exercised muscle correlative to DOMS was assessed using the FP meter (SN-402, Navis, Japan) at 1 kg and rated subjectively using a visual analogue scale (VAS) that has a 100-mm line with “no pain” on one end and “extremely sore” on the other. The points of measurements were the proximal, the middle and the distal points of medial gastrocnemius (MG) and lateral gastrocnemius (LG), the middle points between MG and LG, and the middle and the distal points of soleus (SOL). The ankle active ROM was assessed using the goniometer. The tenderness of the exercised muscle and the ankle active ROM were assessed before, immediately after 24 h and 48 h, 72 h, 96 h and 168 h after and exercise. The blood and urine samples were collected before and 2 h, 4 h, 24 h and 48 h, 72 h and 96 h after the exercise. Participants were supposed to urinate 2 h before each sampling, and the urine samples were collected in measuring cylinders.

Blood and urine sampling and analyses

Approximately 12 ml of blood was drawn by standard venipuncture technique from the antecubital vein. Blood samples were collected into serum separation tubes and vacutainers containing heparin and EDTA. A portion of whole blood was used to measure haemoglobin, haematocrit and complete blood cell counts using an automatic blood cell counter (PocH100i, Sysmex, Kobe, Japan). The serum separation tubes were left to clot at room temperature for 30 min, and the vacutainers containing EDTA for plasma separation were immediately centrifuged at 1000×G for 10 min. Serum and plasma samples were then removed and stored at –80°C for later analyses. Serum CK, AST, ALT, LDH and ALD activities were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Urine samples were centrifuged immediately at 1000×G for 10 min to remove sediments, and the supernatants were stored at –80°C for later analyses. Urinary concentration of creatinine was measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the plasma and urine concentrations of H-FABP, I-FABP, L-FABP and NGAL (Human H-FABP, I-FABP, L-FABP and NGAL ELISA kit, Hycult biotechnology, Uden, The Netherlands), IL-17A (Human IL-17A ELISA kit, Gen-Probe Diaclone SAS, Besancon, France), IL-23 (Human IL-23, R&D Systems, Inc. MN, USA), nerve growth factor (NGF) (Human Nerve Growth Factor ELISA kit, Cusabio Biotech Co. Ltd., Wuhan, China), E-selectin (Soluble), sL-selectin and sP-selectin (Life technologies Co. CA, USA) and 8-isoprostane (Detroit R&D, Inc., MI, USA). The measurements were performed according to the instructions for each ELISA kit using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). The urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Statistical analysis

Data are presented as mean ± SD. Statistical validation was made using Friedman's test. When significant time effects were evident, multiple comparisons were analyzed with Scheffe test. Associations between data were analyzed with Pearson's correlation coefficient (r). Statistical significance was accepted at $p<0.05$.

RESULTS

Changes in the tenderness of the exercised muscle, and the ankle joint active range of motion (ROM)

As shown in Table 1, tenderness of the exercised muscle developed on subsequent days after calf-raise exercise and was principally sensed on the MG. It increased significantly 72 h (proximal MG; $p<0.05$, middle MG; $p<0.01$) after exercise compared with the pre-exercise values. The ankle joint active ROM in the dorsal flexion decreased significantly at 48 h ($p<0.05$) and 72 h ($p<0.01$). The lowest ROM was shown at 72 h after exercise in dorsal flexion, whereas there was no significant difference in plantar flexion compared with the pre-exercise values.

Changes in the muscle and the organ damage markers
As shown in Table 2, CK and ALD activities significantly increased at 72 h after exercise ($p<0.05$), thereafter, they remained elevated for 96 h after exercise ($p<0.01$). LDH activity significantly increased at 96 h after exercise ($p<0.01$). However, AST and ALT activities showed no significant difference when compared with pre-exercise values. As listed in Table 3, plasma concentrations of H-FABP, I-FABP and L-FABP were below the detectable levels of the assays and urinary concentrations exhibited no significant change. Plasma and urinary NGAL were not significantly different compared with pre-exercise values.

Changes in the pro-inflammatory cytokines and other indicators
As shown in Table 4, plasma concentration of IL-17A was not significantly changed and urinary excretion rate of IL-17A was below the detectable level. Plasma concentration of IL-23 was below the detectable level, whereas urinary excretion rate of IL-23 showed no significant change. Plasma sE-selectin concentration was below the detectable level, and sL-selectin and sP-selectin concentrations were not significantly different. Plasma concentration of 8-isoprostane was below the detectable level, and urinary excretion rate of 8-isoprostane and plasma concentration of NGF did not change significantly.

Relationships between enzyme activities and the tenderness of exercised muscle and the ankle active range of motions (ROM)
As shown in Table 5, there were no significant correlations between the tenderness of proximal MG 72 h and CK, LDH and ALD for the percent changes of the peak values. The tenderness of middle MG 72 h was significantly correlated with ALD 72 h ($r=0.78$, $p<0.05$). The dorsal flexion of ankle active ROM was not significantly correlated to the tenderness of MG or to CK, LDH and ALD.

DISCUSSION

Muscle-derived leaking enzymes such as CK, AST, ALT, LDH and ALD have been used in many studies as indirect markers of exercise-induced muscle damage. However, there are generally poor correlations between DOMS and muscle damage indicators (27). In this study, CK and ALD were significantly increased earlier than LDH. Also, ALD showed relatively lower interindividual variations and was significantly correlated with tenderness of middle MG at 72 h, whereas there were only moderate, non-significant correlations between tenderness and the other leaking enzyme activities. These results suggest that ALD rather than CK and LDH might be a more accurate and objective muscle damage indicator.

There are a few studies that have investigated the organ damage markers, FABPs and NGAL following exercise-induced muscle damage. FABPs are present in almost all tissues (50), but H-FABP is present in skeletal muscle (47). Sorichter et al. reported that plasma H-FABP and myoglobin increased earlier (30 min) than CK (2 h) following 20 min of downhill running (39). These results suggest that plasma H-FABP concentrations reflect exercise-induced muscle damage earlier, but plasma H-FABP seems to have no advantage in view of its similarity of changes in the appearance of myoglobin. Also, the change might be attributed

Table 1. Changes in the tenderness of exercised muscle and active range of motion (ROM).

	unit	Pre	0 h	24 h	48 h	72 h	96 h	168 h
medial gastrocnemius								
proximal	mm	16 ± 9	21 ± 10	35 ± 20	40 ± 31	54 ± 24*	36 ± 18	20 ± 15
middle	mm	19 ± 10	23 ± 12	34 ± 18	48 ± 24	64 ± 22**	49 ± 18	23 ± 14
distal	mm	20 ± 11	19 ± 14	30 ± 14	45 ± 23	49 ± 31	40 ± 19	19 ± 12
medial/lateral gastrocnemius								
proximal	mm	15 ± 15	14 ± 12	26 ± 16	24 ± 18	25 ± 23	19 ± 15	11 ± 14
middle	mm	16 ± 13	15 ± 12	18 ± 10	25 ± 24	26 ± 28	16 ± 15	11 ± 11
distal	mm	15 ± 9	16 ± 11	23 ± 15	21 ± 15	24 ± 18	18 ± 15	15 ± 14
lateral gastrocnemius								
proximal	mm	11 ± 12	8 ± 10	15 ± 13	14 ± 11	26 ± 24	18 ± 15	8 ± 12
middle	mm	14 ± 12	15 ± 12	18 ± 13	26 ± 18	39 ± 29	26 ± 19	10 ± 13
distal	mm	13 ± 10	10 ± 11	15 ± 12	19 ± 16	21 ± 17	16 ± 14	6 ± 11
soleus								
middle	mm	21 ± 14	18 ± 13	24 ± 15	18 ± 10	21 ± 14	14 ± 12	14 ± 15
distal	mm	7 ± 8	10 ± 10	11 ± 11	7 ± 11	9 ± 12	10 ± 14	6 ± 8
ROM of ankle joint								
plantar flexion	degree of an angle	33 ± 4	34 ± 4	32 ± 3	32 ± 10	32 ± 5	32 ± 6	33 ± 4
dorsal flexion	degree of an angle	-25 ± 5	-20 ± 5	-20 ± 5	-15 ± 4*	-12 ± 7**	-16 ± 5	-23 ± 7

Data are presented as means ± SD (n=9). Statistics: * $p<0.05$, ** $p<0.01$, significantly different from each Pre values. before exercise (Pre), immediately post-exercise (0 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), 168 hour post-exercise (168 h), range of motion (ROM).

Table 2. Changes in the muscle damage markers following the calf-raise exercise.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
CK	U/l	131.7 ± 94.0	133.0 ± 84.2	139.3 ± 81.4	172.6 ± 150.7	766.1 ± 1474.0	3245 ± 4648*	6069 ± 6498**
AST	U/l	19.4 ± 5.9	18.4 ± 5.1	18.6 ± 4.8	19.2 ± 7.0	29.3 ± 26.2	75.9 ± 88.1	126.2 ± 130.2
ALT	U/l	16.8 ± 6.0	16.3 ± 5.9	15.8 ± 5.0	16.0 ± 6.3	17.7 ± 7.1	27.8 ± 19.6	40.0 ± 29.4
LDH	U/l	139.8 ± 26.9	137.8 ± 24.7	138.1 ± 21.9	137.9 ± 26.9	146.3 ± 24.7	187.8 ± 79.9	241.7 ± 103.8**
ALD	U/l	3.7 ± 0.7	3.7 ± 0.1	3.8 ± 0.6	3.8 ± 0.6	6.2 ± 6.1	21.0 ± 26.7*	47.2 ± 44.7**

Data are presented as means ± SD (n=9). Statistics: * $p < 0.05$, ** $p < 0.01$, significantly different from each Pre values. before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aldolase (ALD).

Table 3. Changes in plasma and urinary novel markers of organ damage.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
H-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
H-FABP-U	pg/min	1242 ± 970	1156 ± 1114	1106 ± 1011	3261 ± 4243	2559 ± 3815	3857 ± 4835	3072 ± 2523
I-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
I-FABP-U	pg/min	45.8 ± 47.8	53.7 ± 35.7	87.9 ± 95.2	54.8 ± 48.2	81.9 ± 83.0	37.8 ± 31.8	40.5 ± 19.2
L-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
L-FABP-U	pg/min	3988 ± 3280	5323 ± 5031	4378 ± 2609	6846 ± 6631	5847 ± 6369	5539 ± 5480	6727 ± 3595
NGAL-P	ng/ml	13.2 ± 5.0	14.3 ± 7.7	13.8 ± 8.2	14.2 ± 9.6	13.6 ± 7.0	17.1 ± 12.9	15.5 ± 11.2
NGAL-U	ng/min	0.36 ± 0.51	0.76 ± 1.13	0.35 ± 0.20	0.39 ± 0.38	0.52 ± 0.55	0.35 ± 0.33	0.35 ± 0.29

Data are presented as means ± SD (n=9). before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), not detected below the detection limits (ND), plasma (P), urine (U), heart (H), Intestine (I), liver (L), fatty acid-binding protein (FABP), neutrophil gelatinase-associated lipocalin (NGAL).

Urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Table 4. Changes in plasma and urinary inflammatory biomarkers.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
IL-17A-P	pg/ml	31.1 ± 36.7	29.9 ± 35.1	29.1 ± 33.4	27.8 ± 32.7	30.9 ± 31.8	33.3 ± 33.5	30.1 ± 29.9
IL-17A-U	pg/min	ND	ND	ND	ND	ND	ND	ND
IL-23-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
IL-23-U	pg/min	0.9 ± 1.2	1.2 ± 1.5	1.4 ± 1.8	1.4 ± 2.0	1.3 ± 2.0	0.8 ± 1.3	0.4 ± 0.4
8-isoprostane-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
8-isoprostane-U	pg/min	243.9 ± 151.9	501.3 ± 397.2	443.7 ± 293.5	792.5 ± 867.7	688.0 ± 969.8	267.1 ± 116.3	490.5 ± 372.3
sE-selectin-P	ng/ml	ND	ND	ND	ND	ND	ND	ND
sL-selectin-P	ng/ml	1341.0 ± 158.0	1348.0 ± 254.1	1276.0 ± 250.9	1263.0 ± 353.1	1370.7 ± 275.9	1440.0 ± 208.7	1332.0 ± 133.9
sP-selectin-P	ng/ml	32.7 ± 2.3	31.9 ± 9.1	33.6 ± 10.9	31.6 ± 5.3	29.1 ± 7.3	27.9 ± 5.5	30.8 ± 8.5
Nerve growth factor-P	pg/ml	2.4 ± 3.1	4.2 ± 5.9	2.6 ± 2.7	9.7 ± 17.6	9.3 ± 17.7	6.1 ± 8.2	9.7 ± 21.1

Data are presented as means ± SD (n=9). before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), not detected below the detection limits (ND), plasma (P), urine (U), soluble Endothelial (sE), soluble Leukocyte-Endothelial (sL), soluble Platelet (sP). Urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Table 5. Pearson's correlation coefficient matrix of muscle damage markers, tenderness of medial gastrocnemius and ankle active range of motion in dorsal flexion.

	CK-72 h	CK-96 h	LDH-96 h	ALD-72 h	ALD-96 h	PMG-72 h	MMG-72 h	dROM-48 h	dROM-72 h
CK-72 h		0.24	0.32	0.94	0.48	0.22	0.59	-0.34	-0.43
CK-96 h	0.24		0.94	0.38	0.93	-0.04	0.13	0.19	-0.05
LDH-96 h	0.32	0.94		0.46	0.95	-0.04	0.20	0.31	0.13
ALD-72 h	0.94	0.38	0.46		0.64	0.37	0.78*	-0.15	-0.25
ALD-96 h	0.48	0.93	0.95	0.64		0.06	0.42	0.22	0.04
PMG-72 h	0.22	-0.04	-0.04	0.37	0.06		0.37	0.25	0.03
MMG-72 h	0.59	0.13	0.20	0.78*	0.42	0.37		0.02	0.12
dROM-48 h	-0.34	0.19	0.31	-0.15	0.22	0.25	0.02		0.89
dROM-72 h	-0.43	-0.05	0.13	-0.25	0.04	0.03	0.12	0.89	

All data are calculated as percent changes for the pre-exercise values.

Statistics: * Significant correlation between serum leaking enzyme activity and muscle tenderness score ($p<0.05$), creatine kinase (CK), lactate dehydrogenase (LDH), aldolase (ALD), proximal medial gastrocnemius (PMG), middle medial gastrocnemius (MMG), ankle range of motion in dorsal flexion (dROM), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h).

to not only eccentric exercise-induced local muscle damage, but also systemic factors such as dynamic exercise-induced haemoconcentration. In this study, one-leg calf-raise exercise caused severe muscle damage as shown by the precise local assessment of the muscle symptoms, but we could not detect any changes in FABPs and NGAL nor any correlations with the symptoms. In any case, these organ damage markers are not considered to be indicative of muscle damage resulting from eccentric exercise loading on the calf, thus they cannot be used as local muscle damage markers at least.

Endurance exercise induces peripheral blood neutrophilia (41-44), and enhances the capacity of neutrophils to produce ROS (29, 42-44). In a study using myeloperoxidase (MPO) knockout mice, exercise-induced muscle damage was facilitated by MPO-containing neutrophils and their activating factors such as proinflammatory cytokines (25). On the other hand, Maruhashi et al. reported that the antioxidant capacity was affected by the type and intensity of exercise, specifically, low-load eccentric exercise did not reduce antioxidant capacity, but conversely low-load concentric exercise temporarily reduced antioxidant capacity (20). We investigated neutrophil activation-related markers such as selectins, 8-isoprostane, IL-17A and IL-23, but they were not significantly changed, suggesting that inflammatory responses and oxidative stress were not changed, at least, in the examined markers following one-leg calf-raise exercise. The enhanced eccentric exercise-induced neutrophil migratory activity independent of ROS production and MPO degranulation observed in our previous study (14) might be due to the mobilization of functionally different heterogeneous neutrophils possibly from the bone marrow reserve (42, 44). Since we could not detect any significant changes of a wide range of proinflammatory and oxidative stress markers in this exercise mode other than neutrophil mobilization and migration (14), it is necessary to focus more on the involvement of this earlier step of inflammation as the underlying mechanisms and the point of target for potential preventive countermeasures against exercise-induced muscle damage in the future studies.

In conclusion, it is confirmed in the present study that not only are serum CK but also ALD activities more reliable indicators for exercise-induced muscle damage than the other examined variables, but there are lower correlations with muscle symptoms, and novel organ damage markers of FABPs and NGAL could not be alternative indicators for muscle disruption, neutrophil mobilization and migration. Also, we could not detect any perturbations of novel proinflammatory cytokines and soluble adhesion molecules, and the inflammatory mechanisms are still not clear. Therefore, further research is needed to determine whether there are more sensitive indicators including urinary biomarkers as non-invasive assessment of exercise-induced muscle damage and the underlying mechanisms as well.

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REFFERENCES

1. Armstrong, R. B. Mechanisms of exercise-induced delayed onset muscular soreness: a brief review. Med. Sci. Sports Exerc. 16: 529-538. 1984.
2. Beaton, L. J., D. A. Allan, M. A. Tarnopolsky, P. M. Tiidus, and S. M. Phillips. Contraction-induced muscle damage is unaffected by vitamin E supplementation. Med. Sci. Sports Exerc. 34: 798-805. 2002.
3. Beaton, L. J., M. A. Tarnopolsky, and S. M. Phillips. Variability in estimating eccentric contraction-induced muscle damage and inflammation in humans. Can. J. Appl. Physiol. 27: 516-526. 2002.
4. Beaton, L. J., M. A. Tarnopolsky, and S. M. Phillips. Contraction-induced muscle damage in humans following calcium channel blocker administration. J. Physiol. 544: 849-859. 2002.
5. Brancaccio, P., N. Maffulli, R. Buonauro, and F. M. Limongelli. Serum enzyme monitoring in sports medicine. Clin. Sports Med. 27(1): 1-18. 2008.
6. Cannon, J. G., and B. A. St. Pierre. Cytokines in exertion-induced skeletal muscle injury. Mol. Cell Biochem. 179: 159-167. 1998.
7. Chakraborty, S., S. Kaur, S. Guha, and S. K. Batra. The multifaced roles of neutrophil gelatinase associated lipocalin (NGAL) in inflammation and cancer. Biochim. Biophys. Acta. 1826(1): 129-69. 2012.
8. Chapman, D., M. Newton, P. Sacco, and K. Nosaka. Greater muscle damage induced by fast versus slow velocity eccentric exercise. Int. J. Sports Med. 27: 591-598. 2006.
9. Clarkson, P. M., K. Nosaka, and B. Braun. Muscle function after exercise-induced muscle damage and rapid adaptation. Med. Sci. Sports Exerc. 24: 512-520. 1992.
10. Cowland, J. B., and N. Borregaard. Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics. 45(1): 17-23. 1997.
11. Halawa, A. The early diagnosis of acute renal graft dysfunction: A challenge we face. The role of novel biomarkers. Ann. Transplant. 16(1): 90-98. 2011.
12. Hamada, K., E. Vannier, J. M. Sacheck, A. L. Witsell, and R. Roubenoff. Senescence of human skeletal muscle impairs the local inflammatory cytokine response acute eccentric exercise. FASEB. J. 19: 264-266. 2005.
13. Jones, D., D. Newham, J. Round, and S. Tolfree. Experimental human muscle damage: morphological changes in relation to other indices of damage. J. Physiol. 375: 435-448. 1986.
14. Kanda, K., K. Sugama, H. Hayashida, J. Sakuma, Y. Kawakami, S. Miura, H. Yoshida, Y. Mori, and K. Suzuki. Eccentric exercise-induced delayed-onset muscle soreness and changes in markers of muscle damage and inflammation. Exerc. Immunol. Rev. 19: 74-87. 2013.
15. Lewin, G. R., and L. M. Mendell. Nerve growth factor and nociception. Trends Neurosci. 16: 353-359. 1993.

16. Ley, K., C. Landanna, M. I. Cybulsky, and S. Nourshargh. Getting to the site of inflammation: the leukocyte adhesion cascade update. Nat. Rev. Immunol. 7: 678-689. 2007.
17. MacIntyre, D. L., W. D. Reid, D. M. Lyster, I. J. Szasz, and D. C. Mckenzie. Presence of WBC, decreased strength, and delayed soreness in muscle after eccentric exercise. J. Appl. Physiol. 80: 1006-1013. 1996.
18. MacIntyre, D. L., W. D. Reid, D. M. Lyster, and D. C. Mckenzie. Different effects of strenuous eccentric exercise on the accumulation of neutrophils in muscle in women and men. Eur. J. Appl. Physiol. 81: 47-53. 2000.
19. Malm, C., P. Nyberg, M. Engstrom, B. Sjodin, R. Lenkei, B. Ekblom, and I. Lundberg. Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies. J. Physiol. 529: 243-262. 2000.
20. Maruhashi, Y., K. Kitaoka, Y. Yoshiki, R. Nakamura, A. Okano, K. Nakamura, T. Tsuyama, Y. Shima, and K. Tomita. ROS scavenging activity and muscle damage prevention in eccentric exercise in rats. J. Physiol. Sci. 57: 211-216. 2007.
21. Morrow, J. D., K. E. Hill, R. F. Burk, T. M. Nammour, K. F. Badr, and L. J. Roberts 2nd. A series of prostaglandin F_2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc. Natl. Acad. Sci. USA. Biochemistry. 87: 9383-9387. 1990.
22. Murase, S., E. Terazawa, F. Queme, H. Ota, T. Matsuda, K. Hirate, Y. Kozaki, K. Katanosaka, T. Taguchi, H. Urai, and K. Mizumura. Bradykinin and nerve growth factor play pivotal roles in muscular mechanical hyperalgesia after exercise (delayed-onset muscle soreness). J. Neurosci. 30(10): 3752-3761. 2010.
23. Nguyen, H. X., and J. G. Tidball. Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells *in vitro.* J. Physiol. 547: 125-132. 2003.
24. Nguyen H. X., and J. G. Tidball. Null mutation of gp91phox reduces muscle membrane lysis during muscle inflammation in mice. J. Physiol. 553: 833-841. 2003.
25. Nguyen, H. X., A. J. Lusis, and J. G. Tidball. Null mutation of myeloperoxidase in mice prevents mechanical activation of neutrophil lysis of muscle cell membranes *in vitro and in vivo*. J. Physiol. 565: 403-413. 2005.
26. Nieman, D. C., J. M. Davis, D. A. Henson, J. Walberg-Rankin, M. Shute, C. L. Dumke, A. C. Utter, D. M. Vinci, J. A. Carson, A. Brown, W. J. Lee, S. R. McAnulty, and L. S. McAnulty. Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run. J. Appl. Physiol. 94: 1917-1925. 2003.
27. Nosaka, K., M. Newton, and P. Sacco. Delayed-onset muscle soreness does not reflect the magnitude of eccentric exercise-induced muscle damage. Scand. J. Med. Sci. Sports. 12(6): 337-46. 2002.
28. Paulsen, G., U. R. Mikkelsen, T. Raastad, and J. M. Peake. Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? Exerc. Immunol. Rev. 18: 42-97. 2012.
29. Peake, J. M. Exercise-induced alterations in neutrophil degranulation and respiratory burst activity: possible mechanisms of action. Exerc. Immunol. Rev. 8: 49-100. 2002.
30. Peake, J., K. Nosaka, and K. Suzuki. Characterization of inflammatory responses to eccentric exercise in humans. Exerc. Immunol. Rev. 11: 64-85. 2005.

31. Pedersen, B. K., A. Steensberg, P. Kellller, C. Keller, C. Fischer, N. Hiscock, G. van Hall, P. Plomgaard, and M. Febbraio. Muscle-derived interleukin-6: lipolytic, antiinflammatory and immune regulatory effects. Eur. J. Physiol. 446(1): 9-16. 2003.
32. Peterson, J. M., T. A. Trappe, E. Mylona, F. White, C. P. Lambert, W. J. Evans, and F. X. Pizza. Ibuprofen and acetaminophen: effect on muscle inflammation after eccentric exercise. Med. Sci. Sports Exerc. 35: 892-896. 2003.
33. Proske, U., and T. J. Allen. Damage to skeletal muscle from eccentric exercise. Exerc. Sport Sci. Rev. 33: 98-104. 2005.
34. Raastad, T., B. A. Risoy, H. B. Benestad, J. G. Fjeld, and J. Hallen. Temporal relation between leukocyte accumulation in muscle and halted recovery 10-20 h after strength exercise. J. Appl. Physiol. 95: 2503-2509. 2003.
35. Rosen, S. D. Ligands for L-selectin: homing, inflammation, and beyond. Annu. Rev. Immunol. 22:129-156. 2004.
36. Roudkenar, M. H., R. Halabian, Z. Ghasemipour, A. M. Roushandeh, M. Rouhbakhsh, M. Nekogoftar, Y. Kuwahara, M. Fukumoto, and M. A. Shokrgozar. Neutrophil gelatinase-associated lipocalin acts as a protective factor against H(2)O(2)toxicity. Arch. Med. Res. 39: 560-566. 2008.
37. Roudkenar, M. H., Y. Kuwahara, T. Baba, A. M. Roushandeh, S. Ebishima, S. Abe, Y. Ohkubo, and M. Fukumoto. Oxidative stress induced lipocalin 2 gene expression: addressing its expression under the harmful conditions. J. Radiat. Res (Tokyo). 48: 39-44. 2007.
38. Round, J., D. Jones, and G. Cambridge. Cellular infiltration in human skeletal muscle: exercise induced muscle damage as a model of inflammatory muscle disease. J. Neurol. Sci. 82: 1-11. 1987.
39. Sorichter, S., J. Mair, A. Koller, M. M. Pelsers, B. Puschendorf, and J. F. Glatz. Early assessment of exercise induced skeletal muscle injury using plasma fatty acid binding protein. Br. J. Sports Med. 32: 121-124. 1998.
40. Stupka, N., M. A. Tarnopolsky, N. J. Yardley, and S. M. Phillips. Cellular adaptation to repeated eccentric exercise-induced muscle damage. J. Appl. Physiol. 91: 1669-1678. 2001.
41. Sugama, K., K. Suzuki, K. Yoshitani, K. Shiraishi, and T. Kometani. IL-17, neutrophil activation and muscle damage following endurance exercise. Exerc. Immunol. Rev. 18: 116-127. 2012.
42. Suzuki, K., S. Naganuma, M. Totsuka, K. J. Suzuki, M. Mochizuki, M. Shiraishi, S. Nakaji, and K. Sugawara. Effects of exhaustive endurance exercise and its one-week daily repetition on neutrophil count and functional status in untrained men. Int. J. Sports Med. 17: 205-212. 1996.
43. Suzuki, K., H. Sato, T. Kikuchi, T. Abe, S. Nakaji, K. Sugawara, M. Totsuka, K. Sato, and K. Yamada. Capacity of circulating neutrophils to produce reactive oxygen species after exhaustive exercise. J. Appl. Physiol. 81: 1213-1222. 1996.
44. Suzuki, K., K. Totsuka, S. Nakaji, M. Yamada, S. Kudoh, Q. Liu, K. Sugawara, K. Yamaya, and K. Sato. Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. J. Appl. Physiol. 87: 1360-1367. 1999.
45. Triebel, S., J. Blaser, H. Reinke, and H. Tschesche. A 25 kDa 2-microglobulin-related protein is a component of the 125 kDa form of human gelatinase. FEBS. Lett. 314: 386-388.1992.

46. Uchida, M. C., K. Nosaka, C. Ugrinowitsch, A. Yamashita, E. Martines, A. S. Moriscot, and M. S. Aoki. Effect of bench press exercise intensity on muscle soreness and inflammatory mediators. J. Sports Sci. 27(5): 499-507. 2009.
47. Van Nieuwenhoven, F. A., A. H. Kleine, W. H. Wodzig, W. T. Hermens, H. A. Kragten, J. G. Maessen, C. D. Punt, M. P. van Dieijen, G. I. van der Vusse, and J. F. Glatz. Discrimination between myocardial and skeletal muscle injury by assessment of the plasma ratio of myoglobin over fatty acid-binding protein. Circulation 10: 304-309. 1995.
48. Warren, G. L., D. A. Lowe, and R. B. Armstrong. Measurement tools used in the study of eccentric contraction-induced injury. Sports Med. 27: 43-59. 1999.
49. Woolf, C. J., B. Safieh-Garabedian, Q.-P. Ma, P. Crilly, and J. Winter. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62: 327-331. 1994.
50. Zimmerman, A. W., and J. H. Veerkamp. New insights into the structure and function of fatty acid binding proteins. Cell Mol. Life Sci. 59:1096-1116. 2002.

Exploring the importance of translational regulation in the inflammatory responses by a genome-wide approach

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ABSTRACT

It is widely recognized that exercise has an important role in inflammation regulation. To understand inflammatory mechanisms, extensive studies on the transcriptome and proteome have been conducted. However, interpreting these results is difficult, partly due to technical difficulties that impose some restriction on the accuracy and comprehensiveness of measurements. Here we first mention some limitations of studies involving large scale proteomics and high-throughput transcriptomics and further introduce a newly developed genome-wide translational analysis which may overcome some of the limitations and discover novel cellular dynamics. We then show preliminary results obtained by conducting a genome-wide translational analysis of the early inflammatory response of macrophages in response to lipopolysaccharide (LPS), and discuss the potential to identify novel factors by employing a genome-wide translational analysis.

Key words: translational regulation, translational isoform, up stream open reading frame (uORF), inflammation, macrophages, gene expression

INTRODUCTION

Exercise has numerous benefits for health and physical fitness, and in particular has proven effective in the management of numerous disease states associated with chronic inflammation, including obesity and diabetes. While research has investigated the anti-inflammatory effects of exercise, the complicated mechanisms are yet to be fully understood. In an attempt to understand the anti-inflammatory effect of exercise, and to reveal the underlying molecular mechanisms,

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genome-wide analyses such as microarray, high-throughput mRNA sequencing (mRNA-Seq), and proteomics are promising tools.

Limitation in large-scale proteomics

While large-scale proteomics techniques may provide further insight into molecular mechanisms there are some limitations to consider. One limitation is the underestimation of low-abundance proteins (19, 31). In mass spectrometry-based proteomics, liquid chromatography and/or two-dimensional gel electrophoresis are typically employed to separate proteins at a high-resolution level. On a two-dimensional gel, the appearance of a low-abundance protein can be masked by other high-abundance proteins (19, 31). In the case of proteins associated with cytokine responses, many physiologically meaningful proteins, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α, are produced at very low concentrations. Concentrations of other abundant proteins, such as α_2-macroglobulin and serum albumin, may be up to 12 orders of magnitude higher than those of cytokines (3, 6, 20). Although the sensitivity to detect less abundant proteins can be improved by using an immunoaffinity column to remove highly abundant proteins, efficiency of removal is only 80 to 90 %, meaning that there is still a large excess of 10^{11} proteins (7, 20). In addition, proteins that interact with the target proteins to be removed may also be filtered out, leading to an unfavorable sample bias (7). These issues may lower the possibility to identify less abundant proteins in a large-scale proteomics.

Limitation in microarray/mRNA-Seq

Detection of low-abundance proteins can be achieved by the use of microarray and mRNA-Seq. However, one concern is that these techniques do not account for post-transcriptional modification (11). Many studies compared protein levels and microarray/mRNA-Seq data and showed low correlations between them (R^2 = 0.17 - 0.41), suggesting that the mRNA level is not always sufficient to estimate its protein abundance (8, 10, 18, 28, 29). One study further revealed that this low correlation is mainly due to the ignorance of translational events, reporting a correlation coefficient of R^2 = 0.41 between mRNA and protein levels whereas

Table 1. Advantages and disadvantages of large-scale proteomics, microarray/mRNA-Seq, and ribosome profiling.

	Advantages	Disadvantages
Large-scale proteomics	- Direct quantification	- Underestimation of low-abundance proteins
Microarray / mRNA-Seq	- Genome-wide quantification including low-abundance factors	- Low correlation between mRNA and protein abundance
Ribosome profiling	- Genome-wide quantification including low-abundance factors - Better estimate of protein abundance - Able to identify translational dynamics	- Substantial amount of rRNA and sequence read loss

measured translation rates and protein abundance were very strongly correlated ($R^2 = 0.95$) (22). This finding indicates that translation rates certainly exert significant influence on protein abundance and that post-translation has only a nominal effect (22). Given the above, it is evident that the translation rate regulated by post-transcriptional control is a critical determinant of protein abundance.

Advantages in ribosome profiling

A recently developed strategy, named ribosome profiling, provides a robust measurement of translational profile. Ribosome profiling was first innovated by Ingolia et al., in 2009 (11). This method achieves a technical breakthrough by deep-sequencing ribosome-protected mRNA fragments (RPF) and quantitating ribosome density on mRNA (11). This mRNA-Seq-based ribosome profiling (or RPF sequencing) provides a powerful genome-wide approach that is reproducible and comprehensive qualities which are lacking in large-scale proteomics. The direct measurement of RPF is able to achieve a better estimate of protein abundance and also monitor translational dynamics such as frame-shifted protein synthesis, translational isoform of proteins, and the alteration of translation-dependent protein synthesis without changing mRNA levels (11, 12, 17). Thereby, it is likely that some unrevealed factors attributable to translational regulation can be discovered and that they may have roles in the anti-inflammatory effects of exercise. Here we introduce ribosome profiling (originally from Ingolia et al. (11)) and present preliminary data obtained using this method to investigate the early inflammatory response in macrophages exposed to lipopolysaccharide (LPS).

METHODS

The simplified workflow of ribosome profiling modified for this study is as follows (Fig. 1): First, cultured cells are incubated with cycloheximide (Sigma, St. Louis, MO) to stall translating ribosomal complex on the mRNA. Total RNA is then extracted and digested by RNase If. Because mRNA fragments encompassed by the ribosome are not digested, they are further purified as monosome by sucrose cushion. Gel purification is then carried out to extract RPF. As RNase If digestion dephosphorylates the 5' end and phosphorylates the 3' end, both ends are phosphorylated and dephosphorylated, respectively. Sequence library preparation for RPF is then conducted and sequenced by a next generation sequencer.

Total RNA extraction for mRNA-Seq and RPF sequencing

RAW 264 macrophages (DS Pharma Biomedical, Osaka, Japan) were cultured at a concentration of 2.5 x 10^5 cells/ml in media (DMEM, 2 mM Glutamine, 10% FBS, 100 units penicillin and 100 μg/mL streptomycin) for 24 hours and then harvested. Ensuring cells were approximately 80 ~ 90% confluency, 100 ng/ml LPS (*E. coli* O55:B5; Sigma) or vehicle (DMEM) was added into the media and incubated for 30 min. To stall ribosomal complex on mRNA, cycloheximide (final concentration of 100 μg/ml) was added and cells were incubated for 5 min, followed by washing with PBS (including 100 μg/ml cycloheximide). Cells were lysed in 400 μl lysis buffer (20 mM TrisHCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 μg/ml cycloheximide, TURBO DNase I 25 U/ml

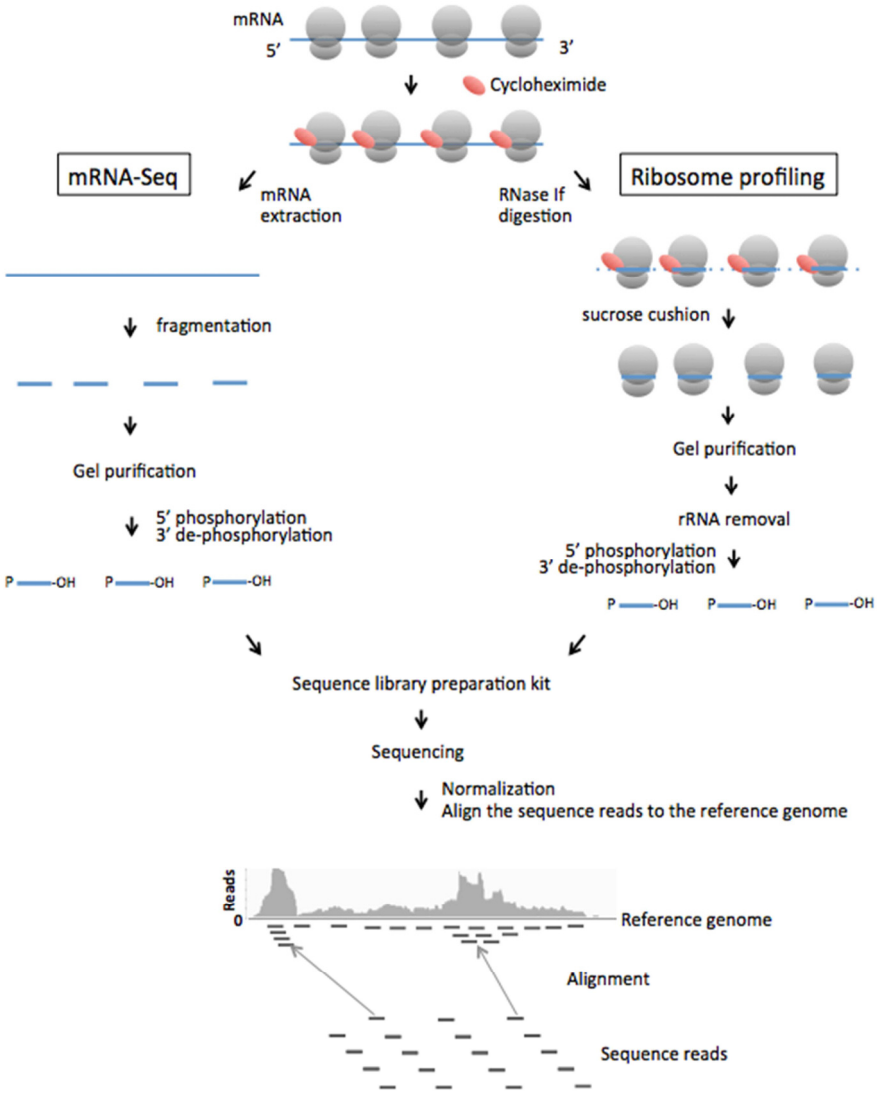

Fig. 1. The concept of ribosome profiling
The simplified workflow of ribosome profiling is shown.

(Ambion, Life Technologies, Carlsbad, CA), 1% Triton X-100 (Sigma)), followed by trituration through a 26-G needle. After centrifugation at 20,000 *g* for 10 min at 4°C, the supernatant was collected.

RNA fragmentation for mRNA-Seq

After purification of total RNA by miRNeasy Mini Kit (Qiagen, Hilden, Ger-

many), poly(A) RNA extraction was carried out by Dynabeads mRNA DIRECT Micro Kit (Ambion). For fragmentation, 2x alkaline buffer (90 mM $NaHCO_3$, 10 mM Na_2CO_3, 2 mM EDTA) was mixed with mRNA and incubated at 80°C for 15 min. The reaction was stopped by adding an ice-cold solution (1.5 µl GlycoBlue (Ambion), 10 µl 3M NaOAc, 48.5 µl RNase-free water) and 150 µl isopropanol, followed by standard precipitation protocols.

Ribosome protected mRNA fragment (RPF) purification

To first digest mRNA which was not protected by the ribosomal complex, 15 µl RNase If (New England Biolabs, Beverly, MA) was added to the total RNA lysate and incubated for 45 min at room temperature. The reaction was stopped by adding 10 µl SUPERase· In (Ambion). While digesting, sucrose cushion buffer (34% sucrose, 20 mM TrisHCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µg/ml cycloheximide, 20 U/ml SUPERase· In) was prepared and loaded into a polycarbonate tube (Beckman Coulter, Palo Alto, CA). The digested sample was then loaded onto the sucrose cushion at a ratio of 1:3. After centrifugation for 4 hours at 400,000 *g*, 4°C in a MLA130 rotor (Beckman Coulter), the pellet was purified by miRNeasy Mini Kit, followed by precipitation as described previously.

Size selection of RPF and fragmented mRNA

The mixture of 2x Novex TBE-Urea sample buffer (Invitrogen, Life Technologies, Carlsbad, CA) and each purified RNA sample, small RNA II Marker (Funakoshi, Tokyo, Japan), and synthesized oligo markers (Greiner, Tokyo, Japan, 32 nucleotide (nt) upper marker: 5'-AUGUACACGGAGUCGAGCUCAACCCGCAACGA, 26nt lower marker: 3'-AUGUACACGGAGUCGACCCGCAACGA, both 3' ends were phosphorylated and both 5' ends were dephosphorylated) was heat-denatured at 70°C for 3 min. Samples were then loaded on a denaturing 15% polyacrylamide TBE-urea gel (Invitrogen) and run for 65 min. The gel was stained with SYBR Gold (Invitrogen) for 5 min. Specific regions (26 ~ 32nt for RPF and 25 ~ 45nt for mRNA-Seq) were excised and disrupted, followed by incubation in 360 µl RNase-free water at 70°C for 10 min. All gel and liquid were transferred into a Spin-X column (Corning, NY), followed by centrifugation at 20,000 *g* for 2 min. Precipitation was carried out as described previously.

rRNA deletion, 3'dephosphorylation, and 5' phosphorylation

Because RPF samples contain a significant amount of ribosomal RNA (rRNA), rRNA removal was conducted using RiboMinus (Invitrogen). After rRNA deletion, the total amount of RPF was reduced to approximately 1/200. To prepare samples for adaptor ligations, both samples (RPF and randomly fragmented mRNA) were 3'-dephosphorylated and 5'-phosphorylated. Dephosphorylation was first carried out at 37°C for 1 hour in a reaction mixture (T4 polynucleotide kinase buffer without ATP, 10 U/µl T4 polynucleotide kinase (New England Biolabs), 20 U/µl SUPERase· In), followed by the addition of 1 mM ATP (New England Biolabs) and immediate 5' phosphorylation for 30 min. Precipitation was then carried out to concentrate samples for sequence library preparation.

Sequence library preparation
Ion Total RNA-Seq Kit v2 (Ion Torrent, Life Technologies, Carlsbad, CA) was used to prepare the sequence library according to the manufacturer's instructions. However, because of the difficulty of differentiating target fragments and primer dimmers mainly coming from reverse transcription PCR, gel purification was conducted to purify a target product. After running the gel, the target region, around 63nt (fragmented sample size: 30nt and ligated adaptors: 33nt), was excised, followed by RNA purification from the gel as described previously and isopropanol precipitation.

Sequencing
For emulsion PCR, the sequence library was processed by Ion PGM Template OT2 200 Kit (Ion Torrent). Sequencing was then carried out by Ion PGM sequencer (Ion Torrent), Ion PGM Sequencing 200 Kit v2 (Ion Torrent) and Ion 318 Chip Kit v2 (Ion Torrent) according to the manufacturer's protocols. Sequenced reads were mapped to reference genomes (mm10 and Coding DNA sequence (CDS) created from RefSeq mRNA). RefSeq mRNA (http://www.ncbi.nlm.nih.gov/refseq/) and mm10 (http://genome.ucsc.edu) genome was obtained. CDS was created by the use of Biomart-MartView (http://www.biomart.org/biomart/martview) (13). As for the reads mapped to multiple locations, the best hit read was selected. Sequenced reads aligned to mm10 were visualized by Integrative Genome Viewer (27).

Data analysis
Sequenced reads aligned to CDS were normalized by CDS length and library size (Reads Per Million per Kilobase of CDS (RPKM)). Unique genes ≥ 15 RPKM in at least one of the four conditions (RPF sequencing without LPS, RPF sequencing LPS 30 min, mRNA-Seq without LPS, mRNA-Seq LPS 30 min) were further analyzed. Pair-wise t tests were used to compare read ratios in 3nt periodicity and *P* values were adjusted using a Bonferroni correction. Pearson's correlation coefficients were also calculated.

RESULTS AND DISCUSSION

Extensive studies have been carried out using proteomics and transcriptomics. In addition to these two major omics, the use of translational profile is now gaining attention. Ribosome profiling, sequencing RPF, now makes it possible to conduct robust measurements of translational dynamics and may reveal novel perspectives of cellular regulation. We conducted a genome-wide translational analysis by ribosome profiling. Our preliminary data suggest the potential to discover novel factors that may have critical roles in regulating inflammatory and anti-inflammatory responses to exercise.

Genome-wide detection of translational dynamics
Large-scale proteomics is limited by its lack of genome-wide detection and reproducibility of results. In the present study, however, mRNA-Seq-based RPF sequencing was reproducible. A very strong correlation coefficient, $R^2 = 0.96$,

was obtained from the two complete biological replicates (both LPS stimulated) including genes with low translation (≥ 15 RPKM). Although there are some sequencing biases depending on genome sequence, these biases are also highly reproducible. These biases were well conserved over two completely different sequencing runs (samples from LPS stimulated and without LPS) (Fig. 2), indicating that they are less likely to influence the quantification of translation between different conditions.

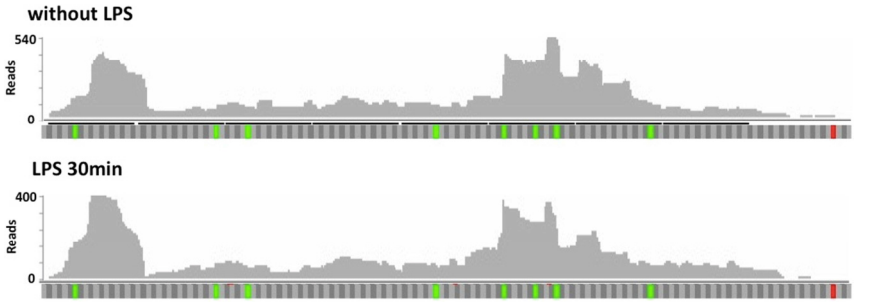

Fig. 2. Reproducibility of mRNA-Seq-based RPF sequencing
Reproducible bias of read coverage in a randomly selected gene in different conditions is shown. The y-axis and x-axis are read coverage and coding DNA sequence (CDS), respectively. The main CDS frame is shown below the x-axis. Light green and red squares indicate a start and stop codon, respectively. The peaks of read coverage show significant fluctuations, indicating sequence bias. However, the similar bias is also observed in a different condition, suggesting that the bias is less likely to influence the quantification of translation.

Using mRNA-Seq-based RPF sequencing more than 1500 genes were detected as moderate to highly translated genes (≥ 100 RPKM). When including those that were less translated (≥ 15 RPKM) nearly 6000 genes were detected. The number of genes detectable largely depends on two factors: sequencer performance and rRNA contamination. We used an Ion PGM sequencer designed to generate up to 10 million reads although if a sequencer capable of producing more reads (e.g., can be 200 million) is used, more genes should be quantified. As reported in previous studies, significant amounts of rRNA (40 ~ 90% of total reads) in the sequence library prevent reads from being sequenced and mapped to actual CDS (9, 11, 26). In our study, approximately half of the mapped reads (~ 48%) came from rRNA. However, we still acquired an adequate number of genes with sufficient reproducibility. Taking advantage of this robust analysis, the importance of translational regulation has been revealed in different fields, such as the mammalian cell cycle (25), nematode developmental transition (24), and maternal to zygote transition in zebrafish (14). In the field of exercise immunology it is now possible to investigate translational regulation induced during and after exercise.

In exercise, translational regulation is dynamic and complex. This is mainly because of different exercise types and the orchestrated signaling cascades of both positive and negative regulators of mammalian target of rapamycin (mTOR) signaling. mTOR signaling is known to play a key role in regulating translation

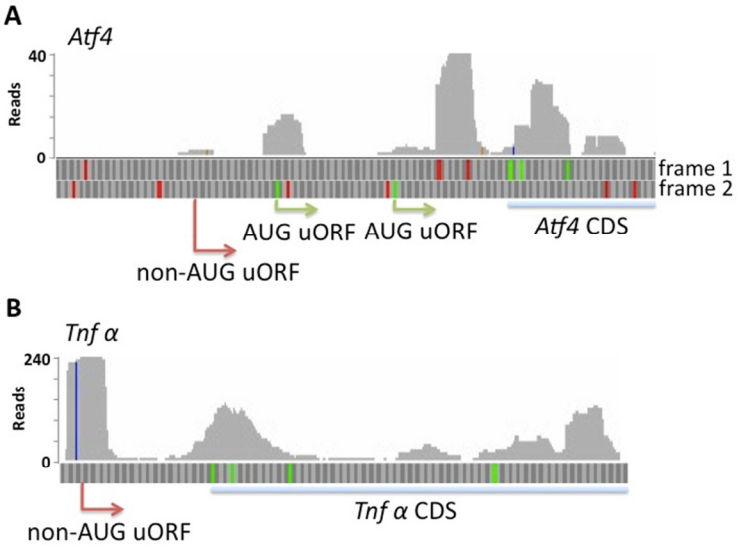

Fig. 3. Upstream open reading frame (uORF) in *Atf4* and *Tnf-α*
Previously identified uORF start sites in Atf4 (A) were also identified. A uORF start site in *Tnf-α* recognized in our study is shown (B). As in Fig. 2, light green and red squares indicate a start and stop codon, respectively. Frame 1 is the main *Atf4* CDS. Although it was difficult to investigate uORFs, it is now possible to monitor the translational dynamics of each uORF depending on different conditions.

by inhibiting a translational inhibitor, eukaryotic initiation factor 4E binding protein (4EBP). A recent study suggests that mTOR has some influence on more than 99% of the translation of mRNAs (26). mTOR in exercise has been well studied focusing especially on its signaling pathway. It is known that resistance exercise upregulates mTOR signaling mediated by protein kinase B (PKB) and concomitantly decreases 4EBP activation, resulting in increased protein synthesis (1). However, in the case of endurance exercise both positive and negative regulators of mTOR signaling are activated. As a positive regulator, extracellular signal-regulated kinase (ERK) 1/2, a member of the mitogen-activated protein kinase (MAPK) family, promotes mTOR signaling by inhibiting a mTOR suppressor complex (30). In turn, protein kinase A (PKA)-mediated activation of AMP-activated kinase (AMPK) shows a mTOR inhibitory effect by promoting the suppressor complex, leading to 4EBP activation (1, 2). Despite the extensive investigation of mTOR signaling cascades, its global impact on translation is still poorly understood. Ribosome profiling has the potential to capture the actual effect of both positive and negative mTOR signaling on each mRNA, and to analyze factors that are translationally activated or suppressed on a global scale.

Other unique features detected by ribosome profiling

In addition to genome-wide detection of translation, unique features of RPF sequencing help to identify totally new cellular regulation such as upstream open

reading frame (uORF), translational isoform of proteins, and frame-shifted protein synthesis (11, 12, 17). uORF is the translated upstream region of an annotated start codon. There are AUG-initiated and non-AUG-initiated uORFs and at least one uORF is found in approximately 50% of transcripts in mammals (16). In our study, many uORFs were recognized, including previously known uORFs, such as activating transcription factor 4 (*Atf4*) (12) and *Tnf-α* (Fig. 3). uORF is known to play a role in translational regulation. Most uORFs negatively regulate translation of the main CDS by reducing ribosomal re-initiation (4, 12, 23). For example, a uORF regulates the translation of β-site amyloid precursor protein cleaving enzyme 1 *(Bace1)*, mediating the excessive accumulation of β amyloid found in the pathology of Alzheimer's disease (32). In normal conditions, the 4th uORF of 6 *Bace1* uORFs is translated. This translation of the 4th uORF reduces the translation rate of *Bace1* CDS, consequently leading to the repression of β amyloid accumulation (32). It was difficult to monitor uORFs on a global scale. As shown in Fig. 3, however, physiologically important uORFs can be discovered by RPF sequencing and it is possible to investigate the expression and regulation of uORFs.

In addition to a suppressive effect on main CDS translation, uORFs regulate the synthesis of distinct protein isoforms. In the case of discs large (Drosophila) homolog-associated protein 3 *(Dlgap3)* (which is involved in mammalian synaptic spasticity), a uORF seems to mediate the alternative translation of *Dlgap3*, resulting in the synthesis of different *Dlgap3* isoforms from one transcript (5). These translational isoforms can be recognized by RPF sequencing. We identified a translational isoform in cluster of differentiation 14 (*Cd14*), derived from N-ter-

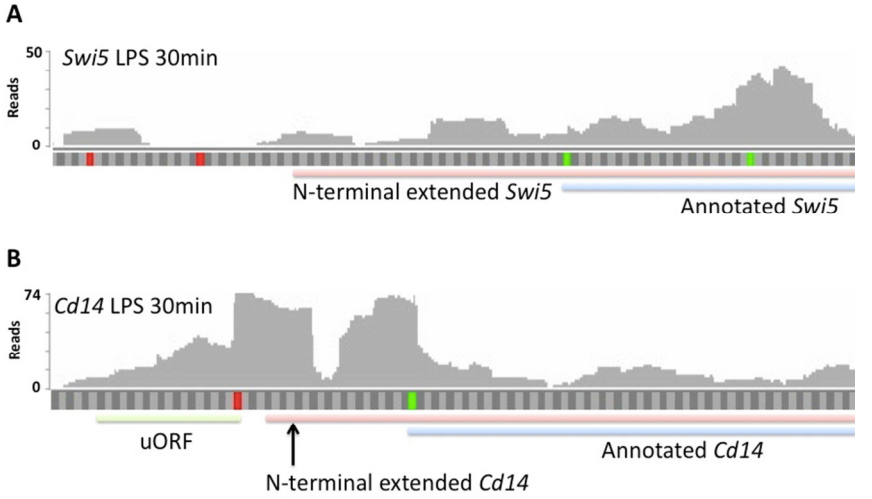

Fig. 4. N-terminal extensions
A previously known N-terminal extension in Swi5 recombination repair homolog (yeast) (*Swi5*) was also identified (A). A N-terminal extension in *Cd14* is shown (B). As in Fig. 2, light green and red squares indicate a start and stop codon, respectively. There was no tool available to detect translational isoforms on a global scale. However, ribosome profiling makes it possible to identify and further investigate them.

minal extension mediated by uORF (Fig. 4). The extension consists of 18 extra amino acids, with poor water solubility estimated by its iso-electric points and peptide length. As CD14 is a co-receptor of toll-like receptor 4, a receptor of LPS, this hydrophobic extension may have a role in regulating signal transition or may have an impact on its turn-over rate from the cellular membrane. As shown here, it is possible to identify translational isoforms by RPF sequencing. It may be interesting to investigate its physiological importance and the dynamics of isoform formation responding to external stimuli such as acute exercise and chronic exercise training.

Another unique feature in RPF sequencing is 3nt periodicity of aligned reads. This may make it possible to discover frame-shifted protein synthesis. A frame-shifted protein synthesis can cause a severe translational malfunction. Although a gene is expressed and the mRNA is associated with the ribosomal complex, frame-shifted translation may synthesize dysfunctional proteins. Contrary to the negative effect, it is also reported that a frame-shifted ribosome results in coding a new frame and synthesizing functional proteins. Ornithine decarboxylase (ODC) antizyme is a negative feedback regulator of ODC which is responsi-

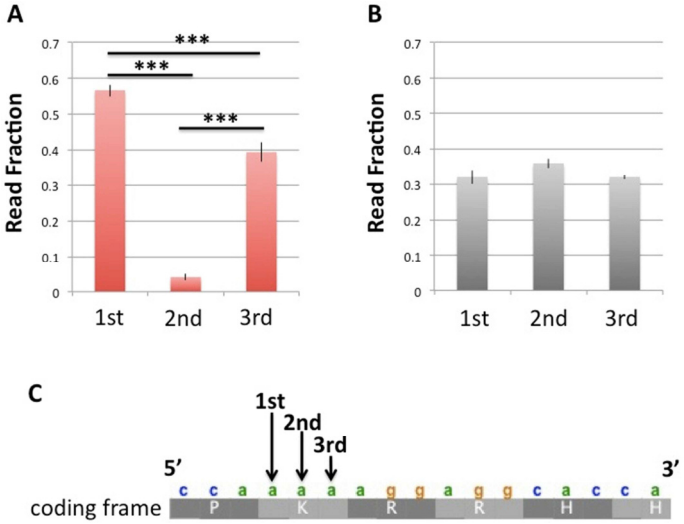

Fig. 5. Characteristics seen in PRF sequencing
A triplet periodicity is seen in RPF sequencing (A) but not seen in mRNA-Seq (B). 1st, 2nd, and 3rd in (A, B) indicate the 5' end of the sequenced reads mapped to 1st, 2nd, and 3rd positions of the codon in a main coding frame (C). Ribosome moves 3nt by 3nt when it translates the codon. Because RPF sequencing captures the mRNA fragments encompassed by translating ribosomes, the sequence reads show 3nt periodicity. In contrast mRNA-Seq sequences randomly fragmented mRNAs (irrelevant to ribosomal movement), and so do not show 3nt periodicity. Reads were randomly selected from the same regions of *Tnf-α* CDS for comparing RPF sequencing and mRNA-Seq. Means ± SD of two biological replicates. *** ($P < 0.01$).

ble for polyamine synthesis (21). The elevated concentration of polyamine triggers a +1 frameshift and frame-shifted protein synthesis of ODC antizyme, promoting the destabilization and degradation of ODC to regulate polyamine levels (15). A frame-shifted protein can gain a function responding to the environment. Such dynamics of frame-shifted protein synthesis can also be detected by RPF sequencing. In RPF sequencing, it is known that the 5' ends of RPF are likely to align mostly to the 1st position of a codon of a main coding frame. In contrast, the 5' ends of the reads from mRNA-Seq equally align to all the three (1st, 2nd, and 3rd) positions of a codon (11, 12). In the present study we also observed this characteristic (Fig. 5). Due to this strong periodicity, it is now possible to monitor the sophisticated dynamics of frame-shifted protein synthesis and its physiological importance (17).

CONCLUSIONS

In this manuscript we introduce a recently developed strategy, named ribosome profiling, in which RPF is deep-sequenced. This strategy makes it possible to conduct a genome-wide translational analysis with precise and accurate measurements at a sub-codon resolution. This robust measurement may reveal novel molecular factors, including translational regulation, uORF, translational isoforms, and frame-shifted translation, which have not been discovered by large-scale proteomics, microarray, or mRNA-Seq. It will be intriguing to investigate the actual roles of these factors in the immune response to exercise.

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REFERENCES

1. Atherton P, Babraj J, Smith K, Singh J, Rennie M, and Wackerhage H. Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation. FASEB J 19: 786-788, 2005.
2. Bolster D, Crozier S, Kimball S, and Jefferson L. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. J Biol Chem 277: 23977-23980, 2002.
3. Bullo M, Garcia-Lorda P, Megias I, and Salas-Salvado J. Systemic inflammation, adipose tissue tumor necrosis factor, and leptin expression. Obes Res 11: 525-531, 2003.

4. Chatterjee S, and Pal JK. Role of 5'- and 3'-untranslated regions of mRNAs in human diseases. Biol Cell 101: 251-262, 2009.
5. Chua JJ, Schob C, Rehbein M, Gkogkas CG, Richter D, and Kindler S. Synthesis of two SAPAP3 isoforms from a single mRNA is mediated via alternative translational initiation. Sci Rep 2: 484, 2012.
6. Dobbs RJ, Charlett A, Purkiss AG, Dobbs SM, Weller C, and Peterson DW. Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism. Acta Neurol Scand 100: 34-41, 1999.
7. Elliott MH, Smith DS, Parker CE, and Borchers C. Current trends in quantitative proteomics. J Mass Spectrom 44: 1637-1660, 2009.
8. Greenbaum D, Colangelo C, Williams K, and Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 4: 117, 2003.
9. Guo H, Ingolia NT, Weissman JS, and Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466: 835-840, 2010.
10. Gygi SP, Rochon Y, Franza BR, and Aebersold R. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19: 1720-1730, 1999.
11. Ingolia NT, Ghaemmaghami S, Newman JR, and Weissman JS. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science 324: 218-223, 2009.
12. Ingolia NT, Lareau LF, and Weissman JS. Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell 147: 789-802, 2011.
13. Kasprzyk A. BioMart: driving a paradigm change in biological data management. Database (Oxford) 2011: bar049, 2011.
14. Lee M, Bonneau A, Takacs C, Bazzini A, Divito K, Fleming E, and Giraldez A. Nanog, Pou5f1 and SoxB1 activate zygotic gene expression during the maternal-to-zygotic transition. Nature 2013.
15. Matsufuji S, Matsufuji T, Miyazaki Y, Murakami Y, Atkins JF, Gesteland RF, and Hayashi S. Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. Cell 80: 51-60, 1995.
16. Medenbach J, Seiler M, and Hentze MW. Translational control via protein-regulated upstream open reading frames. Cell 145: 902-913, 2011.
17. Michel AM, Choudhury KR, Firth AE, Ingolia NT, Atkins JF, and Baranov PV. Observation of dually decoded regions of the human genome using ribosome profiling data. Genome Res 22: 2219-2229, 2012.
18. Nie L, Wu G, and Zhang W. Correlation between mRNA and protein abundance in Desulfovibrio vulgaris: a multiple regression to identify sources of variations. Biochem Biophys Res Commun 339: 603-610, 2006.
19. Ohlendieck K. Skeletal muscle proteomics: current approaches, technical challenges and emerging techniques. Skelet Muscle 1: 6, 2011.
20. Paulo JA, Kadiyala V, Banks PA, Steen H, and Conwell DL. Mass spectrometry-based proteomics for translational research: a technical overview. Yale J Biol Med 85: 59-73, 2012.
21. Pegg AE. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 234: 249-262, 1986.
22. Schwanhausser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, and Selbach M. Global quantification of mammalian gene expression control. Nature 473: 337-342, 2011.

23. Sonenberg N, and Hinnebusch AG. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell 136: 731-745, 2009.
24. Stadler M, and Fire A. Conserved translatome remodeling in nematode species executing a shared developmental transition. PLoS Genet 9: 2013.
25. Stumpf C, Moreno M, Olshen A, Taylor B, and Ruggero D. The Translational Landscape of the Mammalian Cell Cycle. Mol Cell 2013.
26. Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, and Sabatini DM. A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485: 109-113, 2012.
27. Thorvaldsdottir H, Robinson JT, and Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 14: 178-192, 2013.
28. Tian Q, Stepaniants SB, Mao M, Weng L, Feetham MC, Doyle MJ, Yi EC, Dai H, Thorsson V, Eng J, Goodlett D, Berger JP, Gunter B, Linseley PS, Stoughton RB, Aebersold R, Collins SJ, Hanlon WA, and Hood LE. Integrated genomic and proteomic analyses of gene expression in Mammalian cells. Mol Cell Proteomics 3: 960-969, 2004.
29. Washburn MP, Koller A, Oshiro G, Ulaszek RR, Plouffe D, Deciu C, Winzeler E, and Yates JR, 3rd. Protein pathway and complex clustering of correlated mRNA and protein expression analyses in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 100: 3107-3112, 2003.
30. Williamson D, Kubica N, Kimball S, and Jefferson L. Exercise-induced alterations in extracellular signal-regulated kinase 1/2 and mammalian target of rapamycin (mTOR) signalling to regulatory mechanisms of mRNA translation in mouse muscle. J Physiol 573: 497-510, 2006.
31. Wittmann-Liebold B, Graack HR, and Pohl T. Two-dimensional gel electrophoresis as tool for proteomics studies in combination with protein identification by mass spectrometry. Proteomics 6: 4688-4703, 2006.
32. Zhou W, and Song W. Leaky scanning and reinitiation regulate BACE1 gene expression. Mol Cell Biol 26: 3353-3364, 2006.

Alkaloids and athlete immune function: Caffeine, theophylline, gingerol, ephedrine, and their congeners

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ABSTRACT

Plant alkaloids are found in foods, beverages, and supplements consumed by athletes for daily nutrition, performance enhancement, and immune function improvement. This paper examined possible immunomodulatory roles of alkaloids in exercise contexts, with a focus on human studies. Four representative groups were scrutinized: (a) caffeine (guaranine, mateine); (b) theophylline and its isomers, theobromine and paraxanthine; (c) ginger alkaloids including gingerols and shogaol; and (d) ephedra alkaloids such as ephedrine and pseudoephedrine. Emerging or prospective alkaloid sources (Goji berry, Noni berry, and bloodroot) were also considered. Human in vitro and in vivo studies on alkaloids and immune function were often conflicting. Caffeine may be immunomodulatory in vivo depending on subject characteristics, exercise characteristics, and immune parameters measured. Caffeine may exhibit antioxidant capacities. Ginger may exert in vivo anti-inflammatory effects in certain populations, but it is unclear whether these effects are due to alkaloids or other biochemicals. Evidence for an immunomodulatory role of alkaloids in energy drinks, cocoa, or ephedra products in vivo is weak to nonexistent. For alkaloid sources derived from plants, variability in the reviewed studies may be due to the presence of unrecognized alkaloids or non-alkaloid compounds (which may themselves be immunomodulatory), and pre-experimental factors such as agricultural or manufacturing differences. Athletes should not look to alkaloids or alkaloid-rich sources as a means of improving immune function given their inconsistent activities, safety concerns, and lack of commercial regulation.

Keywords not in title: cocoa; ephedra; ginger; guarana; Sanguinaria

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FOREWORD

Many beverages, foods, and supplements consumed by athletes as ergogenic aids or health enhancers contain alkaloids (25, 38, 107). Alkaloids are simplistically characterized as nitrogen-based basic molecules, typically containing one heterocyclic ring, and originating from botanical or fungal sources. Examples include caffeine, morphine, and nicotine. Molecules such as adrenaline (epinephrine) and 5-hydroxytryptamine (serotonin) fit the structural definition of alkaloids, but are not considered members because they are produced only by animals; some amino acids and nucleic acids also technically fit the chemical description but are excluded based on historical conventions.

Several alkaloids have documented or purported immunomodulatory effects in humans (35, 57, 64, 131). For athletes experiencing immunodepression consequent to their intense training regimens (45, 49, 128), alkaloid-containing supplements may be an appealing nutritional countermeasure. Rather than encyclopedically canvass all known alkaloids consumed by athletes, this review will instead focus on the evidence supporting (or refuting) claims of immunomodulation from four representative alkaloids or alkaloid sources (Figure 1): caffeine (including pure and plant sources, such as guarana, mate, and tea); theophylline, including its isomers theobromine and paraxanthine (from cocoa); ephedrine and pseudoephedrine (from ephedra); and gingerols, shogaols, and paradols (from ginger). These four groups are diverse in their efficacy, safety, indicated uses, botanical origins, nutritional sources, current usage patterns, and current knowledge bases. After reviewing these four groups, some emerging or prospective alkaloid-rich herbs are discussed, together with the need for an interdisciplinary approach when studying alkaloids and athlete immune function.

To identify literature appropriate to the review, searches were conducted in both PubMed and Google Scholar from late July-early September 2013 as illustrated in Figure 2. All possible combinations were searched, resulting in a total of 220 unique permutations (or 440 independent searches since two databases were utilized). To further limit the scope of the review, only studies that were written in English, published in peer-reviewed journals or books, and pertaining to human data or a review of human data were considered for inclusion. Resulting hits were screened manually for relevance to the topic of alkaloids and athlete immune function; thus, papers pertaining to ergogenic benefits, cross-reactions with other substances, and doping regulations were generally omitted. Other sources may be consulted for a broad overview of performance-enhancing substances (4, 36), specific herbs used by athletes (25) and in athlete immune function (108), or molecular aspects of immunomodulatory herbs used by athletes (105).

CAFFEINE

Caffeine (Figure 1) is a methylxanthine alkaloid that can be ingested in pill or supplement form, or as a component of beverages including those that consumers readily associate with plants (Table 1), energy drinks, or soft drinks (Table 2). **Guaranine** and **mateine** are the same molecule as caffeine, named after the plant taxa from which they were originally isolated (Table 1). Caffeine is

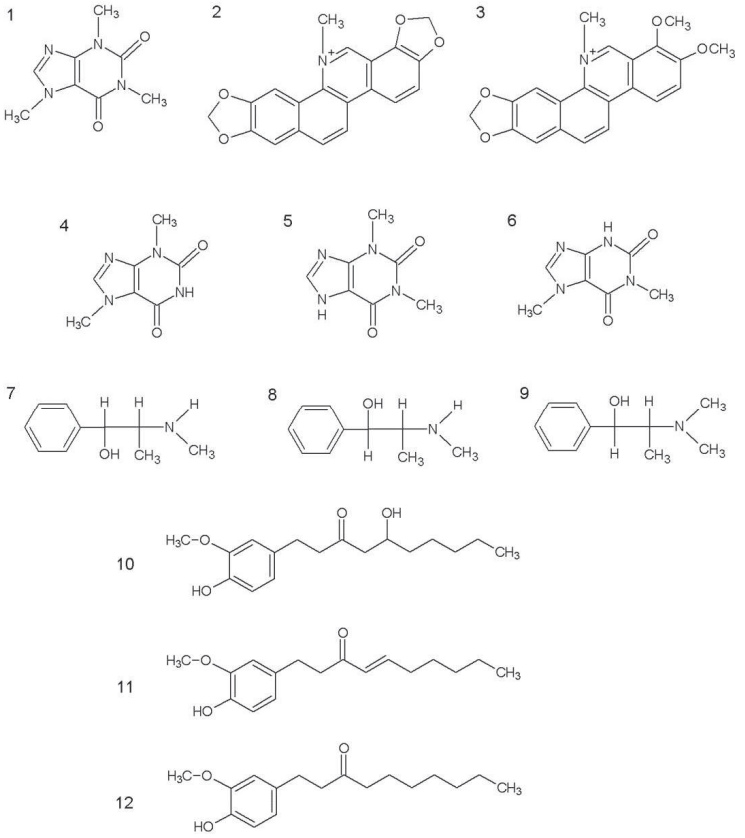

Figure 1. Representative alkaloids discussed in this review: caffeine (1); bloodroot alkaloids including sanguinarine (2) and chelerythrine (3); coca alkaloids including theobromine (4) and its congeners theophylline (5) and paraxanthine (6); ephedra alkaloids including ephedrine (7) and its congeners pseudoephedrine (8) and methylephedrine (9); and ginger alkaloids including [6]-gingerol (10) and its congeners [6]-shogaol (11) and [6]-paradol (12).

a central nervous stimulant and modulator of cardiovascular function (4). It may improve endurance performance for athletes such as adult runners at moderate doses ranging such as 2-3 mg/kg body weight (115). In human (15, 114) and mouse (60) models, the effects of caffeine are greater when abstinence from caffeine occurs at least 7 days prior to dosing, or in non-users.

A comprehensive review of the immunomodulatory effects of caffeine was published in 2006 by Horrigan et al. (57). The authors determined that studies employing *in vitro* concentrations of ≤100 μM were relevant to *in vivo* human contexts. Human *in vitro* data suggested that caffeine:

- increased or had no effect on free radical production by neutrophils at physiological doses from 10-100 μM, contingent on cell pretreatment (116, 117);

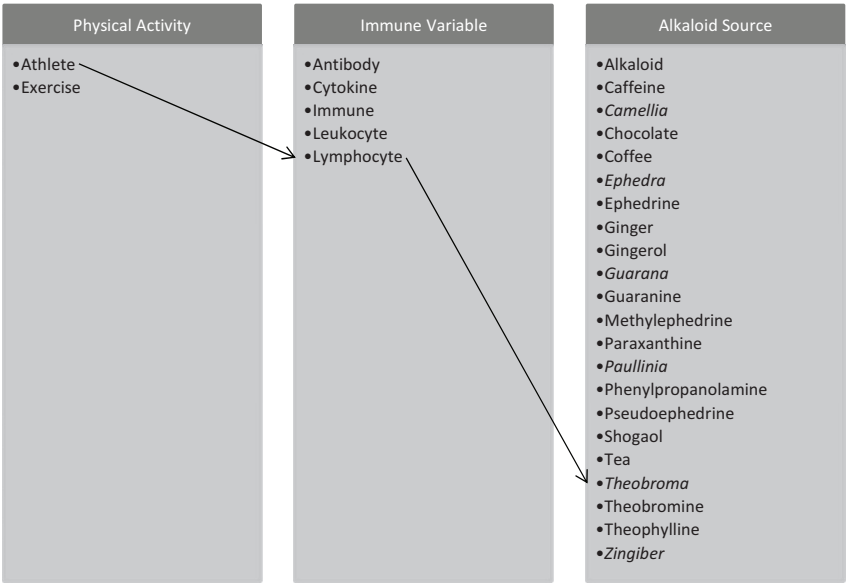

Figure 2. Search strings used in this review. A search string consisted of three terms, one representing physical activity (column 1), another representing an immune variable (column 2), and the third representing an alkaloid source or name (column 3). All possible combinations of terms from each column were used in the searches. One example search string (athlete + lymphocyte + *Theobroma*) is indicated by the arrows.

- reduced peripheral blood mononuclear cell (PBMC) proliferation at physiological concentrations of 25-100 μM (103);
- reduced whole blood production of tumour necrosis factor (TNF) and interleukin (IL)-10 at borderline physiological levels (100 μM or 200 μg/mL, with no effects at lower doses) and had no effect on IL-1β or IL-12 (43, 56, 81).

Horrigan et al. (57) concluded that caffeine exerts an anti-inflammatory pattern in humans, and that the presence or absence of effects on some immune parameters may be a function of dosing differences. With the exception of one study by Bishop et al. (16), all human data in the 2006 review were from *in vitro* studies and consequently did not address possible immunomodulatory roles of caffeine in exercise or sport contexts.

Since that review, many *in vivo* studies have deepened our understanding of the immunomodulatory effects of caffeine in humans, and several have been conducted in athletic contexts. Those that examined possible effects of caffeine administered in "pure" form (either as capsules or dissolved in beverages) in exercise studies are summarized in Table 3. [Table 3 shows only post-exercise differences between caffeine-treated versus placebo groups, and does not indicate what effects exercise had independently of treatment. This convention will be followed for all subsequent tables]. Subjects given caffeine prior to exercise general-

Table 1. Common plant sources of caffeine in human nutrition. *Formerly Sterculiaceae.

Common Name	Scientific Name	Botanical Family	Caffeine Name
Chocolate	*Theobroma cacao* L.	Malvaceae*	Caffeine
Coffee	*Coffea* spp. L.	Rubiaceae	Caffeine
Guarana	*Paullinia cupana* Kunth	Sapindaceae	Guaranine
Kola	*Cola* spp. Schott & Endl.	Malvaceae	Caffeine
Mate, Yerba	*Ilex paraguariensis* A. St. Hil.	Aquifoliaceae	Mateine
Tea	*Camellia sinensis* (L.) Kuntze	Theaceae	Caffeine

Table 2. Caffeine content from a representative sample of energy drinks from central Iowa on September 12, 2013. Representative soft drinks and sports drinks are shown for comparison. Note differences in serving sizes across beverages; more than one serving may be included in a container. Though not included in the Table, chocolate milk or other cocoa-flavoured beverages contain trace amounts of caffeine from the cocoa, typically <10 mg/serving (112), plus other alkaloids such as theobromine. Some beverages contained additional alkaloid sources: ED1, ED3, ED6, ED9, and ED10 contained ginseng and guarana; ED4 contained guarana; and ED2 contained acacia.

Beverage	Serving Size (mL)	Calories (kcal)	Caffeine (mg/serving)
ENERGY DRINKS			
ED1	240	110	71
ED2	473	220	160
ED3	240	110	80
ED4	473	200	260
ED5	240	110	77
ED6	240	140	160
ED7	240	120	142
ED8	250	130	80
ED9	473	50	160
ED10	237	0	104
SOFT DRINKS			
SO1	355	140	34
SO2	355	170	54
SO3	355	150	38
SPORTS DRINKS			
SP1	355	80	0
SP2	360	0	0

ly exhibited unchanged or increased circulating leukocytes and lymphocytes (or specific subpopulations) post-exercise, compared to exercised but placebo-treated controls, with the exception of T-cells. Post-exercise natural killer (NK) cell activation, neutrophil activity, and plasma IL-6 and IL-10 were normally increased in caffeine-treated compared to placebo-treated subjects. Few of the outcomes from Table 3 are directly comparable to the *in vitro* data reviewed by Horrigan et al. (57), but the findings of increased neutrophil activity in response to caffeine stimulation is congruent, whilst the findings on IL-10 production are opposing.

Several of the studies in Table 3 that were conducted by Bishop and colleagues also included plasma catecholamine measurements and reported that caffeine augmented adrenaline (epinephrine) levels (16, 17, 42, 125–127), but not noradrenaline (norepinephrine) levels (42). Separately, Laurent and colleagues (65) reported that highly-trained males given 6 mg/kg caffeine 90 minutes prior to 120 minutes of cycling at 65% VO_2max demonstrated increased post-exercise plasma adrenaline, cortisol, and β-endorphin levels compared to placebo-treated controls. These findings together indicate that caffeine increases sympathetic nervous system activity via the hypothalamo-pituitary-adrenocortical (HPA) axis (92). The consequences of the interactions between caffeine, the HPA axis and its products, and the immune system are beyond the scope of this review but have been discussed elsewhere (42).

Studies that examined the immunomodulatory effects of beverages or supplements containing caffeine (alongside other possibly immunomodulatory ingredients) in exercise contexts are presented in Table 4. Several factors make it difficult to compare the results of Table 3 to Table 4 effectively. These include: differences in outcomes reported; relatively low number of studies in Table 4 versus Table 3; use of both trained and untrained subjects in Table 4 versus solely trained subjects in Table 3; chronic dosing in all Table 4 studies versus acute dosing in all Table 3 studies; lower caffeine dose to body weight ratio in Table 4 studies (where reported) compared to Table 3 studies (expounded below); and the fact that the studies from Table 4 utilized preparations with other potentially immunomodulatory compounds besides caffeine.

As represented in Table 4, many of the beverages, foods, or supplements consumed by athletes for their caffeine content frequently contain other non-caffeine and/or non-alkaloid immunomodulatory compounds. Understanding how these compounds modulate athlete immune function relative to caffeine or other alkaloids (independently, additively, synergistically, and so forth) is problematic yet important. These immunomodulatory compounds often come from plant sources.

Tea *(Camellia sinensis)* serves as a good model for examining this dilemma because it contains caffeine plus other non-alkaloid bioactive phytochemicals, including its relatively well-studied flavonoid subclasses. One flavonoid subclass present in tea is the flavanols, represented by catechins and epicatechins (often referred to simply as polyphenols, or procyanidins or tannins if they are oligomerized; epigallocatechin-3-gallate [EGCG] is the most abundant). Plasma or *in vitro* concentrations of ≤10 μM flavanols are considered physiologically relevant (84, 134), and leukocytes treated with flavanols at these concentrations exhibit anti-inflammatory, antioxidant, or T-cell-modulating effects (12, 30, 134). The cardiovascular benefits of tea consumption have been linked to these phenomena (133). If green tea leaves are processed into black or oolong tea, the green tea catechins oxidize and dimerize into theaflavins; theaflavins maintain similar antioxidant properties as catechins (67). A second flavonoid subclass present in tea is the flavonols (with an "o" instead of an "a"). The flavonol quercetin has anti-inflammatory and antioxidant effects *in vivo* that can sometimes offset the deleterious effects of exercise depending on context (74, 87, 88). Plasma or *in vitro* concentrations ranging from 0.5-1.5 μM are considered physiologically relevant and may be seen in as little as one dietary dose of quercetin, though even higher levels

Table 3. Immunomodulatory effects of pure caffeine in exercise contexts. *Cells were additionally stimulated *in vitro* with antigen (Pediacel vaccine).

Study	Subjects	Caffeine Dose	Exercise	Immunological Outcomes in Caffeine-Treated Subjects compared to Placebo-Treated Controls
Bassini-Cameron et al. **(13)**	22 ♂ soccer athletes	5 mg/kg acute dose pre-exercise	45-min of variable distance running + intermittent recovery test	↑ circulating lymphocytes and monocytes; ↑ circulating segmented neutrophils (only when combined with exercise)
Bishop et al. **(16)**	8 endurance-trained ♂	6 mg/kg acute dose pre-exercise	90-min cycling at 70% VO_2max	↓ total numbers of circulating CD4 and CD8 T-cells, but ↑ in CD4 and CD8 T-cell activation (based on ↑ CD69 expression)
Bishop et al. **(17)**	11 endurance-trained ♂	6 mg/kg acute dose pre-exercise	90-min cycling at 70% VO_2max	↑ sIgA concentrations and secretion rates
Fletcher & Bishop **(40)***	12 endurance-trained ♂ cyclists	2 or 6 mg/kg acute dose pre-exercise	90-min cycling at 70% VO_2max	↑ NK cell activation; ↑ circulating NK cells (high dose only)
Fletcher & Bishop **(41)***	15 healthy ♂	6 mg/kg acute dose pre-exercise (in 1× or 3× boluses)	6 15-min bouts of intermittent shuttle running	↑ NK cell activation (1× bolus only); ↑ circulating NK cells
Fletcher & Bishop **(42)***	9 endurance-trained ♂ cyclists	6 mg/kg acute dose pre-exercise	90-min cycling at 70% VO_2max	↓ CD4 and CD8 T-cell activation; ↑ NK cell activation
Machado et al. **(71)**	20 soccer athletes	4.5 mg/kg acute dose pre-exercise	6 sets of 10 sprints (with active recovery)	No differences in circulating leukocytes, lymphocytes, basophils, eosinophils, monocytes, neutrophils
Machado et al. **(72)**	15 ♂ soccer athletes	4.5 mg/kg acute dose pre-exercise	3 sets of 10 repetitions at 10-RM of bench press, pullover, biceps curl, triceps extension, leg extension, prone leg curl	No differences in circulating leukocytes, lymphocytes, eosinophils, monocytes, neutrophils
Mahdavi et al. **(73)**	26 ♀ basketball athletes	5 mg/kg acute dose pre-exercise	Single 30-second Wingate test	No differences in circulating leukocytes, lymphocytes, granulocytes
Pereira et al. **(94)**	15 soccer athletes	5.5 mg/kg acute dose pre-exercise	12 10-sprint sets	No differences in circulating leukocytes, lymphocytes, neutrophils
Tauler et al. **(118)**	33 running-trained ♂	6 mg/kg acute dose pre-exercise	15-km competitive run	↑ circulating leukocytes and neutrophils; ↑ plasma IL-6 and IL-10; ↑ plasma antioxidants
Vimercatti et al. **(123)**	15 trained ♂	4.5 or 5.5 mg/kg acute dose pre-exercise	60-min treadmill running at 65% VO_2max	No differences in circulating leukocytes
Walker et al. **(125)**	19 trained ♂ cyclists	6 mg/kg acute dose pre-exercise	90-min cycling at 70% VO_2max	↑ circulating lymphocytes; ↑ post-exercise fMLP-stimulated response in neutrophils; no effect on PMA-stimulated neutrophil oxidative burst
Walker et al. **(126)**	9 trained ♂ cyclists	6 mg/kg acute dose pre-exercise	90 min cycling at 70% VO_2max + time trial equivalent to 30-min cycling at 70% VO_2max	↓ circulating lymphocytes; no differences in circulating leukocytes or neutrophils; no differences in post-exercise fMLP-stimulated response in neutrophils
Walker et al. **(127)**	12 trained ♂ cyclists	6 mg/kg acute dose pre-exercise +/- carbohydrate drink	120-min cycling at 65% VO_2max	↑ post-exercise fMLP-stimulated response in neutrophils; ↑ IL-6 immediately post-exercise; variable effects on circulating leukocytes, lymphocytes, and neutrophils contingent on whether carbohydrate drink was co-ingested

Table 4. Immunomodulatory effects of caffeine-containing commercial beverages or supplements in exercise contexts. No sources examining the effects of coffee in the context of athlete immune function were retrieved by the search stratagem, though coffee is an obvious source of nutritional caffeine. Plasma protein carbonyls are indicators of oxidative burden. * EC = epicatechin; EGC = epigallocatechin, EGCG = epigallocatechin gallate.

Study	Subjects	Caffeine Source	Exercise	Immunological Outcomes in Caffeine-Treated Subjects compared to Placebo-Treated Controls
Arent et al. **(6)**	18 weight-trained ♂	Black tea extract containing 40% theaflavins and 30% catechins (total polyphenols exceeding 95%) for 9 days; caffeine content not specified	2 × 30-second Wingate test + 8 10-second cycling intervals	No differences in plasma IL-6
Hismiogullari et al. **(54)**	10 moderately active ♂	1 energy drink prior in between the first and secondn exercise bouts 150 mg caffeine, 80 mg taurine, 11 g carbohydrate, other ingredients	3 sets of 50 maximal knee contractions on an isokinetic dynamometer	↑ circulating basophils; no differences in circulating leukocytes, lymphocytes, eosinophils, monocytes, and neutrophils
Lockwood et al. **(69)**	36 sedentary ♂	1 energy drink/day containing 200 mg caffeine /serving and including ginger, green tea, and guarana for 10 weeks	10 weeks of cross-training	↓ circulating monocytes (only in non-exercising supplement-treatment group; values within normal reference ranges)
Narotzki et al. **(85)**	22 obese elderly (13♂, 9♀)	3 servings of brewed green tea (1.5 g tea/sachet) + 1 vitamin E capsule/day for 12 weeks; caffeine content not specified	12 weeks of 30-min walking 6× per week	↓ plasma protein carbonyls; no differences in CRP
Nieman et al. **(86)**	31 trained individuals (18♂, 13♀)	40 g/day of a green tea-blueberry polyphenol soy protein complex (PSPC) containing 344 mg caffeine and 1001 mg flavanols (79% being EC, EGC, and EGCG)* for 17 days	3 consecutive days of 150-min running at 70% VO_2max; exercise started at Day 14 of supplementation	No differences in leukocyte counts, plasma protein carbonyls, or plasma CRP, IL-6, IL-8, and MCP-1

may be common in some sites including the gut (31, 55, 135). Beyond the flavonoids, other compounds in tea also exert immunomodulatory effects. Theanine is an amino acid found in green tea which is a precursor for glutamate. Glutamate is one of the three amino acid components of glutathione (GSH; the other two being cysteine and glycine), important for maintaining antioxidant status. Cystine-theanine supplementation (700 mg cystine +280 mg theanine) has been shown to reduce exercise-induced changes in plasma C-reactive protein (CRP), circulating leukocytes and neutrophils, and NK cell activity, but not plasma IL-6 or IL-8 levels (62, 82, 83).

Several other plant sources are included in energy or sports drinks for their caffeine content. Guarana *(Paullinia cupana)* seeds contain higher caffeine levels than coffee leaves, in addition to the alkaloid theobromine (discussed below) and non-alkaloid flavanols (discussed above) (18, 98). Yerba mate *(Ilex paraguariensis)* is also valued for its caffeine content and includes different flavonoids, chlorogenic acid and associated derivatives, and triterpene saponins, some of which may possess antioxidant capacities (5, 20). Ginseng *(Panax ginseng* C. A. Meyer and sister species; Araliaceae) is another common ingredient in energy drinks: it contains the alkaloids **ginsenine** and various manifestations of **carbaline,** but is better known for its bioactive non-alkaloid ginsenosides. A thorough discussion of ginseng and athlete immune function may be found elsewhere (105). Thus, for caffeine-containing beverages that derive their caffeine partially

Table 5. Immunomodulatory effects of cocoa supplements in exercise contexts. F_2-isoprsotanes are a marker of oxidative stress. *Twenty males were included in the total study, but only ten exercised.

Study	Subjects	Chocolate Treatment	Exercise	Immunological Outcomes in Cocoa-Treated Subjects compared to Placebo-Treated Controls
Allgrove et al. **(3)**	20 healthy ♂	40 g dark chocolate bar (41.6 mg caffeine, 267 mg theobromine, 15.6 mg catechin, 38.7 mg epicatechin, and 44.4 mg other polyphenols) twice daily for 2 weeks + an acute dose 2 hours pre-exercise	90-min cycling at 60% VO_2max plus 30-seconds of cycling at 90% VO_2max every 10 min	↓ plasma F_2-isoprostanes; no differences in plasma IL-1Ra, IL-6, and IL-10
Davison et al. **(33)**	14 healthy ♂	100 g dark chocolate bar (668 mg theobromine, 104 mg caffeine, 97 mg epicatechin, 39 mg catechin) pre-exercise	150-min cycling at 60% VO_2max	↑ antioxidant status; no differences in plasma IL-6, leukocytosis, neutrophilia, or neutrophil degranulation
Macdonald et al. **(70)**	21 rowers (10 ♂, 11♀)	1 chocolate-flavoured "meal replacement beverage" or 1 chocolate-flavoured milk beverage compared to 2 other beverages post-exercise; alkaloid content not stated	90-min rowing at 60-70% VO_2max with ≤ 5 5-min increased pace bouts	↓ plasma IL-6 in the cocoa beverage trials post-exercise and ↓↓ plasma IL-6 in the chocolate milk trials 6-hours post-exercise
McBrier et al. **(78)**	7 recreationally active ♂ (anaerobically trained)	330 mL of a "cocoa-based protein drink" post-exercise; alkaloid content not stated	30-min run at 75% HRmax (10% downhill grade)	No differences in plasma IL-6, IL-8, or CRP
Singh et al. **(113)**	16 healthy ♂ (8 trained, 8 untrained)	7 days of "enriched cocoa polyphenol supplements" (no other details provided) pre-exercise	60-min cycling at 70% VO_2max	No differences in circulating platelets or platelet activation
Wiswedel et al. **(132)**	10 sedentary ♂*	100 mL of either "high" (187 mg) or "low" (14 mg) flavanols and procyanidins cocoa drink pre-exercise; alkaloid content not stated	10-min cycling at 100% HRmax after warm-up	↓ plasma F_2-isoprostanes

or fully from plant sources, it is impossible to ascertain which immunomodulatory effects are due to caffeine, other alkaloids, or non-alkaloid components. Regarding cognitive or ergogenic benefits ascribed to energy drinks, McLellan and Lieberman concluded that caffeine and possibly guarana (essentially another source of caffeine), but not other alkaloid ingredients within the beverages, were responsible for observed effects (79).

The average dose of caffeine in the studies from Table 3 was 5.3 mg/kg body mass. Assuming a 75-kg athlete, the average dose of caffeine in the studies from Table 4 (where disclosed) was 3.1 mg/kg body mass. The average dose of caffeine in the Table 2 commercial energy drinks was 1.7 mg/kg body mass if athletes consumed only the serving size; however, six of the energy drinks in Table 2 contain two servings per can, so if the athlete consumes the entire can then the average dose of caffeine from Table 2 becomes 2.6 mg/kg body mass. Stated another way, an athlete consuming one energy drink from Table 2 is only ingesting half the caffeine that subjects in the studies from Table 3 consumed; however, this calculation does not necessarily account for total alkaloid content nor does it consider possibly physiologically relevant synergistic effects between caffeine and other ingredients (46, 53).

Altogether, the data from Table 3 suggest caffeine is often immunoneutral but may be immunomodulatory (most frequently immunostimulatory) when consumed as a stand-alone supplement by trained individuals. The data from Table 4

suggest caffeine-containing supplements may be immunoneutral but may improve antioxidant capacity in exercise contexts; however, the precise role of caffeine versus other alkaloids versus non-alkaloid compounds in such preparations on *in vivo* immunomodulation is essentially uncharted territory.

THEOPHYLLINE AND ITS ISOMERS

Theophylline and its isomers **theobromine** and **paraxanthine** (Figure 1) are all alkaloids originally characterized from cocoa *(Theobroma cacao)* but subsequently found in many other plants like kola and tea. Moreover, they are the natural metabolites of caffeine in the human body (7). Chocolate and cocoa (referred together as "cocoa" hereafter) are the main sources of these alkaloids for athletes, yet cocoa beverages and foods contain other naturally-occurring phytochemicals (61) including caffeine and non-alkaloid compounds such as flavanols (catechin, epicatechin and proanthocyanidins—discussed previously), flavonols (quercetin—discussed previously), plus other nonflavonoids like chlorogenic acid and its derivatives. Reviews of the immunomodulatory and cardioprotective effects of cocoa in non-exercise contexts have been published elsewhere (8, 24, 37, 48).

Studies investigating the immunomodulatory effects of cocoa in exercise contexts are presented in Table 5. No conclusions can be drawn about the effects of cocoa alkaloids on athlete immune function because alkaloid composition was only reported in two studies from Table 5 and was never correlated to immune variables. Though athletes in both studies ingested therapeutically efficacious concentrations of theobromine, those in the Davison et al. study (33) ingested only a single pre-exercise bolus while those in the Allgrove et al. study (3) were treated for two weeks. Davison et al. (33) also looked at neuroendocrine correlates but found no association between cocoa treatment and HPA stress hormones including cortisol and adrenocorticotropic hormone (ACTH). Apart from Table 5, no additional studies were found testing isolated theophylline or its congeners on athlete immune function.

Since Table 5 has few studies to draw conclusions from, the literature on *in vitro* effects or *in vivo* non-exercise studies of theophylline and its congeners on human leukocytes may shed some light on these compounds' possible immunomodulatory roles. Plasma theophylline levels of 10-20 μg/mL or 55-110 μM are considered therapeutically relevant, though recent recommendations have suggested a narrower range of 10-15 μg/mL to avoid side effects (35, 90, 136). In human monocytes or cultured macrophages, *in vitro* theophylline treatment:

- at 1.8μg/mL inhibited monocyte → dendritic cell maturation (137);
- at 2.5-20 μg/mL reduced IL-13 mRNA expression in a dose-dependent manner (136);
- at 5-20 μg/mL reduced free radical production in monocytes by inhibiting phosphodiesterases (27);
- and at 30-50 μM reduced production of IL-6 and TNF even in the presence of sambutol (an IL-6 inducer) (35).

In human eosinophils, 18 μg/mL *in vitro* theophylline treatment increased peroxisome proliferator-activated receptor-γ (PPARγ) mRNA and protein levels (120).

Table 6. Immunomodulatory effects of ginger supplements in exercise contexts. *Thirty-two males were included in the study, but only 16 exercised and only 8 of those 16 received ginger supplement plus exercise. †The raw ginger contained 8.2 mg 6-gingerol, 1.3 mg 8-gingerol, 1.9 mg 10-gingerol, 2.2 mg 6-shogaol. ‡The heated ginger contained 2.8 mg 6-gingerol, 1.0 mg 8-gingerol, 1.6 mg 10-gingerol, 2.6 mg 6-shogaol. §Sixty women enrolled in the study, but 20 were given a cinnamon supplement and are not considered here; 49 completed the study, but it is unclear what the final numbers per group were.

Study	Subjects	Ginger Treatment	Exercise	Immunological Outcomes in Ginger-Treated Subjects compared to Placebo-Treated Controls
Atashak et al. **(9)**	16 obese, sedentary ♂*	4 250-mg ginger root capsules/day for 10 weeks; alkaloid content not stated	Progressive resistance training program including chest press, leg press, lateral pulldown, triceps pushdown, knee extension, seated row, bicep curl, and abdominal curl	↓ plasma CRP
Ayaz et al. **(11)**	20 obese ♀ with breast cancer	4 750-mg ginger root capsules/day for 6 weeks; alkaloid content not stated	40-80 min water aerobics (individualized per subject) 4 days/week for 6 weeks	↓ plasma CRP and IL-6
Black et al. **(19)**	74 individuals (73% ♀)	2 g raw ginger† or 2 g heated ginger‡ for 11 days	18 eccentric contractions of the elbow flexors	No differences in plasma prostaglandin E_2
Mashhadi et al. **(75)**	40 athletic ♀§	3 g ginger powder/day for 8 weeks; alkaloid content not stated	Acute bout of resistance training exercises (specific to the sport of each subject)	No differences in plasma IL-6

Finally, in human leukocytes *in vitro* treatment with 5 μM theophylline reduced production of the leukotrienes B_4 and C_4 (111). Regarding *in vivo* effects, chronic obstructive pulmonary disease (COPD) patients treated with 400 mg/d theophylline (which should correspond to plasma theophylline levels of ~10-15 μg/mL) demonstrated reduced sputum levels of IL-8, TNF, and neutrophils (58). In COPD patients treated with standard steroid therapy or standard steroid therapy plus low-dose theophylline (200 mg/d), patients in the latter group had greater declines in plasma TNF and IL-8 than the former (32). Thus, non-exercise *in vitro* and *in vivo* studies both show efficacy of theophylline as an immunomodulator at therapeutic or even subtherapeutic concentrations.

GINGEROLS AND CONGENERS

The major category of alkaloids from ginger *(Zingiber officinale* Roscoe; Zingiberaceae) are the **gingerols**, a series of chemical homologues (Figure 1) important in both flavouring and bioactivity, and structurally analogous to capsaicin (110). When ginger is dried, these compounds are dehydrated to **shogaols. Paradols** are also present, though in more minor quantities. Ginger supplements are indicated for various conditions including cardiovascular diseases, diabetes, and gut disorders (23, 76) because of anti-inflammatory, antioxidant, antimicrobial properties ascribed to their alkaloids. Ginger has also been suggested for pain management, though this application remains unproven (119). Volatiles, including mono- and sesquiterpenes, are also important co-occurring chemicals (23).

Anti-inflammatory effects have been shown in human *in vitro* studies, as reviewed elsewhere (28).

Although no plasma thresholds have been declared as therapeutic standards, some studies have looked as plasma responses to ginger supplementation in humans. After human subjects were given a single oral bolus of 2 g ginger supplement containing ~135 mg gingerols and related congeners, peak plasma concentrations of free gingerol (9.5 ng/mL) and free shogaol (13.6 ng/mL) were observed 1 hour post-consumption, with peak concentrations of numerous metabolites also observed at that time (138). Separately, Zick et al. (139) dosed human subjects with anywhere from 100 mg-2g standardized ginger supplement (containing ~98.4 mg gingerol and related congeners per 2 g ginger). They found no free plasma gingerols or shogoals; however, peak metabolites of these compounds were observed between 45-120 minutes, with peak concentrations of gingerol metabolites at 0.5-1.7 ng/mL and shogaol metabolites at 0.2 ng/mL. From these limited findings, it appears that plasma concentrations of gingerols or related congeners peak 1-2 hours post-supplementation and can vary markedly.

Studies that examined the immunomodulatory effects of ginger supplementation in exercise contexts are shown in Table 6. Atashak et al. and Avaz et al. showed reduced plasma CRP and/or IL-6 (9, 11), whereas Black et al. and Mashhadi et al. showed no effect of ginger supplementation on IL-6 or PGE_2 (19, 75). Across Table 6, subjects were diverse in terms of sex and fitness levels. Subjects in the first two studies were clinically obese whereas subjects in the latter two studies were not, possibly suggesting that any anti-inflammatory effects of ginger may only be manifested in overweight individuals. Whether these effects are due to gingerols, paradols, shogaols, their congeners, or other compounds remains to be ascertained. Given the lack of data from humans, it is interesting to note that both *in vitro* and *in vivo* rodent models also show anti-inflammatory effects from ginger supplementation, as reviewed elsewhere (66). For example, at concentrations of 5-50 μM, gingerols block NF-κB and MAPK activation, c-Jun N-terminal kinase (JNK) phosphorylation, and TNF-α expression in *in vitro* or mouse skin models (59, 63).

EPHEDRINE AND CONGENERS

Ephedrine and **pseudoephedrine** (Figure 1) are stereoisomers first isolated from Ma Huang or the ephedra plant (*Ephedra sinica* Stapf.; Ephedraceae [formerly Gnetaceae]) of traditional Chinese medicine (TCM) (80), but are found in all members of the genus including the North American species Mormon tea (*Ephedra viridis* Coville). **Methylephedrine** and other congeners are structurally similar and also naturally-occurring in this genus, whereas **phenylpropanolamine** is a synthetic analogue of ephedrine. These compounds are described as sympathomimetic alkaloids because their *in vivo* actions are similar to amphetamine and include tachycardia, hypertension, and smooth muscle relaxation (hence their application in cough syrups, decongestants, and weight loss products) (89). Normal human plasma concentrations after one or a couple doses of ephedra extract range from 80-400 ng/mL, with lower concentrations being more common (52, 101, 130). Other alkaloids present in ephedra include caffeine, theobromine, and

theophylline (discussed earlier). Ephedrine and ephedrine-like alkaloids act synergistically when combined with drugs such as aspirin (129) or other alkaloids such as caffeine (51), as is often the case in energy drinks. Additional ephedra phytochemicals may exert immunomodulatory activities—for example, carbohydrates from the plant may dose-dependently inhibit complement components C2 and C9 in human sera (68).

There are no *in vivo* studies of the effects of these compounds on athlete immune function. Human *in vitro* studies are ample and may provide some insight on possible effects. In a study by Wilasrusmee et al. (131), PBMC stimulated *in vitro* with an aqueous extract of *Ephedra sinica* dried powder (ephedrine quantity not provided) showed no differences in cellular proliferation, IL-2 production, or IL-10 production compared to controls. In a separate study by Attard and Vella (10), human PBMC were stimulated *in vitro* with pure ephedrine across ten-fold dilutions ranging from 0.69-69 μg/mL ephedrine or aqueous-benzene extracts from different *Ephedra fragilis* aerial parts standardized to 69 μg/mL ephedrine content. PBMC proliferation increased, but not significantly. A comparison of these results to rodent models has been given elsewhere (107). Regarding isolated compounds, Fiebich et al. (39) demonstrated that when Jurkat T-cells were treated with pseudoephedrine *in vitro* at doses ranging from 1-5 mM, the following were diminished: transcriptional activity of NFAT, NF-κB, and AP-1; c-Jun activation; and TNF and IL-2 gene transcription. In a study by Watson et al. (129), ephedrine and phenylpropanolamine (both at 50 μmol/L, administered independently) inhibited platelet aggregation when administered *in vitro* to human platelet-rich plasma. Given the absence of data, little can be concluded about the effects of ephedra alkaloids on athlete immune function. Limited research suggests they may have anti-inflammatory effects, but this needs to be demonstrated convincingly *in vivo*.

EMERGING ALKALOID SOURCES

This review has so far considered only those alkaloids or alkaloid-rich plants that are appreciably consumed by athletes. Several other plants, either obscure or only recently surging in popularity, also contain alkaloids that may be therapeutic immunomodulators relevant to athletes.

Noni berry (*Morinda citrifolia* L.; Rubiaceae) is escalating in popularity among Western athletes, though it has long been used by Polynesians. One of its primary bioactive compounds is the alkaloid **xeronine.** Reviews have concluded that noni berry has immunomodulatory (e.g., cytokine- and cannabinoid receptor-modulating) activities in mice (91) and marginal anticancer properties (21), though the molecules responsible for the latter (possibly alkaloids) are yet to be determined. Noni berry presents a diverse array of phytochemicals including alkaloids, other flavonoids, phenylpropanoids, and triterpenoids (93). Much work remains to determine the bioactive role of noni berry alkaloids (50), or whether noni might be efficacious for bolstering athlete immune function.

Goji berry or wolfberry (*Lycium barbarum* L. and *Lycium chinense* Mill.; Solanaceae) supplements and juices are also growing in popularity among Western athletes (97). Along with carotenoids, sterols, and the organic acid taurine,

Table 7. Effects of 50% ethanol bloodroot extracts of proximal, middle, or distal rhizomes on in vitro cytokine production by human PBMC (previously unpublished work). PBMC were isolated from the blood of eight healthy adult donors, plated at 1.0×10^6 cells per well, and cultured with LPS or extract as detailed elsewhere (95, 107). Cytokine production was determined by ELISA. *Significant ($p<0.005$) differences compared to solvent vehicle (media) control. †Significant ($p<0.005$) differences compared to both solvent vehicle and LPS controls.

	Solvent Vehicle	LPS (no extract)	Proximal (no LPS)	Middle (no LPS)	Distal (no LPS)	Proximal + LPS	Middle + LPS	Distal + LPS
IL-6	15.12 ± 1.74	1176.07 ± 112.03*	11.49 ± 3.98	8.52 ± 2.64	9.2 ± 1.86	11.35 ± 1.19	13.07 ± 1.47	11.45 ± 1.88
IL-8	1487.22 ± 157.52	2022.69 ± 72.99*	17.97 ± 2.08†	18.07 ± 1.32†	16.68 ± 1.99†	17.42 ± 1.74†	16.7 ± 2†	15.61 ± 2.32†
IL-10	9.73 ± 1.54	675.4 ± 100.5*	15.13 ± 2.5	14.44 ± 2.02	15.32 ± 2.69	17.26 ± 2.12	16.46 ± 2.88	13.34 ± 1.88
TNF	12.76 ± 2.65	1222.73 ± 142.04*	11.77 ± 1.71	11.34 ± 2.03	9.98 ± 1.12	13.44 ± 3.53	9.24 ± 1.77	7.6 ± 1.49

Goji berries also contain alkaloids including small amounts of **atropine.** Using influenza-infected rats, Ren et al. (100) showed that wolfberry supplementation for 4 weeks (containing 530 mg/g wolfberry fruit) augmented the immune response to infection and spurred T-cell responses by upregulating IL-2 production. Du et al. (34) performed a similar study with similar results, and showed that wolfberry supplementation improved activity and maturation of antigen-presenting dendritic cells, which in turn improved antigen-specific T-cell proliferation and CD4 T-cell production of IL-4 and IFN-γ *in vitro.* While these results suggest a possible immunotherapeutic role for Goji berry in athletes, human tests still need to be conducted (including safety determinations). It is presently unclear which Goji berry components produced these effects.

Bloodroot (*Sanguinaria canadensis* L., Papaveraceae) produces a phalanx of benzophenanthridine alkaloids. While **sanguinarine** and **chelerythrine** (Figure 1) are the most frequently researched, others are present in lesser quantities. Bloodroot alkaloids are found in greatest concentrations in the rhizomes (underground stems) and increase in concentration from proximal to distal (Figure 3). When human PBMC were stimulated *in vitro* with 50% ethanol extracts produced from different rhizome regions, the extracts had no effect on IL-6, IL-10, and TNF production, but suppressed IL-8 production compared to media-treated controls (Table 7). When PBMC were co-stimulated with both bloodroot extract and lipopolysaccharide (LPS), cells produced lower levels of all cytokines compared to cells treated with LPS alone. In two separate pilot studies in athletes, the same or identically produced extracts reduced *in vitro* TNF and IL-10 production and PBMC proliferation, regardless of the effects of exercise (a graded treadmill VO_2max test or 90 minutes of cycling at 85% ventilatory threshold), and also abrogated the effects of LPS or phytohaemagluttinn (PHA) co-stimulation (107). Observed effects are unlikely to be due to toxicity, as two groups have shown sanguinarine or sanguinarine-containing extracts are toxic to human cancerous cells but promote proliferation of healthy human PBMC *in vitro* (2, 104). Together, these data may suggest an immunosuppressive effect of bloodroot rhizome extracts, which would be counterproductive to athletes trying to offset the immunodepressive effects of intense training. However, in a previous study where subjects completed both a graded cycling VO_2max test and 90 minutes of cycling at 85% ventilatory threshold, 50% ethanol flower extracts (but not 50% ethanol root extracts) stimulated post-exercise *in vitro* PBMC production of TNF,

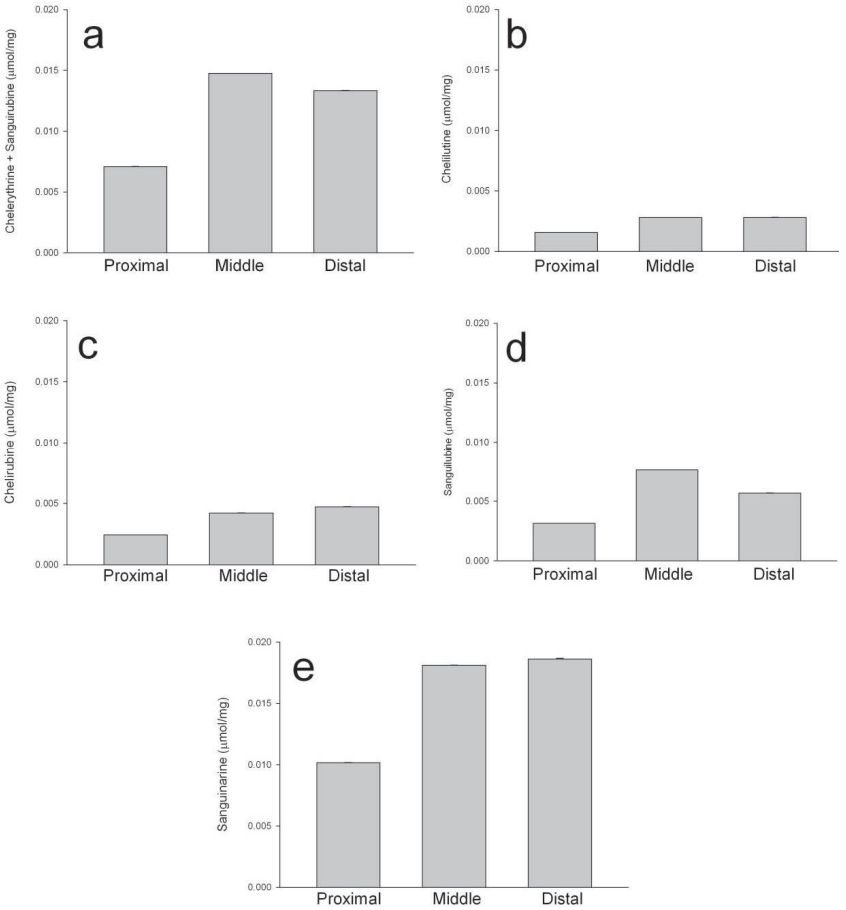

Figure 3. Alkaloid composition of 50% ethanol extracts produced from the proximal, middle, and distal portions of bloodroot rhizomes (previously unpublished work). Plants were harvested from an upland ravine in south Story County, Iowa, April 2010. Rhizome regions were demarcated by measuring the length of each individual rhizome and dividing it by thirds. Extraction and LC/MS were conducted as detailed elsewhere (107). All extracts were tested in triplicate. Assayed alkaloids include chelerythrine and sanguirubine, which co-eluted and were indistinguishable (a); chelilutine (b); chelirubine (c); sanguilutine (d); and sanguinarine (e). Standard deviation bars are present but invisible at this scale; by ANOVA, alkaloid content differed significantly for every possible comparison (all $p<0.001$).

IL-1β, and IL-10 following both exercise bouts (106). The data in Figure 3, Table 7, and previous studies collectively suggest that bloodroot ethanol extracts may be immunostimulatory or immunosuppressive depending on which plant organ is harvested. The roles of its alkaloid constituents are less clear, sometimes correlating negatively or not at all with immune effects; for example, even though alka-

loid content differed significantly across the three rhizome extracts for all alkaloids (Figure 3; $p<0.001$), there were no significant differences in immune effects across the three extracts (Table 7).

CONCLUSIONS AND INTERDISCIPLINARY VIEWPOINTS

As the previous examples of green tea and bloodroot have shown, plant-based alkaloid sources are replete with known immunomodulatory alkaloids, other known non-alkaloid immunomodulatory compounds, and a panoply of unknown (or unattended) potentially immunomodulatory compounds. Plants can vary considerably in biochemical composition from species to species, interindividually, or even intraindividually (discussed below). In a previous issue of *EIR*, we proposed a framework to help account for these factors experimentally from an exercise immunologist's viewpoint using an interdisciplinary structure including botanical, chemical, and clinical considerations (108), and that framework has subsequently been expanded into the "seed to stomach" model to address manufacturing and consumer factors (109). The "seed to stomach" model is a useful tool for considering the current state of research regarding alkaloids and athlete immune function.

Based on the search stratagem used for this review, there were a much greater number of studies examining possible *in vivo* immunomodulatory roles of caffeine in human exercise contexts than other alkaloids or alkaloid sources. Only a handful of sources were uncovered examining possible *in vivo* immunomodulatory roles of cocoa or ginger in human exercise contexts. None were found for ephedra. However, the search stratagem used in this review may not have uncovered all germane references, particularly those written in non-English languages. This review did not address the library of rodent exercise studies: these may prove to be applicable to human exercise contexts in the future.

Human *in vitro* and *in vivo* studies on alkaloids and immune function are disjointed, making it difficult to form generalizations about the roles of alkaloids in athlete immune function. There is some evidence that caffeine can serve as an immunostimulatory, immunoneutral, or immunodepressing agent, depending on subject and exercise characteristics and immune variables measured, though no clear patterns emerge from the reviewed studies. It may also exhibit antioxidant properties. Ginger may exert anti-inflammatory effects, but it is unclear whether these effects are due to alkaloids or other biochemicals. Evidence for an immunomodulatory role of marker alkaloids in multi-compound preparations such as energy drinks, cocoa, or ephedra products is weak to nonexistent. For alkaloid sources derived from plants, variability in the reviewed studies may be due to the presence of other alkaloids or non-alkaloids, which may be immunomodulatory themselves. Pre-experimental factors such as growth or manufacturing differences may also play a part.

From an athlete's or coach's perspective, the data on alkaloids and athlete immune function are inconsistent and inconclusive. Although the health benefits of several of the plants in this review appear well-founded and have been discussed above, including cocoa (8), ginger (23), and green tea (102), it is unclear what role (if any) alkaloids are playing. For many athletes, proper diet is the best

nutrition and additional supplementation is often unnecessary (77, 121). Other caveats forewarn consideration of alkaloids as nutraceuticals. Safety and/or overdosing concerns are well-documented for sources such as caffeine (1, 47) and bloodroot (29, 124) and has led to the prohibition or regulation of ephedrine and its congeners by governments and sports regulatory agencies (96, 121). The sports supplement industry is poorly regulated and supplements may not contain adequate safety warnings (99). Package labels may not accurately reflect actual ingredients, even if manufacturers' claims of standardization are provided (22, 44). The preponderance of studies on alkaloids and athlete immune function have used males exclusively, so it is unclear whether the same outcomes would accrue with female athletes, though it appears caffeine has similar pharmacokinetics in both sexes (14, 46).

From the vantage points of botany and chemistry, the range of immune effects arising from alkaloids is logical given their structural diversity. The plants used in sports supplements are also evolutionarily diverse (105). A lack of correlation between marker components and supplement immune activity is reported for other medicinal plant genera, such as *Echinacea* (122) and *Pueraria* (26), so is perhaps unsurprising for the plant sources in this review. Exacerbating the problem are botanical or chemical factors that occur prior to or during extract preparation; summarily, these factors include planning factors (species selection, genetic variables), field factors (environmental conditions during growth), production factors (harvesting and manufacturing methods), and post-production factors (storage conditions) (109). Though it is pragmatically impossible for any research team to simultaneously attend to all these factors within a single study, future exercise immunology endeavors can add to the knowledge base by either controlling or accounting for a handful of these factors. For exercise immunologists who are already juggling kinesiology, immunology, physiology, and related disciplines in their experiments, widening the interdisciplinary approach may be daunting or even unmanageable. In such a situation it may even be beneficial to recruit scientists from other disciplines to the team.

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REFERENCES

1. Abbott PJ. Caffeine: a toxicological overview. Med. J. Aust. 145: 518–521, 1986.
2. Ahmad N, Gupta S, Husain MM, Heiskanen KM, Mukhtar H. Differential antiproliferative and apoptotic response of sanguinarine for cancer cells versus normal cells. Clin. Cancer Res. 6: 1524–1528, 2000.
3. Allgrove J, Farrell E, Gleeson M, Williamson G, Cooper K. Regular dark chocolate consumption's reduction of oxidative stress and increase of free-fatty-acid mobilization in response to prolonged cycling. Int. J. Sport Nutr. Exerc. Metab. 21: 113–123, 2011.

4. Angell PJ, Chester N, Sculthorpe N, Whyte G, George K, Somauroo J. Performance enhancing drug abuse and cardiovascular risk in athletes: implications for the clinician. Br. J. Sports Med. 46 Suppl 1: i78–84, 2012.
5. Arçari DP, Porto VB, Rodrigues ERV, Martins F, Lima RJ de, Sawaya ACHF, Ribeiro ML, Carvalho P de O. Effect of mate tea (Ilex paraguariensis) supplementation on oxidative stress biomarkers and LDL oxidisability in normo- and hyperlipidaemic humans. J. Funct. Foods 3: 190–197, 2011.
6. Arent SM, Senso M, Golem DL, McKeever KH. The effects of theaflavin-enriched black tea extract on muscle soreness, oxidative stress, inflammation, and endocrine responses to acute anaerobic interval training: a randomized, double-blind, crossover study. J. Int. Soc. Sports Nutr. 7: 11, 2010.
7. Arnaud MJ. Pharmacokinetics and metabolism of natural methylxanthines in animal and man. Handb. Exp. Pharmacol. : 33–91, 2011.
8. Arranz S, Valderas-Martinez P, Chiva-Blanch G, Casas R, Urpi-Sarda M, Lamuela-Raventos RM, Estruch R. Cardioprotective effects of cocoa: clinical evidence from randomized clinical intervention trials in humans. Mol. Nutr. Food Res. 57: 936–947, 2013.
9. Atashak S, Peeri M, Azarbayjani MA, Stannard SR, Haghighi MM. Obesity-related cardiovascular risk factors after long- erm resistance training and ginger supplementation. J. Sports Sci. Med. 10: 685–691, 2011.
10. Attard E, Vella K. Effects of ephedrine and Ephedra fragilis crude extracts on human peripheral lymphocytes. Pharmacogn. Res. 1: 38–42, 2009.
11. Ayaz A, Roshan VD. Effects of 6-weeks water-based ntermittent exercise with and without Zingiber officinale on pro-inflammatory markers and blood lipids in overweight women with breast cancer. J. Appl. Pharm. Sci. 2: 218–224, 2012.
12. Babu PVA, Liu D. Green tea catechins and cardiovascular health: an update. Curr. Med. Chem. 15: 1840–1850, 2008.
13. Bassini-Cameron A, Sweet E, Bottino A, Bittar C, Veiga C, Cameron L-C. Effect of caffeine supplementation on haematological and biochemical variables in elite soccer players under physical stress conditions. Br. J. Sports Med. 41: 523–530; discussion 530, 2007.
14. Beaudoin M-S, Allen B, Mazzetti G, Sullivan PJ, Graham TE. Caffeine ingestion impairs insulin sensitivity in a dose-dependent manner in both men and women. Appl. Physiol. Nutr. Metab. Physiol. Appliquée Nutr. Métabolisme 38: 140–147, 2013.
15. Bell DG, McLellan TM. Exercise endurance 1, 3, and 6 h after caffeine ingestion in caffeine users and nonusers. J. Appl. Physiol. 93: 1227–1234, 2002.
16. Bishop NC, Fitzgerald C, Porter PJ, Scanlon GA, Smith AC. Effect of caffeine ingestion on lymphocyte counts and subset activation in vivo following strenuous cycling. Eur. J. Appl. Physiol. 93: 606–613, 2005.
17. Bishop NC, Walker GJ, Scanlon GA, Richards S, Rogers E. Salivary IgA responses to prolonged intensive exercise following caffeine ingestion. Med. Sci. Sports Exerc. 38: 513–519, 2006.
18. Bittencourt LS, Machado DC, Machado MM, Dos Santos GFF, Algarve TD, Marinowic DR, Ribeiro EE, Soares FAA, Barbisan F, Athayde ML, Cruz IBM. The protective effects of guaraná extract (Paullinia cupana) on fibroblast NIH-3T3 cells exposed to sodium nitroprusside. Food Chem. Toxicol. 53: 119–125, 2013.

19. Black CD, Herring MP, Hurley DJ, O'Connor PJ. Ginger (Zingiber officinale) reduces muscle pain caused by eccentric exercise. J. Pain 11: 894–903, 2010.
20. Bracesco N, Sanchez AG, Contreras V, Menini T, Gugliucci A. Recent advances on Ilex paraguariensis research: minireview. J. Ethnopharmacol. 136: 378–384, 2011.
21. Brown AC. Anticancer activity of Morinda citrifolia (Noni) fruit: a review. Phytother. Res. 26: 1427–1440, 2012.
22. Buell JL, Franks R, Ransone J, Powers ME, Laquale KM, Carlson-Phillips A, National Athletic Trainers' Association. National Athletic Trainers' Association position statement: evaluation of dietary supplements for performance nutrition. J. Athl. Train. 48: 124–136, 2013.
23. Butt MS, Sultan MT. Ginger and its health claims: molecular aspects. Crit. Rev. Food Sci. Nutr. 51: 383–393, 2011.
24. Castell M, Perez-Cano FJ, Bisson J-F. Clinical Benefits of Cocoa: An Overview. In: Chocolate in Health and Nutrition, edited by Watson RR, Preedy VR, Zibadi S. Humana Press, 2013, p. 265–275.
25. Chen CK, Muhamad AS, Ooi FK. Herbs in exercise and sports. J. Physiol. Anthropol. 31: 4, 2012.
26. Cherdshewasart W, Sutjit W. Correlation of antioxidant activity and major isoflavonoid contents of the phytoestrogen-rich Pueraria mirifica and Pueraria lobata tubers. Phytomedicine 15: 38–43, 2008.
27. Chorostowska-Wynimko J, Kus J, Skopińska-Rózewska E. Theophylline inhibits free oxygen radicals production by human monocytes via phosphodiesterase inhibition. J. Physiol. Pharmacol. 58 Suppl 5: 95–103, 2007.
28. Chrubasik S, Pittler MH, Roufogalis BD. Zingiberis rhizoma: a comprehensive review on the ginger effect and efficacy profiles. Phytomedicine 12: 684–701, 2005.
29. Cienki JJ, Zaret L. An Internet misadventure: bloodroot salve toxicity. J. Altern. Complement. Med. 16: 1125–1127, 2010.
30. Coimbra S, Castro E, Rocha-Pereira P, Rebelo I, Rocha S, Santos-Silva A. The effect of green tea in oxidative stress. Clin. Nutr. 25: 790–796, 2006.
31. Conquer JA, Maiani G, Azzini E, Raguzzini A, Holub BJ. Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. J. Nutr. 128: 593–597, 1998.
32. Cosio BG, Iglesias A, Rios A, Noguera A, Sala E, Ito K, Barnes PJ, Agusti A. Low-dose theophylline enhances the anti-inflammatory effects of steroids during exacerbations of COPD. Thorax 64: 424–429, 2009.
33. Davison G, Callister R, Williamson G, Cooper KA, Gleeson M. The effect of acute pre-exercise dark chocolate consumption on plasma antioxidant status, oxidative stress and immunoendocrine responses to prolonged exercise. Eur. J. Nutr. 51: 69–79, 2012.
34. Du X, Wang J, Niu X, Smith D, Wu D, Meydani SN. Dietary Wolfberry Supplementation Enhances the Protective Effect of Flu Vaccine against Influenza Challenge in Aged Mice. J. Nutr. 144: 224-229, 2014.
35. Ezeamuzie CI, Shihab PK. Interactions between theophylline and salbutamol on cytokine release in human monocytes. J. Pharmacol. Exp. Ther. 334: 302–309, 2010.
36. Fernandez MMF, Hosey RG. Performance-enhancing drugs snare nonathletes, too. J. Fam. Pract. 58: 16–23, 2009.

37. Fernández-Murga L, Tarín JJ, García-Perez MA, Cano A. The impact of chocolate on cardiovascular health. Maturitas 69: 312–321, 2011.
38. Ferreira MP, Gendron F, McClure K, Kindscher K. North American Bioactive Plants for Human Health and Performance. Glob. J. Res. Med. Plants Indig. Med. 1: 568–582, 2012.
39. Fiebich BL, Collado JA, Stratz C, Valina C, Hochholzer W, Muñoz E, Bellido LM. Pseudoephedrine inhibits T-cell activation by targeting NF-κB, NFAT and AP-1 signaling pathways. Immunopharmacol. Immunotoxicol. 34: 98–106, 2012.
40. Fletcher DK, Bishop NC. Effect of a high and low dose of caffeine on antigen-stimulated activation of human natural killer cells after prolonged cycling. Int. J. Sport Nutr. Exerc. Metab. 21: 155–165, 2011.
41. Fletcher DK, Bishop NC. Effect of a single and repeated dose of caffeine on antigen-stimulated human natural killer cell CD69 expression after high-intensity intermittent exercise. Eur. J. Appl. Physiol. 111: 1329–1339, 2011.
42. Fletcher DK, Bishop NC. Caffeine ingestion and antigen-stimulated human lymphocyte activation after prolonged cycling. Scand. J. Med. Sci. Sports 22: 249–258, 2012.
43. Van Furth AM, Seijmonsbergen EM, Langermans JA, van der Meide PH, van Furth R. Effect of xanthine derivates and dexamethasone on Streptococcus pneumoniae-stimulated production of tumor necrosis factor alpha, interleukin-1 beta (IL-1 beta), and IL-10 by human leukocytes. Clin. Diagn. Lab. Immunol. 2: 689–692, 1995.
44. Gilroy CM, Steiner JF, Byers T, Shapiro H, Georgian W. Echinacea and truth in labeling. Arch. Intern. Med. 163: 699–704, 2003.
45. Gleeson M. Can nutrition limit exercise-induced immunodepression? Nutr. Rev. 64: 119–131, 2006.
46. Graham TE. Caffeine and exercise: metabolism, endurance and performance. Sports Med. 31: 785–807, 2001.
47. Graham TE. Caffeine, coffee and ephedrine: impact on exercise performance and metabolism. Can. J. Appl. Physiol. 26 Suppl: S103–119, 2001.
48. Gu Y, Lambert JD. Modulation of metabolic syndrome-related inflammation by cocoa. Mol. Nutr. Food Res. 57: 948–961, 2013.
49. Gunzer W, Konrad M, Pail E. Exercise-induced immunodepression in endurance athletes and nutritional intervention with carbohydrate, protein and fat-what is possible, what is not? Nutrients 4: 1187–1212, 2012.
50. Gupta RK, Patel AK. Do the health claims made for Morinda citrifolia (Noni) harmonize with current scientific knowledge and evaluation of its biological effects. Asian Pac. J. Cancer Prev. 14: 4495–4499, 2013.
51. Haller CA, Benowitz NL. Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids. N. Engl. J. Med. 343: 1833–1838, 2000.
52. Haller CA, Jacob P, Benowitz NL. Short-term metabolic and hemodynamic effects of ephedra and guarana combinations. Clin. Pharmacol. Ther. 77: 560–571, 2005.
53. Haskell CF, Dodd FL, Wightman EL, Kennedy DO. Behavioural effects of compounds co-consumed in dietary forms of caffeinated plants. Nutr. Res. Rev. 26: 49–70, 2013.
54. Hismiogullari AA, Punduk Z, Hismiogullari SE, Yavuz O. The acute effect of commercial "energy drink" on hematological, biochemical and strength parameters induced by eccentric contractions. Isokinet. Exerc. Sci. 21: 247–252, 2013.

55. Hollman PC, vd Gaag M, Mengelers MJ, van Trijp JM, de Vries JH, Katan MB. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. Free Radic. Biol. Med. 21: 703–707, 1996.
56. Horrigan LA, Kelly JP, Connor TJ. Caffeine suppresses TNF-alpha production via activation of the cyclic AMP/protein kinase A pathway. Int. Immunopharmacol. 4: 1409–1417, 2004.
57. Horrigan LA, Kelly JP, Connor TJ. Immunomodulatory effects of caffeine: friend or foe? Pharmacol. Ther. 111: 877–892, 2006.
58. Iiboshi H, Ashitani J-I, Katoh S, Sano A, Matsumoto N, Mukae H, Nakazato M. Long-term treatment with theophylline reduces neutrophils, interleukin-8 and tumor necrosis factor-alpha in the sputum of patients with chronic obstructive pulmonary disease. Pulm. Pharmacol. Ther. 20: 46–51, 2007.
59. Isa Y, Miyakawa Y, Yanagisawa M, Goto T, Kang M-S, Kawada T, Morimitsu Y, Kubota K, Tsuda T. 6-Shogaol and 6-gingerol, the pungent of ginger, inhibit TNF-alpha mediated downregulation of adiponectin expression via different mechanisms in 3T3-L1 adipocytes. Biochem. Biophys. Res. Commun. 373: 429–434, 2008.
60. Kaplan GB, Greenblatt DJ, Kent MA, Cotreau-Bibbo MM. Caffeine treatment and withdrawal in mice: relationships between dosage, concentrations, locomotor activity and A1 adenosine receptor binding. J. Pharmacol. Exp. Ther. 266: 1563–1572, 1993.
61. Katz DL, Doughty K, Ali A. Cocoa and chocolate in human health and disease. Antioxid. Redox Signal. 15: 2779–2811, 2011.
62. Kawada S, Kobayashi K, Ohtani M, Fukusaki C. Cystine and theanine supplementation restores high-intensity resistance exercise-induced attenuation of natural killer cell activity in well-trained men. J. Strength Cond. Res. 24: 846–851, 2010.
63. Kim SO, Kundu JK, Shin YK, Park J-H, Cho M-H, Kim T-Y, Surh Y-J. [6]-Gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-kappaB in phorbol ester-stimulated mouse skin. Oncogene 24: 2558–2567, 2005.
64. Lantz RC, Chen GJ, Sarihan M, Sólyom AM, Jolad SD, Timmermann BN. The effect of extracts from ginger rhizome on inflammatory mediator production. Phytomedicine 14: 123–128, 2007.
65. Laurent D, Schneider KE, Prusaczyk WK, Franklin C, Vogel SM, Krssak M, Petersen KF, Goforth HW, Shulman GI. Effects of caffeine on muscle glycogen utilization and the neuroendocrine axis during exercise. J. Clin. Endocrinol. Metab. 85: 2170–2175, 2000.
66. Leiherer A, Mündlein A, Drexel H. Phytochemicals and their impact on adipose tissue inflammation and diabetes. Vascul. Pharmacol. 58: 3–20, 2013.
67. Leung LK, Su Y, Chen R, Zhang Z, Huang Y, Chen ZY. Theaflavins in black tea and catechins in green tea are equally effective antioxidants. J. Nutr. 131: 2248–2251, 2001.
68. Ling M, Piddlesden SJ, Morgan BP. A component of the medicinal herb ephedra blocks activation in the classical and alternative pathways of complement. Clin. Exp. Immunol. 102: 582–588, 1995.
69. Lockwood CM, Moon JR, Smith AE, Tobkin SE, Kendall KL, Graef JL, Cramer JT, Stout JR. Low-calorie energy drink improves physiological response to exercise in previously sedentary men: a placebo-controlled efficacy and safety study. J. Strength Cond. Res. 24: 2227–2238, 2010.

70. Macdonald SL, Perry TL, Rose MC. Glycaemic, insulinaemic, and immune responses to commercially available beverages consumed during recovery from rowing training. Eur. J. Sport Sci. 10: 337–345, 2010.
71. Machado M, Antunes WD, Tamy ALM, Azevedo PG, Barreto JG, Hackney AC. Effect of a Single Dose of Caffeine Supplementation and Intermittent-interval Exercise on Muscle Damage Markers in Soccer Players. J. Exerc. Sci. Fit. 7: 91–97, 2009.
72. Machado M, Koch AJ, Willardson JM, dos Santos FC, Curty VM, Pereira LN. Caffeine does not augment markers of muscle damage or leukocytosis following resistance exercise. Int. J. Sports Physiol. Perform. 5: 18–26, 2010.
73. Mahdavi R, Daneghian S, Homayouni A, Jafari A. Effects of caffeine supplementation on oxidative stress, exercise-induced muscle damage and leukocytosis. Pharm. Sci. 18: 117–182, 2012.
74. Malaguti M, Angeloni C, Hrelia S. Polyphenols in exercise performance and prevention of exercise-induced muscle damage. Oxid. Med. Cell. Longev. 2013: 825928, 2013.
75. Mashhadi NS, Ghiasvand R, Askari G, Feizi A, Hariri M, Darvishi L, Barani A, Taghiyar M, Shiranian A, Hajishafiee M. Influence of ginger and cinnamon intake on inflammation and muscle soreness endued by exercise in Iranian female athletes. Int. J. Prev. Med. 4: S11–15, 2013.
76. Mashhadi NS, Ghiasvand R, Askari G, Hariri M, Darvishi L, Mofid MR. Anti-oxidative and anti-inflammatory effects of ginger in health and physical activity: review of current evidence. Int. J. Prev. Med. 4: S36–42, 2013.
77. Maughan R. The athlete's diet: nutritional goals and dietary strategies. Proc. Nutr. Soc. 61: 87–96, 2002.
78. McBrier NM, Vairo GL, Bagshaw D, Lekan JM, Bordi PL, Kris-Etherton PM. Cocoa-based protein and carbohydrate drink decreases perceived soreness after exhaustive aerobic exercise: a pragmatic preliminary analysis. J. Strength Cond. Res. 24: 2203–2210, 2010.
79. McLellan TM, Lieberman HR. Do energy drinks contain active components other than caffeine? Nutr. Rev. 70: 730–744, 2012.
80. Mehendale SR, Bauer BA, Yuan C-S. Ephedra-containing dietary supplements in the US versus ephedra as a Chinese medicine. Am. J. Chin. Med. 32: 1–10, 2004.
81. Meiners I, Hauschildt S, Nieber K, Münch G. Pentoxyphylline and propentophylline are inhibitors of TNF-alpha release in monocytes activated by advanced glycation endproducts. J. Neural Transm. 111: 441–447, 2004.
82. Murakami S, Kurihara S, Koikawa N, Nakamura A, Aoki K, Yosigi H, Sawaki K, Ohtani M. Effects of oral supplementation with cystine and theanine on the immune function of athletes in endurance exercise: randomized, double-blind, placebo-controlled trial. Biosci. Biotechnol. Biochem. 73: 817–821, 2009.
83. Murakami S, Kurihara S, Titchenal CA, Ohtani M. Suppression of exercise-induced neutrophilia and lymphopenia in athletes by cystine/theanine intake: a randomized, double-blind, placebo-controlled trial. J. Int. Soc. Sports Nutr. 7: 23, 2010.
84. Nakagawa K, Okuda S, Miyazawa T. Dose-dependent incorporation of tea catechins, (-)-epigallocatechin-3-gallate and (-)-epigallocatechin, into human plasma. Biosci. Biotechnol. Biochem. 61: 1981–1985, 1997.
85. Narotzki B, Reznick AZ, Navot-Mintzer D, Dagan B, Levy Y. Green Tea and Vitamin E Enhance Exercise-Induced Benefits in Body Composition, Glucose Homeostasis, and Antioxidant Status in Elderly Men and Women. J. Am. Coll. Nutr. 32: 31–40, 2013.

86. Nieman DC, Gillitt ND, Knab AM, Shanely RA, Pappan KL, Jin F, Lila MA. Influence of a Polyphenol-Enriched Protein Powder on Exercise-Induced Inflammation and Oxidative Stress in Athletes: A Randomized Trial Using a Metabolomics Approach. PLoS ONE 8: e72215, 2013.
87. Nieman DC, Laupheimer MW, Ranchordas MK, Burke LM, Stear SJ, Castell LM. A-Z of nutritional supplements: dietary supplements, sports nutrition foods and ergogenic aids for health and performance--Part 33. Br. J. Sports Med. 46: 618–620, 2012.
88. Nieman DC. Quercetin's bioactive effects in human athletes. Curr. Top. Nutraceutical Res. 8: 33–44, 2010.
89. Norton RP, Lehnhard RA, Kearns CF, McKeever KH. Ephedra increases VO2, impairs thermoregulation and cardiovascular function, but does not enhance exercise performance. Comp. Exerc. Physiol. 9: 109–117, 2013.
90. Ohta K, Fukuchi Y, Grouse L, Mizutani R, Rabe KF, Rennard SI, Zhong N-S. A prospective clinical study of theophylline safety in 3810 elderly with asthma or COPD. Respir. Med. 98: 1016–1024, 2004.
91. Palu AK, Kim AH, West BJ, Deng S, Jensen J, White L. The effects of Morinda citrifolia L. (noni) on the immune system: its molecular mechanisms of action. J. Ethnopharmacol. 115: 502–506, 2008.
92. Patz MD, Day HEW, Burow A, Campeau S. Modulation of the hypothalamo-pituitary-adrenocortical axis by caffeine. Psychoneuroendocrinology 31: 493–500, 2006.
93. Pawlus AD, Kinghorn DA. Review of the ethnobotany, chemistry, biological activity and safety of the botanical dietary supplement Morinda citrifolia (noni). J. Pharm. Pharmacol. 59: 1587–1609, 2007.
94. Pereira R, Machado M, Sampaio-Jorge F, Barreto JG, Lazo Osorio RA. Intermittent exercise and caffeine supplementation: effects on the white blood cells. Rev. Int. Med. Cienc. Act. Física El Deporte 8: 353–361, 2008.
95. Perera MADN, Robinson JR, Shearn CT, Noble TM, Hallam JE, Kohut ML, Senchina DS. Effects of bloodroot (Sanguinaria canadensis L.) rhizome ethanol extracts on cytokine production by blood mononuclear cells during flowering and fruiting. J. Herb. Med. doi: 10.1016/j.hermed.2013.11.001.
96. Rados C. Ephedra ban: no shortage of reasons. FDA Consum. 38: 6–7, 2004.
97. Ranchordas MK, Burd NA, Godfrey RJ, Senchina DS, Stear SJ, Burke LM, Castell LM. A-Z of nutritional supplements: dietary supplements, sports nutrition foods and ergogenic aids for health and performance: Part 43. Br. J. Sports Med. 47: 399–400, 2013.
98. Rath M. Energy drinks: what is all the hype? The dangers of energy drink consumption. J. Am. Acad. Nurse Pract. 24: 70–76, 2012.
99. Raynor DK, Dickinson R, Knapp P, Long AF, Nicolson DJ. Buyer beware? Does the information provided with herbal products available over the counter enable safe use? BMC Med. 9: 94, 2011.
100. Ren Z, Na L, Xu Y, Rozati M, Wang J, Xu J, Sun C, Vidal K, Wu D, Meydani SN. Dietary supplementation with lacto-wolfberry enhances the immune response and reduces pathogenesis to influenza infection in mice. J. Nutr. 142: 1596–1602, 2012.
101. Roman MC. Determination of ephedra alkaloids in urine and plasma by HPLC-UV: collaborative study. J. AOAC Int. 87: 15–24, 2004.

102. Rosen T. Green tea catechins: biologic properties, proposed mechanisms of action, and clinical implications. J. Drugs Dermatol. 11: e55–60, 2012.
103. Rosenthal LA, Taub DD, Moors MA, Blank KJ. Methylxanthine-induced inhibition of the antigen- and superantigen-specific activation of T and B lymphocytes. Immunopharmacology 24: 203–217, 1992.
104. Senchina DS, Flinn GN, McCann DA, Kohut ML, Shearn CT. Bloodroot (Sanguinaria canadensis L., Papaveraceae) enhances proliferation and cytokine production by human peripheral blood mononuclear cells in an in vitro model. J. Herbs Spices Med. Plants 15: 45–65, 2009.
105. Senchina DS, Hallam JE, Cheney DJ. Multidisciplinary perspectives on mechanisms of activity of popular immune-enhancing herbal supplements used by athletes. Front. Biol. 8: 78–100, 2013.
106. Senchina DS, Hallam JE, Dias AS, Perera MA. Human blood mononuclear cell in vitro cytokine response before and after two different strenuous exercise bouts in the presence of bloodroot and Echinacea extracts. Blood Cells. Mol. Dis. 43: 298–303, 2009.
107. Senchina DS, Hallam JE, Thompson NM, Nguyen NA, Robinson JR, Perera MA. Alkaloids and endurance athletes. Track Cross Ctry. J. 2: 2–18, 2012.
108. Senchina DS, Shah NB, Doty DM, Sanderson CR, Hallam JE. Herbal supplements and athlete immune function--what's proven, disproven, and unproven? Exerc. Immunol. Rev. 15: 66–106, 2009.
109. Senchina DS. Athletics and herbal supplements. Am. Sci. 101: 134–141, 2013.
110. Shao X, Lv L, Parks T, Wu H, Ho C-T, Sang S. Quantitative analysis of ginger components in commercial products using liquid chromatography with electrochemical array detection. J. Agric. Food Chem. 58: 12608–12614, 2010.
111. Shimizu T, Kristjánsson S, Wennergren G, Hansson GC, Strandvik B. Inhibitory effects of theophylline, terbutaline, and hydrocortisone on leukotriene B4 and C4 generation by human leukocytes in vitro. Pediatr. Pulmonol. 18: 129–134, 1994.
112. Shively CA, Tarka SM Jr. Methylxanthine composition and consumption patterns of cocoa and chocolate products. Prog. Clin. Biol. Res. 158: 149–178, 1984.
113. Singh I, Quinn H, Mok M, Southgate RJ, Turner AH, Li D, Sinclair AJ, Hawley JA. The effect of exercise and training status on platelet activation: do cocoa polyphenols play a role? Platelets 17: 361–367, 2006.
114. Van Soeren MH, Graham TE. Effect of caffeine on metabolism, exercise endurance, and catecholamine responses after withdrawal. J. Appl. Physiol. 85: 1493–1501, 1998.
115. Stear SJ, Castell L, Burke L, Spriet LL. BJSM reviews: A–Z of nutritional supplements: dietary supplements, sports nutrition foods and ergogenic aids for health and performance Part 6. Br. J. Sports Med. 44: 297 –298, 2010.
116. Sullivan GW, Carper HT, Mandell GL. Pentoxifylline modulates activation of human neutrophils by amphotericin B in vitro. Antimicrob. Agents Chemother. 36: 408–416, 1992.
117. Sullivan GW, Luong LS, Carper HT, Barnes RC, Mandell GL. Methylxanthines with adenosine alter TNF alpha-primed PMN activation. Immunopharmacology 31: 19–29, 1995.
118. Tauler P, Martínez S, Moreno C, Monjo M, Martínez P, Aguiló A. Effects of caffeine on the inflammatory response induced by a 15-km run competition. Med. Sci. Sports Exerc. 45: 1269–1276, 2013.

119. Terry R, Posadzki P, Watson LK, Ernst E. The use of ginger (Zingiber officinale) for the treatment of pain: a systematic review of clinical trials. Pain Med. 12: 1808–1818, 2011.
120. Usami A, Ueki S, Ito W, Kobayashi Y, Chiba T, Mahemuti G, Oyamada H, Kamada Y, Fujita M, Kato H, Saito N, Kayaba H, Chihara J. Theophylline and dexamethasone induce peroxisome proliferator-activated receptor-gamma expression in human eosinophils. Pharmacology 77: 33–37, 2006.
121. Vernec A, Stear SJ, Burke LM, Castell LM. A–Z of nutritional supplements: dietary supplements, sports nutrition foods and ergogenic aids for health and performance: Part 48. Br. J. Sports Med. 47: 998–1000, 2013.
122. Vimalanathan S, Arnason JT, Hudson J. Anti-inflammatory activities of Echinacea extracts do not correlate with traditional marker components. Pharm. Biol. 47: 430–435, 2009.
123. Vimercatti NS, Zovico PVC, Carvalho AS, Barreto JG, Machado M. Two doses of caffeine do not increase the risk of exercise-induced muscle damage or leukocytosis. Phys. Educ. Sport 52: 96–99, 2008.
124. Vlachojannis C, Magora F, Chrubasik S. Rise and fall of oral health products with Canadian bloodroot extract. Phytother. Res. 26: 1423–1426, 2012.
125. Walker GJ, Caudwell P, Dixon N, Bishop NC. The effect of caffeine ingestion on neutrophil oxidative burst responses following prolonged cycling. Int. J. Sport Nutr. Exerc. Metab. 16: 24–35, 2006.
126. Walker GJ, Dziubak A, Houghton L, Prendergast C, Lim L, Bishop NC. The effect of caffeine ingestion on human neutrophil oxidative burst responses following time-trial cycling. J. Sports Sci. 26: 611–619, 2008.
127. Walker GJ, Finlay O, Griffiths H, Sylvester J, Williams M, Bishop NC. Immunoendocrine response to cycling following ingestion of caffeine and carbohydrate. Med. Sci. Sports Exerc. 39: 1554–1560, 2007.
128. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, Simon P. Position statement. Part one: Immune function and exercise. Exerc. Immunol. Rev. 17: 6–63, 2011.
129. Watson R, Woodman R, Lockette W. Ephedra alkaloids inhibit platelet aggregation. Blood Coagul. Fibrinolysis 21: 266–271, 2010.
130. Wei F, Zhang M, Feng Y-Q. Combining poly (methacrylic acid-co-ethylene glycol dimethacrylate) monolith microextraction and on-line pre-concentration-capillary electrophoresis for analysis of ephedrine and pseudoephedrine in human plasma and urine. J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 850: 38–44, 2007.
131. Wilasrusmee C, Siddiqui J, Bruch D, Wilasrusmee S, Kittur S, Kittur DS. In vitro immunomodulatory effects of herbal products. Am. Surg. 68: 860–864, 2002.
132. Wiswedel I, Hirsch D, Kropf S, Gruening M, Pfister E, Schewe T, Sies H. Flavanol-rich cocoa drink lowers plasma F(2)-isoprostane concentrations in humans. Free Radic. Biol. Med. 37: 411–421, 2004.
133. Wolfram S. Effects of green tea and EGCG on cardiovascular and metabolic health. J. Am. Coll. Nutr. 26: 373S–388S, 2007.
134. Wong CP, Nguyen LP, Noh SK, Bray TM, Bruno RS, Ho E. Induction of regulatory T cells by green tea polyphenol EGCG. Immunol. Lett. 139: 7–13, 2011.

135. Van der Woude H, Gliszczyńska-Swigło A, Struijs K, Smeets A, Alink GM, Rietjens IMCM. Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans. Cancer Lett. 200: 41–47, 2003.
136. Yao P-L, Tsai M-F, Lin Y-C, Wang C-H, Liao W-Y, Chen JJW, Yang P-C. Global expression profiling of theophylline response genes in macrophages: evidence of airway anti-inflammatory regulation. Respir. Res. 6: 89, 2005.
137. Yasui K, Kondo Y, Wada T, Yashiro M, Tsuge M, Morishima T. Theophylline inhibits the differentiation of human monocyte into dendritic cell potentially via adenosine receptor antagonism. Clin. Exp. Allergy 39: 1857–1865, 2009.
138. Yu Y, Zick S, Li X, Zou P, Wright B, Sun D. Examination of the pharmacokinetics of active ingredients of ginger in humans. AAPS J. 13: 417–426, 2011.
139. Zick SM, Djuric Z, Ruffin MT, Litzinger AJ, Normolle DP, Alrawi S, Feng MR, Brenner DE. Pharmacokinetics of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol and conjugate metabolites in healthy human subjects. Cancer Epidemiol. Biomark. Prev. 17: 1930–1936, 2008.

Altered immune response to exercise in patients with chronic fatigue syndrome/myalgic encephalomyelitis: A systematic literature review

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ABSTRACT

An increasing number of studies have examined how the immune system of patients with Chronic Fatigue Syndrome (CFS), or myalgic encephalomyelitis, responds to exercise. The objective of the present study was to systematically review the scientific literature addressing exercise-induced immunological changes in CFS patients compared to healthy control subjects. A systematic literature search was conducted in the PubMed and Web of science databases using different keyword combinations. We included 23 case control studies that examined whether CFS patients, compared to healthy sedentary controls, have a different immune response to exercise. The included articles were evaluated on their methodological quality. Compared to the normal response of the immune system to exercise as seen in healthy subjects, patients with CFS have a more pronounced response in the complement system (i.e. C4a split product levels), oxidative stress system (i.e. enhanced oxidative stress combined with a delayed and reduced anti-oxidant response), and an alteration in the immune cells' gene expression profile (increases in post-exercise interleukin-10 and toll-like receptor 4 gene expression), but not in circulating pro- or anti-inflammatory cytokines. Many of

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these immune changes relate to post-exertional malaise in CFS, a major characteristic of the illness. The literature review provides level B evidence for an altered immune response to exercise in patients with CFS.

Keywords: fatigue, pain, genetics, oxidative stress, complement system, cytokine, inflammation, exercise, physical activity

INTRODUCTION

Chronic fatigue syndrome (CFS), or myalgic encephalomyeltitis, is a condition defined by the 1994 Center for Disease Control and Prevention definition (11, 15). The major symptom is fatigue, of new or definite onset (not been lifelong), lasting for 6 months or longer (11, 15). CFS is diagnosed by exclusion of any other medical condition which might explain the symptoms (e.g. untreated hypothyroidism, sleep apnea, narcolepsy, major depressive disorder, bipolar affective disorders, schizophrenia, anorexia nervosa, bulimia nervosa, severe obesity, etc.) (11, 15). In addition, four or more of the following symptoms must be present for 6 months or longer: impaired memory or concentration; sore throat; tender lymph nodes (cervical or axillary); muscle pain; pain in multiple joints without joint swelling or redness; headaches of a new kind or greater severity; unrefreshing sleep and post-exertional malaise lasting more than 24 hours (11). Symptoms are often exacerbated during and after physical activities (54).

The presence of symptoms like a sore throat, tender lymph nodes, and low-grade fever, as well as flu-like symptoms including widespread muscle pain and severe fatigue, has inspired researchers to search for immune abnormalities in patients with CFS. Several immune abnormalities have been reported in CFS patients: decreased natural killer cell activity (44), altered functional B cell subset populations (3), alterations in cytokine production (52), alterations in inflammatory markers (25), increased nitrosative and oxidative stress pathways (25-27), upregulation of various aspects of the 2'-5' oligoadenylate synthetase/RNase L pathway (28, 32, 43, 50, 51), among others. However, many of the observed abnormalities were not confirmed by others, resulting in inconsistent findings across studies (12, 31). This probably relates to the heterogeneous nature of the illness, the use of different diagnostic criteria for diagnosing CFS, the different laboratory methods used for measuring immune function (i.e. different assays, specimens or stimuli), and the fluctuating nature of CFS.

Besides resting immune function, the immune system of patients with CFS might respond differently to exercise as compared to what we see in healthy, sedentary controls. The 2011 International Consensus Document regarding Myalgic Encephalomyelitis acknowledges the importance of malaise following exercise for the diagnosis of CFS (8). Indeed, in CFS patients too vigorous exercise (1, 18, 20) or a sudden increase in activity (2) frequently triggers a severe increase in symptoms. The severe exacerbation of symptoms following exercise, as seen in CFS patients, is one of the core features of the illness (38, 47).

In addition, the (normal) effects of exercise on the body's immune system are well established, and an ever-growing volume of scientific publications speaks to the rapid growth in understanding of exercise immunology (56). An increasing number of studies have examined the response of the immune system to exercise in CFS patients (e.g. (18, 19, 22, 23, 35, 37, 47)), yet the abnormal immune responses to exercise in CFS patients has not been conclusively defined and it remains unclear whether exercise-induced immune abnormalities can be regarded as biomarker for CFS.

Hence, the objective of the present study was to systematically review the scientific literature addressing exercise-induced immunological changes in CFS patients compared to healthy control subjects. Specifically the review examined whether CFS patients, compared to healthy sedentary controls, have a different immune response to exercise. Thus, the review intends to answer the following question: does the immune system of patients with CFS respond differently to exercise as compared to healthy sedentary controls? It is hypothesized that CFS patients show a more pronounced immune response to exercise as compared to healthy sedentary controls.

METHODS

This systematic review is reported following the PRISMA-guidelines (Preferred Reporting Items for Systematic reviews and Meta-Analyses), which is an updated statement addressing the conceptual and methodological issues of the original QUOROM Statement (29).

Search strategy

The aim of this systematic review was to answer the research question that was formulated using the PICO model; "Do CFS patients (P) have a different immune response (O) to exercise (I) compared to healthy people (C)?". To answer the research question a systematic search of the existing literature up to August 2013 was conducted on the electronic databases PubMed and Web of Science, and is reported following the PRISMA guidelines (21). The search strategy was based on a combination of search terms and Mesh terms, which were derived from the "PICO" research question. Therefore all search terms from "P" (combined with OR) were combined with the possible search terms from "I" (combined with OR) and "O", using the boolean term 'AND'. The used search and Mesh terms, and the construct of the search strategy are presented in Table 1. No filters were used during the search strategy.

Study selection

To be included in this systematic review, papers had to fulfill predefined inclusion criteria regarding type of report, topic and population studied. Only full text clinical reports / (original) research reports (= *type of repor*t) which examined exercise induced immunological changes (= *topic*) in adult (≥18 years) CFS patients and compared these with the results of healthy controls (= *population studied*) were eligible. No language, publication date, or publication status restrictions were imposed, and all clinical study designs were eligible. Non-clini-

Table 1. The search terms used for the literature review.

Patient		AND	Intervention		AND	Outcome	
Keywords Pubmed	**Keywords Web of Science**		**Keywords Pubmed**	**Keywords Web of Science**		**Keywords Pubmed**	**Keywords Web of Science**
Chronic fatigue syndrome [Mesh]	Chronic fatigue syndrome		Exercise [Mesh]	Exercise		Immune system phenomena [Mesh]	Immune system phenomena
	Myalgic encephalomyelitis			Physical exercise		Immune system	Immune system
	Postviral fatigue syndrome			Aerobic exercise		Immune function	Immune function
	Chronic fatigue disorder		Motor activity [Mesh]	Motor activities		Immunology	Immunology
				Physical activity		Immunity	Immunity
				Locomotor activity		Cytokines [Mesh]	Cytokines
			Exercise therapy [Mesh]	Exercise therapy		Lymphokines [Mesh]	Lymphokines
						Lymphocytes [Mesh]	Lymphocyte
							Lymphoid cells
						Leukocytes [Mesh]	Leukocyte
							White blood cell
						Complement System Proteins [Mesh]	Complement system proteins
							Complement protein
							Complement
						Natural Killer T-cells [Mesh]	Natural killer t-cell
							NKT Cells
						T-Lymphocytes, Cytotoxic [Mesh]	Cytotoxic t-lymphocyte
							Cell-mediated lympholytic cells
						Cytotoxicity, Immunologic [Mesh]	Immunologic cytotoxicity
						Gene expression [Mesh]	Gene expression
						Gene expression profiling [Mesh]	Gene expression profiling
						Apoptosis [Mesh]	Apoptosis
							Programmed cell death, type I
							Intrinsic pathway apoptosis
							Extrinsic pathway apoptosis
						Necrosis [Mesh]	Necrosis
						Autophagy [Mesh]	Autophagy
						Biological Markers [Mesh]	Biological marker
							Biomarkers
							Clinical marker
							Immunologic marker
							Viral marker
							Serum marker
							Biochemical marker
							Laboratory marker
						Laboratory tests	Laboratory tests

cal reports such as reviews, abstracts, posters, letters to the editor, and editorials were excluded. First, the title and abstract of all citations retrieved using the search strategy were screened using the inclusion criteria. If it was not clear from the abstract whether the study was eligible for study inclusion, the full-text was consulted. The full text versions of all papers that met the inclusion criteria were retrieved for quality assessment and data extraction.

Qualification of searchers

The literature was searched and screened independently by AN and JVO. The risk of bias was assessed by 3 researchers (AN, JVO and JN), who were blinded from each other's assessment. AN holds a bachelor degree in rehabilitation sciences, and was trained by the final author. JVO and JN hold a PhD degree and have published several systematic literature reviews.

Risk of bias assessment

Each study that fulfilled the inclusion criteria was assessed for methodological quality using the evaluation 'Evidence Base Richtlijn Ontwikkeling' (EBRO) criteria for case-control studies as recommended by The Dutch Cochrane Centre (http://dcc.cochrane.org/sites/dcc.cochrane.org/files/uploads/patient-controleonderzoek.pdf). The evaluation criteria for case-control studies assess 6 items. First it is assessed whether an adequate definition of the case group is given (C1), and secondly if this is also the case for the control group (C2). Therefore we assessed whether the total sample size and demographical characteristics were described for each group. For the case group of CFS patients the fulfilled CFS diagnostic criteria had to be mentioned. In case only sedentary or moderately active subjects were included in the control group, a description of the authors' understanding of sedentary or moderately active was expected. The 3rd item controls for exclusion of selection bias (*C3*), which implied that studied groups needed to be representative of the general population. This item was scored negative when the included age range was not representative for the general adult population or when only male CFS patients were recruited. A negative assessment was given in case the patient sample was recruited solely from one sort of setting such as a specific hospital department or only through a patient support group, as combining a variety of recruitment procedures is recommended to prevent recruitment bias (33). The 4th item entails a clear description of the exposure and an adequate method for assessment (*C4*), The 5th item was related to blinding of the involved assessors (*C5*). In case an assessor could influence the results, non-blinding or failure to mention blinding was penalized. This included that saliva and blood processing / analyses was performed by personnel blinded to the subject's health status. As a final point identification or accounting for confounders was assessed (*C6*). Possible confounders related to exercise performance and immunology are body mass index (BMI), physical activity levels, and menstrual phase. Furthermore, pooling of gender data has been identified as an important source of bias in studies of exercise physiology in CFS patients (42).

When a study fulfilled a criterion a positive score was given, when a criterion was applicable but the study did not comply with the criterion a negative was given. When no adequate information was present regarding an applicable criterion, that

item was scored as negative. If a criterion was not applicable to the design and purpose of the study it was not scored. Fulfillment of the criteria was assessed by 3 independent, blinded researchers (JVO, JN and AN) using a score sheet. After rating the selected articles, the results of all three researchers were compared, and the amount of agreement (in percentages) between the 3 researchers was calculated. In case of disagreement, the final risk of bias score was the score which was given by 2 out of the 3 researchers. Finally, a total score for methodological quality was computed by adding up the scores for each of the related criteria which were applicable. The maximum total score that could be achieved was 6, and was also presented as a percentage to facilitate comparison between studies. In table 2 the risk of bias scores of the studies are presented. Levels of evidence were defined based on study design and quality, according to the EBRO-guidelines.

Table 2. Risk of bias and level of evidence of the exercise immunology studies in CFS patients.

References	Criteria						Total quality score	Total quality %	Level of evidence
	1	2	3	4	5	6			
Cannon et al. 1999 (7)	+	+	+	+	-	+	5	83,3	B
Light et al. 2012 (22)	+	-	+	+	+	+	5	83,3	B
Jammes et al. 2009 (17)	+	+	+	+	-	-	4	66,7	B
Jammes et al. 2012 (16)	+	+	+	+	-	-	4	66,7	B
LaManca et al. 1999 (19)	+	+	-	+	-	+	4	66,7	B
Light et al. 2009 (23)	+	-	-	+	+	+	4	66,7	B
White et al. 2012 (59)	+	-	+	+	-	+	4	66,7	B
Nijs et al. 2010 (37)	+	+	+	+	-	-	4	66,7	B
Robinson et al. 2010 (41)	+	+	-	+	-	+	4	66,7	B
Jammes et al. 2005 (18)	+	+	-	+	-	+	4	66,7	B
Smylie et al. 2013 (45)	+	+	-	+	-	+	4	66,7	B
Suarez et al. 2010 (49)	+	+	-	+	-	+	4	66,7	B
Thambirajah et al. 2008 (53)	+	+	-	+	-	-	3	50,0	B
Peterson et al. 1994 (39)	+	+	-	+	-	-	3	50,0	B
White et al. 2010 (58)	+	+	-	+	-	-	3	50,0	B
Broderick et al. 2013 (4)	+	-	-	+	-	+	3	50,0	B
Cannon et al. 1997 (6)	+	+	-	-	-	+	3	50,0	B
Whistler et al. 2005 (57)	+	+	-	-	-	-	2	33,3	B
Sorensen et al. 2003 (47)	+	+	-	-	-	-	2	33,3	B
Lloyd et al. 1994 (24)	-	-	-	+	-	+	2	33,3	B
Sorensen et al. 2009 (46)	-	+	-	-	-	-	1	16,7	B
Gupta et al. 1999 (13)	-	-	-	-	+	-	1	16,7	B
Steineau et al. 2004 (48)	-	-	-	-	-	-	0	0,0	B

Criterion 1: adequate definition of the case group
Criterion 2: adequate definition of the control group
Criterion 3: exclusion of selection bias
Criterion 4: exposure is clearly defined and method for assessment of exposure is adequate
Criterion 5: blinded assessor
Criterion 6: identification or accounting for confounders
0: criterion not fulfilled; 1: criterion fulfilled; NA: criterion not applicable

RESULTS

Study selection

The search of the databases provided a total of 584 citations. After removing duplicates 254 studies remained. Of these, 231 studies were discarded after reviewing the title and abstract or when necessary the full text paper. More specifically, studies were excluded because they had a study type (n=80), population (n=139), or topic (n=217) offline with the predefined selection criteria (figure 1). Twenty-three studies met all inclusion criteria.

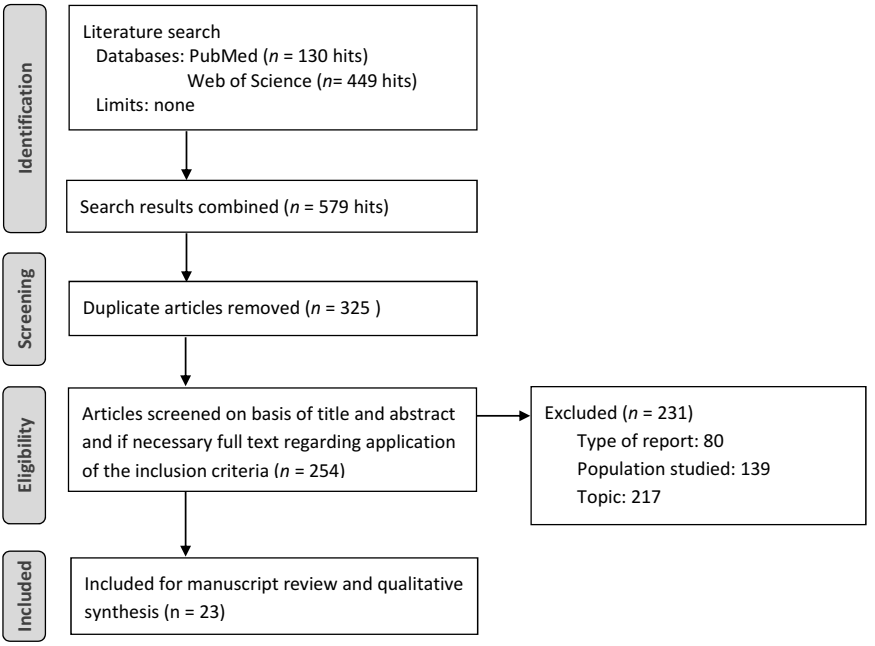

Figure 1. PRISMA flow diagram of the literature search and study selection.

Risk of bias and level of evidence

The agreement between the three raters was 70.3 % (97/138), 73.2 % (101/138) and 75.4 % (104/138). All three raters agreed on the scores of 83 out of 138 items. An adequate definition of the case group was given in 19 out of 23 studies, and for the control group this was the case in 16 studies (table 2). As different criteria exist for the diagnosis of CFS, and for the definition of sedentary or moderate activity levels, authors should at least refer to the criteria which were used. There was a risk of selection bias in 17 studies because the included subjects were not representative for the population which was studied. The authors failed to mention from where the subjects were recruited or they recruited solely from one sort of setting. The studies by Gupta et al. 1999 (13), Light et al. 2009 (23), Sorensen

et al. 2009 (46), and Steinau et al. 2004 (48) describe that (a part of) the included subjects were selected from a previous study, but fail to mention why exactly these subjects were selected. Most studies (17 out of 23) provided a clear description of the physical exercise which was performed by the subjects and the methods for assessing outcome measures. Failure of blinding formed an important source of bias in 20 studies. In most cases, it was not mentioned whether blood samples were coded and stored anonymously, and whether blood processing / analyses were performed by personnel blinded to the subject's health status. We assume that this was the case in many studies, but that authors simply failed to mention whether they prevented this form of assessment bias. Eleven studies did not prevent or account for important confounders related to exercise immunology. The most frequent cause of penalization was pooling of gender data. The EBRO-classification assigns a B score for individual studies which use a case-control study design. Because all the included studies were comparative studies without randomization of the study subjects, an evidence level B was applicable for all studies.

Study characteristics

All included studies applied a case-control design, comparing CFS patients with healthy controls. In each of the included studies, immune variables were measured at rest (pre-exercise) and following one bout of exercise, with post-exercise measurements performed immediately and/or up to days following exercise. All studies examined the acute effects of exercise on the immune system (i.e. the effects of one exercise bout on the immune system). None of the studied applied a true experimental design, or studied the effects of exercise *therapy* on the immune system in CFS patients.

ARTICLE RESULTS and DISCUSSION

The main study findings are summarized below and discussed together in view of the risk of bias scores. Given the focus of the review, only the study findings addressing exercise immunology in CFS patients versus controls are presented. Other findings, like baseline (resting) differences in immune function or differences in exercise physiology unrelated to the body's immune system, are not presented. Study findings are organized in 4 major categories, namely cytokines, complement system, oxidative stress and leukocyte gene expression.

Cytokines

The cytokine response to exercise of CFS patients in comparison with healthy controls has been examined thoroughly (6, 7, 13, 17, 19, 24, 37, 39, 41, 45, 47, 58). Lloyd et al. did not find alterations in the blood level of interferon-γ, interferon-α, interleukin-1β, or tumour necrosis factor-α at baseline, during, and up to 24 hours following 30 minutes of submaximal isometric (hand-grip) exercise (24). They compared twelve male CFS patients with 13 male matched healthy controls (24). Similar findings were reported in the Peterson et al. study, who failed to detect alterations in serum cytokine levels of interleukin-1β, interleukin-6, or tumour necrosis factor-α in any of the participants (CFS-patients or healthy) com-

paring levels at rest with values immediately and 40 minutes following walking on a horizontal treadmill at a speed of 1 mph for a maximum of 30 minutes or until exhaustion (39). This is surprising given the well-established cytokine response to strenuous exercise in healthy people (56, 60), questioning the validity of the study. The risk of bias assessment of these studies revealed rather low scores of 33% and 50% for the Lloyd et al. (24) and Peterson et al. (39) studies respectively. Still, the lack of alterations in blood interleukin-1β in response to exercise in CFS patients was later confirmed in a larger study (n=22 per group) performing quantitative *in vitro* detection of human interleukin-1β using two different assays (37). Others were similarly unable to find alterations of interleukin-1β in response to exercise in CFS patients (7, 39, 47). The Peterson et al. study, however, did detect serum transforming growth factor-β differences at rest between CFS patients and healthy controls, with higher values in the CFS group, but this cytokine did not respond to exercise either (39).

Others used a moderate whole-body exercise task (working both arms and legs) for 25 minutes to examine whether the flare in symptoms, up to 48 hours following exercise, was related to changes in peripheral blood cytokines and CD40 ligand (cluster of determination 40 ligand) of CFS patients (58). CD40 ligand is a pro-inflammatory marker, linked to platelet activation. Increased levels have been linked to cardiovascular diseases, while low levels may be related to increased risk for opportunistic infections (which are often seen in CFS (36)). CD40 ligand was lower in the CFS patients versus healthy controls, a difference that remained following exercise, and the level of CD40 ligand decreased similarly in both groups following exercise (58). Besides from changes in red blood cell count, which are of less relevance to this literature review, no major changes in cytokine response to exercise were observed between CFS and healthy controls (58). However, when dividing the CFS group into high and low symptom flares following exercise, it was found that CFS patients with high symptom flares had a pattern of pro- (interleukin-6, interleukin-1β, interleukin-12) as well as anti-inflammatory cytokines (interleukin-10, interleukin-13) at 8 hours post-exercise compared to the low symptom flare group. Still, the findings from this study should be interpret with caution, as not all participants in the control group were sedentary, the control group differed in gender distribution and body mass index from the CFS group, and the cytokine changes were not controlled for these possible confounders. Pooling of gender data has been identified as an important source of bias in studies addressing exercise physiology in CFS patients (42).

In a small (n=6 male subjects per group), but otherwise methodologically sound study, the response of interleukin-6, its soluble receptors (sIL-6R and sgp130) and F2-isoprostanes to submaximal exercise were studied (41). Interleukin-6 increased from rest to the end of the exercise and returned to resting values 24 hours post-exercise, but there were no group X time interactions, indicating that the increase in interleukin-6 in response to exercise was similar in both groups (41). Likewise, there were no group X time interactions for sIL-6R, sgp130 and F2-isoprostanes. Another study examined the interleukin-6 response to exercise in CFS, this time using 30 minutes of fatiguing non-dominant limb exercise (13). In the CFS group, and not in the control group, spontaneous interleukin-6 produc-

tion by monocytes increased following exercise. Phytohemagglutinin-induced (for lymphocytes) and lipopolysaccharide-induced (for monocytes) production of interleukin-6 did not differ following exercise across groups. Given the small sample size (5 CFS patients vs. 4 healthy controls) and the low risk of bias score (17 %), the weight of these findings is limited.

Two similar studies by Cannon, et al. (6, 7) used an exercise protocol involving 15 minutes of stepping on and off a platform, and compared peripheral blood mononuclear cells' secretion of interleukin-1β, interleukin-1 receptor antagonist, soluble interleukin-1 receptor type II (6), secretion of interleukin-6 in unstimulated cultures and interleukin-6 secretion in lipopolysaccharide-stimulated cultures (7). However, they were unable to find exercise-induced changes in any of the immune parameters studied, including α2-macroglobulin (6, 7). The exercise challenge might have been too low to enable immune alterations; the lack of exercise-induced symptom-increases in the CFS group (7) supports this notion.

In response to 20 minutes of steady-state stationary cycling at 70% of the subject's predicted maximum workload, patients with CFS (n=32), compared to healthy controls (n=29), did not show statistically significant differences in pro-inflammatory (interleukin-1β, interleukin-6, tumour necrosis factor-α, interferon-α) or anti-inflammatory cytokines (interleukin-10) detected in peripheral blood mononuclear cells (47). However, there was a trend of an increase in pro-inflammatory cytokines in the CFS group at 6 hours post-exercise, whereas at the same time, the values for pro-inflammatory cytokines decreased in the control group (47).

The latter study findings are in line with those reported by La Manca et al. 1999 (19). A rigorous study – the risk of bias assessment yielded a score of 67 % - investigated the differences in cytokines, leukocytes, granulocytes, monocytes and lymphocyte subsets in response to a graded treadmill exercise until exhaustion in 20 CFS patients and 14 sedentary controls (19). Blood samples were collected up to 24 hours post-exercise. Although changes in peripheral lymphocytes' cytokines were found from pre- to post-exercise, there were no time X group interactions for any of the cytokines (interleukin-2, -4, -10, -12, interferon-γ, and tumour necrosis factor-α) examined (19). The same applies to the leukocytes, granulocytes, monocytes and lymphocyte subsets (including T-helper cells, suppressor T, cytotoxic T, and natural killer cells) studied: most of them responded acutely to exercise, returning to baseline values relatively quickly (after 1 to 24 hours), but again no time X group interactions were found (19). Interestingly, in another rigorous but small (n=9 CFS versus 9 controls) study, venous levels of interleukin-6 and tumour necrosis factor-α increased in response to maximal cycling exercise in the healthy controls, but remained unchanged in the CFS group (17), suggesting a depressed cytokine response to exercise in the CFS group. The oxidative stress findings of that study are presented below.

Finally, Smylie et al. studied the cytokine response to a peak graded exercise stress test in patients with CFS (n=22), Gulf War Illness (n=30) and healthy controls (n=30) (45). They correctly separated sexes for running the data analysis, but focused on searching an immune signature / biomarker among the 16 cytokines

examined at baseline, at peak effort and 4 hours post-exercise. Therefore they did not report time X group interactions, making the report less appropriate for the present review.

To summarize the findings addressing the cytokine response of CFS patients to exercise, from the available literature data there is moderate evidence that CFS patients have a normal circulating cytokine (e.g. interleukin-1β, interleukin-6, interleukin-10, tumour necrosis factor-α) response to exercise. Exercise does not result in abnormally higher levels of pro- or anti-inflammatory cytokines in patients with CFS.

Complement system

Nijs et al. (described above) compared the immune response of CFS patients (n=22) and sedentary, healthy controls (n=22) to two types of exercise: a submaximal bicycle exercise and a self-paced, physiologically limited bicycle exercise (37). The study was primarily interested in examining the changes in blood elastase level and complement C4a split product levels in people with CFS versus healthy sedentary control subjects. Elastase is a proteolytic enzyme produced by monocytes and neutrophils during the inflammatory response. In a previous study of people with CFS it was found that baseline elastase level was predictive of the respiratory exchange ratio and the oxygen uptake at the anaerobic threshold (35). In this exercise immunology study, neither type of exercise altered blood levels of elastase activity, interleukin-1, or complement C4a split product levels in people with CFS or healthy sedentary control subjects. However, the change in complement C4a level was strongly related to the increase in pain and fatigue 24 hours following the self-paced, physiologically limited exercise (37). Post-exercise elastase activity level and the change in elastase activity level were inversely related to the fatigue increase one hour following the self-paced, physiologically limited exercise (37). These findings suggest that subtle alterations in blood elastase activity level and complement C4a split product levels account in part of post-exertional malaise in people with CFS.

These findings are in line with those by Sorensen et al. (47), who reported statistically significant correlations between the increase in C4a and total symptom score, as well as with individual symptoms like headache, joint problems and cognitive difficulty in CFS patients. Moreover, they showed that people with CFS respond to an exercise challenge with increased expression of the lectin pathway (C4 and mannan-binding lectin serine protease 2) in peripheral blood mononuclear cells, resulting in significant increase of C4a split product, but not C3a or C5a (46, 47). The fact that Nijs et al. (37) did not find changes in C4a are not in contradiction with the earlier reports. Firstly, in the study by Sorensen et al. the increase in complement C4a split product became apparent only at 6 hours after exercise (47). In the study by Nijs et al. (37) peripheral blood levels of C4a were measured only at 1 hour after exercise, a time point at which Sorensen and colleagues were unable to find changes in circulating C4a levels either (47).

In the Sorensen et al. study, the eosinophilic cationic protein, a protein released during eosinophil degranulation and consequently related to inflammation, responded differently to exercise in CFS patients compared to healthy controls (47). In CFS patients, eosinophilic cationic protein levels decreased from baseline to post-exercise, followed by an increase 6 hours later and that remained at 24 hours post-exercise. Healthy controls had a similar acute decrease of eosinophilic cationic protein levels post-exercise, but the values did not increase to the same extent in the next hours as the CFS group (significant group-by-time interaction) (47).

Taken together, moderate evidence suggests that CFS patients, compared to healthy controls, respond to strenuous exercise with a slow (not earlier than 6 hours post-exercise) but stronger increase in blood complement C4a split product levels. Importantly, these alterations appear of clinical importance as two independent studies have confirmed the relationship between altered complement response and post-exertional malaise in patients with CFS. Findings such as altered eosinophilic cationic protein response to exercise in CFS require replication.

Oxidative stress

The oxidative stress response to exercise in patients with CFS has been the subject of five studies (16-18, 41, 53). As mentioned above, Robinson et al. did not find group X time interactions for F2-isoprostanes (41) (a marker of lipid peroxidation), but they only included 6 patients per group. In 2005, Jammes et al. were the first to report a dysfunctional oxidative stress response in CFS patients (18). They studied the oxidative stress response in venous blood of 15 CFS patients and 11 healthy controls to a maximal graded bicycle stress test, and found an earlier and longer increase in thiobarbituric acid reactive substances, a byproduct of lipid peroxidation and reflecting oxidative stress damage. Also an enhanced post-exercise decrease in the antioxidant ascorbic acid was found in those with CFS, although glutathione levels did not respond differently to exercise compared to the healthy controls (18).

In a later study Jammes et al. again found an early and longer increase in oxidative stress response to strenuous exercise in CFS patients compared to healthy controls (17). Thiobarbituric acid reactive substances were accentuated and increased early following exercise in the CFS group compared to the healthy controls (17). They also reported a delayed and reduced increase of ascorbic acid and heat shock proteins 27 and 70 following exercise (17), which implies a reduced anti-oxidant status post-exercise in the CFS group. This was confirmed more recently in a similar study by the same group, this time using a much larger sample (n=43 CFS patients and n=23 healthy, sedentary controls) (16). Another group used treadmill exercise for 18 minutes to study the heat shock protein response to exercise in 6 CFS patients and 7 healthy controls; heat shock proteins 27, 60, 70 and 90 were determined in peripheral blood mononuclear cells of the study partic-

ipants before, immediately after, and 1 and 7 days following the exercise challenge (53). A group X time interaction was only found for heat shock protein 27, as heat shock protein 27 remained unchanged in the healthy people, and declined following exercise in the CFS group (53).

Interestingly, Jammes et al. (16) found that exercise-induced oxidative stress levels were higher in those CFS patients who had regularly taken part in sports activity (> 6 hours per week) for more than 6 years prior to CFS onset, and/or suffered from a severe infection (peritonitis, pneumonia or encephalomyelitis) within 3-4 months preceding CFS onset (16).

One study examined the nitrosative stress response of female CFS patients (n=44) versus healthy women (n=25) to two bicycle exercise bouts (one maximal test to exhaustion and a second personalized submaximal bout), and found that nitric oxide metabolites (nitrates) become much higher post-exercise among the CFS patients (49). This was true for both conditions: the maximal and submaximal exercise bout. These findings require replication.

Summarizing the findings in relation to oxidative stress, cumulating evidence indicates that oxidative stress following exercise occurs earlier and lasts longer in CFS patients, and also that the anti-oxidant response post-exercise is delayed and reduced. However, nearly all studies come from the same laboratory and hence require replication.

Leukocyte gene expression

Immune response to exercise can be studied at the cellular level, at the protein level, but also at the gene level. Six studies have examined immune cell gene responses to exercise in patients with CFS (4, 22, 23, 48, 57, 59). The most important findings are summarizes below.

Whistler, et al. studied blood mononuclear cell gene expression in response to 20 minutes of steady-state bicycle exercise at 70% of the predicted maximum work load in 5 women with CFS and 5 healthy controls (57). They first identified 21 genes as being differentially expressed in response to exercise in healthy subjects (as a normal response to exercise). When comparing the gene response of CFS patients with the normal response, they identified differences in exercise-responsive genes in CFS subjects before and after exercise. A lower expression of the identified genes, as observed in response to exercise in the CFS group, may have a subtle effect on immune functioning (57). More specifically, gene regulation in chromatin structure was the most obvious change following exercise in CFS patients, and they also observed that the complement pathway showed significant differences between CFS and control subjects after exercise (57). This is important as it shows that exercise results in a stronger complement activation in CFS patients, not only at the protein level (i.e. gene product level), but also at the transcript level. In addition, nucleosome assembly, cytoplasmic vesicles, membrane

transport, and G protein-coupled receptor ontologies were found in those with CFS.

The differences in gene expression suggest important perturbation in biochemical activity, including ion transport and ion channel activity of lymphocyte and monocyte peripheral blood fractions from CFS subjects in response to exercise (57). It suggests that immune cells of CFS do not respond normally to an exercise challenge. However, the findings should be interpreted with caution, as this small scale study had a high risk of bias assessment score (33%). Hence, these findings should be viewed as pilot data, as is the case with the report by Steinau et al., who studied only one CFS patient versus one matched healthy control (48).

Three additional gene expression exercise immunology studies in CFS patients obtained higher risk of bias assessment scores (table 2). Light et al. studied gene expression in leukocytes (venous blood samples) obtained from 19 CFS patients and 16 healthy controls 48 hours before, and up to 48 hours following, 25 minutes of submaximal whole-body exercise (combined arm and leg cycle ergometer) (23). Although baseline (resting) mRNA levels were similar in both groups, in response to exercise they found group differences for metabolite detecting genes, adrenergic genes, and immune genes (23). More specifically, and in line with the hypothesis of the present review, CFS patients showed larger post-exercise increases in interleukin-10 and Toll-like receptor 4 gene expression. Toll-like receptors are important for the activation of both the innate and acquired (specific) immune system.

The same group later partly confirmed these findings in a larger study (n=48 CFS patients and n=49 healthy controls), using the same protocol as the previous study (22). This time, exercise in CFS patients led to increased expression of certain sensory ion channel, adrenergic and immune genes, which do not occur in healthy controls. Addressing the genes closely related to immune function, the previous finding of greater post-exercise increases in Toll-like receptor 4 gene expression in the CFS group was not confirmed, even though they confirmed the larger increase in interleukin-10 gene expression (22). No changes in interleukin-6 or CD14 mRNA were observed in either study.

Thus, although none of the studies summarized above found meaningful changes following exercise in the circulating anti-inflammatory cytokine interleukin-10 (19, 47, 58), two similar studies from the same group reported increased interleukin-10 mRNA (gene expression) in peripheral leukocytes following exercise in CFS patients (22, 23). No changes in interleukin-6 mRNA were observed in either study.

The discrepancies across studies are most likely due to the different sample sizes, range of ages, differences in disease severity, and possibly partly due to medication differences as the more recent study allowed pain medication and anticonvulsant medication use. Importantly the post-exercise increases in interleukin-10 mRNA in the CFS group correlated with increases in post-exercise pain and fatigue (22), pointing to its clinical relevance in relation to post-exertional malaise in CFS.

The larger post-exercise interleukin-10 gene expression increases in CFS patients in response to exercise might indicate too strong and prolonged anti-inflammatory action following exercise, which increases the risk of opportunistic infections. Opportunistic infections have increased prevalence rates in patients with CFS compared to healthy people (5, 30, 36). However, it remains to be established whether exercise responses at the gene level have implications for immune function as well. For instance, although an abnormally high post-exercise interleukin-10 increase was found at the gene level in CFS patients (22, 23), blood levels of interleukin-10 did not change following exercise in CFS patients (19, 47, 58).

Still, the clinical importance of the differences in gene expression following exercise was further substantiated in a similar study from the same group, applying similar methods for comparing CFS (n=22) with healthy controls (n=23) and patients with relapsing-remitting multiple sclerosis (n=20), another chronic illness characterized by chronic fatigue (and to a lesser extend pain) (59). Toll-like receptor 4 gene expression differed between CFS and multiple sclerosis, with a post-exercise decrease in the latter group (59). Unfortunately, all three studies (22, 23, 59) from this group pooled gender data.

Gender is an issue when interpreting the findings of a study that examined gene expression of peripheral blood mononuclear cells of male subjects (n=7 CFS, n=20 Gulf War Illness, and n=22 healthy, sedentary veterans) in response to a standard, maximal graded cycling stress test (4). Given their focus on Gulf War Illness rather than CFS, the choice for male participants is understandable, but it limits the external validity of the study findings for CFS patients (even though the patients with Gulf War Illness complied with the CDC criteria for the diagnosis of CFS as well). Although differences were found between groups (e.g. subdued cell cycle progression and immune signaling in CFS), no time X group interactions were found, indicating the gene expression of peripheral blood mononuclear cells does not respond differently between male CFS patients, patients with Gulf War Illness and healthy sedentary controls (4).

Summarizing the findings of gene expression profiling of CFS patients in response to exercise, there is moderate evidence that CFS patients showed larger post-exercise interleukin-10 and Toll-like receptor 4 gene expression increases, which accounts in part for post-exertional malaise. Although compelling, these findings have been confirmed solely by the same laboratory and hence require replication by independent researchers.

RESEARCH AGENDA

In addition to the above outlined need for replicating findings in independent laboratories, several other recommendations for further research can be formulated based on the study of the scientific literature in this area (table 3 summarizes these recommendations).

First, it is important to make a distinction between the effects from therapeutic interventions using exercise *therapy* in CFS (e.g. reference (55)) and findings from studies examining the exercise immunology*/physiology* of people with CFS (e.g. references (37, 47)). The latter often use one bout of exercise to examine the acute response to (often very strenuous) exercise. Such exercise physiology studies provide us with valuable information on the biology of post-exertional malaise of CFS, but the exercise response may be very different in longer-term low-intensity exercise programs. These are two distinct issues. Studies examining the effects of exercise therapy on immune function in CFS patients are essentially lacking. Given the compelling findings addressing acute responses of the immune system to exercise in CFS patients as reviewed here, this is an important avenue for future research in this area.

All studies examined here, used standardized exercise protocols. Physical activities like walking long distances or cycling are not applicable, or possible, for all CFS patients. In addition, such studies were often conducted in laboratory settings. Hence, such studies have limited ecological validity. Therefore, there is a need to study exercise immunology using physically demanding functional tasks for CFS patients, like stair climbing and ironing, rather than graded bicycle or treadmill tests. Stair climbing has been used for studying CFS patients (14, 34), but not from an exercise immunology perspective. It remains to be established whether the observed exercise immunology abnormalities (e.g. increased oxidative stress response, enhanced complement activation) are specific for (sub)maximal exercise, or can be extrapolated to activities of daily living.

Table 3. Research agenda for exercise immunology in patients with chronic fatigue syndrome.

	Recommendations for future studies addressing exercise immunology
1.	Examining whether exercise responses at the gene level have implications for immune function in CFS patients.
2.	Examining whether tissue cytokines (e.g. inflammatory cytokines in muscle tissue) respond different to exercise in CFS patients.
3.	Replication of the findings of enhanced oxidative stress response to exercise in CFS patients.
4.	Examining the effects of exercise therapy, rather than acute exercise bouts, on immune function in CFS patients.
5.	Examining whether immune responses to exercise account for post-exertional malaise in CFS.
6.	Examining the immune response to daily life physical activities in CFS patients.
	Methodological recommendations
1.	Apply a true experimental design (randomized cross-over study design)
2.	Use blinded assessments / assessors.
3.	Do not pool gender data.
4.	Apply appropriate statistical methods for a priori determination of the required sample size.
5.	Identify and if required account for possible confounders when designing the study, or the very least when (re)analyzing the data.

Previous exercise immunology studies in the field of CFS used case-control rather than experimental designs. This implies that previous observations regarding exercise immunology in CFS patients did not control for potential bias due to emotional stressors or the fluctuating nature of CFS. Therefore, it is recommended that future studies apply a true experimental design (randomized study design) controlling for emotional stressors.

Only 4 (13, 22, 23, 59) of the 23 studies used blinded assessors, and 3 out of 4 studies are from the same group. Traditionally, this represents an important shortcoming, but in the area of exercise immunology one can question its importance. If a standardized exercise protocol is used in all comparison groups, as is the case in all studies included in this review, then little bias is to be expected from a lack of blinding of the assessors. After all, the outcomes are laboratory analyses, and (blood) samples are typically coded and transferred blindly to the laboratory for analysis. A more important shortcoming is the sample size. The majority of the studies had less that 20 subjects in the CFS group, and only one study (37) based the sample size on an a priori sample size estimation. It is advised that future studies in the field use established statistical methods for a priori determination of the required sample size.

The analysis for blood samples or mRNA extraction varied between studies. This may account for some of the discrepancies across studies. From the available literature it is concluded that CFS patients have a normal circulating cytokine response (e.g. interleukin-1β, interleukin-6, interleukin-10, tumour necrosis factor-α) to exercise (7, 17, 19, 24, 37, 39, 41, 47, 58). Still, tissue cytokines and their response to exercise have hardly been studied in CFS patients. For instance, it would be worthwhile examining whether pro- and anti-inflammatory cytokines in working muscles of CFS patients respond different to (local) exercise than in healthy sedentary controls.

To date, few studies have carefully examined whether exercise-induced immune changes in patients with CFS account for the symptom exacerbations as typically seen in these patients (i.e. post-exertional malaise). As outlined in the introduction section, post-exertional malaise is now increasingly recognized as a prominent characteristic of CFS. Future studies are advised to explore this relevant feature.

Finally, pooling of gender data has been identified as an important source of bias in studies addressing exercise physiology in CFS patients (42), and exercise immunology more in particular (45). Despite this important methodological finding, pooling of gender data remains a common shortcoming in exercise immunology studies in CFS patients, and should be addressed in future work. In general, it is recommended that future studies account for potential confounders when designing the studies and when analyzing the data. Few studies have reanalyzed the dataset accounting for possible confounding factors, as reflected by the low number of studies (12 out of 23) fulfilling criterion 6 (identification or accounting for confounders) in the risk of bias assessment score sheet (table 2).

CONCLUSION

The intention of this review was to answer the following question: does the immune system of patients with CFS respond differently to exercise as compared to healthy sedentary controls? Based on the available research data, our hypothesis that CFS patients show a more pronounced immune response to exercise as compared to healthy sedentary controls can be confirmed. Indeed, the literature review provides evidence for an altered immune response to exercise in patients with CFS. More specifically, compared to the normal response of the immune system to exercise as seen in healthy subjects, patients with CFS have a more pronounced response in the complement system (i.e. C4a split product levels), oxidative stress system (i.e. an enhanced oxidative stress combined with a delayed and reduced anti-oxidant response), and the immune cells' gene expression profile (larger post-exercise interleukin-10 and toll-like receptor 4 gene expression increases), but not in circulating pro- or anti-inflammatory cytokines. Many of these immune changes relate to post-exertional malaise in CFS, a major characteristic of the illness. Future research in this area should apply a true experimental design, extend findings to other tissues than blood samples, control for covariates, and examine the immune response of CFS patients following long-term exercise therapy.

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REFERENCES

1. Bazelmans E, Bleijenberg G, Voeten MJ, van der Meer JW, and Folgering H. Impact of a maximal exercise test on symptoms and activity in chronic fatigue syndrome. Journal of psychosomatic research 59: 201-208, 2005.
2. Black CD, O'Connor P J, and McCully KK. Increased daily physical activity and fatigue symptoms in chronic fatigue syndrome. Dyn Med 4: 3, 2005.
3. Bradley AS FB, Bansal AS. Altered functional B cell subset populations in patients with chronic fatigue syndrome compared to healthy controls. Clin Exp Immunol 172: 73-80., 2013.
4. Broderick G, Ben-Hamo R, Vashishtha S, Efroni S, Nathanson L, Barnes Z, Fletcher MA, and Klimas N. Altered immune pathway activity under exercise challenge in Gulf War Illness: an exploratory analysis. Brain, behavior, and immunity 28: 159-169, 2013.
5. Buchwald D, Ashley RL, Pearlman T, Kith P, and Komaroff AL. Viral serologies in patients with chronic fatigue and chronic fatigue syndrome. Journal of medical virology 50: 25-30, 1996.

6. Cannon JG, Angel JB, Abad LW, Vannier E, Mileno MD, Fagioli L, Wolff SM, and Komaroff AL. Interleukin-1 beta, interleukin-1 receptor antagonist, and soluble interleukin-1 receptor type II secretion in chronic fatigue syndrome. Journal of clinical immunology 17: 253-261, 1997.
7. Cannon JG, Angel JB, Ball RW, Abad LW, Fagioli L, and Komaroff AL. Acute phase responses and cytokine secretion in chronic fatigue syndrome. Journal of clinical immunology 19: 414-421, 1999.
8. Carruthers BM, van de Sande MI, De Meirleir KL, Klimas NG, Broderick G, Mitchell T, Staines D, Powles AC, Speight N, Vallings R, Bateman L, Baumgarten-Austrheim B, Bell DS, Carlo-Stella N, Chia J, Darragh A, Jo D, Lewis D, Light AR, Marshall-Gradisbik S, Mena I, Mikovits JA, Miwa K, Murovska M, Pall ML, and Stevens S. Myalgic encephalomyelitis: International Consensus Criteria. Journal of internal medicine 270: 327-338, 2011.
9. Demettre E, Bastide L, D'Haese A, De Smet K, De Meirleir K, Tiev KP, Englebienne P, and Lebleu B. Ribonuclease L proteolysis in peripheral blood mononuclear cells of chronic fatigue syndrome patients. The Journal of biological chemistry 277: 35746-35751, 2002.
10. Englebienne P, Herst CV, De Smet K, D'Haese A, and De Meirleir K. Interactions between RNase L ankyrin-like domain and ABC transporters as a possible origin for pain, ion transport, CNS and immune disorders of chronic fatigue immune dysfunction syndrome. Journal of Chronic Fatigue Syndrome 8: 83-102, 2001.
11. Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, and Komaroff A. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. Annals of internal medicine 121: 953-959, 1994.
12. Gerrity TR, Papanicolaou DA, Amsterdam JD, Bingham S, Grossman A, Hedrick T, Herberman RB, Krueger G, Levine S, Mohagheghpour N, Moore RC, Oleske J, and Snell CR. Immunologic aspects of chronic fatigue syndrome. Report on a Research Symposium convened by The CFIDS Association of America and co-sponsored by the US Centers for Disease Control and Prevention and the National Institutes of Health. Neuroimmunomodulation 11: 351-357, 2004.
13. Gupta S, Aggarwal S, and Starr A. Increased production of interleukin-6 by adherent and non-adherent mononuclear cells during 'natural fatigue' but not following 'experimental fatigue' in patients with chronic fatigue syndrome. International journal of molecular medicine 3: 209-213, 1999.
14. Heins M, Knoop H, Nijs J, Feskens R, Meeus M, Moorkens G, and Bleijenberg G. Influence of Symptom Expectancies on Stair-Climbing Performance in Chronic Fatigue Syndrome: Effect of Study Context. International journal of behavioral medicine 2012.
15. Holmes GP, Kaplan JE, Gantz NM, Komaroff AL, Schonberger LB, Straus SE, Jones JF, Dubois RE, Cunningham-Rundles C, Pahwa S, and et al. Chronic fatigue syndrome: a working case definition. Annals of internal medicine 108: 387-389, 1988.
16. Jammes Y, Steinberg JG, and Delliaux S. Chronic fatigue syndrome: acute infection and history of physical activity affect resting levels and response to exercise of plasma oxidant/antioxidant status and heat shock proteins. Journal of internal medicine 272: 74-84, 2012.

17. Jammes Y, Steinberg JG, Delliaux S, and Bregeon F. Chronic fatigue syndrome combines increased exercise-induced oxidative stress and reduced cytokine and Hsp responses. Journal of internal medicine 266: 196-206, 2009.
18. Jammes Y, Steinberg JG, Mambrini O, Bregeon F, and Delliaux S. Chronic fatigue syndrome: assessment of increased oxidative stress and altered muscle excitability in response to incremental exercise. Journal of internal medicine 257: 299-310, 2005.
19. LaManca JJ, Sisto SA, Zhou XD, Ottenweller JE, Cook S, Peckerman A, Zhang Q, Denny TN, Gause WC, and Natelson BH. Immunological response in chronic fatigue syndrome following a graded exercise test to exhaustion. Journal of clinical immunology 19: 135-142, 1999.
20. Lapp CW. Exercise limits in chronic fatigue syndrome. The American journal of medicine 103: 83-84, 1997.
21. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, and Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. Journal of clinical epidemiology 62: 23, 2009.
22. Light AR, Bateman L, Jo D, Hughen RW, Vanhaitsma TA, White AT, and Light KC. Gene expression alterations at baseline and following moderate exercise in patients with Chronic Fatigue Syndrome and Fibromyalgia Syndrome. Journal of internal medicine 271: 64-81, 2012.
23. Light AR, White AT, Hughen RW, and Light KC. Moderate Exercise Increases Expression for Sensory, Adrenergic, and Immune Genes in Chronic Fatigue Syndrome Patients But Not in Normal Subjects. J Pain 2009.
24. Lloyd A, Gandevia S, Brockman A, Hales J, and Wakefield D. Cytokine production and fatigue in patients with chronic fatigue syndrome and healthy control subjects in response to exercise. Clin Infect Dis 18: S142-146, 1994.
25. Maes M. Inflammatory and oxidative and nitrosative stress pathways underpinning chronic fatigue, somatization and psychosomatic symptoms. Current opinion in psychiatry 22: 75-83, 2009.
26. Maes M. An intriguing and hitherto unexplained co-occurrence: Depression and chronic fatigue syndrome are manifestations of shared inflammatory, oxidative and nitrosative (IO&NS) pathways. Progress in neuro-psychopharmacology & biological psychiatry 35: 784-794, 2011.
27. Maes M, Mihaylova I, Kubera M, and Bosmans E. Not in the mind but in the cell: increased production of cyclo-oxygenase-2 and inducible NO synthase in chronic fatigue syndrome. Neuro endocrinology letters 28: 463-469, 2007.
28. Meeus M, Nijs J, McGregor N, Meeusen R, De Schutter G, Truijen S, Fremont M, Van Hoof E, and De Meirleir K. Unravelling intracellular immune dysfunctions in chronic fatigue syndrome: interactions between protein kinase R activity, RNase L cleavage and elastase activity, and their clinical relevance. In vivo (Athens, Greece) 22: 115-121, 2008.
29. Moher D, Liberati A, Tetzlaff J, and Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Annals of internal medicine 151: 264-269, W264, 2009.
30. Nasralla M, Haier J, and Nicolson GL. Multiple mycoplasmal infections detected in blood of patients with chronic fatigue syndrome and/or fibromyalgia syndrome. Eur J Clin Microbiol Infect Dis 18: 859-865, 1999.

31. Natelson BH HM, Ponzio NM. Evidence for the presence of immune dysfunction in chronic fatigue syndrome. Clinical and diagnostic laboratory immunology 9: 747-752, 2002.
32. Nijs J, and Fremont M. Intracellular immune dysfunction in myalgic encephalomyelitis/chronic fatigue syndrome: state of the art and therapeutic implications. Expert opinion on therapeutic targets 12: 281-289, 2008.
33. Nijs J, Inghelbrecht E, Daenen L, Hachimi-Idrissi S, Hens L, Willems B, Roussel N, Cras P, Wouters K, and Bernheim J. Recruitment bias in chronic pain research: whiplash as a model. Clinical rheumatology 30: 1481-1489, 2011.
34. Nijs J, Meeus M, Heins M, Knoop H, Moorkens G, and Bleijenberg G. Kinesiophobia, catastrophizing and anticipated symptoms before stair climbing in chronic fatigue syndrome: an experimental study. Disability and rehabilitation 2012.
35. Nijs J, Meeus M, McGregor NR, Meeusen R, de Schutter G, van Hoof E, and de Meirleir K. Chronic fatigue syndrome: exercise performance related to immune dysfunction. Medicine and science in sports and exercise 37: 1647-1654, 2005.
36. Nijs J, Nicolson GL, De Becker P, Coomans D, and De Meirleir K. High prevalence of Mycoplasma infections among European chronic fatigue syndrome patients. Examination of four Mycoplasma species in blood of chronic fatigue syndrome patients. FEMS immunology and medical microbiology 34: 209-214, 2002.
37. Nijs J, Van Oosterwijck J, Meeus M, Lambrecht L, Metzger K, Fremont M, and Paul L. Unravelling the nature of postexertional malaise in myalgic encephalomyelitis/chronic fatigue syndrome: the role of elastase, complement C4a and interleukin-1beta. Journal of internal medicine 267: 418-435, 2010.
38. Ohashi K, Yamamoto Y, and Natelson BH. Activity rhythm degrades after strenuous exercise in chronic fatigue syndrome. Physiology & behavior 77: 39-44, 2002.
39. Peterson PK, Sirr SA, Grammith FC, Schenck CH, Pheley AM, Hu S, and Chao CC. Effects of mild exercise on cytokines and cerebral blood flow in chronic fatigue syndrome patients. Clinical and diagnostic laboratory immunology 1: 222-226, 1994.
40. Player MR, and Torrence PF. The 2-5A system: modulation of viral and cellular processes through acceleration of RNA degradation. Pharmacology & therapeutics 78: 55-113, 1998.
41. Robinson M, Gray SR, Watson MS, Kennedy G, Hill A, Belch JJ, and Nimmo MA. Plasma IL-6, its soluble receptors and F2-isoprostanes at rest and during exercise in chronic fatigue syndrome. Scandinavian journal of medicine & science in sports 20: 282-290, 2010.
42. Sargent C, Scroop GC, Nemeth PM, Burnet RB, and Buckley JD. Maximal oxygen uptake and lactate metabolism are normal in chronic fatigue syndrome. Medicine and science in sports and exercise 34: 51-56, 2002.
43. Shetzline SE, and Suhadolnik RJ. Characterization of a 2',5'-oligoadenylate (2-5A)-dependent 37-kDa RNase L: azido photoaffinity labeling and 2-5A-dependent activation. The Journal of biological chemistry 276: 23707-23711, 2001.
44. Siegel SD, Antoni MH, Fletcher MA, Maher K, Segota MC, and Klimas N. Impaired natural immunity, cognitive dysfunction, and physical symptoms in patients with chronic fatigue syndrome: preliminary evidence for a subgroup? Journal of psychosomatic research 60: 559-566, 2006.
45. Smylie AL, Broderick G, Fernandes H, Razdan S, Barnes Z, Collado F, Sol C, Fletcher MA, and Klimas N. A comparison of sex-specific immune signatures in Gulf War illness and chronic fatigue syndrome. BMC Immunol 14: 1471-2172, 2013.

46. Sorensen B, Jones JF, Vernon SD, and Rajeevan MS. Transcriptional control of complement activation in an exercise model of chronic fatigue syndrome. Mol Med 15: 34-42, 2009.
47. Sorensen B, Streib JE, Strand M, Make B, Giclas PC, Fleshner M, and Jones JF. Complement activation in a model of chronic fatigue syndrome. The Journal of allergy and clinical immunology 112: 397-403, 2003.
48. Steinau M, Unger ER, Vernon SD, Jones JF, and Rajeevan MS. Differential-display PCR of peripheral blood for biomarker discovery in chronic fatigue syndrome. J Mol Med 82: 750-755, 2004.
49. Suarez A, Guillamo E, Roig T, Blazquez A, Alegre J, Bermudez J, Ventura JL, Garcia-Quintana AM, Comella A, Segura R, and Javierre C. Nitric oxide metabolite production during exercise in chronic fatigue syndrome: a case-control study. J Womens Health 19: 1073-1077, 2010.
50. Suhadolnik RJ, Peterson DL, O'Brien K, Cheney PR, Herst CV, Reichenbach NL, Kon N, Horvath SE, Iacono KT, Adelson ME, De Meirleir K, De Becker P, Charubala R, and Pfleiderer W. Biochemical evidence for a novel low molecular weight 2-5A-dependent RNase L in chronic fatigue syndrome. J Interferon Cytokine Res 17: 377-385, 1997.
51. Suhadolnik RJ, Reichenbach NL, Hitzges P, Sobol RW, Peterson DL, Henry B, Ablashi DV, Muller WE, Schroder HC, Carter WA, and et al. Upregulation of the 2-5A synthetase/RNase L antiviral pathway associated with chronic fatigue syndrome. Clin Infect Dis 18 Suppl 1: S96-104, 1994.
52. Swanink CM, Vercoulen JH, Galama JM, Roos MT, Meyaard L, van der Ven-Jongekrijg J, de Nijs R, Bleijenberg G, Fennis JF, Miedema F, and van der Meer JW. Lymphocyte subsets, apoptosis, and cytokines in patients with chronic fatigue syndrome. The Journal of infectious diseases 173: 460-463, 1996.
53. Thambirajah AA, Sleigh K, Stiver HG, and Chow AW. Differential heat shock protein responses to strenuous standardized exercise in chronic fatigue syndrome patients and matched healthy controls. Clinical and investigative medicine Medecine clinique et experimentale 31: E319-327, 2008.
54. Van Oosterwijck J, Nijs J, Meeus M, Lefever I, Huybrechts L, Lambrecht L, and Paul L. Pain inhibition and postexertional malaise in myalgic encephalomyelitis/chronic fatigue syndrome: an experimental study. Journal of internal medicine 268: 265-278, 2010.
55. Wallman KE, Morton AR, Goodman C, Grove R, and Guilfoyle AM. Randomised controlled trial of graded exercise in chronic fatigue syndrome. The Medical journal of Australia 180: 444-448, 2004.
56. Walsh NP, Gleeson M, Shephard RJ, Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, and Simon P. Position statement. Part one: Immune function and exercise. Exerc Immunol Rev 17: 6-63, 2011.
57. Whistler T, Jones JF, Unger ER, and Vernon SD. Exercise responsive genes measured in peripheral blood of women with chronic fatigue syndrome and matched control subjects. BMC physiology 5: 5, 2005.
58. White AT, Light AR, Hughen RW, Bateman L, Martins TB, Hill HR, and Light KC. Severity of symptom flare after moderate exercise is linked to cytokine activity in chronic fatigue syndrome. Psychophysiology 47: 615-624, 2010.

59. White AT, Light AR, Hughen RW, Vanhaitsma TA, and Light KC. Differences in metabolite-detecting, adrenergic, and immune gene expression after moderate exercise in patients with chronic fatigue syndrome, patients with multiple sclerosis, and healthy controls. Psychosomatic medicine 74: 46-54, 2012.
60. Zhao G, Zhou S, Davie A, and Su Q. Effects of moderate and high intensity exercise on T1/T2 balance. Exerc Immunol Rev 18: 98-114, 2012.

Exercise-induced leukocyte apoptosis

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ABSTRACT

Physical exercise is well known to affect leukocyte numbers and function. While regular exercise training has been shown to enhance specific immune functions, acute bouts of intensive exercise often lead to a pro-inflammatory response accompanied by a transient lymphocytopenia and neutrophilia. It can be assumed, that lymphocytopenia can be attributed at least partially to an enhanced lymphocyte apoptosis. In contrast, regulation of neutrophil apoptosis after exercise remains controversial since studies demonstrated both an up-regulation as well as a down-regulation of cell death. However, these discrepancies may be due to differences in exercise protocols, subjects' fitness levels, and to different methodological approaches.

Two major signalling pathways of exercise induced apoptosis have been identified. First the external receptor mediated pathway using death receptors, and second the internal, oxidative-mediated pathway which encompasses the mitochondria. Potential apoptosis modulating mediators are reactive oxygen species (ROS), glucocorticoids and cytokines which are part of the systemic inflammatory response evoked after acute intensive exercise.

Finally, the physiological impact and clinical relevance of leukocyte apoptosis will be discussed. On the one hand, exercise-induced apoptosis might be a mechanism to remove activated and potentially autoreactive immune cells. On the other hand, apoptosis might be a regulatory mechanism which is necessary for tissue reorganization and adaptational training processes.

Keywords: cell death, lymphocytes, neutrophils, extrinsic apoptosis pathway, intrinsic apoptosis pathway, reactive oxygen species, glucocorticoids.

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1. Apoptosis

Apoptosis is of fundamental importance to guarantee a balance between the generation of new cells and removal of damaged or aged cells. Thereby, apoptosis is a complex process of cell death that allows cells to die in a well controlled fashion. Apoptotic processes play an important role during development and in maintaining tissue homeostasis. Besides physiological cell turnover, the role of apoptosis has also been demonstrated during a variety of pathophysiologicalconditions, for example: during inflammatory response, after cellular damage or in preventing neoplastic diseases (9). The process of apoptosis is highly orchestrated and cells have specific intracellular signalling pathways necessary for cell death. The specific induction-pathways of apoptosis strongly depend on the cell type, the presence or absence of specific death promoting or cell stabilizing signals, and the extent of cell damage or dysfunction (29).
A common characteristic of apoptosis is the elimination of cells without lysis or necrosis which is known to result in local inflammatory reactions. The typical morphological changes during apoptosis include membrane blebbing, DNA fragmentation and cellular component degradation.
Therefore, apoptosis offers the opportunity to remove cells without any collateral damage in adjacent tissues. Based on the nature of the apoptotic stimuli, two main pathways for apoptosis induction have been differentiated: an extrinsic pathway which is initiated by the binding of a ligand. These ligands are more frequently expressed after specific physiologic stimuli and bind to a death promoting receptor. The second main pathway is known as the intrinsic pathway, which is mainly triggered by damage to the cell or single components (23).

1.1 The extrinsic pathway of apoptosis

The extrinsic pathway of apoptosis is initiated by specific ligands which bind todeath receptors. Death receptors are membrane receptors which transduce apoptotic signals from the extracellular space into the cytoplasm. The ligands for these receptors form a family of related cytokines collectively named as the TNF family. The ligands are known to act in both an autocrine and a paracrine way to induce the trimerization of their respective cell surface receptors (65).

A major signalling pathway for the extrinsic induction of apoptosis is the Fas-receptor (FasR)/Fas ligand (FasL) pathway (64). After binding of FasL to FasR, the receptor is trimerized. The Fas adaptor protein, Fas-associated death domain protein (FADD), binds to the trimerized Fas cytoplasmatic region through the interaction of the death domains. Next pro-caspase-8, which is an important executing protease, is recruited to FADD. The Fas-receptor, FADD and pro-caspase-8 form a functional death-inducing signalling complex (DISC). This process induces self activation of caspase-8, which is released into the cytosol and activates the downstream effector caspase 3, which cleaves cellular targets and induces apoptosis (32,50).

1.2 The intrinsic pathway of apoptosis

Besides the extrinsic pathway, a second route can initiate apoptosis in response to specific stimuli. Because one of the first steps in this pathway is the permeabiliza-

tion of the outer membrane of the mitochondria, this pathway is also known as the mitochondrial pathway. Several stimuli like ionizing radiation, oxidative or heat stress, osmotic changes or cytotoxic drugsare able to provoke induction of the intrinsic pathway. In this framework, the life and death of cells is largely controlled by proteins of the Bcl-2 family. Within this family of proteins, three subfamilies exist represented by Bcl-2 like molecules, Bax like molecules and BH3-molecules (7). It is assumed that the Bcl-2 proteins mediate their effects at least in part by regulating mitochondrial morphology and/or function. The pro-apoptotic Bax protein may form channels in the outer mitochondrial membrane, whereas Bcl-2 interferes with that mechanism and maintains the integrity of mitochondria (1). As previously mentioned, mitochondria are central regulators of the intrinsic apoptosis pathway. After permeabilization of the mitochondrial membrane, cytochrome c is released into the cytoplasm which recruits the caspase adaptor molecule Apaf-1 and the apoptosis initiator molecule procaspase-9 (22). Consequently, cytochrome c, Apaf-1 and caspase-9 form a holoenzyme complex called an apoptosome. They proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed (14).

In many cell types both apoptosis pathways are not fully separated. Instead, there is some crosstalk between the extrinsic and intrinsic apoptosis pathway. The crosstalk is known to be caspase-8-mediated which induces a cleavage of the Bcl-2 family member Bid (38). Bid mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cleaved Bid activates Bax and causes oligomer formation on the mitochondrial membrane, loss of mitochondrial transmembrane potential and triggers the intrinsic pathway of apoptosis (71).

2. Leukocyte apoptosis

Apoptosis is a central mechanism in all leukocyte subclasses to govern their lifespan and to support their cellular function. In the case of an inflammatory response cells develop a certain apoptosis resistance to increase their cell population (clonal expansion), while after termination of the immune reaction these cells have to be deleted effectively through controlled cell death (clonal contraction) (32). Because the topic of exercise and leukocyte apoptosis was up to now mainly focused on lymphocytes and neutrophils, this review will also focus on these cell types as central themes.

2.1 Lymphocyte apoptosis

T and B cells are the central actors of the adaptive immune response. During physiological conditions, apoptosis plays an important role in the maintenance of lymphocyte homeostasis and the elimination of aged or potentiallyself-reactive cells.In the thymus, apoptosis is a part of the immature T cell development. Thereby, immature thymocytes expressing either self-reactive (TCRs) or non-functional T cell receptors are eliminated. Cells with functional TCRs migrate out

of the thymus as naïve T cells. During infection some of these cells recognize foreign antigens, become activated and rapidly expand. Some of their descendants differentiate into effector cells. Mature T cells can further differentiate into cytotoxic T cells, helper T cells, or regulatory T cells. Mostly, proliferation and differentiation are accompanied by a significant shortened lifespan (71). Hence, the expression of death receptors is a general feature of activated lymphocytes rendering them susceptible to apoptosis. This homeostatic mechanism ensures that all cellular components of the adaptive immune system are submitted to control through a specific form of the extrinsic apoptosis pathway, the activation-induced cell death (AICD) (32,34). After peak expansion phase, it is estimated that roughly 90% of T cells are eliminated by AICD. While earlier studies concluded that there is a major role for CD95 in AICD, recent studies proposed that Bcl-2-interacting mediator of cell death (BIM) is also a main regulator in the contracton phase of the T cell response. However, it is assumed that a failure in this system could predispose to autoimmunity due to the persistence of potentially cross-reactive activated T cells (32).

2.2 Neutrophil apoptosis

Neutrophils are known to be key regulators of inflammatory responses. During inflammatory conditions, cytokines such as G-CSF mobilize neutrophils from bone marrow. Activated neutrophils adhere to the vascular wall and transmigrate to the extracellular space along concentration gradients of chemokines. Locally, neutrophils initiate immune reactions by phagocytosis of pathogens, the production of ROS and the release of protease enzymes. Moreover, neutrophils amplify the inflammatory response and direct other immune cells to inflammatory sites by production of various cytokines and chemokines (6). Therefore, they are inflammatory effectors as well as immunoregulatory cells. Since ROS and proteases damage cells, the removal of neutrophils from inflamed tissue is recognized as a cardinal step in the resolution of inflammation. Physiological cell death of neutrophils occurs by both, necrosis as well as apoptosis. While necrosis leads to a release of cellular contents into extracellular space, this process ampilfies inflammation. By contrast, apoptosis induces the stepwise release of cell fragments which are cleared by neighbouring phagocytic cells.

Similarly to lymphocytes, caspases are crucial for the initiation and execution of cell death. Activaton of caspases in neutrophils is also induced by both the extrinsic and intrinsic apoptosis pathway (12). In general, several mechanisms and signalling pathways involved in the regulation of neutrophil apoptosis have many similaritieswith those described in lymphocytes. However, apoptosis in neutrophils shows also some peculiarities. In neutrophils, apoptosis is an invitable and early event in unstimulated cells, but their progression can be delayed by several cytokines and other inflammatory mediators. LPS, CRP, leukotriene B4, IL-8, GM-CSF and G-CSF are known to inhibit or delay neutrophil apoptosis, although the complete mechanisms of these effects are unclear. It is assumed that this process is PI 3-kinase and ERK-dependend. Recent studies suggest that a reduced expression of pro-apoptotic Bax and a stabilisation of Mcl-1 expression play crucial roles in delay of apoptosis during inflammatory conditions (12). A transient delay of apoptosis enables the cells to initate their immune response effectively. In the absence of an inflammatory stimulus they remain after maturation 2-6 days before undergoing spontaneous apoptosis (12,15).

Beside the common apoptosis pathways recent studies described an additional neutrophil-specific cell death process described as NETosis. Thereby, dying cells generate neutrophil extracellular traps (NETs) which are extracellular structures composed of chromatin and granule proteins that bind or kill pathogens. This form of cell death depends strongly on the generation of ROS by NADPH oxidase. On the one hand, NETosis seems to be an important defence mechanism of the innate immunity. On the other hand, evidence suggests that NET formation participates in pathogenesis of several autoimmune and inflammatory disorders, with proposed involvement in chronic lung disease, sepsis, and vascular disorders. However, the biological significance of NETs is just beginning to be explored (24).

3. Exercise and apoptosis

Exercise is a type of physiological stress which has a substantial effect on leukocyte life span. The amounts of several hormones, cytokines and other factors which might influence cellular survival are increased or decreased in organs, tissues and peripheral blood during exercise (20,32,42). However, the initiation of apoptosis depends on both the critical balance between pro-survival and pro-apoptotic factors as well as the intracellular protection systems contributing to apoptosis resistance (5).

3.1 Exercise and apoptosis of circulating lymphocytes

Exercise has a marked impact on numbers and functions of lymphocytes in blood. While numbers of circulating lymphocytes significantly increase during exercise, it is followed by a post-exercise lymphopenia (58). It is believed that lymphopenia is the result of at least two different processes. On the one hand lymphocytes are redistributed into various tissues and organs (29,30). On the other hand cells die by apoptosis (42,46). These processes are thought to run in parallel and their relative magnitude seemsto depend on the mode of exercise.

Exercise intensity is assumed to be a main effector of exercise induced lymphocyte apoptosis. It has been repeatedly demonstrated that intensive exercise significantly increases both percentage as well as total numbers ofcirculating apoptotic lymhocytes (28,46). Regarding endurance exercise, an increase of apoptosis was observed after ultra-marathon (2), marathon run (46,47), intensive treadmill running (47), intensive ergometer cycling (70), and triathlon (35). In contrast, moderate exercise did not or only marginally affected lymphocyte apoptosis (28,46). It was further found that an increase of lymphocyte apoptosis occurs after exceeding a threshold of 40-60% of VO_{2max} (49). It can be speculated, that the concentration of potential apoptosis mediators are gradiently expressedwith increasing exercise intensity and that they induce apoptosis after exceeding a specific threshold (47,51). Similarly, the effect of exercise duration can be postulated. After the athlete exceeds a specific duration of exercise, expression of several potential mediators is amplified and might exceed a death inducing threshold (70,71).

Besides endurance exercise, it was also shown that lymphocyte apoptosis increased also after resistance exercise (27,61). Similarly to endurance exercise, an increased rate of apoptosis was mainly related to the intensity of resistance exercise. In this regard, intensity means weight loads of 75% of the one repetition

maximum or above, while rest-interval length seemed to have only minimal effects (27,61).
There is less information available about lymphocyte apoptosis after short bouts of exercise. Only a study by Friedman et al. (2012) found no increase of apoptotic cells after repeated high-intensity "Wingate cycle bouts". Therefore, it can be suggested that not only exercise intensity, but also a minimum of exercise duration has to be exceeded to induce a significant increase of apoptosis (51,77).

3.2 Exercise and apoptosis of tissue lymphocytes

Because the blood compartment represents only a small fraction of total lymphocytes, some studies assessed lymphocyte apoptosis inside organs or tissues. Using mouse models, increased lymphocyte apoptosis was presented for several lymphphatic organs. At first, the group of Hoffman-Goetz demonstrated increased apoptosis of both intestinal lymphocytes (ILs) as well as for lymphocytes in thymus and in spleen after 90 min of intensive treadmill running in mice (20,21,63). Similarly, our group showed that exercise-induced lymphocyte apoptosis is a systemic phenomenon which was also observed in lung, lymph nodes, bone marrow and Peyer's patches after treadmill running at an intensity corresponding to 80% of VO_{2max}. Thereby, the extent of apoptosis, the kinetics and the inducing mechanisms seemed to have a certain tissue specificity. While an early and strong increase of apoptosis was observed in Peyer's patches, delayed smaller changes were found in lung, bone marrow and lymph nodes after exercise. As shown for apoptosis in human circulating lymphocytes, no increase of lymphocyte tissue apoptosis was found at moderate exercise intensities (28).

3.3 Effect of trainings status on lymphocyte apoptosis

Apoptosis sensitivity of lymphocytes seems to be inversely related to the athlete's training status (47,60). Mooren et al. (2004) analyzed subgroups of athletes after a marathon run and found that programmed cell death occurred only in less trained, but not in well trained athletes. Similarly, Peters et al. (2006) did not find changes of apoptosis in well trained athletes after prolonged exercise despite a significant lympopenia. In support of these data similar observations were published about tissue lymphocytes in mice. Accordingly, the number of intestinal CD4 lymphocytes decreased after an acute bout of exercise in non-trained mice, but not in mice with 4 month access to a running wheel (10).
Regarding potential mechanisms Avula et al. (2001) demonstrated that lymphocytes seem to decrease apoptosis sensititvity in response to repeated stress (3). In detail, it was demonstrated that mouse splenic lymphocytes from trained mice were less sensitive to H_2O_2 induced apoptosis compared to cells from non-trained mice indicating the up-regulation of cellular anti-oxidative defence mechanisms by regular exercise training (3). These observations support the hypothesis that regular physical activity may prevent stress-induced suppression of the immune system (17).

3.4 Exercise and neutrophil apoptosis

There are conflicting data about the effects of exercise on neutrophil apoptosis. Syu et al. (2011) demonstrated that acute incremental exercise test induced an oxidative state in neutrophils which resulted in acceleration of spontaneous neu-

trophil apoptosis (74). The same group presented evidence that repeated moderate exercise (30 min a day, 5 days a week at 60% of maximal workload) delayed neutrophil apoptosis (72). The latter observation was proved by a recent study of our group. Here a significant delay of neutrophil apoptosis after marathon run, intensive endurance and downhill running as well as intensive resistance exercise was presented (49). In this regard, it has to be considered that acute exercise mobilizes immature non-segmented neutrophils from the bone marrow which might affect relative numbers of apoptotic cells in the circultation (79). However, also the *"in vitro"* spontaneous apoptosis was demonstrated as being significantly delayed after intensive exercise protocols. In contrast, the delay of apoptosis was not observed after moderate exercise protocols suggesting also an intensity dependent mechanism (49).

Regarding NETosis or neutrophil NET formation, Syu et al. (2013) demonstrated an acute bout of severe exercise facilitated NET formation in inactive subjects, while they did not observe changes in trained subjects (75). In sedentary subjects increased NET formation was accompanied by increased ROS production and a reduced mitochondrial membrane potential. A comparison of these results to previous data is difficult since subjects completed a short incremental exercise protocol in this study. However, these data implicate that apoptosis regulation also depends on subjects' training status indicating an effect of training adaptation on neutrophils cell death (75).

Only limited data were available regarding the effect of exercise on tissue neutrophils. Lagranha et al. (2004) obtained neutrophils of exercised rats by intraperitoneal lavage after injection of oyster glycogen solution. Here an increase in DNA fragmentation, chromatin condensation, and phosphatidylserine externalization was demonstrated. In addition, oral glutamine supplementation partially prevented the exercise-induced apoptosis in neutrophils. However, the differing results to human studies might be dueto the pre-treatment and source of the cells (33).

3.5 Apoptosis signalling pathways during exercise

There is evidence that exercise induces lymphocyte apoptosis by both, the intrinsic as well as the extrinsic pathways. For peripheral human lymphocytes, it was shown that intensive treadmill running, marathon running and intensive resistance training lead to an up-regulation of CD95 receptors (28,46,47) and partly of CD95 ligands (47). Furthermore, it is assumed that exercise also induces a shift to lymphocyte subpopulations with a higher density of CD95-receptors on their surface (46). Further evidence for Fas induced apoptosis comes from animal studies. After intensive treadmill running in mice the increase of apoptotic lymphocytes in tissues was accompanied by increased expression of either FasR or FasL, respectively. The critical role of Fas signaling in apoptosis induction was supported by the absence or reduction of apoptosis signalling after treadmill running of Fas-deficient MRL/lpr-mice (28).

Experiments with intestinal lymphocytes suggest that also the intrinsic pathway of apoptosis is addressed by exercise. Here apoptosis after exercise was accompanied by mitochondrial membrane depolarization, an increase of cytosolic cytochrome c, and a significant reduction of Bcl-2 protein content (62).

A possible crosslink of both pathways is indicated by a recent study of our group. It was found that increased lymphocyte apoptosis after resistance exercise was

accompanied by an up-regulation of FasR as well as a significant reduction in cellular Bcl-2, followed by a loss of mitochondrial membrane potential. However, further evidence for a crosslink of the intrinsic and extrinsic apoptosis pathway remains to be shown (27).
Regarding the mechanisms of neutrophil apoptosis modulation after exercise only little information is available. Mooren et al. (2012) showed that delayed neutrophil apoptosis was neither accompanied by changes of mitochondrial membrane potential nor by death receptor/ligand expression. Instead, apoptosis delay was accompanied by enhanced intracellular calcium transients and decreased glutathione levels. Further details for potential signaling pathways come from Su et al. (2011) who showed a collective up-regulation of the iNOS-NO-cGMP-Mcl-1 pathway after exercise (72). Taken together, it can be assumed that both the extrinsic as well as the intrinsic pathways are involved in exercise induced leukocyteapoptosis. Their predominant role seems to depend on the compartment, the exercise protocol and of the mediators of apoptosis induction.

3.6 Mediators of exercise-induced apoptosis

Intensive exercise is accompanied by changes of the expression of numerous cytokines, hormones, growth factors and the oxidative status. All these factors are known to potentially mediate either accelerated death or prolonged survival of leukocytes (Figure 1). Often apoptosis regulation depends on the proper balance between pro-apoptotic and anti-apoptotic factors and on the cell type and tissue.

3.6.1 Reactive oxygen species (ROS)

Intensive exercise is known to affect the balance between the production of free radicals and their depletion by antioxidant defense mechanisms. A major source of free radicals during exercise might be the high turnover rate of the mitochondrial transport chain due to higher energetic demands, ischemia reperfusion injury and the mobilisation or activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) carrying cells (11). ROS are known to affect certain pathways of lymphocyte apoptosis. On the one hand, ROS reduce cellular Bcl-2 and depolarize the outer layer of the mitochondrial membrane (11). On the other hand, ROS crosslink the intrinsic with the extrinsic apoptosis pathway by increasing Fas expression (11,15).
The role of oxidative stress in mediating exercise induced apoptosis mainly comes from animal studies. Here it was repeatedly demonstrated that exercise-induced lymphocyte apoptosis was accompanied by markers of oxidative stress including decreased intracellular glutathione concentrations (63) or formation of malondialdehyde (MDA), an intermediate product of lipid peroxidation (25). More direct evidence for a major role of ROS for apoptosis comes from studies which used the application of antioxidants like the ROS scavenging agent N-acetylcysteine (NAC). Lin et al. (1999) demonstrated that antioxidant administration inhibits exercise-induced thymocyte apoptosis in rats (37). Similarly, Quadrilatero and Hoffman-Goetz (2004) showed that application of NAC prevented exercise-induced intestinal lymphocyte apoptosis by maintaining intracellular glutathione levels and reducing mitochondrial membrane depolarization. However, further studies indicated that apoptosis inducing mechanisms during exercise

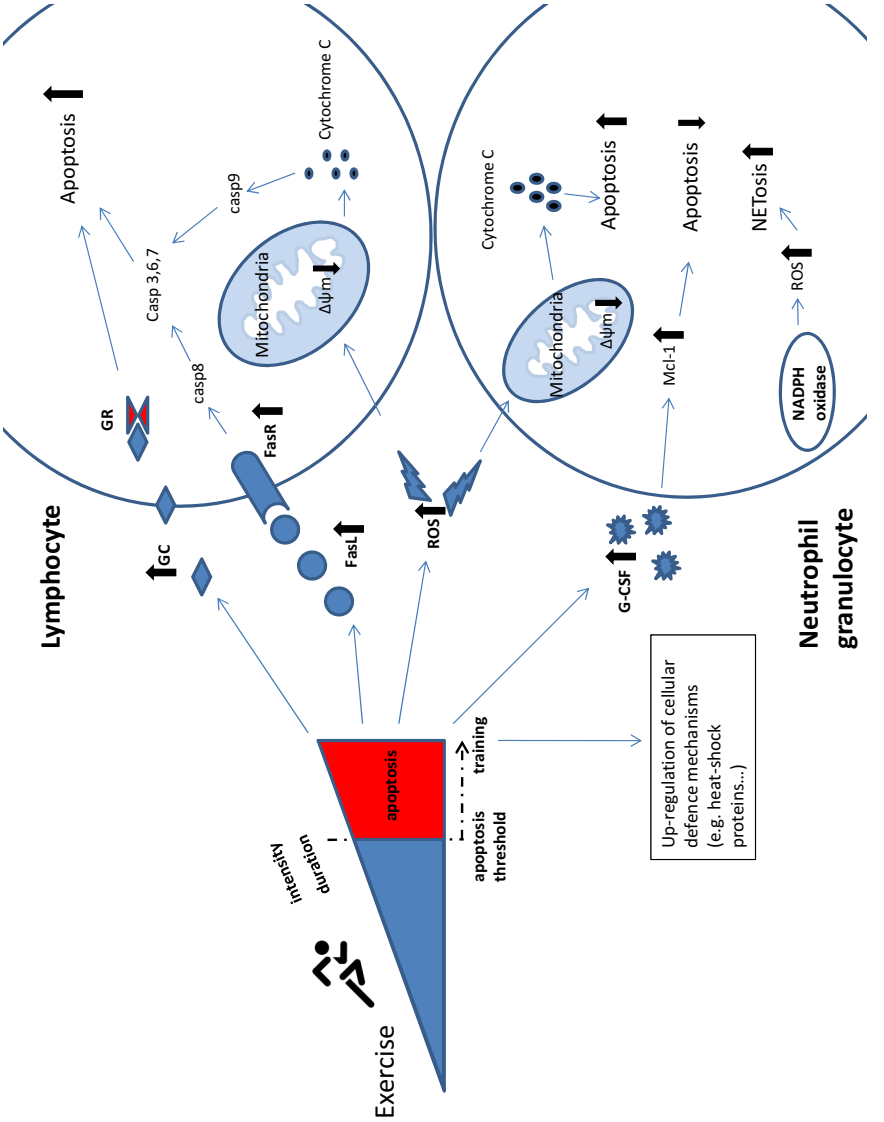

Figure 1: Schematic figure of pathways of exercise-induced lymphocyte and neutrophil apoptosis (GC=glucocorticoids, GR = glucocorticoid receptor, casp = caspase, Δψm = change in mitochondrial membrane potential, ROS = reactive oxygen species, FasL = fas ligand, FasR = fas receptor).

might be tissue specific. While application of NAC prevented exercise-induced T cell apoptosis in spleen and bone marrow, it was ineffective in lymph nodes (28). It can be speculated that the increased apoptosis resistance of trained individuals might be the result of an increased antioxidant defence. In this regard, it was demonstrated that cells initiate the expression of antioxidant enzymes and heat shock proteins (HSPs) in response to repeated exercise stress which exhibit protective effects against cellular stressors induced by acute bouts of exercise (16).
There is some evidence that ROS have divergent roles in neutrophils' cell cycle-physiology. On the one hand ROS production mediates the process of NETosisafter intensive exercise (75). On the other hand, it was demonstrated that ROS production was associated with the activation of pro-survival signals (43). Neutrophils have a specific antioxidant enzyme activity profile, probably related to the lower numbers of mitochondria and the production of high amounts of ROS during the oxidative burst (12). Accordingly, Fadeel et al. (1998) demonstrated that activated neutrophils showed an increased ROS production and suppressed caspases (15). Additionally, in neutrophils increased ROS generation is followed by NF-κ-B activation and translocation, which is a strong anti-apoptotic signal through the expression of proteins of the Bcl-2 family. Otherwise, if ROS production exceeds the neutrophil's natural defences, sustained perturbation of this balance may result in apoptotic cell death via the intrinsic pathway of apoptosis (15).

3.6.2 Glucocorticoids

Glucocorticoids (GC) are known to induce apoptosis in monocytes, macrophages and T cells. (13). Thereby, GCs act via binding to their intracellular receptors known as the glucocorticoid receptors (GRs). Prior to activation by interaction with its ligand, GRs are associated with heat shock protein 90 (HSP90). Upon hormone binding, a conformational change occurs where HSP90 dissociates from the receptor molecule and allows the receptor hormone complex to migrate into the nucleus. Here, it can transactivate genes that are involved in apoptosis. A first step in apoptosis induction is mitochondrial dysfunction followed by the relase of cytochrome c into the cytosol. Further steps in GC induced apoptosis involved the activation of the caspase cascade (13). Our group demonstrated that lymphocyte apoptosis after resistance training correlated with levels of plasma cortisol. Furthermore, an *in vitro* approach elucidated that exercise-concentrations of hydrocortisone induced lymphocyte apoptosis which can be attenuated by adding mifepristone (MIF), a glucocorticoid receptor blocker. Simultaneously to apoptosis induction, a significant decrease of mitochondrial membrane potential, a reduction of Bcl-2, and an upregulation of FasR were observed. Therefore, it can be speculated whether the extrinsic and intrinsic pathways were crosslinked after cortisol induced apoptosis during exercise (27). For intestinal lymphocytes Quadrilatero et al. (2005) demonstrated no major role for GCs for intestinal lymphocyte apoptosis in mice. In contrast, for thymocytes in rats it was demonstrated that DNA fragmentation after exercise was decreased in MIF treated rats after exercise (8).
Considering the role for GCs on peripheral lymphocyte apoptosis, a possible effect of the time of day when exercise is performed has to be discussed. Because cortisol levels follow a circadian rhythm, time of day might have an impactupon the rate of lymphocyte apoptosis (26). However, up to now there are no data available regarding circadian rhythm and apoptosis.

3.6.3 Further signalling pathways

Catecholamines and oestrogen hormones were considered to affect exercise-induced lymphocyte apoptosis. Marra et al. (2005) found that both adrenaline (epinephrine) and noradrenaline (norepinephrine) application in mice resulted in fewer apoptotic intestinal lymphocytes compared to control mice given saline. Similarly, it was demonstrated that neither alpha- nor beta-antagonism prevented exercise-induced cell loss in the intestine (41). Therefore, it can be assumed that catecholamines are not responsible for apoptosis in tissue lymphocytes.

It is known that oestrogen hormones (E2) protect lymphocytes from apoptosis *in vitro*. Therefore, it was tested if gender or menstrual cycle phase influences exercise-induced lymphocyte apoptosis. However, up to now neither gender specifty nor menstrual cycle effects for exercise-induced lymphocytes apoptosis have been found (53).

Since intensive exercise induces an intensity dependent systemic inflammatory response, Neubauer et al. (2008) investigated if exercise-induced apoptosis or DNA damage might be a consequence of inflammatory processes (58). In order to test this hypothesis, they tried to find relationships between DNA damage in lymphocytes and different systemic inflammatory markers in athletes after an Ironman triathlon race. However, no clear relationship was found. In addition, our group analysed the effect of exercise concentrations of inflammatory mediators on *"in vitro"* lymphocyte apoptosis. Both C-reactive protein as well as IL-6 failed to affect apoptosis of isolated lymphocytes (27).

It was previously demonstrated that the immune perturbations during exercise can be reduced by nutritional supplementation of carbohydrates. However, supplementation of carbohydrates failed to alter the apoptotic response after 60min of cycling on an ergometer at 80% of VO_{2max} (52).

Regarding inflammatory factors, granulocyte-colony stimulating factor (G-CSF) has been identified to have a major role in delaying neutrophil apoptosis after exercise (49). More precisely, it was found that a delay of spontaneous apoptosis *in vitro* could be induced by incubation of neutrophils in post-exercise serum. Addition of anti-G-CSF antibody to post-exercise serum was effective in reversing its apoptosis delaying effect (49). A role of G-CSF in apoptosis modulation is also supported by Su et al. (2011). They recently showed that exercise up-regulated Mcl-1 proteins in neutrophils which are known to be stabilized by G-CSF (72).

4. Methodological aspects of apoptosis measurement

There are some variations in percentage and total numbers of apoptotic cells in studies on exercise-induced leukocyte apoptosis. However, besides the use of subjects of different training status and the use of various exercise protocols, some of these variations might be the result of sensitivity issues related to the methodology used to assess cell death or apoptosis. Therefore, some methodological aspects have to be discussed.

It was described that the morphological characterization by microscopy to be the gold standard for identification of apoptotic cells (18). Using this method, characteristics of apoptotic cells such as nuclear condensation, nuclear fragmentation, and membrane blebbing were used to quantify cell death. However, this method is described as time consuming and error prone since there is a lack of objectivity

and reproducibility. In this context, Navalta et al. (2011) investigated the intra- and inter-rater reliability of morphological apoptosis evaluation in trained and untrained observers (53). It was found that a single trained observer get the highest reliability in apoptosis assessment.

Morphological analyses make it difficult to identify changes in large populations. Most exercise studies measured apoptosis by flow cytometry using different fluorescence markers which target hallmarks of apoptosis. Common methods are represented by the TUNEL (Terminal Deoxynucleotide Transferased UTP Nick End Labeling) assay for detection of DNA fragmentation, the Annexin V assay for surface phosphatidylserine (PS) exposure, and fluorogeniccaspase substrates to detect caspase activation (19).

Comparing the different methods, it is assumed that morphological methods yield a greater apoptotic index compared to those employing biochemical markers (55). Therefore, it can be proposed that an objective evaluation by morphological method in conjunction with biochemical marker assessment might be the better strategy compared to using a single method (19). A similar conclusion was drawn by Navalta et al. (2010) who described an image-based morphological approach by which computer software assesses the characteristics associated with lymphocyte apoptosis (55). Here automated analysis of the cellular morphology helps to overcome the lack of objectivity. They also compared morphological, image-based, and biomarker methods and got remarkble differences in percentage of apoptotic cells after an exercise intervention. Therefore, they recommended an *in vivo* method to be the best way to measure apoptosis after exercise conditions because exercise rapidly changes the internal cellular environment. However, such a method is currently not available. As a conclusion they discussed the possibility of combining methods such as morphological analysis with current biochemical marker methods. In this regard, a promising method is represented by the usage of multispectral image-based flow cytometry which couples the quantitative advantage of flow cytometry with the accuracy of morphology-based algorithms (18,19).

Recently, Navalta et al. (2011) presented a rapid and minimally invasive procedure for the analysis of the actual status lymphocyte apoptosis in athletes. In detail, whole blood taken from a finger stick sample is added to an antibody panel (56). Following the incubation period, red blood cells are lysed and samples are analysed. The usefulness, validity and reliability of this method has to be evaluated in future studies using morphological and biochemical methods in parallel.

5. Physiological relevance of apoptosis during exercise conditions

Finally, the physiological function of exercise-induced leukocyte apoptosis has to be discussed. In early studies lymphocyte apoptosis is often considered to contribute to post-exercise lymphopenia. In this regard, it was often assumed that increased apoptosis is associated with a loss of immunological competence. This idea is supported by studies which demonstrated a transient increase of upper respiratory tract infections (URTIs) after prolonged intensive exercise (59). However, the precise role of apoptosis in a clinical context remains to be shown. Similarly, the contribution of apoptosis to post-exercise lymphopenia is still discussed. While some studies demonstrated that lymphopenia was directly accompanied by

increased apoptosis (46), others found a decrease of lymphocytes without changes of total numbers of apoptotic cells (66,68). As previously mentioned, it is assumed that lymphopenia is the result of both lymphocyte migration as well as apoptosis. Therefore, their relative magnitude might depend on the different exercise protocols applied in these studies (29,30).

Beside detrimental effects on immunity researchers also considered that lymphocyte apoptosis is a regulatory mechanism to remove senescent, activated or potentially autoreactive lymphocytes. In this context, Simpson et al. (2010) noted that lymphocytes, which are mobilized into blood during exercise, are mainly senescent cells. Given that these cells mainly die by apoptosis, it can be speculated that exercise-induced lymphocyte apoptosis creates "vacant space" for newly functional lymphocytes to occupy and expand the naïve T-cell repertoire (68). A similar conclusion came from Goon et al. (2008) who demonstrated that long term Tai Chi exercise increased both lymphocyte apoptosis as well as proliferation (22). Therefore, it can be assumed that exercise promotes both cell death as well as cell production. The idea that exercise increases not only cell death, but cell turnover in general, is also supported by different studies which showed that exercise mobilizes hematopoietic progenitor cells from bone marrow (4). It can be speculated that an increased turnover of cells might be part of exercise-induced adaptation processes.

The physiological function of neutrophil apoptosis during exercise is also still unclear. From several acute and chronic inflammatory conditions it is known that a delay in neutrophil apoptosis contributes to the development of neutrophilia (43). However, it remains to be shown whether such a process is also operative during neutrophilia after exercise conditions. Further it can be speculated that life span is prolonged by the inflammatory mileu to take part in the damage repair processes as part of the adaptation to regular exercise training (39). In this regard, it is known that especially after eccentric exercise protocols neutrophils are rapidly mobilized into the circulation and migrate into the damaged muscle tissue (60). Therefore, it can be speculated that neutrophils survival is prolonged in order to amplify the repair process in muscle tissues.

Taken together, it is clearly demonstrated that exercise modulates leukocyte apoptosis depending on the exercise intensity. Thereby, apoptosis seemed to be induced by both the extrinsic as well as the intrinsic pathway depending on the mediators and the cell compartment. In case of lymphocytes, it was shown that ROS and glucocorticoids are major mediators of increased cell death, while neutrophils' life span seems to be affected by the inflammatory reaction evoked by exercise. In future studies, researchers should focus more on the physiological impact and clinical relevance of the transient alterations of leukocyte apoptosis. Additionally, the methods employed in future studies should be improved, for example, by using morphological methods in addition to biochemical markers.

REFERENCES

1. Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC. Inhibition of Baxchannel-forming activity by Bcl-2. Science 1997; 277: 370–372.
2. Atamaniuk J, Stuhlmeier KM, Vidotto C, Tschan H, Dossenbach-Glaninger A, Mueller M. Effects of ultra-marathon on circulating DNA and mRNA expression of pro- and anti-apoptotic genes in mononuclear cells. Eur J Appl Physiol. 2008 Nov;104(4):711-7. doi: 10.1007/s00421-008-0827-2.
3. Avula CP, Muthukumar AR, Zaman K, McCarter R, FernandesG.Inhibitory effects of voluntary wheel exercise on apoptosis in splenic lymphocyte subsets of C57BL/6 mice. J Appl Physiol. 2001 Dec;91(6):2546-52.
4. Bonsignore MR, Morici G, Santoro A, Pagano M, Cascio L, Bonanno A, Abate P, Mirabella F, Profita M, Insalaco G, Gioia M, Vignola AM, Majolino I, Testa U, Hogg JC.Circulating hematopoietic progenitor cells in runners.J Appl Physiol. 2002 Nov;93(5):1691-7.
5. Bortner CD, Scoltock AB, Sifre MI, Cidlowski JA.Osmotic stress resistance imparts acquired anti-apoptotic mechanisms in lymphocytes.J Biol Chem. 2012 Feb 24;287(9):6284-95.
6. Cassatella MA. Neutrophil-derived proteins: selling cytokines by the pound. Adv Immunol 1999;73:369-509.
7. Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell. 2001, 8(3):705-11.
8. Concordet JP, Ferry A. Physiological programmed cell death in thymocytes is induced by physical stress (exercise). Am J Physiol. 1993 Sep;265(3 Pt 1):C626-9.
9. Curtin JF, Cotter TG.Live and let die: regulatory mechanisms in Fas-mediated apoptosis. Cell Signal. 2003 Nov;15(11):983-92
10. Davidson SR, Hoffman-Goetz L.Freewheel running selectively prevents mouse CD4+ intestinal lymphocyte death produced after a bout of acute strenuous exercise. Brain Behav Immun. 2006 Mar;20(2):139-43.
11. Denning TL, Takaishi H, Crowe SE, Boldogh I, Jevnikar A, Ernst PB.Oxidative stress induces the expression of Fas and Fas ligand and apoptosis in murine intestinal epithelial cells. Free RadicBiol Med. 2002 Dec 15;33(12):1641-5
12. Dibbert B, Weber M, Nikolaizik WH, Vogt P, Schöni MH, Blaser K, Simon HU. Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: a general mechanism to accumulate effector cells in inflammation.ProcNatlAcadSci U S A. 1999;96(23):13330-5.
13. Distelhorst CW.Recent insights into the mechanism of glucocorticosteroid-induced apoptosis.Cell Death Differ. 2002 Jan;9(1):6-19.
14. EarnshawWC.Apoptosis. A cellular poison cupboard. Nature. 1999, 397(6718):387-9.
15. Fadeel B, Ahlin A, Henter JI, Orrenius S, Hampton MB. Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. Blood 1998;92(12):4808-18.
16. Fehrenbach E, Northoff H.Free radicals, exercise, apoptosis, and heat shock proteins.ExercImmunol Rev. 2001;7:66-89.

17. Fleshner M. Physical activity and stress resistance: sympathetic nervous system adaptations prevent stress-induced immunosuppression.Exerc Sport Sci Rev. 2005 Jul;33(3):120-6.
18. Friedman RA, Navalta JW, Fedor EA, Kell HB, Lyons TS, Arnett SW, Schafer MA. Repeated high-intensity Wingate cycle bouts influence markers of lymphocyte migration but not apoptosis. ApplPhysiolNutrMetab. 2012 Apr;37(2):241-6. doi: 10.1139/h11-156.
19. Henery S, George T, Hall B, Basiji D, Ortyn W, Morrissey P.Quantitative image based apoptotic index measurement using multispectral imaging flow cytometry: a comparison with standard photometric methods. Apoptosis. 2008 Aug;13(8):1054-63
20. Hoffman-Goetz L, Zajchowski S, Aldred A.Impact of treadmill exercise on early apoptotic cells in mouse thymus and spleen. Life Sci. 1999;64(3):191-200.
21. Hoffman-Goetz L, Quadrilatero J.Treadmill exercise in mice increases intestinal lymphocyte loss via apoptosis. ActaPhysiol Scand. 2003 Nov;179(3):289-97.
22. Goon JA, Noor Aini AH, Musalmah M, YasminAnum MY, Wan Ngah WZ. Long term Tai Chi exercise reduced DNA damage and increased lymphocyte apoptosis and proliferation in older adults. Med J Malaysia. 2008 Oct;63(4):319-24.
23. Ishizaki Y, Cheng L, Mudge AW, Raff MC.Programmed cell death by default in embryonic cells, fibroblasts, and cancer cells. MolBiol Cell. 1995, 6(11):1443-58.
24. Kaplan MJ, Radic M. Neutrophil extracellular traps: double-edged swords of innate immunity.J Immunol. 2012 Sep 15;189(6):2689-95.
25. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.Br J Cancer. 1972 Aug;26(4):239-57.
26. Korsmeyer SJ. Programmed cell death and the regulation of homeostasis. Harvey Lect. 1999-2000, 95:21-41.
27. Krüger K, Agnischock S, Lechtermann A, Tiwari S, Mishra M, Pilat C, Wagner A, Tweddell C, Gramlich I, Mooren FC..Intensive resistance exercise induces lymphocyte apoptosis via cortisol and glucocorticoid receptor-dependent pathways. J Appl Physiol. 2011;110(5):1226-32.
28. Krüger K, Frost S, Most E, Völker K, Pallauf J, Mooren FC. Exercise affects tissue lymphocyte apoptosis via redox-sensitive and Fas-dependent signaling pathways. Am J PhysiolRegulIntegr Comp Physiol. 2009 May;296(5):R1518-27.
29. Krüger K, Lechtermann A, Fobker M, Völker K, Mooren FC. Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. Brain Behav Immun. 2008 Mar;22(3):324-38
30. Krüger K, Mooren FC. T cell homing and exercise. ExercImmunol Rev.2007;13:37-54.
31. Kraemer WJ, Fleck SJ, Maresh CM, Ratamess NA, Gordon SE, Goetz KL, Harman EA, Frykman PN, Volek JS, Mazzetti SA, Fry AC, Marchitelli LJ, Patton JF. Acute hormonal responses to a single bout of heavy resistance exercise in trained power lifters and untrained men. Can J Appl Physiol.1999;l24: 524–537.
32. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. Nat Rev Immunol. 2007, 7(7):532-42.
33. Lagranha CJ, Senna SM, de Lima TM, Silva E, Doi SQ, Curi R, Pithon-Curi TC. Beneficial effect of glutamine on exercise-induced apoptosis of rat neutrophils. Med Sci Sports Exerc. 2004 Feb;36(2):210-7.

34. Lenardo M, Chan KM, Hornung F, McFarland H, Siegel R, Wang J et al. Mature T lymphocyte apoptosis—immune regulation in a dynamic and unpredictable antigenic environment. Ann Rev Immunol 1999; 17: 221–53.
35. Levada-Pires AC, Cury-Boaventura MF, Gorjão R, Hirabara SM, Puggina EF, Pellegrinotti IL, DominguesFilho LA, Curi R, Pithon-Curi TC. Induction of lymphocyte death by short- and long-duration triathlon competitions.Med Sci Sports Exerc. 2009 Oct;41(10):1896-901.
36. Levada-Pires AC, Fonseca CE, Hatanaka E, Alba-Loureiro T, D'Angelo A, Velhote FB, Curi R, Pithon-Curi TC. The effect of an adventure race on lymphocyte and neutrophil death.Eur J Appl Physiol. 2010 Jun;109(3):447-53.
37. Lin YS, Kuo HL, Kuo CF, Wang ST, Yang BC, Chen HI. Antioxidant administration inhibits exercise-induced thymocyte apoptosis in rats. Med Sci Sports Exerc. 1999 Nov;31(11):1594-8.
38. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. 1998, 94(4):481-90.
39. Malm C, Nyberg P, Engstrom M, Sjodin B, Lenkei R, Ekblom B, Lundberg I. Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies. J Physiol. 2000 Nov 15;529 Pt 1:243-62
40. Marra S, Burnett M, Hoffman-Goetz L. Intravenous catecholamine administration affects mouse intestinal lymphocyte number and apoptosis. J Neuroimmunol. 2005 Jan;158(1-2):76-85.
41. Marra S, Hoffman-Goetz L. Beta-adrenergic receptor blockade during exercise decreases intestinal lymphocyte apoptosis but not cell loss in mice. Can J Physiol-Pharmacol. 2004 Jul;82(7):465-73
42. Mars M, Govender S, Weston A, Naicker V, Chuturgoon A.High intensity exercise: a cause of lymphocyte apoptosis? BiochemBiophys Res Commun. 1998 Aug 19;249(2):366-70.
43. Melley DD, Evans TW, Quinlan GJ. Redox regulation of neutrophil apoptosis and the systemic inflammatory response syndrome. ClinSci (Lond) 2005;108(5):413-623.
44. Mooren FC, Lechtermann A, Pospiech S, Fromme A, Thorwesten L, Völker K. Decoupling of intracellular calcium signaling in granulocytes after exhaustive exercise. Int J Sports Med 2001;22(5):323-8.
45. Mooren FC, Lechtermann A, Fromme A, Thorwesten L, Völker K. Alterations in intracellular calcium signaling of lymphocytes after exhaustive exercise. Med Sci Sports Exerc 2001;33(2):242-8.
46. Mooren FC, Bloming D, Lechtermann A, Lerch MM, Volker K. Lymphocyte apoptosis after exhaustive and moderate exercise. J Appl Physiol. 2002;93(1):147-53.
47. Mooren FC, Lechtermann A, Völker K. Exercise-induced apoptosis of lymphocytes depends on training status. Med Sci Sports Exerc. 2004, 36(9):1476-83.
48. Mooren FC, Lechtermann A, Fobker M, Brandt B, Sorg C, Völker K, Nacken W. The response of the novel pro-inflammatory molecules S100A8/A9 to exercise. Int J Sports Med.2006;27(9):751-8.
49. Mooren FC, Völker K, Klocke R, Nikol S, Waltenberger J, Krüger K. Exercise delays neutrophil apoptosis by a G-CSF-dependent mechanism.J Appl Physiol. 2012 Oct;113(7):1082-90
50. Nagata S, Golstein P.The Fas death factor. Science.1995, 267(5203):1449-56.

51. Navalta JW, Sedlock DA, Park KS. Effect of exercise intensity on exercise induced lymphocyte apoptosis. Int J Sports Med. 2007;28(6):539-42.
52. Navalta JW, McFarlin BK, Lyons S, Arnett SW, Schafer MA.Cognitive awareness of carbohydrate intake does not alter exercise-induced lymphocyte apoptosis. Clinics (Sao Paulo). 2011;66(2):197-202
53. Navalta, J.W., B.K. McFarlin, T.S. Lyons, A.R. Ramos, S.W. Arnett and M.A. Schafer. Intra- and Interrater Reliability of Morphologically Evaluated Lymphocyte Apoptosis in Trained and Untrained Observers. Biology of Sport 28, 2011: 223-226.
54. Navalta JW, Sedlock DA, Park KS, McFarlin BK.Neither gender nor menstrual cycle phase influences exercise-induced lymphocyte apoptosis in untrained subjects. ApplPhysiolNutrMetab. 2007 Jun;32(3):481-6.
55. Navalta JW, Mohamed R, El-Baz A, McFarlin BK, Lyons TS. Exercise-induced immune cell apoptosis: image-based model for morphological assessment. Eur J Appl Physiol. 2010 Sep;110(2):325-31.
56. Navalta J, McFarlin B, Simpson R, Fedor E, Kell H, Lyons S, Arnett S, Schafer M.Finger-stick blood sampling methodology for the determination of exercise-induced lymphocyte apoptosis.J Vis Exp. 2011 Feb 24;(48).
57. Navalta JW, McFarlin BK, Lyons TS.Does exercise really induce lymphocyte apoptosis?Front Biosci (Elite Ed). 2010 Jan 1;2:478-88.
58. Neubauer O, Reichhold S, Nersesyan A, König D, Wagner KH. Exercise-induced DNA damage: is there a relationship with inflammatory responses? ExercImmunol Rev. 2008;14:51-72.
59. Nieman DC.Exercise, infection, and immunity. Int J Sports Med. 1994, 15 Suppl 3:S131-41.
60. Peake J, Nosaka K, Suzuki K.Characterization of inflammatory responses to eccentric exercise in humans.ExercImmunol Rev. 2005;11:64-85.
61. Pereira GB, Prestes J, Tibana RA, Shiguemoto GE, Navalta J, Perez SE.Acute resistance training affects cell surface markers for apoptosis and migration in CD4□ and CD8□ lymphocytes.Cell Immunol. 2012 Oct;279(2):134-9
62. Peters EM, Van Eden M, Tyler N, Ramautar A, Chuturgoon AA.Prolonged exercise does not cause lymphocyte DNA damage or increased apoptosis in well-trained endurance athletes. Eur J Appl Physiol. 2006 Sep;98(2):124-31.
63. Quadrilatero J, Hoffman-Goetz L.N-Acetyl-L-cysteine prevents exercise-induced intestinal lymphocyte apoptosis by maintaining intracellular glutathione levels and reducing mitochondrial membrane depolarization. BiochemBiophys Res Commun. 2004 Jul 2;319(3):894-901.
64. Quadrilatero J, Hoffman-Goetz L. Mouse thymocyte apoptosis and cell loss inresponse to exercise and antioxidant administration. Brain Behav Immun. 2005;19(5):436-44.
65. Siegel RM, Lenardo MJ. Apoptosis signaling pathways. CurrProtocImmunol. 2002, Chapter 11:Unit 11.9C.
66. Simpson RJ, Florida-James GD, Whyte GP, Black JR, Ross JA, Guy K. Apoptosis does not contribute to the blood lymphocytopenia observed after intensive and downhill treadmill running in humans. Res Sports Med. 2007 Jul-Sep;15(3):157-74.
67. Simpson RJ, Cosgrove C, Chee MM, McFarlin BK, Bartlett DB, Spielmann G, O'Connor DP, Pircher H, ShielsPG.Senescent phenotypes and telomere lengths of peripheral blood T-cells mobilized by acute exercise in humans. ExercImmunol Rev. 2010; volume and pages.

68. Simpson RJ.Aging, persistent viral infections, and immunosenescence: can exercise "make space"? Exerc Sport Sci Rev. 2011 Jan;39(1):23-33.
69. Smilios I, Pilianidis T, Karamouzis M, Parlavantzas A, Tokmakidis SP. Hormonal responses after a strength endurance resistance exercise protocol in young and elderly males. Int J Sports Med.2007;28: 401–406.
70. Steensberg A, Morrow J, Toft AD, Bruunsgaard H, Pedersen BK.Prolonged exercise, lymphocyte apoptosis and F2-isoprostanes. Eur J Appl Physiol. 2002 May;87(1):38-42.
71. Strasser A, Pellegrini M. T-lymphocyte death during shutdown of an immune response. Trends Immunol. 2004, 25(11):610-5.
72. Su SH, Jen CJ, Chen HI.NO signaling in exercise training-induced anti-apoptotic effects in human neutrophils. BiochemBiophys Res Commun. 2011 Feb 4;405(1):58-63. doi: 10.1016/j.bbrc.2010.12.123.
73. Suzuki K, Nakaji S, Yamada M, Totsuka M, Sato K, Sugawara K.Systemic inflammatory response to exhaustive exercise. Cytokine kinetics.ExercImmunol Rev. 2002;8:6-48
74. Syu GD, Chen HI, Jen CJ. Severe exercise and exercise training exert opposite effects on human neutrophil apoptosis via altering the redox status. PLoS One. 2011;6(9):e24385.
75. Syu GD, Chen HI, Jen CJ.Acute severe exercise facilitates neutrophil extracellular trap formation in sedentary but not active subjects.Med Sci Sports Exerc. 2013 Feb;45(2):238-44.
76. Sureda A, Tauler P, Aguiló A, Cases N, Llompart I, Tur JA, Pons A.Influence of an antioxidant vitamin-enriched drink on pre- and postexercise lymphocyte antioxidant system. Ann NutrMetab.2008;52: 233–240.
77. Tanimura Y, Shimizu K, Tanabe K, Otsuki T, Yamauchi R, Matsubara Y, Iemitsu M, Maeda S, Ajisaka R.Exercise-induced oxidative DNA damage and lymphocytopeniain sedentary young males. Med Sci Sports Exerc. 2008 Aug;40(8):1455-62. doi: 10.1249/MSS.0b013e31817242cf.
78. Tuan TC, Hsu TG, Fong MC, Hsu CF, Tsai KK, Lee CY, Kong CW. Deleterious effects of short-term, high-intensity exercise on immune function: evidence from leucocyte mitochondrial alterations and apoptosis. Br J Sports Med. 2008 Jan;42(1):11-5.
79. Yamada M, Suzuki K, Kudo S, Totsuka M, Nakaji S, Sugawara K.Raised plasma G-CSF and IL-6 after exercise may play a role in neutrophil mobilization into the circulation. J Appl Physiol (1985). 2002 May;92(5):1789-94.

Exercise Immunology Meets MiRNAs

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ABSTRACT

A large body of evidence indicates modified expression of protein-coding genes in response to different kinds of physical activity. Recent years have exposed another level of regulation of cellular processes mediated by non-coding RNAs. MicroRNAs (miRNAs) are one of the largest families of non-coding RNAs. MiRNAs mediate post-transcriptional regulation of gene expression. The amount of data supporting the key role of miRNAs in the adaptation of the immune and other body systems to exercise steadily grows. MiRNAs change their expression profiles after exercise and seem to be involved in regulation of exercise-responsive genes in immune and other cell types. Here we discuss existing data and future directions in the field.

Key words: exercise, immunology, miRNA, non-coding RNA, inflammation

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INTRODUCTION

The adaptation to exercise affects virtually all body systems. The immune system is among the systems most responsive to exercise. The regulation of a plethora of physiological processes is now known to be mediated by non-coding RNAs, in particular, miRNAs. These small ~22 nt RNAs are involved in post-transcriptional regulation of gene expression. Estimates are, that more than half of the human protein-coding genes are under miRNA regulation (40), which means that miRNAs are involved in nearly all major mechanisms controlling body processes.

A large amount of recent data demonstrates that miRNAs are essential for the normal activity and development of the immune system (reviewed in (15, 69, 83, 90, 119); see below). Likewise, rapidly increasing evidence indicates a role of miRNAs in the function of skeletal muscle, cardiovascular system, and other body systems. These findings promoted the research on the role of miRNAs in the adaptation to exercise. The field is young: 69 out of 76 publications on "miRNAs and exercise" found in Pubmed in August 2013 were published since 2010. However, the data obtained in this recently started trend suggest that miRNAs play a key role in mechanisms controlling the adaptation to exercise. Exercise rapidly changes the cellular levels of many miRNAs in the immune system, skeletal muscle, and cardiovascular system. These changes modulate the expression of target genes, driving short-term and long-term adaptations.

This review discusses current data on the role of miRNAs in the body adaptation to exercise with particular attention to the immune system and miRNAs in the bloodstream since they are likely involved in post-transcriptional regulation of gene expression in the cells of the immune system.

MiRNA Biogenesis and Function

MiRNAs are key factors of gene expression that regulate a variety of processes including development, cell proliferation, differentiation, apoptosis, and different metabolic pathways. The number of described miRNAs steadily grows, and the MiRBase currently includes more than 2500 human miRNAs (release 20;(58)). MiRNA genes are located in introns of protein-coding genes, introns and exons of non-coding RNA genes, and occasionally in exons of protein-coding genes (19, 38). They are transcribed by RNA polymerase II (67, 137), although certain miRNA genes downstream of Alu repeats are transcribed by RNA polymerase III (17). The primary transcripts (called pri-miRNAs) contain a ~70 nt hairpin (called pre-miRNA), which includes the miRNA sequence. Pri-miRNA is recognized by the 'microprocessor'complex composed of RNase III Drosha and RNA-binding protein DGCR8, which processes it into pre-miRNA (38, 66, 92). Certain pre-miRNAs localized within short introns are processed by splicing machinery, where the spliceosome and debranching enzyme function as the microprocessor. Such miRNAs are known as miRtrons (107, 136). Surprisingly, miRNAs can be derived from other small RNAs, e.g. tRNAs, Y RNAs, and small nucleolar RNAs (snoRNAs) (30, 78, 97, 115). The transcription and processing of miRNAs are controlled by a variety of cofactors (38, 59).

Exportin 5 mediates pre-miRNA export to the cytoplasm (145), where they are further processed by another RNase III, Dicer, in a complex with the RNA-

binding protein TRBP. The resulting ~22 nt double-stranded molecules associate with one of the Ago family proteins, the main component of the RNA-induced silencing complex (RISC). One of the duplex strands is degraded and the mature miRNA in the RISC complex can interact with the complementary target mRNA (38). Such interaction represses the translation or initiates mRNA deadenylation and degradation (47). MiRNAs also have non-canonical functions: upregulation of mRNA translation (44, 93, 130), transcriptional gene silencing (13, 53), and even signal transduction through the binding to Toll-like receptors, which makes miRNAs a kind of hormones ((31, 32, 68), see below, c-miRNAs.). The biogenesis, functions, and regulation of miRNA activity have been reviewed in detail elsewhere (38, 47, 59, 92, 94).

Short length and imperfect complementarity with mRNA allow each miRNA to have hundreds of targets (10). MiRNA binding sites can be found in the 3'-UTR and occasionally in the 5'-UTR (65, 77, 93) as well as in the coding regions of mRNAs (39). An mRNA can have several binding sites for the same or different miRNAs, which allows complex translational regulation (40).

The majority of miRNAs are expressed in a broad range of tissues; however, some miRNAs demonstrate pronounced tissue-specific profiles (62). At the same time, each cell type has a specific miRNA expression profile. For instance, different leukocyte lineages demonstrate different miRNA expression profiles (11, 63, 98, 134); moreover, several miRNAs specific for the skeletal muscle are even considered as a special subset of miRNAs termed 'myomiRs' (54).

MiRNAs can be found not only in the cell but also in various body fluids such as plasma/serum, milk, saliva, and urine. Such miRNAs proved resistant to nucleases and were called circulating miRNAs (c-miRNAs). They have been identified in apoptotic bodies, shedding vesicles, and exosomes as well as in complex with high-density lipoprotein (57, 126). However, a big part of c-miRNAs can be found in the vesicle-free fraction in complex with Ago proteins (7, 127). There is conflicting information on the proportion of free versus vesicle-bound miRNAs (41, 127). The mechanisms of c-miRNAs emergence and their possible physiological role still remain matters of speculation (16). *In vitro* experiments demonstrated that exosomes secreted by one cell type can be absorbed by other cell types, where the exosomal miRNAs can modulate gene expression (126). This proposes c-miRNAs as a new type of messengers that allow distant cells to communicate in the body. This is particularly significant for immune cells since most of them continuously interact with body fluids.

C-miRNAs proved to be good markers for many pathologies and physiological conditions (16). In particular, plasma levels of certain c-miRNAs change after physical activity (8, 9, 110, 128). This is of both, fundamental and practical interest and can be used for functional assessment and injury diagnosis of organs after exercise of different intensities (see below, c-miRNAs). Thus, c-miRNA monitoring can be widely used in sports medicine.

MiRNAs and Immune System

The amount of data on the role of miRNAs in immune functions rapidly grows (1199 out of 1549 publications on "miRNAs and immune system" found in Pubmed in August 2013 were published since 2010). To date, the function of dozens of miRNAs has been determined and their key role in the regulation of

immune system development and function has been established. Below we describe the major progress in the field, while the interested reader can consult specific reviews (15, 37, 69, 83, 90, 119).

The critical importance of miRNAs for the immune function was demonstrated in a series of works in which miRNA biogenesis was affected. For instance, a specific deletion of Dicer in a T-cell lineage impaired T cell development and T helper differentiation and induced autoimmune diseases (71, 85, 148). The ablation of Dicer in early B cell progenitors suppressed the transition from pro-B to pre-B cells (56). Specific deletion of Dicer in activated B cells induced multiple abnormalities in the immune response to pathogen and impaired germinal center B-cell formation (141). Natural killer (NK) cells with Dicer deletion demonstrated impaired maturation, survival, turnover, and other defects (14, 120). Thus, miRNAs are required for normal development and function of the immune system.

To date, miRNA expression profiles are available for many cell types of innate and adaptive immune system: monocytes/macrophages, dendritic cells, neutrophils, eosinophils, NK cells, and different T and B cell types (2, 11, 33, 34, 63, 74, 82, 98, 106) Out of more than 2500 described human miRNAs (58), 150-600 species can be usually identified in each cell type of the immune system, 20 or 30 of which are the most abundant (11, 74, 82, 135). Immune cells have specific miRNA expression profiles, which can change during cellular development (11, 82, 106); and the concentrations of the same miRNA species at different differentiation stages can vary by a factor of 1000 (55). Interestingly, new miRNAs are still being described in the immune system (11, 34, 98).

Simultaneously with the large-scale transcriptome studies, individual miRNA species are being actively explored with focus on their function in specific cell types of the immune system (reviewed in (15, 72, 117)). MiRNAs proved to be involved in a variety of cellular pathways so that they seem to mediate all significant events in immune cells. For instance, miRNAs control the differentiation of naive T-helper cells into Th1, Th2, Th17, T-regulatory, and follicular helper T cells (T_{fh} cells) (114). In particular, the normal differentiation of T_{fh} cells requires the miRNA cluster miR-17-92 (12, 51). MiRNAs also mediate the maintenance of naive T-helper cells in undifferentiated state. MiR-125b is more abundant in naive T-helper cells than in other T cells and suppresses the expression of genes underlying the differentiation of naive T-helper cells (namely, interferon-γ, interleukin (IL)-2 receptor-β, IL-10 receptor-α, and transcriptional repressor Blimp-1), and thus maintains the undifferentiated state of these cells (106).

Interestingly, miRNA processing can change as the immune cells develop. For instance, the sequences of mature miRNAs can be shifted by one or two nucleotides at different stages of T cell development. In particular, the nucleotide sequence of miR-17 expressed in DN3 thymocytes corresponds to the canonical one, while the same miRNA expressed in DN4 and DP thymocytes is shifted 3' by one nucleotide. Cells of all other stages expressed miR-17 that was shifted 3' by two nucleotides. These shifts indicate different processing of pre-miR-17 in the course of T cell development (55). Since the seed region (the region completely complementary to the mRNA target nucleotides 2 to 7/8) (10) is short, even a single-nucleotide shift changes two thirds of the predicted miR-17 targets and the seed sequence becomes identical to that of miR-302a or miR-106a (55).

Thus, alternative miRNA processing during T cell development contributes extra variation to the regulation of miRNA targets.

The range of biochemical pathways controlled by miRNAs in immune cells is particularly wide. For instance, miRNAs control antigen presentation (e.g., miR-148/152 (75)), T-cell receptor signaling (e.g., miR-181a (70)), Toll-like receptor signaling (e.g., let-7e (4)) and cytokine production (e.g., miR-146a (43)). MiRNAs are required for normal proliferation of activated lymphocytes; for instance, miR-182 promotes clonal expansion of activated T helper cells (118) and miR-181b decreases excessive DNA damage accompanying somatic hypermutation and class switch recombination in activated B cells, thus preventing their malignant transformation (25). MiRNAs are involved in both inflammatory and anti-inflammatory responses (90).

Notably, cellular miRNAs can have an effect on the viral life cycle through the regulation of viral genome replication, while viral miRNAs in turn can have an effect on the host cell (95). Different immune abnormalities, in particular, malignant and autoimmune diseases, demonstrate altered miRNA profiles, which can point to the contribution of miRNAs to the development of these diseases (26, 96).

MiRNAs and Response of Peripheral Blood Leukocytes to Exercise

Publications from two groups have demonstrated that acute exercise changes the expression profile of many miRNAs in circulating leukocytes (100, 101, 102, 125). In a series of studies Dr. Dan Cooper and coworkers used miRNA microarrays to study the changes in miRNA expression in untrained subjects immediately after brief bouts of heavy exercise in circulating neutrophils (102), peripheral blood mononuclear cells (PMBCs: T, B, and NK cells and monocytes) (101), and circulating NK cells alone (100). In all cases, expression profiles changed for 20-40 miRNAs (Table S1). Considering that leukocytes express several hundreds of miRNA species (11, 74, 82, 135), this finding supports the specificity of the observed response. Note that most of these miRNAs are not among the most abundant species in neutrophils and PMBCs (88, 131, 134, 135). The authors also linked their miRNA data to corresponding exercise-induced mRNAs by identifying potential mRNA targets for each miRNA and selecting those which were also changed. Resulting biochemical pathways are considered candidates for being under the control of exercise-induced miRNAs. In neutrophils they include ubiquitin-mediated proteolysis, Jak-STAT signaling, and Hedgehog signaling. All these pathways mediate inflammatory response (102). Twelve pathways have been identified in PBMCs including TGF-β signaling and MAPK signaling (101). Exercise-activated pathways in NK cells are predominantly associated with cancer and cell communication: p53 signaling, melanoma, glioma, and prostate cancer, as well as adherens junction and focal adhesion (100).

Unfortunately, parallel expression data on miRNA and mRNA were only available in the NK cell study, while the data for neutrophils and PBMCs were obtained in separate experiments, which can compromise the authenticity of the identified miRNA-mRNA pairs. Moreover, different experimental procedures (ten 2-minute bouts of cycle ergometer exercise for miRNA assay and 30 minutes of constant cycle ergometer exercise for mRNA assay) were used in the study on neutrophils, and the experiments with PBMCs were carried out on individuals of

different age (men with a mean age of 22 years and late pubertal boys with a mean age of 17 years). Nevertheless, in sum, these findings are at least compatible with the assumption that many exercise-induced mRNA changes are under the control of miRNAs.

Attempting to increase the depth of understanding, our group analyzed whole blood samples taken from highly trained athletes after a 30-minute treadmill test at 80% maximal oxygen uptake (moderate test, MT) (125). The whole blood approach allows fast, precise timing and minimizes artifacts. Samples were analyzed for miRNA and mRNA before and immediately after exercise, as well as 30 minutes, and 60 minutes into recovery. This allowed us to identify four dynamically regulated networks with four differentially expressed miRNAs and their validated mRNA targets. All of them displayed anti-correlated expression profiles for both, immediate post-exercise time point and recovery period. These miRNAs included miR-21 and its targets TGFBR3, PDGFD, and PPM1L; miR-24-2 and its targets MYC and KCNJ2; miR-27a and its target ST3GAL6; as well as miR-181a and its targets ROPN1L and SLC37A3 (125). All target genes are involved in processes highly relevant to exercise response including immune function, apoptosis, membrane traffic of proteins, and transcription regulation. These data are in good accordance with the findings by Cooper's group and support the assumption that miRNAs regulate key pathways of the immune response to exercise.

The number of differentially expressed miRNAs was higher in the studies by Cooper's group (several dozens) as compared to our study (five) (125). Possible reasons for this discrepancy are numerous, ranging from different numbers of subjects, different exercise procedures, different microarray systems, different cell populations and work up procedures to different fitness level of subjects. Indeed, the microarray used by our group contained four times less miRNAs, but, nevertheless, did contain two thirds of those identified by Cooper's group. Some other reasons also need to be discussed in more details.

Use of whole blood may mask mild expression changes in minor leukocyte populations like NK cells, and can also cause changes through cellular shifts, including subpopulations (125). On the other hand, analysis in isolated cell populations (100, 101, 102) is influenced by manipulation and time delay inherent to the sorting procedure. While this may still suggest that true exercise-induced miRNA changes do exist, we have to acknowledge that even within isolated cell populations, shifts in subpopulations do occur (also discussed in (100, 101, 102)) and may be responsible for a substantial part of the expression changes observed. At present this question cannot be unequivocally answered. We do however believe that the dynamic regulation of mRNA-miRNA pairs as shown in (125) would be hard to explain by a cellular shift. But finally, no matter, if shifts in leukocyte populations / subpopulations are involved or not, miRNA (and of course mRNA) expression data do mirror the actual activation status of the peripheral blood and therefore deliver valuable biological information.

While exercise procedures differed between the two groups (100, 101, 102, 125), the total duration (30 minutes) and intensity (80 vs. 76-77% of VO_2 max) were somewhat comparable. However, the highly trained athletes investigated by our group did not cross the anaerobic threshold while the less trained probands investigated by Cooper's group, did. Crossing the individual anaerobic threshold

(IAT) is associated with major physical stress, which might require the induction of more and different miRNA species than work just below the threshold. Trying to get more pertinent information on this question we decided to perform an additional ramp test to exhaustion (RTE, as described in (109); duration ~15 minutes/including 4-5 minutes above IAT) with the same athletes that had performed the 30-minute moderate test (MT) (125). Whole blood samples collected before and immediately after the test were analyzed for miRNA expression (as described in (109)). Results are given in Table 1 together with results from the MT (recalculated for 2 time points, disregarding recovery, in order to create optimal comparability between our data sets and the results described by Cooper`s group).

Table 1. MiRNAs differentially expressed in whole blood leukocytes before and after exercise tests. MiRNA species with a 1.5-fold or greater expression difference are boldfaced

MiRNA name and fold change[1]	Experiment participants	Exercise type	Time points of blood sampling
mir-24 ↑2.0 **mir-27a ↑1.5** **mir-181b ↑1.5** mir-23a ↑1.3	8 highly trained male athletes with a mean age of 21.7±2.6 years	Moderate test: 30 min treadmill running at 80% VO2 max (as described in (109, 125).	Immediately before and after exercise test
mir-181a ↑1.5 **mir-181b ↑1.5** **mir-101 ↓1.5** mir-142 ↓1.4 mir-29a ↓1.4 mir-124 ↓1.3 mir-29c ↓1.3 mir-223 ↓1.2 mir-30d ↑1.2 mir-130b ↓1.2		Ramp test to exhaustion: 15 min treadmill test with an incremental step protocol until exhaustion (as described in (109)).	

[1] Only the miRNA genes with significant differential expression are listed (false discovery rate < 0.05). The miRNA set for the MT is not identical to that published previously (125) since the samples collected 30 and 60 min after exercise were excluded.

Indeed, expression of a greater number of miRNA species was altered in RTE than in MT. To our knowledge, this is the first report that miRNAs in peripheral leukocytes can change in such a short time following any external stimulus. Numbers of expressed miRNAs in RTE were, however not excessive and clearly less than those identified in the studies by Cooper's group (which also included work above IAT). We think that, apart from crossing the anaerobic threshold, the mere fact that our group investigated highly trained athletes may also make a difference. Trained athletes are known to be able to regulate their body functions more efficiently than non-athletes, be it above or below IAT. They may therefore generally need less miRNAs to be induced than non-athletes.

One more observation we made when analyzing our results may be of interest: three of the four miRNAs found elevated after MT, namely, mir-23a, mir-24, and mir-27a, belong to the same cluster. They are processed from a common precursor and often coregulated (22). The members of this cluster are involved in a plethora of biological processes including haematopoiesis, angiogenesis, cell proliferation, and cardiac hypertrophy (reviewed in (22)). Analysis of their target gene set suggests the involvement of this cluster in several immune-related pathways, e.g. T-cell receptor signaling, and TGF-beta pathways (22). It will be very interesting to unveil the function of this cluster in directing the exercise response in peripheral blood leukocytes.

The sets of differentially expressed miRNAs differ between leukocyte types; however, certain miRNAs proved common for two or three sets (Table 2). Notice that the direction of changes can be opposite in some cases. For instance, miR-223 level increased in neutrophils but decreased in NK cells (Table 2). This could indicate different interpretation of the same external signal by different cell types either upregulating or downregulating the same gene; however, as in other cases, shifts in subpopulations cannot be excluded with certainty.

It is of interest that quite some exercise-induced miRNAs identified in leukocytes demonstrated a similar response in the skeletal muscle, heart, or plasma(Table 2). Moreover, only a few out of hundreds of miRNA species coincided in all microarray studies, which might indicate a critical role for them in adaptation to exercise. For instance, Keller et al. identified 21 miRNA species expression of which changed after exercise in muscle (52), and seven of them demonstrated differential expression in leukocytes, too (Table 2). Although the direction of changes was different in some cases and the sets of their targets likely differ in leukocytes and muscle, these miRNAs can be assumed to be involved in the universal adaptive response to exercise.

Data on the role of individual miRNAs in specific immune functions are increasing but still vastly fragmentary. Thus, a comprehensive discussion of possible roles of indentified miRNAs is difficult. Still, miRNAs of the miR-181 family - miR181a and miR181b - may be worthwhile discussing in more detail, since their differential expression was observed in all our tests in athletes as well as in two out of three tests in non-athletes (101, 102), (Table 2).

Since miR-181 expression was affected in different leukocyte types irrespective of the exercise intensity and training level (Table 2), its involvement in the regulation of some fundamental adaptive changes in the immune system seems likely. MiR-181a suppresses the inflammatory response induced by oxidized low-density lipoprotein in dendritic cells (139), and miR-181b suppresses the NF-kappa B-mediated inflammatory response in endothelial cells *in vivo* (121). On the other hand, Xie et al. demonstrated increased levels of miR-181a in whole blood leukocytes during the early inflammatory response, and proposed that miR-181a upregulation can compensatorily limit hyperinflammatory reactions (140). In a similar way, exercise-induced miR-181 may be interpreted as compensatory anti-inflammatory reaction to primary inflammatory stimuli caused by exercise. Indeed, exercise-induced immune reactions used to be viewed as primary inflammatory reactions followed by anti-inflammatory counter-reactions. This has recently been challenged (oral communication, ISEI meeting, Newcastle, Australia, 2013, and Asghar Abbasi, *Brain Behavior and Immunity*, in press).

Table 2. Overview of miRNAs changed in two or more cell types after exercise. Human, mouse, and rat miRNAs are prefixed with hsa-, mmu-, and rno-, respectively, following the nomenclature adopted in miRBase (58). WBL - whole blood leukocytes, FC - fold change.

MiRNA	Leukocytes, FC				Muscle	C-miRNA
	WBL	Neutr.[1]	PBMC[2]	NK[3]	FC, Muscle name, Ref.	FC, Ref.
hsa-miR-7			↑1.4	↑2.2		
hsa-miR-15a hsa-miR-15b			↑1.3		↓1.6 (Vastus lat.;(52))	
hsa-mir-16 rno-mir-16		↓1.23			↓1.36 (Soleus;(36))	
hsa-miR-21* hsa-miR-21 mmu-mir-21			↑1.5		↑2.3 (Gastrocn.;(5))	↑2.6(8)
hsa-mir-23a mmu-mir-23	↑1.3 MT				↓6.2 (Quadriceps;(108))	
hsa-miR-26a hsa-miR-26b			↑1.2		↓1.8 (Vastus lat.;(24)) ↓1.6 (Vastus lat.;(52))	
hsa-miR-27a rno-mir-27a	↑1.5 MT				↑2.0 (Heart;(35))	
hsa-miR-29a rno-miR-29a hsa-miR-29b hsa-miR-29c rno-mir-29c	↓1.4 RTE ↓1.3 RTE			↑2.0 ↑3.5 ↑2.3	↓2.0 (Vastus lat.;(24)) ↑1.5 (Heart;(116)) ↓1.6 (Vastus lat.;(52)) ↑2.18 (Heart;(116))	
hsa-miR-30d hsa-miR-30e	↑1.2 RTE			↑2.1		
hsa-mir-101	↓1.5 RTE				↓2.0 (Vastus lat.;(52))	
hsa-miR-107 mmu-mir-107		↓1.26			↑1.56 (Quadriceps;(108))	
hsa-miR-125a		↑1.22	↓1.3		↑1.6 (Vastus lat.;(52))	
hsa-miR-126		↓1.53	↓1.3	↓3.2		↑4.0(128)
hsa-miR-130a hsa-mir-130b	↓1.2 RTE	↓1.61	↓1.2	↓2.9		
hsa-miR-142	↓1.4 RTE			↑2.5		
hsa-miR-145		↑1.22	↓1.3			
hsa-miR-151-5p		↓1.60	↓1.3	↓2.8		
hsa-miR-181a hsa-miR-181a2* hsa-mir-181b mmu-mir-181	↑1.5 RTE ↑1.5MT,RTE	↑1.64	↑1.4 ↑2.0 ↑1.7		↑1.37 (Quadriceps;(108))	↑1.5(9)
hsa-miR-199a-3p hsa-miR-199a-5p			↓1.3 ↓1.3	↓3.1 ↓2.9		
hsa-miR-221			↓1.2	↓2.1		↑5.8(8)
hsa-miR-223	↓1.2 RTE	↑1.29		↓2.9		
hsa-miR-338			↑1.4	↑2.2	↓1.6 (Vastus lat.; (52))	
hsa-miR-363		↓1.34	↑1.5	↑2.1		
hsa-mir-451			↓3.8		↓4.0 (Vastus lat.; (52)) ↑4.0 (Vastus lat.; (24))	
hsa-miR-652			↓1.2	↓2.2		

[1]according to (102); [2]according to (101); [3]according to (100)

It was proposed that the reaction to exercise may be more of a direct, preemptive anti-inflammatory reaction induced by IL-6 or other mediators, including miRNAs, and miR-181 is certainly a candidate for playing a critical role in such an immediate anti-inflammatory response to exercise.

MiR-181 expression also increased in muscle after acute endurance exercise (108) and during regeneration (86). MiR-181 is known to repress the repressor (Hox-A11) of MyoD and thus mediates myoblast differentiation and muscle regeneration (86). Finally, miR-181 also shows increased plasma levels following acute exercise (9). Together with its protective, anti-inflammatory impact all this prompts us to propose that miR-181 may have a central, multiple role in the adaptation to exercise.

MiRNA and Skeletal Muscle Response to Exercise

Muscle and vascular function are not the focus of this review. Nevertheless, we like to highlight some current facts and findings in this field, honouring the fact that all exercise-related gene expression probably starts in the muscle.

Although more than 150 miRNA species are expressed in the muscle (81, 122), up to 25% of muscle miRNA population correspond to just a few muscle-specific miRNAs (81) (miR-1, miR-133, miR-206, and miR-499), collectively called myomiRs; myomiRs also include less abundant miR-208 and miR-486 (54). Thus, most studies in the field are focused on the identification of changes in the expression of myomiRs (largely miR-1, miR-133, and miR-206) after various forms of exercise. Indeed, adaptation to exercise proved to affect myomiR levels. Somewhat unexpectedly, changes were, however, only moderate in general and even undetectable in some of the studies (Table S2). There is a trend towards increased levels of miR-1, and miR-133 during acute endurance exercise (89, 108) and decreased levels with chronic endurance exercise (52, 89) or with resistance exercise (27, 80, 84), (Table S2). A central function of myomiRs, in particular miR-1, is obviously long term regulation / adaptation of protein synthesis and muscle size. Validated targets of miR-1 include components of the insulin-like growth factor 1(IGF1) pathway (29), and the decrease of miR-1 with chronic endurance exercise and with both, acute and chronic resistance exercise, is therefore a plausible adaptation to the increased need for protein synthesis and muscle growth/regeneration. As mentioned above, significant changes of myomiRs were not seen in all experiments. In a study with high responders and low responders to resistance exercise (low muscle mass gain), no significant changes in expression of myomiRs was observed. Instead, some non-myomiRs (mir-451↑, miR-378↓, miR-29a↓, and miR-26a↓) were changed in low responders only, possibly in a compensatory effort (24).

Age-related loss of muscle function is accompanied by hampered transition of the miR1 precursor pri-miR-1 to miR-1 and by a failure to modify mature miR1 expression in response to a single bout of resistance exercise in elderly men (27). On the other hand, decreased miR1 expression was demonstrated in elderly men following prolonged resistance training (84). Thus, elderly men may just need more time for adaptive changes to occur. In any case, age-related changes can at least partly be relieved by training.

MyomiRs also control the expression of key myogenic transcription factors and regulators such as Pax3, and Pax7 during satellite cell proliferation and dif-

ferentiation (reviewed in (147)), and injection of myomiRs into injured rat muscle could accelerate regeneration (87). Thus myomiRs seem to have control functions in both, adaptation to exercise and regeneration of muscle.

A row of studies has investigated the consequences of enforced physical inactivity, using different experimental settings. MyomiRs miR-1, miR-133, miR206 (Table S2), mir208b and miR499 and some others were down-regulated in response to inactivity (3, 49, 81, 104), and miR208b and miR499 seem to be involved in the slow (type 1) to fast (type 2) fibre switch which accompanies muscular atrophy (81). Altogether results in this field are inconsistent and sometimes contradictory, likely due to different experimental settings. Spontaneous reinnervation following denervation and replacement of muscle fibres by fibroblasts are some of the problems encountered in those experiments.

Intriguingly, compared to myomiRs, a number of non-myomiR miRNAs showed much more pronounced differential expression in response to exercise (Table S3). Identified reactive miRNAs include both, species of low (e.g. miR-183 and miR-189 (113)) and high (e.g. miR-23 (113)) abundance. Since they are not muscle-specific, they seem to control vital functions which muscle cells share with some or all other cells. Low-level transcripts are fairly often involved in crucial processes like cell division. Thus this might also be the case for exercise modulated low-level transcripts. On the other hand, non-muscle-specific high abundance miRNAs may well be involved in fast adaptive metabolic reactions to contractile activity. Adaptation of miRNA expression to chronic endurance or resistance training shares some changes and differs in others. Three out of four miRNAs differentially expressed after resistance training, namely miR-26, miR-29, and miR-451 (24) were also changed after endurance training (52). While miR-26 and miR-29 decreased in both, miR-451 was increased after resistance and decreased after endurance exercise. Thus, there are common exercise-related and specific exercise type-related regulations of miR expression, which, together with myomiRs, can form the necessary network to govern adaptation and regeneration.

Exploration of the adaptive significance of individual miRNAs is still in its infancy. Existing data point to a role of miR-696, miR-23 and miR-494 in mitochondrial biogenesis through different pathways (5, 108, 144). PCG1 (Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha) is a critical factor of mitochondrial biogenesis and a predicted target for miR-696 and miR-23 (5, 108). Its regulation by miR-696 was also confirmed by hyperexpression experiments (5). Transcription factors TFA and Foxj3 which also control mitochondrial amplification are validated targets for miR-494 (144).

When looking closer to those miRNAs which show high or very high expression changes due to exercise, we realized that they are often species-specific, taxon-specific or are synthesized via non-canonical ways (unpublished observation). MiR-616 seems to be human/primate-specific while miR-680, miR-696, miR-705, and miR-709 are mouse-specific (MirBase; http://www.mirbase.org, Table S3). MiR-720 is presumably synthesized from a tRNA (111). Recent data indicate that tRNAs can give rise to shorter miRNA-like molecules which are involved in gene silencing and most actively synthesized during stress (115). MiR-680 is encoded in the LTR of mouse retrotransposon ERVB4 (according to the annotation of the genome browser at the University of California,

http://genome.ucsc.edu). The significance of these findings is not clear at present. Possibly it has to do with the need for fast action which is associated with physical activity. It is also possible that the small size of miRNAs makes them a perfect tool of nature to gain species-specific adaptation to exercise out of a pathway which is common to all mammals. In any case, a significant fraction of exercise-associated miRNA expression changes is observed in species-specific or non-canonically synthesized miRNAs.

Chronic exercise is associated with decreased blood pressure, increased capillary numbers and physiological hypertrophy of the heart. Studies on the role of miRNAs in these adaptational processes have just started. All were conducted in rats submitted to 10 weeks of moderate training (swimming) (23, 35, 36, 116). First results indicate massive involvement of miRs in cardiac hypertrophy. 87 of 349 miRs studied were altered, among these, miR-1 and mir-133 (decreased) and members of the miR-29 family (increased) (116). These changes promote muscle fibre growth (myomiRs) and decreased collagen synthesis (miR-29). Increases in miR-27 and miR-126 and decrease in miR-16 may have critical roles in angiogenesis by targeting negative regulators of vascular endothelial growth factor (VEGF) pathway (miR126), and by reducing miR-16 dependent inhibition of angiogenesis (VEGF, its receptor VEGFR2 and FGF receptor 1 are all validated targets of miR16) (23, 36). MiR-27 probably targets angiotensin converting enzyme (ACE), and this may lead to decreased blood pressure (35). Finally, miR-27 also regulates the inflammatory response (20). Altogether, the results in this field need confirmation to prove that they are indeed an adequate mirror of vascularization.

MiRNAs Might Contribute to the Beneficial Effect of Exercise in Different Diseases

Exercise is known to be beneficial in a plethora of diseases. Recent data increasingly indicate the involvement of miRNAs in the beneficial effect of exercise. For instance, increased expression of miR-21 and decreased expression of miR-15a was observed in rats with spinal cord injury after post-injury cycling exercise. It was accompanied by the corresponding changes in the expression of their target genes: the mRNA levels of proapoptotic genes PTEN and PDCD4 decreased, while that of anti-apoptotic factor Bcl-2 increased (73).

Spontaneously hypertensive rats demonstrated increased levels of miR-16 and miR-21 and decreased level of miR-126 in the soleus muscle relative to control. Some of their targets showed anti-correlated expression. Exercise training normalized the expression of these miRNAs to levels similar to controls (36). Exercise training also normalized the expression of certain targets of these miRNAs; for instance, the levels of anti-apoptotic factor Bcl-2 and proangiogenic factor VEGF (targeted by miR-16) increased (36).

Microarray analysis of the whole blood in patients with coronary arterial disease after coronary artery bypass graft surgery and exercise rehabilitation program demonstrated increased expression of miR-92a and miR-92b. At the same time, the mRNA level of the respiratory chain component NDUFA1 and proapoptotic factor CASP3, which are predicted targets of these miRNAs, decreased (124).

Thus, miRNAs are obviously mediators of antiapoptotic and proangiogenic

effects of exercise. At present we do not know how these findings relate to exercise-induced lymphocyte apoptosis.

Circulating MiRNAs and Exercise

Emerging data indicate changes in the blood levels of c-miRNAs after exercise (see Table S4). Uhlemann et al. evaluated human plasma concentrations of miR-126 and miR-133 before and after different exercises and found elevated concentrations of these miRNAs following a marathon (miR-126 and miR-133) and after resistance exercise (miR-133) (128). Banzet et al. likewise found increased levels of miR-133 and other myomiRs (miR-1, miR-208b, and miR-499) in plasma after downhill walking (9), Table S4. Thus, increased plasma levels of these miRNAs may be used as markers for injury of muscle (miR-133) and endothelial cells (miR-126). Mir-133 may serve as a convenient replacement of creatine phosphokinase (CPK), while miR-126 could be the first available marker for endothelial damage (128).

It should be noted that not all published data on the subject are in agreement. For instance, Sawada et al. (110) observed no changes in the serum level of myomiRs in humans after acute resistance exercise, while Uhlemann et al. (128) reported myomiR differential expression after a similar exercise. Different percentage of eccentric load, different time points, and different numbers of probands can explain this inconsistency.

Apparently, c-miRNA concentrations change after exercise not only through cell damage. Changed plasma levels of miR-149*, miR-146a, and miR-221 were demonstrated three days after resistance exercise, while levels of myomiRs remained unaltered (110). Further, increased plasma levels of c-miRNAs miR-181b and miR-214 in absence of elevated myomiRs or CPK were reported immediately after uphill exercise (9). This suggests that the increased levels of these miRNAs resulted from active secretion rather than cell damage. Baggish et al. studied exhaustive exercise tests before and after a 90-day period of rowing training (8). Eight miRNAs involved in angiogenesis, inflammation, muscle contractility, and adaptation to hypoxia were analyzed. Four patterns of c-miRNA response to exercise have been revealed. (i) The levels of miR-146a and miR-222 increased after acute exhaustive exercise before and after sustained exercise training; (ii) miR-21 and miR-221 levels increased after acute exhaustive exercise only before sustained exercise training; (iii) miR-20a level increased after sustained exercise training but not after acute exhaustive exercise; and (iv) miR-133a, miR-328, and miR-220 levels remained unaltered after all tests (8), (Table S4). The unchanged levels of miR-133a likely indicate the absence of muscle damage, while the different patterns of c-miRNA response observed point to the existence of different control mechanisms not associated with cell damage.

C-miRNA quantification can be used to predict the risk of cardiovascular diseases. Microarray data show that healthy individuals with low maximal oxygen uptake have increased levels of circulating miR-21, miR-210, and miR-222 (18). Low maximal oxygen uptake is indicative of a predisposition to cardiovascular diseases (138); accordingly, the identified miRNAs can serve as convenient noninvasive markers of risk for these diseases (18).

Thus, the first studies in the field demonstrated that exercise-induced changes in plasma/serum levels of c-miRNAs can result from both, cell damage

or independent mechanisms. The contribution of cell damage seems to increase with exercise load (128). Rapid and substantial (several-fold) increase in c-miRNA levels after intense exercise in the absence of tissue damage markers in plasma (8) can indicate the peak release of previously synthesized miRNAs into the bloodstream, while stable changes in c-miRNA levels after long-term exercise training suggest modified basal expression and/or secretion of miRNAs.

Table 3. C-miRNAs described in relation to exercise and possible relevance for the immune system.

MiRNA name, fold change and source		Presumed immune-related effects[a]	Number of hits[b]
miR-1	↑4.0 (9)	Anti-inflamm., anti-astmathic, anti-prolif.(42, 76, 123)	16
miR-20a	↑ 3.0 (8)	Unknown	0
miR-21	↑ 2.6 (8)	Both pro- and anti-inflamm., oncogenic (32, 60, 91, 99, 142)	93
miR-126	↑4.0 (128)	Pro-angiogenic; anti-inflamm., pro-astmathic (pro-Th2) (6, 79, 103, 105)	29
miR-133	↑8.9 (128), ↑4,8 (9), NC (8)	Unknown; found in inflammatory vesicles (46)	2
miR-146a	↑3.0 (8), ↓2.0 (110)	Anti-inflamm.; possible counterpart of mir-21 in regul. of inflamm. (21, 50, 112, 143)	17
miR-149*	↑2.3 (110)	Unknown (rare passenger strand of anti-inflamm. mir-149)	0
miR-181b	↑1,5 (9)	Anti-inflamm., controls autoimmunity (48, 132)	4
miR-208	↑11,5 (9)	Unknown	0
miR-214	↑1,8 (9)	Prolif. stim, pro-oncogen. (105, 146)	6
miR-221	↑ 5.8 (8) ↓ 2.0 (110)	Anti-inflamm., anti-angiogenic (28, 45, 129)	13
miR-222	↑2.4 (8)	Anti-inflamm. (105)	9

[a] as judged from described effects of corresponding cellular miRs (personal interpretation of literature data)

[b] hits found in PubMed search for name of miRNA and inflammation

NC = no change

In most cases, the cell source(s) of c-miRNAs remain unclear. The bulk of miRNAs are expressed in several cell types albeit at different rates (62). For instance, miR-133 is abundant in the skeletal muscle and heart but also detectable in the brain (61) and brown adipose tissue (133). MiR-126, typical of endothelium, is also expressed in the liver, haematopoietic cells, and some other tissues (62). Accordingly, direct identification of the c-miRNA source is nearly impossible except in case of overt cell damage (64). Further studies on the mechanisms of miRNA secretion and the nature of circulating miRNA-containing complexes may help to address these questions.

To date, the effect of altered c-miRNA concentrations on body systems remains largely unclear. Possible effects can be deduced from validated targets of these miRNAs; in the case of exercise, these are largely associated with muscle function, angiogenesis, inflammation, and oxidative stress, i.e., the processes primarily affected by exercise. Here, the question, how circulating miRNAs may find and enter

their target cells, needs to be discussed. Studies mentioned above evaluated c-miRNAs without considering their carrier. The nature of the carrier (high-density lipoprotein-particles, exosomes, apoptotic bodies, shedding vesicles or vesicle-free Ago protein bound miRNAs) may dramatically influence the cell/organ-specific targeting and even biological effects.

To our knowledge, at present, no clear proof is available to show which biological effects can be attributed to exercise-induced c-miRNA expression. In spite of all the imponderabilities mentioned above, it seems, however, reasonable to assume, that they have identical or similar effects as their cellular counterparts. On this basis, Table 3 presents the presumed effects of exercise-induced c-miRNAs as judged from literature data with focus on immune related effects. The majority of exercise-induced miRNAs has predominantly anti-inflammatory effects rather than inflammatory ones. Since exercise-induced gene (mRNA) expression also has a remarkable anti-inflammatory bias (1) this concordance makes likely that the protective generalized reaction of the body to exercise is organized by help of miRNAs or co-organized together with hormones at a very early stage.

Intriguingly, it has recently been shown that c-miRNAs can also induce biological effects without directly interacting with mRNA. Tumour cells can use extracellular miRNAs as ligands to Toll-like receptors (TLR7 in mice and TLR8 in human) of macrophages and thereby modulate the immune response to their favor (31, 32, 68). Although, up to now, this new and exciting hormone-like mechanism has only been demonstrated for cancer and for neurodegenerative processes, it may well be functional under physiological conditions as well. Modulation of immune functions by exercise is one of the fields to be investigated in this context.

Altogether we are convinced that the future will see c-miRNAs as useful markers for exercise-related damage or malfunction and will also expose an important biological role for them in exercise immunology.

Concluding Remarks and Future Directions

Recent data indicate that miRNAs are an essential element in the adaptation of the immune system and other systems to exercise. Progress in the field requires flanking progress in related fields, namely, detailed data on the role of each miRNA in the function of different cell types. Such data are actively generated now, and since miRNAs are a hotspot in molecular biology, further data expansion can be expected in the nearest future. The improvement of technical approaches is also desirable, in particular, rapid sorting of blood cells and preservation of isolated RNA. RT-PCR is widely used in miRNA studies discussed here but it allows only a small number of miRNA species to be detected; in the cases when microarray technology is used, microarray data files are not always made publicly available. Wide application of large-scale screening methods and publication of comprehensive microarray data will undoubtedly accelerate the progress in exercise immunology.

As of now, only limited numbers of exercise physiology studies involving miRNAs have been carried out on human and animal groups of different size, age, and sex, using different exercise designs, all of which makes correct comparison of results and data meta-analysis largely impossible. Small sample size in the

studies and small changes in the studied parameters (often below 50%), coupled with significant individual variation in gene expression levels, open the door to misinterpretations. In this context, the development of uniform study designs by the scientific community is of primary importance.

Other classes of non-coding RNAs can also be expected to mediate the reaction of our immune system to acute or chronic exercise. For instance, well-studied tRNAs unexpectedly proved to mediate stress responses (115), and exercise is of course known to be a kind of stress. Moreover, the involvement of another group of non-coding RNAs, snoRNAs, in the reaction of leukocytes to exercise has recently been demonstrated by our group (109). We expect many more exciting discoveries about the role of miRs & Co in exercise immunology.

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Table S1. MiRNAs differentially expressed in different leukocyte types before and after exercise test. MiRNA species with a 1.5-fold or greater expression difference are boldfaced.

Leukocyte type	Neutrophils		PBMC		NK cells
MiRNA name and fold change	**hsa-miR-485-3p ↑2.92** **hsa-miR-520d-3p ↑2.82** **hsa-miR-181b ↑1.64** **hsa-miR-130a ↓1.61** **hsa-miR-151-5p ↓1.60** **hsa-miR-1238 ↑1.58** **hsa-miR-193a-3p ↑1.58** **hsa-miR-1225-5p ↑1.55** **hsa-miR-126 ↓1.53** hsa-miR-20a ↓1.24 hsa-miR-106a ↓1.39 hsa-miR-20b ↓1.29 hsa-miR-17 ↓1.40 hsa-miR-93 ↓1.24 hsa-miR-130b ↓1.41 hsa-miR-16 ↓1.23 hsa-let-7i ↓1.25 hsa-miR-107 ↓1.26 hsa-miR-185 ↓1.28 hsa-miR-18a ↓1.35 hsa-miR-18b ↓1.29 hsa-miR-194 ↓1.26 hsa-miR-22 ↓1.28	hsa-miR-363 ↓1.34 hsa-miR-660 ↓1.23 hsa-miR-96 ↓1.28 hsa-miR-125a-5p ↑1.22 hsa-miR-145 ↑1.22 hsa-miR-197 ↑1.38 hsa-miR-212 ↑1.44 hsa-miR-223 ↑1.29 hsa-miR-340* ↑1.25 hsa-miR-365 ↑1.37 hsa-miR-505 ↑1.24 hsa-miR-629* ↑1.44 hsa-miR-638 ↑1.36 hsa-miR-939 ↑1.40 hsa-miR-940 ↑1.39	**hsa-miR-451 ↓3.8** **hsa-miR-181a* ↑2.0** **hsa-miR-181b ↑1.7** **hsa-miR-486-5p ↓1.7** **hsa-miR-363 ↑1.5** **hsa-miR-1225-5p ↑1.5** **hsa-miR-21* ↑1.5** hsa-miR-181a ↑1.4 hsa-miR-181c ↑1.2 hsa-miR-338-3p ↑1.4 hsa-miR-26b ↑1.2 hsa-miR-132 ↑1.3 hsa-miR-15a ↑1.3 hsa-miR-939 ↑1.3 hsa-miR-7 ↑1.4 hsa-miR-140-5p ↑1.3 hsa-miR-940 ↑1.3 hsa-miR-125b ↓1.4 hsa-let-7e ↓1.4 hsa-miR-320 ↓1.2 hsa-miR-151-5p ↓1.3 hsa-miR-31 ↓1.3 hsa-miR-125a-5p ↓1.3	hsa-miR-99b ↓1.4 hsa-miR-652 ↓1.2 hsa-miR-151-3p ↓1.3 hsa-miR-130a ↓1.2 hsa-miR-126 ↓1.3 hsa-miR-199b-3p ↓1.3 hsa-miR-23b ↓1.2 hsa-miR-221 ↓1.2 hsa-miR-199a-5p ↓1.3 hsa-miR-584 ↓1.3 hsa-miR-145 ↓1.3	**hsa-let-7e ↓2.6** **hsa-miR-126 ↓3.2** **hsa-miR-126* ↓2.9** **hsa-miR-130a ↓2.9** **hsa-miR-151-5p ↓2.8** **hsa-miR-199a-3p ↓3.1** **hsa-miR-199a-5p ↓2.9** **hsa-miR-221 ↓2.1** **hsa-miR-223 ↓2.9** **hsa-miR-326 ↓2.3** **hsa-miR-328 ↓2.3** **hsa-miR-652 ↓2.2** **hsa-miR-142-3p ↑3.0** **hsa-miR-142-5p ↑2.5** **hsa-miR-192 ↑2.2** **hsa-miR-29a ↑2.0** **hsa-miR-29b ↑3.5** **hsa-miR-29c ↑2.3** **hsa-miR-30e ↑2.1** **hsa-miR-338-3p ↑2.2** **hsa-miR-363 ↑2.1** **hsa-miR-590-5p ↑2.6** **hsa-miR-7 ↑2.2**
Experiment participants	Eleven healthy men 19–30 years old of average fitness (non-athletes)		Twelve healthy men with a mean age of 22±1 years of average fitness (non-athletes)		Eleven healthy men 20–29 years old of average fitness (non-athletes)
Exercise type	20 min of exercise consisting of 10 2-min bouts of constant work rate cycle ergometry with a 1-min rest interval between each bout (total test time is 30 min) at 76-77% peak VO2				
Time points of blood sampling	Immediately before and after exercise test				
Reference	(102)		(101)		(100)

Table S2. Differential expression of myomiRs in the skeletal muscle after different exercise types and forced immobility.

Exercise type	MiRNA name[1] and fold change			Species	Experiment participants	Muscle studied	Exercise modality	Experiment duration	Time points of expression analysis	Reference
	miR-1	mir-133a	mir-206							
Chronic endurance	↓1.5	↓1.3	↓2.0	Human	10 trained males with a mean age of 30.5 years	Vastus lateralis	Cycle ergometer	12 weeks	Before and 3-5 days after training	(89)
	↓1.6	↓1.9	NC[2]	Human	24 sedentary males with a mean age of 23 years	Vastus lateralis	Cycle ergometer	6 weeks	Before and one day after training	(52)
	NC[3]	NC	NC	Mouse	8-week-old mice	Gastrocnemius	Treadmill running	4 weeks	Experimental and control mice were analyzed one day after experiment	(5)
Acute endurance	↑ 1.3	↑1.4	NC	Human	10 trained males with a mean age of 30.5 years	Vastus lateralis	Cycle ergometer	60 min	Before and immediately after exercise	(89)
	↑1.4	NC	ND	Mouse	7 mice of 4 month old in experimental and control groups each	Quadr. femoris	Treadmill running	90 min	Experimental and control mice were analyzed 3 h after exercise	(108)
Resistance	↓1.7	NC	NC	Human	6 sedentary males with a mean age of 29 years	Vastus lateralis	Leg extension, essential amino acid solution	8 sets of 10 repetitions	Immediately before and 3 h after exercise	(27)
	↓1.3	ND	ND	Human	14 males and 13 females with a mean age of 80.1 years	Vastus lateralis	Resistance exercise or eccentric ergometer sessions	12 weeks	Before and 2-3 days after training	(84)
	NC	NC	NC	Human	17 untrained males of18-30 years old	Vastus lateralis	Rotating split-body resistance training program	12 weeks	Before and 2 days after training	(24)
	↓2.0	↓2.0	NC	Mouse	10-week-old mice	Plantaris	*In vivo* model of muscle hypertrophy	1 week	Experimental and control mice were analyzed after experiment	(80)

Resting muscle	↓1.1	↓1.1	ND	Human	12 physically active males with a mean age of 26.2 years	Vastus lateralis	Bed rest	7 days	Before and after experiment	(104)
	NC[3]	NC	NC	Mouse	14-week-old mice	Gastrocnemius	Hind limb fixation	5 days	Fixed and free limb muscles	(5)
	NC	NC	NC	Rat	3 6-month-old rats in two experimental and one control groups, each	Soleus	Hind limb suspension	2 and 7 days	Experimental and control rats were analyzed after experiment	(81)
	NC (trend toward a decrease)	NC (trend toward a decrease)	↓2.0	Mouse	4 77-day-old mice in experimental and control groups each	Gastrocnemius	Space flight	~12 days	Experimental and control mice were analyzed ~4 h after landing	(3)
	↓~3.3	↓~3.3	NC	Rat	Adult animals	Soleus	Denervation	4 weeks	Experimental and control rats were analyzed after experiment	(49)

[1]The data on the most often studied myomiRs are presented.
[2]The data on miRNAs with a 1.5-fold or greater expression difference are reported in the original paper.
[3]The data on miRNAs with a twofold or greater expression difference are reported in the original paper.
NC, no change
ND, not determined

Table S3. MiRNA species with most pronounced expression changes in muscle after exercise. Taxon-specific miRNAs are boldfaced.

MiRNA name	mir-183	mir-189	mir-432*	mir-451	mir-589	**mir-616**	mir-21	**mir-696**	**mir-709**	mir-720	mir-23	mir-451	**mir-680**	**mir-696**	**mir-705**	mir-762
Fold change	↑2.0	↑2.0	↑2.3	↓4.0	↓3.5	↑2.0	↑2.3	↓2.9	↓2.3	↓2.0	↓6.2	↑4.0	↑2.3	↑2.1	↑2.1	↑2.1
Reference	(52)						(5)				(108)	(24)	(5)			
Exercise type	Chronic endurance										Acute endurance	Resistance	Immobilization			

Table S4. Differential expression of miRNAs in human plasma and serum after exercise.

MiRNA name	Fold Change	Experiment participants	Exercise type	Time points of expression analysis and c-miRNA source	Refer-ence
miR-1	↑4.0	9 recreationally active males of 27-36 years old	30-min downhill walking	Before and 6 h after exercise; plasma	(9)
miR-20a	↑ 3.0	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
miR-21	↑ 1.9	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **prior** to sustained exercise training	Before and immediately after exercise; plasma	(8)
	↑ 2.6	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
miR-126	↑3.4	22 male marathon runners with a mean age of 56.8±5.2 years	Marathon race	Before and immediately after exercise; plasma	(128)
	↑2.0	13 healthy individuals (7 males and 6 females) with a mean age of 30.4±2.0 years	Single symptom-limited exercise test	Before and 5 min after exercise; plasma	(128)
	↑4.0	13 healthy well trained males with a mean age of 32.4±2.3 years	Cycle ergometer for 4 h below the anaerobic threshold	Before and immediately after exercise; plasma	(128)
	NC	11 trained subjects (4 males and 7 females) with a mean age of 37±2 years	Singular resistance training with additional eccentric loads (lat pulldown, leg press and butterfly)	Before and immediately after exercise; plasma	(128)
miR-133	↑8.9	22 male marathon runners with a mean age of 56.8±5.2 years	Marathon race	Before and immediately after exercise; plasma	(128)
	NC	13 healthy individuals (7 males and 6 females) with a mean age of 30.4±2.0 years	Single symptom-limited exercise test	Before and 5 min after test; plasma	(128)
	NC	13 healthy well trained males with a mean age of 32.4±2.3 years	Cycle ergometer for 4 h below the anaerobic threshold	Before and 1 h after exercise; plasma	(128)
	↑2.1	11 trained subjects (4 males and 7 females) with a mean age of 37±2 years	Singular resistance training with additional eccentric loads (lat pulldown, leg press and butterfly)	Before and immediately after exercise; plasma	(128)
	NC	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance and chronic endurance (90 days of rowing) exercises	Before and immediately after exercises; plasma	(8)
	↑4.8	9 recreationally active males of 27-36 years old	30-min downhill walking	Before and 6 h after exercise; plasma	(9)
miR-146a	↑3.0	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **prior** to 90-day exercise training	Before and immediately after exercise; plasma	(8)
	↑3.0	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
	↑7.5	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **after** 90-day exercise training	Before 90-day exercise training and immediately after acute exercise; plasma	(8)
	↓ 2.0	3 physically active males	Acute resistance exercise (bench press and bilateral leg press)	Before and 3 days after exercise; serum	(110)
miR-149*	↑2.3	3 physically active males	Acute resistance exercise (bench press and bilateral leg press)	Before and 1 day after exercise; serum	(110)

miR-181b	↑1.5	9 recreationally active males of 27-36 years old	30-min uphill walking	Before and immediately after exercise; plasma	(9)
miR-208	↑11.5	9 recreationally active males of 27-36 years old	30-min downhill walking	Before and 6 h after exercise; plasma	(9)
miR-214	↑1.8	9 recreationally active males of 27-36 years old	30-min uphill walking	Before and immediately after exercise; plasma	(9)
miR-221	↑ 3.6	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **prior** to 90-day exercise training	Before and immediately after exercise; plasma	(8)
	↑ 5.8	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
	↓ 2.0	3 physically active males	Acute resistance exercise (bench press and bilateral leg press)	Before and 3 days after exercise; serum	(110)
miR-222	↑2.5	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **prior** to 90-day exercise training	Before and immediately after exercise; plasma	(8)
	↑2.4	10 competitive male athletes with a mean age of 19.1±0.6 years	Rowing for 90 days	Before and after 90 days of exercise training; plasma	(8)
	↑4.0	10 competitive male athletes with a mean age of 19.1±0.6 years	Acute endurance exercise (cycle ergometer) **after** 90-day exercise training	Before 90-day exercise training and immediately after acute exercise; plasma	(8)

NC, no change

REFERENCES

1. Abbasi A, Hauth M, Walter M, Hudemann J, Wank V, Niess AM and Northoff H. Exhaustive exercise modifies different gene expression profiles and pathways in LPS-stimulated and un-stimulated whole blood cultures. Brain, Behavior, and Immunity, 2013.
2. Allantaz F, Cheng DT, Bergauer T, Ravindran P, Rossier MF, Ebeling M, Badi L, Reis B, Bitter H, D'Asaro M, Chiappe A, Sridhar S, Pacheco GD, Burczynski ME, Hochstrasser D, Vonderscher J and Matthes T. Expression profiling of human immune cell subsets identifies miRNA-mRNA regulatory relationships correlated with cell type specific expression. PLoS One 7: e29979, 2012.
3. Allen DL, Bandstra ER, Harrison BC, Thorng S, Stodieck LS, Kostenuik PJ, Morony S, Lacey DL, Hammond TG, Leinwand LL, Argraves WS, Bateman TA and Barth JL. Effects of spaceflight on murine skeletal muscle gene expression. J Appl Physiol (1985) 106: 582-595, 2009.
4. Androulidaki A, Iliopoulos D, Arranz A, Doxaki C, Schworer S, Zacharioudaki V, Margioris AN, Tsichlis PN and Tsatsanis C. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity 31: 220-231, 2009.
5. Aoi W, Naito Y, Mizushima K, Takanami Y, Kawai Y, Ichikawa H and Yoshikawa T. The microRNA miR-696 regulates PGC-1{alpha} in mouse skeletal muscle in response to physical activity. Am J Physiol Endocrinol Metab 298: E799-806, 2010.

6. Arner E, Mejhert N, Kulyte A, Balwierz PJ, Pachkov M, Cormont M, Lorente-Cebrian S, Ehrlund A, Laurencikiene J, Heden P, Dahlman-Wright K, Tanti JF, Hayashizaki Y, Ryden M, Dahlman I, van Nimwegen E, Daub CO and Arner P. Adipose tissue microRNAs as regulators of CCL2 production in human obesity. Diabetes 61: 1986-1993, 2012.
7. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF and Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 108: 5003-5008, 2011.
8. Baggish AL, Hale A, Weiner RB, Lewis GD, Systrom D, Wang F, Wang TJ and Chan SY. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. J Physiol 589: 3983-3994, 2011.
9. Banzet S, Chennaoui M, Girard O, Racinais S, Drogou C, Chalabi H and Koulmann N. Changes in circulating microRNAs levels with exercise modality. J Appl Physiol (1985) 115: 1237-1244, 2013.
10. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 136: 215-233, 2009.
11. Basso K, Sumazin P, Morozov P, Schneider C, Maute RL, Kitagawa Y, Mandelbaum J, Haddad J, Jr., Chen CZ, Califano A and Dalla-Favera R. Identification of the human mature B cell miRNome. Immunity 30: 744-752, 2009.
12. Baumjohann D, Kageyama R, Clingan JM, Morar MM, Patel S, de Kouchkovsky D, Bannard O, Bluestone JA, Matloubian M, Ansel KM and Jeker LT. The microRNA cluster miR-17 approximately 92 promotes TFH cell differentiation and represses subset-inappropriate gene expression. Nat Immunol 14: 840-848, 2013.
13. Benhamed M, Herbig U, Ye T, Dejean A and Bischof O. Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. Nat Cell Biol 14: 266-275, 2012.
14. Bezman NA, Cedars E, Steiner DF, Blelloch R, Hesslein DG and Lanier LL. Distinct requirements of microRNAs in NK cell activation, survival, and function. J Immunol 185: 3835-3846, 2010.
15. Bissels U, Bosio A and Wagner W. MicroRNAs are shaping the hematopoietic landscape. Haematologica 97: 160-167, 2012.
16. Boon RA and Vickers KC. Intercellular transport of microRNAs. Arterioscler Thromb Vasc Biol 33: 186-192, 2013.
17. Borchert GM, Lanier W and Davidson BL. RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 13: 1097-1101, 2006.
18. Bye A, Rosjo H, Aspenes ST, Condorelli G, Omland T and Wisloff U. Circulating microRNAs and aerobic fitness--the HUNT-Study. PLoS One 8: e57496, 2013.
19. Cai X, Hagedorn CH and Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10: 1957-1966, 2004.
20. Chen WJ, Yin K, Zhao GJ, Fu YC and Tang CK. The magic and mystery of microRNA-27 in atherosclerosis. Atherosclerosis 222: 314-323, 2012.
21. Cheng HS, Sivachandran N, Lau A, Boudreau E, Zhao JL, Baltimore D, Delgado-Olguin P, Cybulsky MI and Fish JE. MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways. EMBO Mol Med 5: 949-966, 2013.
22. Chhabra R, Dubey R and Saini N. Cooperative and individualistic functions of the microRNAs in the miR-23a~27a~24-2 cluster and its implication in human diseases. Mol Cancer 9: 232, 2010.

23. D. A. Silva ND J, Fernandes T, Soci UP, Monteiro AW, Phillips MI and EM DEO. Swimming training in rats increases cardiac MicroRNA-126 expression and angiogenesis. Med Sci Sports Exerc 44: 1453-1462, 2012.
24. Davidsen PK, Gallagher IJ, Hartman JW, Tarnopolsky MA, Dela F, Helge JW, Timmons JA and Phillips SM. High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression. J Appl Physiol (1985) 110: 309-317, 2011.
25. de Yebenes VG, Belver L, Pisano DG, Gonzalez S, Villasante A, Croce C, He L and Ramiro AR. miR-181b negatively regulates activation-induced cytidine deaminase in B cells. J Exp Med 205: 2199-2206, 2008.
26. Di Leva G and Croce CM. miRNA profiling of cancer. Curr Opin Genet Dev 23: 3-11, 2013.
27. Drummond MJ, McCarthy JJ, Fry CS, Esser KA and Rasmussen BB. Aging differentially affects human skeletal muscle microRNA expression at rest and after an anabolic stimulus of resistance exercise and essential amino acids. Am J Physiol Endocrinol Metab 295: E1333-1340, 2008.
28. El Gazzar M and McCall CE. MicroRNAs distinguish translational from transcriptional silencing during endotoxin tolerance. J Biol Chem 285: 20940-20951, 2010.
29. Elia L, Contu R, Quintavalle M, Varrone F, Chimenti C, Russo MA, Cimino V, De Marinis L, Frustaci A, Catalucci D and Condorelli G. Reciprocal regulation of microRNA-1 and insulin-like growth factor-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. Circulation 120: 2377-2385, 2009.
30. Ender C, Krek A, Friedlander MR, Beitzinger M, Weinmann L, Chen W, Pfeffer S, Rajewsky N and Meister G. A human snoRNA with microRNA-like functions. Mol Cell 32: 519-528, 2008.
31. Fabbri M, Paone A, Calore F, Galli R and Croce CM. A new role for microRNAs, as ligands of Toll-like receptors. RNA Biol 10: 169-174, 2013.
32. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, Lovat F, Fadda P, Mao C, Nuovo GJ, Zanesi N, Crawford M, Ozer GH, Wernicke D, Alder H, Caligiuri MA, Nana-Sinkam P, Perrotti D and Croce CM. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Proc Natl Acad Sci U S A 109: E2110-2116, 2012.
33. Fayyad-Kazan H, Rouas R, Fayyad-Kazan M, Badran R, El Zein N, Lewalle P, Najar M, Hamade E, Jebbawi F, Merimi M, Romero P, Burny A, Badran B and Martiat P. MicroRNA profile of circulating CD4-positive regulatory T cells in human adults and impact of differentially expressed microRNAs on expression of two genes essential to their function. J Biol Chem 287: 9910-9922, 2012.
34. Fehniger TA, Wylie T, Germino E, Leong JW, Magrini VJ, Koul S, Keppel CR, Schneider SE, Koboldt DC, Sullivan RP, Heinz ME, Crosby SD, Nagarajan R, Ramsingh G, Link DC, Ley TJ and Mardis ER. Next-generation sequencing identifies the natural killer cell microRNA transcriptome. Genome Res 20: 1590-1604, 2010.
35. Fernandes T, Hashimoto NY, Magalhaes FC, Fernandes FB, Casarini DE, Carmona AK, Krieger JE, Phillips MI and Oliveira EM. Aerobic exercise training-induced left ventricular hypertrophy involves regulatory MicroRNAs, decreased angiotensin-converting enzyme-angiotensin ii, and synergistic regulation of angiotensin-converting enzyme 2-angiotensin (1-7). Hypertension 58: 182-189, 2011.

36. Fernandes T, Magalhaes FC, Roque FR, Phillips MI and Oliveira EM. Exercise training prevents the microvascular rarefaction in hypertension balancing angiogenic and apoptotic factors: role of microRNAs-16, -21, and -126. Hypertension 59: 513-520, 2012.
37. Fernando TR, Rodriguez-Malave NI and Rao DS. MicroRNAs in B cell development and malignancy. J Hematol Oncol 5: 7, 2012.
38. Finnegan EF and Pasquinelli AE. MicroRNA biogenesis: regulating the regulators. Crit Rev Biochem Mol Biol 48: 51-68, 2013.
39. Forman JJ, Legesse-Miller A and Coller HA. A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. Proc Natl Acad Sci U S A 105: 14879-14884, 2008.
40. Friedman RC, Farh KK, Burge CB and Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92-105, 2009.
41. Gallo A, Tandon M, Alevizos I and Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One 7: e30679, 2012.
42. Georgantas RW, Streicher K, Greenberg SA, Greenlees L, Zhu W, Brohawn P, Higgs BW, Czapiga M, Morehouse C, Amato A, Richman L, Jallal B, Yao Y and Ranade K. Inhibition of myogenic MicroRNAs-1, 133, and 206 by inflammatory cytokines links inflammation and muscle degeneration in adult inflammatory myopathies. Arthritis Rheum, 2013.
43. He Y, Sun X, Huang C, Long XR, Lin X, Zhang L, Lv XW and Li J. MiR-146a Regulates IL-6 Production in Lipopolysaccharide-Induced RAW264.7 Macrophage Cells by Inhibiting Notch1. Inflammation, 2013.
44. Henke JI, Goergen D, Zheng J, Song Y, Schuttler CG, Fehr C, Junemann C and Niepmann M. microRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J 27: 3300-3310, 2008.
45. Hu G, Gong AY, Liu J, Zhou R, Deng C and Chen XM. miR-221 suppresses ICAM-1 translation and regulates interferon-gamma-induced ICAM-1 expression in human cholangiocytes. Am J Physiol Gastrointest Liver Physiol 298: G542-550, 2010.
46. Hulsmans M and Holvoet P. MicroRNA-containing microvesicles regulating inflammation in association with atherosclerotic disease. Cardiovasc Res 100: 7-18, 2013.
47. Huntzinger E and Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet 12: 99-110, 2011.
48. Hutchison ER, Kawamoto EM, Taub DD, Lal A, Abdelmohsen K, Zhang Y, Wood WH, 3rd, Lehrmann E, Camandola S, Becker KG, Gorospe M and Mattson MP. Evidence for miR-181 involvement in neuroinflammatory responses of astrocytes. Glia 61: 1018-1028, 2013.
49. Jeng SF, Rau CS, Liliang PC, Wu CJ, Lu TH, Chen YC, Lin CJ and Hsieh CH. Profiling muscle-specific microRNA expression after peripheral denervation and reinnervation in a rat model. J Neurotrauma 26: 2345-2353, 2009.
50. Kanaan Z, Barnett R, Gardner S, Keskey B, Druen D, Billeter A and Cheadle WG. Differential microRNA (miRNA) expression could explain microbial tolerance in a novel chronic peritonitis model. Innate Immun 19: 203-212, 2013.
51. Kang SG, Liu WH, Lu P, Jin HY, Lim HW, Shepherd J, Fremgen D, Verdin E, Oldstone MB, Qi H, Teijaro JR and Xiao C. MicroRNAs of the miR-17 approximately 92 family are critical regulators of T(FH) differentiation. Nat Immunol 14: 849-857, 2013.

52. Keller P, Vollaard NB, Gustafsson T, Gallagher IJ, Sundberg CJ, Rankinen T, Britton SL, Bouchard C, Koch LG and Timmons JA. A transcriptional map of the impact of endurance exercise training on skeletal muscle phenotype. J Appl Physiol (1985) 110: 46-59, 2011.
53. Kim DH, Saetrom P, Snove O, Jr. and Rossi JJ. MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci U S A 105: 16230-16235, 2008.
54. Kirby TJ and McCarthy JJ. MicroRNAs in skeletal muscle biology and exercise adaptation. Free Radic Biol Med 64: 95-105, 2013.
55. Kirigin FF, Lindstedt K, Sellars M, Ciofani M, Low SL, Jones L, Bell F, Pauli F, Bonneau R, Myers RM, Littman DR and Chong MM. Dynamic microRNA gene transcription and processing during T cell development. J Immunol 188: 3257-3267, 2012.
56. Koralov SB, Muljo SA, Galler GR, Krek A, Chakraborty T, Kanellopoulou C, Jensen K, Cobb BS, Merkenschlager M, Rajewsky N and Rajewsky K. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. Cell 132: 860-874, 2008.
57. Kosaka N, Yoshioka Y, Hagiwara K, Tominaga N, Katsuda T and Ochiya T. Trash or Treasure: extracellular microRNAs and cell-to-cell communication. Front Genet 4: 173, 2013.
58. Kozomara A and Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res, 2013.
59. Krol J, Loedige I and Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11: 597-610, 2010.
60. Kumarswamy R, Volkmann I and Thum T. Regulation and function of miRNA-21 in health and disease. RNA Biol 8: 706-713, 2011.
61. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W and Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol 12: 735-739, 2002.
62. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Muller RU, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De Vita G, Frezzetti D, Trompeter HI, Hornung V, Teng G, Hartmann G, Palkovits M, Di Lauro R, Wernet P, Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, Brownstein MJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M and Tuschl T. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129: 1401-1414, 2007.
63. Larsen MT, Hother C, Hager M, Pedersen CC, Theilgaard-Monch K, Borregaard N and Cowland JB. MicroRNA profiling in human neutrophils during bone marrow granulopoiesis and in vivo exudation. PLoS One 8: e58454, 2013.
64. Laterza OF, Lim L, Garrett-Engele PW, Vlasakova K, Muniappa N, Tanaka WK, Johnson JM, Sina JF, Fare TL, Sistare FD and Glaab WE. Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. Clin Chem 55: 1977-1983, 2009.
65. Lee I, Ajay SS, Yook JI, Kim HS, Hong SH, Kim NH, Dhanasekaran SM, Chinnaiyan AM and Athey BD. New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites. Genome Res 19: 1175-1183, 2009.
66. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S and Kim VN. The nuclear RNase III Drosha initiates microRNA processing. Nature 425: 415-419, 2003.

67. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH and Kim VN. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23: 4051-4060, 2004.
68. Lehmann SM, Kruger C, Park B, Derkow K, Rosenberger K, Baumgart J, Trimbuch T, Eom G, Hinz M, Kaul D, Habbel P, Kalin R, Franzoni E, Rybak A, Nguyen D, Veh R, Ninnemann O, Peters O, Nitsch R, Heppner FL, Golenbock D, Schott E, Ploegh HL, Wulczyn FG and Lehnardt S. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat Neurosci 15: 827-835, 2012.
69. Li J, Wan Y, Ji Q, Fang Y and Wu Y. The role of microRNAs in B-cell development and function. Cell Mol Immunol 10: 107-112, 2013.
70. Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, Braich R, Manoharan M, Soutschek J, Skare P, Klein LO, Davis MM and Chen CZ. miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell 129: 147-161, 2007.
71. Liston A, Lu LF, O'Carroll D, Tarakhovsky A and Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. J Exp Med 205: 1993-2004, 2008.
72. Liu G and Abraham E. MicroRNAs in immune response and macrophage polarization. Arterioscler Thromb Vasc Biol 33: 170-177, 2013.
73. Liu G, Keeler BE, Zhukareva V and Houle JD. Cycling exercise affects the expression of apoptosis-associated microRNAs after spinal cord injury in rats. Exp Neurol 226: 200-206, 2010.
74. Liu X, Wang Y, Sun Q, Yan J, Huang J, Zhu S and Yu J. Identification of microRNA transcriptome involved in human natural killer cell activation. Immunol Lett 143: 208-217, 2012.
75. Liu X, Zhan Z, Xu L, Ma F, Li D, Guo Z, Li N and Cao X. MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIalpha. J Immunol 185: 7244-7251, 2010.
76. Lu TX, Munitz A and Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. J Immunol 182: 4994-5002, 2009.
77. Lytle JR, Yario TA and Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A 104: 9667-9672, 2007.
78. Makarova JA, Ivanova SM, Tonevitsky AG and Grigoriev AI. New functions of small nucleolar RNAs. Biochemistry (Mosc) 78: 638-650, 2013.
79. Mattes J, Collison A, Plank M, Phipps S and Foster PS. Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. Proc Natl Acad Sci U S A 106: 18704-18709, 2009.
80. McCarthy JJ and Esser KA. MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. J Appl Physiol (1985) 102: 306-313, 2007.
81. McCarthy JJ, Esser KA, Peterson CA and Dupont-Versteegden EE. Evidence of MyomiR network regulation of beta-myosin heavy chain gene expression during skeletal muscle atrophy. Physiol Genomics 39: 219-226, 2009.
82. Mildner A, Chapnik E, Manor O, Yona S, Kim KW, Aychek T, Varol D, Beck G, Itzhaki ZB, Feldmesser E, Amit I, Hornstein E and Jung S. Mononuclear phagocyte miRNome analysis identifies miR-142 as critical regulator of murine dendritic cell homeostasis. Blood 121: 1016-1027, 2013.

83. Montagner S, Orlandi EM, Merante S and Monticelli S. The role of miRNAs in mast cells and other innate immune cells. Immunol Rev 253: 12-24, 2013.
84. Mueller M, Breil FA, Lurman G, Klossner S, Fluck M, Billeter R, Dapp C and Hoppeler H. Different molecular and structural adaptations with eccentric and conventional strength training in elderly men and women. Gerontology 57: 528-538, 2011.
85. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A and Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. J Exp Med 202: 261-269, 2005.
86. Naguibneva I, Ameyar-Zazoua M, Polesskaya A, Ait-Si-Ali S, Groisman R, Souidi M, Cuvellier S and Harel-Bellan A. The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. Nat Cell Biol 8: 278-284, 2006.
87. Nakasa T, Ishikawa M, Shi M, Shibuya H, Adachi N and Ochi M. Acceleration of muscle regeneration by local injection of muscle-specific microRNAs in rat skeletal muscle injury model. J Cell Mol Med 14: 2495-2505, 2010.
88. Nelson PT, De Planell-Saguer M, Lamprinaki S, Kiriakidou M, Zhang P, O'Doherty U and Mourelatos Z. A novel monoclonal antibody against human Argonaute proteins reveals unexpected characteristics of miRNAs in human blood cells. RNA 13: 1787-1792, 2007.
89. Nielsen S, Scheele C, Yfanti C, Akerstrom T, Nielsen AR, Pedersen BK and Laye MJ. Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle. J Physiol 588: 4029-4037, 2010.
90. O'Connell RM, Rao DS and Baltimore D. microRNA regulation of inflammatory responses. Annu Rev Immunol 30: 295-312, 2012.
91. O'Neill LA, Sheedy FJ and McCoy CE. MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nat Rev Immunol 11: 163-175, 2011.
92. Okamura K. Diversity of animal small RNA pathways and their biological utility. Wiley Interdiscip Rev RNA 3: 351-368, 2012.
93. Orom UA, Nielsen FC and Lund AH. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell 30: 460-471, 2008.
94. Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 13: 271-282, 2012.
95. Pedersen I and David M. MicroRNAs in the immune response. Cytokine 43: 391-394, 2008.
96. Persengiev SP. miRNAs at the crossroad between hematopoietic malignancies and autoimmune pathogenesis. Discov Med 13: 211-221, 2012.
97. Persson H, Kvist A, Vallon-Christersson J, Medstrand P, Borg A and Rovira C. The non-coding RNA of the multidrug resistance-linked vault particle encodes multiple regulatory small RNAs. Nat Cell Biol 11: 1268-1271, 2009.
98. Polikepahad S and Corry DB. Profiling of T helper cell-derived small RNAs reveals unique antisense transcripts and differential association of miRNAs with argonaute proteins 1 and 2. Nucleic Acids Res 41: 1164-1177, 2013.
99. Quinn SR and O'Neill LA. A trio of microRNAs that control Toll-like receptor signalling. Int Immunol 23: 421-425, 2011.
100. Radom-Aizik S, Zaldivar F, Haddad F and Cooper DM. Impact of brief exercise on peripheral blood NK cell gene and microRNA expression in young adults. J Appl Physiol (1985) 114: 628-636, 2013.

101. Radom-Aizik S, Zaldivar F, Jr., Leu SY, Adams GR, Oliver S and Cooper DM. Effects of exercise on microRNA expression in young males peripheral blood mononuclear cells. Clin Transl Sci 5: 32-38, 2012.
102. Radom-Aizik S, Zaldivar F, Jr., Oliver S, Galassetti P and Cooper DM. Evidence for microRNA involvement in exercise-associated neutrophil gene expression changes. J Appl Physiol (1985) 109: 252-261, 2010.
103. Ren G and Kang Y. A one-two punch of miR-126/126* against metastasis. Nat Cell Biol 15: 231-233, 2013.
104. Ringholm S, Bienso RS, Kiilerich K, Guadalupe-Grau A, Aachmann-Andersen NJ, Saltin B, Plomgaard P, Lundby C, Wojtaszewski JF, Calbet JA and Pilegaard H. Bed rest reduces metabolic protein content and abolishes exercise-induced mRNA responses in human skeletal muscle. Am J Physiol Endocrinol Metab 301: E649-658, 2011.
105. Rippe C, Blimline M, Magerko KA, Lawson BR, LaRocca TJ, Donato AJ and Seals DR. MicroRNA changes in human arterial endothelial cells with senescence: relation to apoptosis, eNOS and inflammation. Exp Gerontol 47: 45-51, 2012.
106. Rossi RL, Rossetti G, Wenandy L, Curti S, Ripamonti A, Bonnal RJ, Birolo RS, Moro M, Crosti MC, Gruarin P, Maglie S, Marabita F, Mascheroni D, Parente V, Comelli M, Trabucchi E, De Francesco R, Geginat J, Abrignani S and Pagani M. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b. Nat Immunol 12: 796-803, 2011.
107. Ruby JG, Jan CH and Bartel DP. Intronic microRNA precursors that bypass Drosha processing. Nature 448: 83-86, 2007.
108. Safdar A, Abadi A, Akhtar M, Hettinga BP and Tarnopolsky MA. miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6J male mice. PLoS One 4: e5610, 2009.
109. Sakharov DA, Maltseva DV, Riabenko EA, Shkurnikov MU, Northoff H, Tonevitsky AG and Grigoriev AI. Passing the anaerobic threshold is associated with substantial changes in the gene expression profile in white blood cells. Eur J Appl Physiol 112: 963-972, 2012.
110. Sawada S, Kon M, Wada S, Ushida T, Suzuki K and Akimoto T. Profiling of circulating microRNAs after a bout of acute resistance exercise in humans. PLoS One 8: e70823, 2013.
111. Schopman NC, Heynen S, Haasnoot J and Berkhout B. A miRNA-tRNA mix-up: tRNA origin of proposed miRNA. RNA Biol 7: 573-576, 2010.
112. Schulte LN, Westermann AJ and Vogel J. Differential activation and functional specialization of miR-146 and miR-155 in innate immune sensing. Nucleic Acids Res 41: 542-553, 2013.
113. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E and Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol 5: R13, 2004.
114. Sethi A, Kulkarni N, Sonar S and Lal G. Role of miRNAs in CD4 T cell plasticity during inflammation and tolerance. Front Genet 4: 8, 2013.
115. Sobala A and Hutvagner G. Transfer RNA-derived fragments: origins, processing, and functions. Wiley Interdiscip Rev RNA 2: 853-862, 2011.

116. Soci UP, Fernandes T, Hashimoto NY, Mota GF, Amadeu MA, Rosa KT, Irigoyen MC, Phillips MI and Oliveira EM. MicroRNAs 29 are involved in the improvement of ventricular compliance promoted by aerobic exercise training in rats. Physiol Genomics 43: 665-673, 2011.
117. Staedel C and Darfeuille F. MicroRNAs and bacterial infection. Cell Microbiol 15: 1496-1507, 2013.
118. Stittrich AB, Haftmann C, Sgouroudis E, Kuhl AA, Hegazy AN, Panse I, Riedel R, Flossdorf M, Dong J, Fuhrmann F, Heinz GA, Fang Z, Li N, Bissels U, Hatam F, Jahn A, Hammoud B, Matz M, Schulze FM, Baumgrass R, Bosio A, Mollenkopf HJ, Grun J, Thiel A, Chen W, Hofer T, Loddenkemper C, Lohning M, Chang HD, Rajewsky N, Radbruch A and Mashreghi MF. The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. Nat Immunol 11: 1057-1062, 2010.
119. Sullivan RP, Leong JW and Fehniger TA. MicroRNA regulation of natural killer cells. Front Immunol 4: 44, 2013.
120. Sullivan RP, Leong JW, Schneider SE, Keppel CR, Germino E, French AR and Fehniger TA. MicroRNA-deficient NK cells exhibit decreased survival but enhanced function. J Immunol 188: 3019-3030, 2012.
121. Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, Hunninghake GM, Vera MP, Blackwell TS, Baron RM and Feinberg MW. MicroRNA-181b regulates NF-kappaB-mediated vascular inflammation. J Clin Invest 122: 1973-1990, 2012.
122. Sylvius N, Bonne G, Straatman K, Reddy T, Gant TW and Shackleton S. MicroRNA expression profiling in patients with lamin A/C-associated muscular dystrophy. FASEB J 25: 3966-3978, 2011.
123. Takyar S, Vasavada H, Zhang JG, Ahangari F, Niu N, Liu Q, Lee CG, Cohn L and Elias JA. VEGF controls lung Th2 inflammation via the miR-1-Mpl (myeloproliferative leukemia virus oncogene)-P-selectin axis. J Exp Med 210: 1993-2010, 2013.
124. Taurino C, Miller WH, McBride MW, McClure JD, Khanin R, Moreno MU, Dymott JA, Delles C and Dominiczak AF. Gene expression profiling in whole blood of patients with coronary artery disease. Clin Sci (Lond) 119: 335-343, 2010.
125. Tonevitsky AG, Maltseva DV, Abbasi A, Samatov TR, Sakharov DA, Shkurnikov MU, Lebedev AE, Galatenko VV, Grigoriev AI and Northoff H. Dynamically regulated miRNA-mRNA networks revealed by exercise. BMC Physiol 13: 9, 2013.
126. Turchinovich A, Samatov TR, Tonevitsky AG and Burwinkel B. Circulating miRNAs: cell-cell communication function? Front Genet 4: 119, 2013.
127. Turchinovich A, Weiz L, Langheinz A and Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic Acids Res 39: 7223-7233, 2011.
128. Uhlemann M, Mobius-Winkler S, Fikenzer S, Adam J, Redlich M, Mohlenkamp S, Hilberg T, Schuler GC and Adams V. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. Eur J Prev Cardiol, 2012.
129. Urbich C, Kuehbacher A and Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. Cardiovasc Res 79: 581-588, 2008.
130. Vasudevan S, Tong Y and Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science 318: 1931-1934, 2007.
131. Vaz C, Ahmad HM, Sharma P, Gupta R, Kumar L, Kulshreshtha R and Bhattacharya A. Analysis of microRNA transcriptome by deep sequencing of small RNA libraries of peripheral blood. BMC Genomics 11: 288, 2010.

132. Vinuesa CG, Rigby RJ and Yu D. Logic and extent of miRNA-mediated control of autoimmune gene expression. Int Rev Immunol 28: 112-138, 2009.
133. Walden TB, Timmons JA, Keller P, Nedergaard J and Cannon B. Distinct expression of muscle-specific microRNAs (myomirs) in brown adipocytes. J Cell Physiol 218: 444-449, 2009.
134. Wang P, Gu Y, Zhang Q, Han Y, Hou J, Lin L, Wu C, Bao Y, Su X, Jiang M, Wang Q, Li N and Cao X. Identification of resting and type I IFN-activated human NK cell miRNomes reveals microRNA-378 and microRNA-30e as negative regulators of NK cell cytotoxicity. J Immunol 189: 211-221, 2012.
135. Ward JR, Heath PR, Catto JW, Whyte MK, Milo M and Renshaw SA. Regulation of neutrophil senescence by microRNAs. PLoS One 6: e15810, 2011.
136. Westholm JO and Lai EC. Mirtrons: microRNA biogenesis via splicing. Biochimie 93: 1897-1904, 2011.
137. Winter J, Jung S, Keller S, Gregory RI and Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 11: 228-234, 2009.
138. Wisloff U, Najjar SM, Ellingsen O, Haram PM, Swoap S, Al-Share Q, Fernstrom M, Rezaei K, Lee SJ, Koch LG and Britton SL. Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. Science 307: 418-420, 2005.
139. Wu C, Gong Y, Yuan J, Zhang W, Zhao G, Li H, Sun A, Zou Y and Ge J. microRNA-181a represses ox-LDL-stimulated inflammatory response in dendritic cell by targeting c-Fos. J Lipid Res 53: 2355-2363, 2012.
140. Xie W, Li Z, Li M, Xu N and Zhang Y. miR-181a and inflammation: miRNA homeostasis response to inflammatory stimuli in vivo. Biochem Biophys Res Commun 430: 647-652, 2013.
141. Xu S, Guo K, Zeng Q, Huo J and Lam KP. The RNase III enzyme Dicer is essential for germinal center B-cell formation. Blood 119: 767-776, 2012.
142. Xu WD, Pan HF, Li JH and Ye DQ. MicroRNA-21 with therapeutic potential in autoimmune diseases. Expert Opin Ther Targets 17: 659-665, 2013.
143. Xue X, Xia W and Wenzhong H. A modeled dynamic regulatory network of NF-kappaB and IL-6 mediated by miRNA. Biosystems 114: 214-218, 2013.
144. Yamamoto H, Morino K, Nishio Y, Ugi S, Yoshizaki T, Kashiwagi A and Maegawa H. MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead box j3. Am J Physiol Endocrinol Metab 303: E1419-1427, 2012.
145. Yi R, Qin Y, Macara IG and Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17: 3011-3016, 2003.
146. Yin G, Chen R, Alvero AB, Fu HH, Holmberg J, Glackin C, Rutherford T and Mor G. TWISTing stemness, inflammation and proliferation of epithelial ovarian cancer cells through MIR199A2/214. Oncogene 29: 3545-3553, 2010.
147. Yin H, Price F and Rudnicki MA. Satellite cells and the muscle stem cell niche. Physiol Rev 93: 23-67, 2013.
148. Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT and Bluestone JA. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. J Exp Med 205: 1983-1991, 2008.

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