Effect of exercise on micronutrient status and stress and immune response

Rieneke Terink

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Thesis

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Chapter 1

General Introduction

Being physically and mentally able to perform physical exertion is a trait that has offered competitive advantages during evolutionary development. Hence, humans possess several physiological mechanisms that not only facilitate physical activity in itself, but also stimulate rapid recovery and adaptation. Recovery and adaptation are particularly relevant when it comes to repeated physical activity, on the short term and longer term. In principle, successful adaptation leads to an increased ability to perform, which is generally associated with increased overall physical and mental fitness. In modern society, the direct necessity to deliver exertion has diminished and is often replaced by physical exercise as a physical activity to enhance or maintain physical fitness, overall health and wellness. More specifically in sports, physical exercise is purposed to train and improve sports performance.

However, in those situations in which there is significant disbalance between the effort at the one hand, and fitness and physiological resilience on the other hand, negative health effects may result. These include an enhanced stress response and effects on the immune system, resulting in increased sensitivity to infection and eventually contributing to symptoms of overtraining. The role of nutrition or specific nutrients in these processes has largely remained unclear which merits further investigation.

What happens during and after exercise: Acute exercise, recovery, and adaptation

Physical exercise can provide an effective manner to improve physical and mental health and well-being. For example, even running once a week has shown to decrease the risk for an early death (1). Exercise induces a range of physiological responses involving different organs, tissues and systems, including the circulatory, endocrine, immune, muscular and nervous system (2). The metabolic and mechanical effects directly following exercise are often referred to as “acute exercise response”. While the recovery after exercise concerns mechanisms to repair, refuel, replenish and to return to homeostasis and longer-term adaptations relate to specific forms of adaptation and growth. See the schematic figure 1 below.

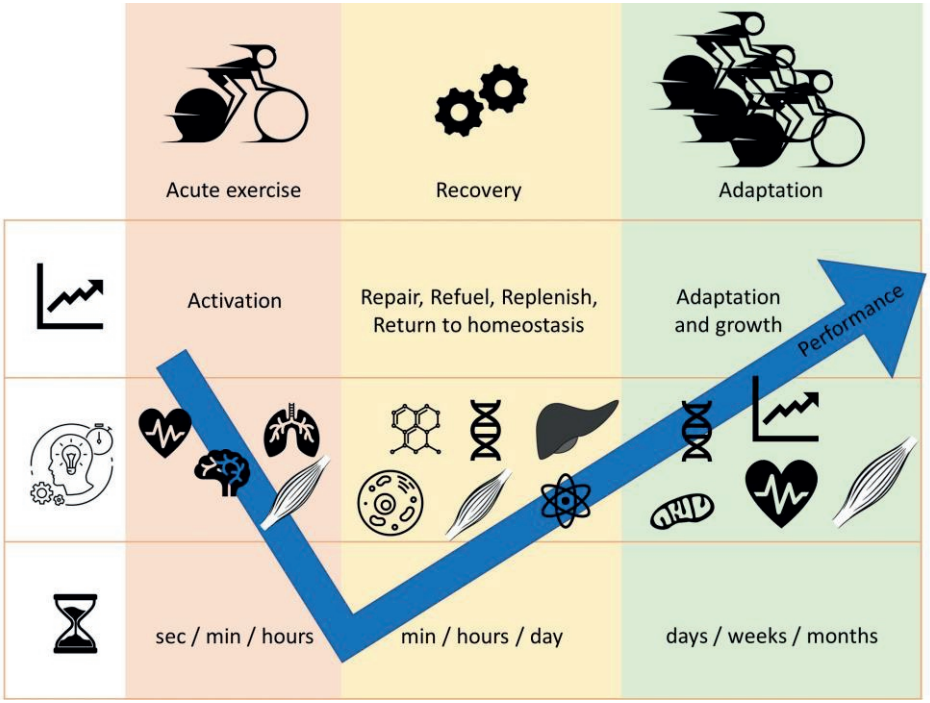

Figure 1: Schematic display of physiological responses to exercise

Acute exercise

Once exercise begins, the sympathetic nervous system is activated, stimulating heart rate, blood flow and oxygen supply to muscles and other tissues involved. Next to this, energy is mobilized via neuronal and humoral pathways [7]. At the same time, the activity of the parasympathetic system is decreased, resulting in decreased functioning of the digestive system, diverted blood flow away from the gastro-intestinal (GI) tract and skin etc. (3). These processes allow exercising more efficiently, they stimulate alertness and block pain signals. The body will use different energy systems depending on the duration and intensity of the exercise. Furthermore, minerals including magnesium (4), potassium (5) and calcium (6) are mobilized and redistributed with the likely purpose to facilitate exercise. When exercise intensity is high, or when exercise is prolonged, fatigue develops at some point. Causes include impaired blood flow, ion imbalance within the muscle (7), nervous fatigue (8), accumulated heat in the brain (9) and depleted energy sources. These factors will finally force the athlete to decrease intensity or even terminate the exercise, a biological response to maintain homeostasis, but not always desirable in competition.

Recovery

Directly after terminating exercise, the main goals of the body are recovery and return to homeostasis. Substrates and fluid used during exercise must be replenished (10). Micronutrient (11, 12) and hormone levels (13), as well as immune cell numbers (14) change during and after acute exercise, and these levels will return to baseline within hours to days after exercise. Furthermore, when exercise was prolonged, strenuous or eccentric, muscles can be damaged, necessitating repair. Muscle damage can occur in the sarcolemma, contractile proteins and connective tissue (15). The subsequent skeletal muscle healing process includes necrosis/degeneration, inflammation, repair and scar-tissue formation (fibrosis) (16). These processes are characterized by in an influx of extracellular calcium (17), an invasion of inflammatory cells (macrophages and T-cells), and secretion of growth factors and cytokines (18). Exercise causes a (short-term) disturbance in cellular homeostasis (19). At the molecular level, exercise affects gene expression, enhances mRNA translation and modulates signaling pathways. This results in the synthesis of specific proteins (20) to allow return to homeostasis and for adaptation and eventually supercompensation.

Adaptation

Longer term, adaptive responses to exercise are called training adaptations. Training adaptations are tightly coupled to the mode, frequency, intensity and duration of exercise performed (21). Furthermore, the majority of training-induced adaptations occur only in those muscles and muscle fibers that have been recruited during the exercise regimen, which is called training specificity (22). Possible training adaptations include muscle hypertrophy (23), increased activity of oxidative enzymes, increased capillary density (24), increased mitochondrial density (25) and improved delivery of oxygen and fuels to the muscle facilitated by changes in the circulatory and respiratory system (10). Such adaptations greatly enhance the oxidative capacity of the endurance-trained muscle and power of the resistance trained muscle. It is beyond the scope of this introduction and thesis to go into more detail about adaptive responses to training. Readers are referred to reviews (21, 26). In the end, athletes aim for specific training adaptations that could enhance their performance.

Modulating the acute exercise response, recovery and (long-term) adaptations: training, rest *and* nutrition

Regular exercise training is needed for training adaptations and these adaptations theoretically lead to improved performance. In this context, exercise training consists of multiple bouts of physical activity performed within a certain time frame, with the aim of achieving long-term goals. Therefore, exercise training concerns the process instead of the single exercise bouts themselves. Training programs are developed to induce the greatest adaptations possible, without inducing overload. The magnitude of adaptive responses is determined by training impulses: volume, but also intensity and frequency (22). A long held view is that training adaptation is directly related to the volume of exercise undertaken (27). However, there is a maximum volume beyond which additional stimuli do not induce further increases in functional capacity. This becomes clear when chronic intensive training and inadequate recovery lead to performance decrement and ultimately the overtraining syndrome (28). When training results in temporary fatigue with subsequent improvements after appropriate recovery, the temporary fatigue is considered functional overreaching (28). This is a desired part of a training program. However, when training overload results in longer term fatigue without performance improvements after considerable recovery time, it is described as non-functional overreaching. When recovery takes as long as months to even a year, the underperformance period is called 'overtraining syndrome' (28). Of course, the latter two situations are highly undesirable and should be avoided, whenever possible. See figure 2 below for the different phases of training and training overload and the accompanying recovery times.

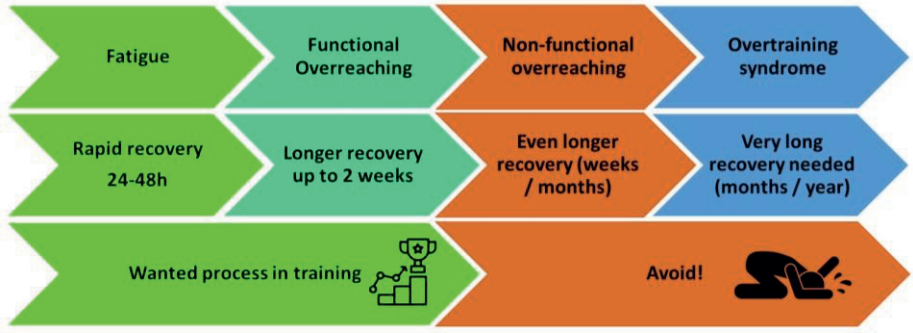

Figure 2. Presentation of the different phases of training, overreaching and overtraining syndrome (adapted and adjusted from Meeusen et al. 2013)

If the rate of recovery is appropriate, a higher training volume and/or intensity is possible without the detrimental effects of overtraining. So, adequate recovery enables high performance with the next exercise session. Or the other way around,

if muscles are not fully repaired, peak muscle force is diminished and blood glucose transport into skeletal muscle cells is decreased, resulting in decreased replenishment of glycogen stores (29). When recovery is insufficient and levels of hormones and micronutrients, as well as immune functionality have not returned to baseline, an increased risk for disease or psychological discomfort is probable (28, 30). Therefore, ways to enhance the recovery process is of high scientific interest. Possible approaches include various nutritional strategies like what, and how much to consume of various nutrients and optimizing timing of intake (31, 32). But also stretching, cooling, and for example the use of compression clothes are ways to enhance the recovery process (33).

Optimal nutritional strategies are not only relevant when it comes to the recovery process, but nutrition is also increasingly used to specifically trigger adaptive mechanisms. For example, studies examining the effects of protein supplementation on endurance training adaptations in healthy young males are undertaken (34). Another example is that studies have demonstrated that proteins and signalling molecules involved in training adaptation may be induced to a greater extent when exercise is performed under low CHO availability. Examples of such proteins and signalling molecules include AMPK (35), p38 mitogen-activated protein kinase (36), p53 (37) and PGC-1a (38). These are molecules involved in energy generating processes (glucose uptake and fatty acid oxidation), myogenesis, and mitochondrial biogenesis (20) and increased protein levels might enhance training adaptation. On the other hand, training with low CHO availability may increase the exercise-induced stress response, resulting in higher cortisol levels and suppressed immune function (39).

Modulating the acute exercise (stress) response can be a way to enhance training adaptation, but it can also result in detrimental effects like overreaching and overtraining when no adequate recovery is considered. Fine tuning this acute exercise response via a personalised nutrition plan is something we will likely see a lot in future (40). In some cases, the exercise response should be enhanced to gain greater training adaptations, while in other scenarios, the response should be reduced to lower the risk for overtraining and infection and possibly shorten recovery time. However, how to monitor this acute exercise stress response, and the impact of nutritional manipulation on this response requires more investigation.

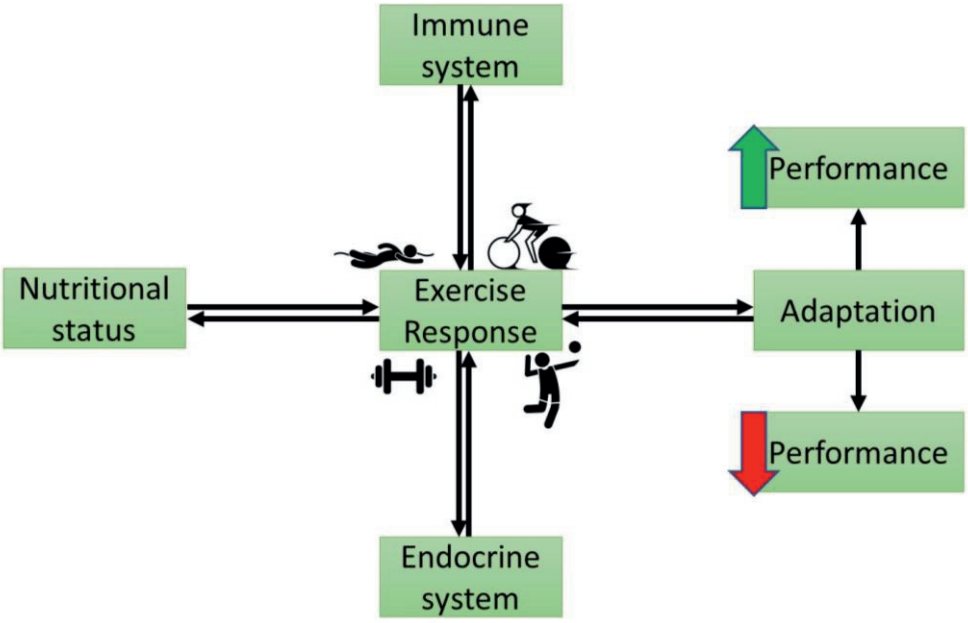

Figure 3 Conceptual representation of this thesis.

Topic of this thesis

In this thesis the acute exercise response takes a central position, see Figure 3. Studying the exercise response is complex, but a better understanding of the acute response, recovery and adaptation can provide new insights into potential markers to monitor training response and training load, resilience and health. Furthermore, such knowledge will be instrumental to evaluate potential nutritional strategies intended to modulate the exercise response and its impact on subsequent adaptation. Figure 3 represents the topics that will be addressed in this PhD thesis, namely nutritional status, the immune and endocrine system. These topics are gaining a lot of interest in exercise studies and are clearly linked to exercise performance and interrelated with one-another.

The central aim of this thesis was to further investigate the exercise responses during acute and repeated exercise and during periods of exercise training. More specifically, this aim was translated into the following sub-questions: 1) how does micronutrient status change during acute and repeated exercise, and 2) how is the stress and immune response affected during repeated exercise and periods of exercise training?

In addition, these two sub-questions are combined together in a dietary intervention study which aimed to study how an exercise-induced stress and immune response can be modulated by nutrition.

Outline of the thesis

In **chapter 2** the effect of an acute bout of exercise on total and ionized magnesium blood levels in healthy well-trained athletes during exercise as well as throughout recovery was investigated. We also wanted to study what happens with ionized and total magnesium levels when people exercise for multiple days and for a longer duration.

As sub-optimal magnesium levels have frequently been reported for older persons, an exercise-induced further decrease could have detrimental effects in this group. Therefore, in **chapter 3**, total and ionized magnesium levels were measured in a unique group of 80+ year olds, who walked ~8 hours per day for four consecutive days as participants of the Nijmegen 4-days Marches.

As described in **chapter 4**, the exercise challenge consisting of four consecutive days of walking was also chosen to study effects of repeated exercise on iron metabolism in middle-aged adults. Iron was taken as subject of investigation because exercise is known to influence its status due to (foot strike) haemolysis and inflammation.

In **chapter 5** cytokine levels during four consecutive days of walking exercise were measured in middle-aged adults. The magnitude of the change of cytokine levels depends on exercise duration and intensity. Previous studies were limited to a single day and insight in the time-course during multiple days of exercise was lacking.

In chapter 6 till 9 the focus is shifted back to athletes. In **chapter 6,** we first investigated the usefulness of salivary and hair cortisol and testosterone levels to measure exercise-response and to monitor training load. The rationale behind using these matrices is that blood measurements are perceived invasive for athletes. The measurement of salivary cortisol and testosterone gains more and more attention when it comes to assessing the (acute) exercise response. By contrast measuring cortisol and testosterone in hair has not yet been published for athletes to our knowledge, while such method could provide a stable indicator of long-term effects. To this end, hair and saliva samples were collected from 10 well-trained male swimmers during 10 consecutive weeks during which they followed a specific training periodization ending with a competition.

Prolonged training load without sufficient recovery can lead to overtraining syndrome. This demands for bio markers that indicate early signs of overtraining. Various exercise models are used to study overtraining, in these models changes in

hormones, nutrient status, immunological changes and psychological changes are assessed. In **chapter 7** various experimental approaches available from the literature that have been used to study overreaching and overtraining are reviewed and discussed. As study designs varied a lot in quality and in protocols used the review provides guidelines/advices for future researchers in the field of overtraining.

Modulating the exercise-induced stress and immune response aims to produce enhancement of adaptation. However, more insight is needed in terms of possible strategies and outcomes. In **chapter 8**, an intervention study was used to investigate to what extent modulation of the exercise-induced stress and immune response via diet is achievable. To this end, we compared the effects of a very low carbohydrate (LC) diet with those of a high carbohydrate diet (HC).

In **chapter 9**, we further investigated this exercise-induced immune response after the two different diets, focussing in more detail on immune cell differentiation and homing patterns.

Finally, in **chapter 10, the general discussion,** results of the studies performed are integrated and discussed. Finally, implications and directions for further research are proposed.

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2+
Magnesium

Chapter 2

Decrease in ionized and total magnesium blood concentrations in endurance athletes following an exercise bout restores within hours – potential consequences for monitoring and supplementation

Decreased ionized and total magnesium after exercise

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ABSTRACT

Magnesium is essential for optimal sport performance, generating an interest to monitor its status in athletes. However, before measuring magnesium status in blood could become routine, more insight into its diurnal fluctuations and effects of exercise itself is necessary. Therefore, we measured the effect of an acute bout of exercise on ionized (iMg) and total plasma magnesium (tMg) in blood obtained from 18 healthy well-trained endurance athletes (age, 31.1 ± 8.1 yr.; VO_2max, 50.9 ± 7.5 ml/kg/min) at multiple time points, and compared this with a resting situation. At both days, 7 blood samples were taken at set time points (8:30 fasted, 11:00, 12:30, 13:30, 15:00, 16:00, 18:30). The control day was included to correct for a putative diurnal fluctuation of magnesium. During the exercise day, athletes performed a 90 minute bicycle ergometer test (70% VO_2max) between 11:00 and 12:30. Whole blood samples were analysed for iMg and plasma for tMg concentrations. Both concentrations decreased significantly after exercise (0.52 ± 0.04 to 0.45 ± 0.03 mmol/L and 0.81 ± 0.07 to 0.73 ± 0.06 mmol/L, respectively, $p < .001$) while no significant decline was observed during that time-interval on control days. Both, iMg and tMg, returned to baseline, on average, 2.5 hours after exercise. These findings suggest that timing of blood sampling to analyse Mg status is important. Additional research is needed to establish the recovery time after different types of exercise to come to a general advice regarding the timing of magnesium status assessment in practice.

KEY WORDS: Micronutrients, Status monitoring, blood analysis

INTRODUCTION

Magnesium (Mg) is an essential micronutrient for health and performance (1). It is involved in numerous metabolic processes (2). In terms of exercise capacity, magnesium is for example crucial for glycolysis (1, 3), protein synthesis and muscular contraction (4), with ATP and calcium both depending on Mg concentrations (4, 5). Research showed that magnesium deficiency can lead to muscle weakness, neuromuscular dysfunction, and muscle cramping (2, 6-11). As a result, performance is highly dependent on adequate magnesium levels (12), generating a demand to monitor its status in athletes to prevent magnesium-related muscular dysfunction. At the same time, studies suggest that Mg supplements are frequently used by athletes (13).

Currently, measurement of total serum or plasma magnesium (tMg) is most commonly used to determine magnesium status (14), but it's reliability is subject to debate. In plasma (or serum), magnesium is present in three forms: 1) the free, ionized form, 2) complexed to anions and, 3) protein bound (mainly to albumin). Since free ionized magnesium (iMg) is the active, directly available form involved in cellular processes, it is suggested that iMg should be the preferable parameter to evaluate Mg status (15, 16). However, iMg is not widely used because its measurement has been technically challenging so far and the availability of suitable devices limited. During the past decade, more devices for iMg measurement have become available (16-18). Free ionized magnesium concentrations in serum/plasma account for approximately 60 -70 % of those for tMg, giving reference ranges according to the literature between 0.46 and 0.60 mmol/L (19).

Another problem with monitoring magnesium status is that exercise itself seems to affect both ionized and total magnesium. Previous studies showed inconclusive results for the effect of exercise on ionized and total magnesium status. A decrease in iMg after an incremental running exercise has been reported (15). Contrarily, a significant increase in iMg was observed after a bicycle ergometer test (20). Furthermore, published studies have presented contradicting effects of a single bout of exercise on *total* serum/plasma magnesium (tMg) concentration as well. Decreases in tMg were found after a marathon (21, 22) and a stepwise treadmill ergometer test (15) while an increase was found after intensive basketball training (23).

None of these studies measured ionized and total magnesium at different time points during an exercise day, thereby hampering evaluation of putative changes in Mg status. Moreover, previous studies did not account for a possibly underlying diurnal

fluctuation. Taken together, changes in magnesium concentrations upon exercise, putative recovery to pre-exercise levels and the influence of diurnal fluctuations remain unclear. This information is crucial to formulate guidelines regarding reliable assessment of magnesium status.

Therefore, the aim of this study was to evaluate the effect of a single bout of exercise on ionized (whole blood) *and* total magnesium (plasma) concentrations in endurance athletes.

Additionally, and hypothesizing that both parameters will decrease after exercise, we wanted to estimate the interval needed to return to baseline.

METHODS

Study population

Nine well-trained male and nine well-trained female athletes (cyclists and triathletes) participated in this study. All athletes trained regularly, for at least 5 hours per week. Participants refrained from exercise the last 24 hours before each study day. Their total plasma magnesium concentration was > 0.70 mmol/L (lower limit of normal), and subjects did not donate blood during the last six weeks prior to the study and did not take any calcium and/or magnesium supplements from the moment they underwent the first preliminary measurement. The study was approved by the Medical Ethical Committee of Wageningen University and all participants gave written informed consent prior to participation. This study was conducted in accordance with the Declaration of Helsinki.

Experimental design

The study had a cross-over design with random assignment to a control day and an exercise day, and at least two weeks between both measuring days. Prior to the study, preliminary measurements were performed. These included a maximal exercise test on a bicycle ergometer (Ergoline GmbH, Bitz, Germany) to establish maximal aerobic capacity (VO_{2max}). After an initial workload of 75 Watt for women and 100 Watt for men, workload was subsequently increased by 35W / 2min until the participant could not maintain the required pedaling frequency of 60 rpm. Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Hoechberg, Germany). In addition, body length (Seca 213 portable stadiometer, Hamburg, Germany), weight (Seca 761 scale), four-point skinfolds thickness (Holtain Tanner/Whitehouse Skinfold Caliper, Crosswell, UK) to estimate body fat percentage (24), and blood total magnesium concentration were measured. Questionnaires about supplement use, sports background, and food intake (Food Frequency Questionnaire, FFQ) (25, 26) were also taken.

At both experimental days, the first blood sample was taken at 08:30 am (overnight fasted state) and subsequent blood samples were taken at 11:00 am, 12:30 pm, 13:30 pm, 15:00 pm, 16:00 pm and 18:30 pm (all non-fasting). Breakfast was provided after the first blood drawing. On the exercise day a 90 minutes bicycle ergometer test at 70% of the athletes' individual VO_{2max} was performed between 11:00 am and 12:30 pm. Participants were allowed to drink plain water during the test, but were not allowed to eat. During the rest of the day, participants were allowed to eat and drink their own lunch and snacks. There was no specific diet prescribed, as

magnesium dietary intake has been shown not to directly affect serum magnesium concentrations (14, 27). However, individual food consumption was approximately the same on both test days, and consisted of typical Dutch breakfast and lunch products, like bread, cheese, yoghurt, fruits and juice. Body weight was measured directly before and after exercise.

Blood sampling

Blood samples were taken through a cannula from the cephalic vein and collected in lithium-heparin tubes (3.5 ml LH PST™ II and 4.0 ml 17 I.U./mL, Becton-Dickinson, New Jersey, America). LH PST™ II samples were centrifuged at 3000 g for 8 minutes at room temperature, and plasma was analyzed the same day for total magnesium concentration (Vista 1500, Siemens HealthCare, USA). Accuracy and precision of the Vista 1500 have been validated. At 0.62 mmol/L and 1.44 mmol/L coefficients of variation (CVs) are 3.0% and 1.7%, respectively. For measurement of ionized magnesium status, whole blood collected in a LH 17 I.U./mL vacutainer was immediately analyzed using the Stat Profile pHOx Plus M analyzer (Nova Biomedical, Waltham, MA, USA) according to the manufacturers' recommendations. Precision testing of the Stat Profile pHOx Plus M Analyzer, prior to this study, showed CVs of 1.33% and 2.38% at 0.53 mmol/L and 0.64 mmol/L, respectively.

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences 22.0 (IBM SPSS version 22.0, Armonk, New York, USA), and the level of significance was set at $p < 0.05$. Data are mean ± SD unless otherwise indicated.
The Shapiro-Wilk test was used to examine normality of the data distribution.
Linear mixed model was used to determine whether ionized and total magnesium changed over time on the exercise day. Data from the control day were used in the model to correct data from the exercise day. A top-down strategy was used to assess the model. With this approach several variables (for example: magnesium concentrations at rest day, sex, age and VO_{2max}) are added to the model. Next, these are deleted one by one when the variable does not contribute significantly to the fit of the model.
A cross-correlation between the time series of ionized and total magnesium was used to determine the cross-correlation coefficients. A Pearson correlation was used to determine the correlation between magnesium intake (estimated with FFQ) and total magnesium concentration in plasma.

RESULTS

Characteristics of the study population

The participants' characteristics are shown in Table 1 as mean ± standard deviation (SD). In spite of the weekly training load, some individual VO_{2max} values were rather modest, as reflected by the mean VO_{2max} of 54.9 ± 6.2 and 46.9 ± 6.8 ml kg^{-1} min^{-1} for men and women, respectively. Likewise, the peak power achieved was rather low for some individuals. At inclusion, (measured during preliminary measurements,) total magnesium concentration was above the lower limit of normal (0.7 mmol/L)(28) in all participants, range: 0.73 – 1.01 mmol/L. Magnesium intake (calculated with FFQ) was not associated with fasted total plasma magnesium or with fasted ionized magnesium levels at 08:30 am ($p > .05$). Subsequent results will be presented for men and women together, as there were no significant differences between men and women concerning magnesium concentrations and changes upon exercise.

Table 1. Participants' characteristics

	Men (n=9)	Women (n=9)
Age (years)	33.6 ± 7.8	28.6 ± 7.9
Height (cm)	185.7 ± 5.9	173.3 ± 8.6
Weight (kg)	79.6 ± 8.6	62.1 ± 9.2
BMI (kg/m2)	23.0 ± 2.0	20.6 ± 1.6
Body fat percentage (%)	14 ± 4	23 ± 4
Plasma tMg (mmol/L)	0.88 ± 0.03	0.87 ± 0.08
Dietary Mg intake (mg)	619 ± 184	479 ± 145
Wmax (Watt)	390 ± 26	281 ± 51
VO_2max (ml/kg/min)	54.9 ± 6.2	46.9 ± 6.8

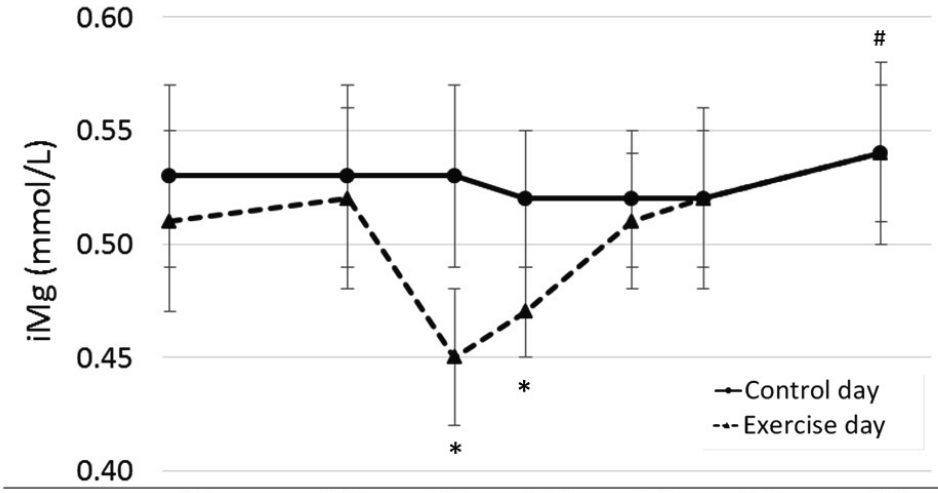
Data is expressed as mean ± SD. BMI, body mass index; plasma tMg , total magnesium concentration in plasma; Dietary Mg intake was estimated from an FFQ; Wmax, maximal power output during maximal effort test; VO_2max, maximal oxygen consumption.

Effect of an acute bout of exercise on ionized and total magnesium

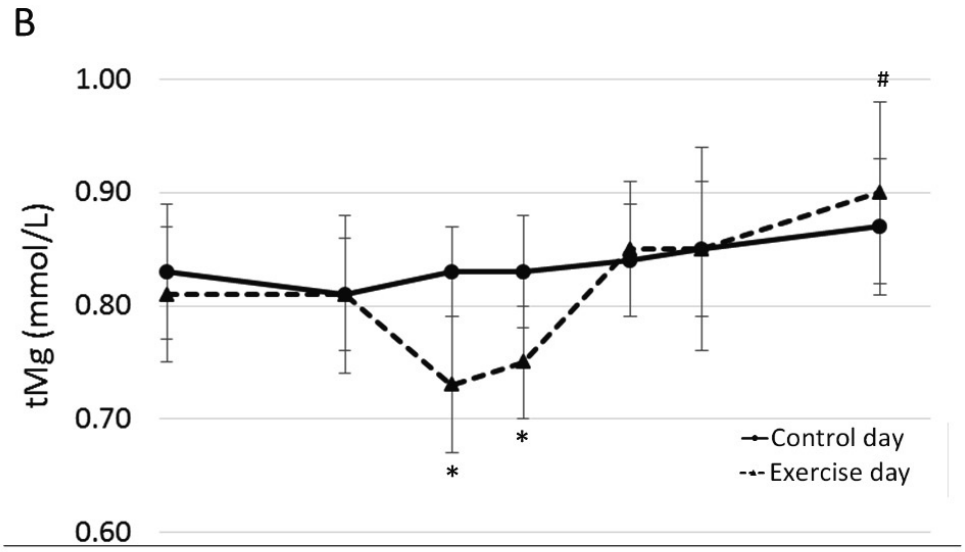
The ionized and total magnesium concentrations at different time points during the exercise day are shown in Figure 1. Mean ionized (whole blood) and total magnesium (plasma) concentrations early in the morning after an overnight fast (8:30 am) were 0.51 ± 0.04 and 0.81 ± 0.06 mmol/L, respectively, with iMg ranging from 0.45 – 0.59 mmol/L and tMg ranging from 0.67 – 0.92 mmol/L. Two of our participants (one male, one female) had tMg values below the lower limit of normal

in the fasted state: 0.67 and 0.69 mmol/L, respectively. Immediately before exercise (11:00 am), ionized and total magnesium concentration did not significantly differ from early morning values (0.52 ± 0.04 and 0.81 ± 0.07 mmol/L, respectively). After exercise (12:30 pm), both total and ionized magnesium concentrations were significantly lower. Ionized magnesium decreased by 0.06 ± 0.03 mmol/L to 0.45 ± 0.03 mmol/L ($p < .001$). Total magnesium decreased by 0.08 ± 0.04 mmol/L to 0.73 ± 0.06 mmol/L ($p < .001$). Ionized and total magnesium concentrations at 13:30 pm, one hour after finishing exercise, were still significantly lower compared to the concentrations before exercise ($p < .001$).

A



	8:30	11:00	12:30	13:30	15:00	16:00	18:30
Control day	0.53 ± 0.04	0.53 ± 0.04	0.53 ± 0.04	0.52 ± 0.03	0.52 ± 0.03	0.52 ± 0.03	0.54 ± 0.03
Exercise day	0.51 ± 0.04	0.52 ± 0.04	0.45 ± 0.03	0.47 ± 0.02	0.51 ± 0.03	0.52 ± 0.04	0.54 ± 0.04



	8:30	11:00	12:30	13:30	15:00	16:00	18:30
Control day	0.83 ± 0.06	0.81 ± 0.05	0.83 ± 0.04	0.83 ± 0.05	0.84 ± 0.05	0.85 ± 0.06	0.87 ± 0.06
Exercise day	0.81 ± 0.06	0.81 ± 0.07	0.73 ± 0.06	0.75 ± 0.05	0.85 ± 0.06	0.85 ± 0.09	0.90 ± 0.08

Fig. 1 – Mean ionized (A) and total (B) magnesium concentrations, during exercise (dotted line) and control (black line) day. * significantly lower concentration compared with pre-exercise concentrations ($p < .001$) # significantly higher concentration compared with all earlier time points. ($p < 0.05$).

Recovery of magnesium concentrations after exercise

At 2.5 hours after exercise (15:00 pm), concentrations seemed to be recovered, as they were no longer significantly different from fasting and pre-exercise values. However, when individual data were analyzed, only 10 participants (out of the 18) were back at their pre-exercise *ionized* magnesium concentration (Δ Post-exercise – Pre-exercise ≥ 0 mmol/L), while 15 participants were back at their pre-exercise *total* magnesium concentration, see Table 2. At six hours after exercise, almost all participants were back at their pre-exercise *total* magnesium concentration, however, 2 participants (one man; one woman) were still below their pre-exercise *ionized* magnesium concentration.

Table 2. Recovery of ionized and total magnesium after exercise

	Ionized magnesium		Total magnesium	
	Number Recovered (n / 18)	Mean difference (Mean ± SD)	Number Recovered (n / 18)	Mean difference (Mean ± SD)
directly after exercise	0 / 18	- 0.06 ± 0.03*	0 / 18	- 0.08 ± 0.04*
1 hour after exercise	1 / 18	- 0.05 ± 0.02*	1 / 18	- 0.06 ± 0.04*
2.5 hours after exercise	10 /18	- 0.007 ± 0.02	15 /18	0.04 ± 0.06
3.5 hours after exercise	13 / 18	0.00 ± 0.02	15 / 18	0.04 ± 0.06
6 hours after exercise	16 / 18	0.03 ± 0.03	18 / 18	0.09 ± 0.05

Differences between pre-exercise and different time points after exercise. Concentrations are recovered when post-exercise – pre-exercise difference ≥ 0 mmol/L. n represents the amount of participants recovered. * significantly lower concentration compared with pre-exercise concentrations ($p < .001$).

Magnesium concentrations during control days

Magnesium concentrations fluctuated during the control day (Figure 1). Ionized and total magnesium concentrations were both significantly higher at the end of the afternoon (18:30 pm). To be certain that exercise-induced changes were caused by exercise only and not due to an underlying diurnal fluctuation, these control day data were used for evaluation of the exercise day data.

Cross-correlation between ionized and total magnesium

A cross correlation analysis revealed that iMg and tMg concentrations were significantly correlated at both the exercise day ($r = 0.728$, $p < .001$) and the control day ($r = 0.405$, $p < .001$). Both ionized and total magnesium concentrations decreased immediately after exercise and were higher at the end of both the exercise and control day. There was no delay between iMg and tMg, as Lag -1 ($r = 0.248$) and Lag 1 ($r = 0.163$) in the cross correlation were lower than Lag 0.

DISCUSSION

To our knowledge this is the first study to measure the effect of an acute bout of exercise on total *and* ionized magnesium in endurance athletes by comparing iMg and tMg 2.5 hours before, and until 6 hours after exercise in comparison to a similar situation (same time points) without exercise. Ionized magnesium has the greatest biological activity, it is readily available and diffusible. In agreement with our hypothesis, the primary finding was that both ionized and total magnesium decreased significantly after exercise. Recovery to baseline levels occurred, on average, 2.5 hours after exercise termination. This implicates that measuring Mg status the day after exercise should be sufficient to obtain reliable insight in its status.

Decreased total and ionized magnesium concentrations after exercise

Measurement of ionized magnesium and total magnesium at consecutive time points following exercise, in comparison with a resting situation has not been performed before. With this setup we were certain that the changes found after an acute bout of exercise were caused by exercise only and not by an underlying diurnal fluctuation. A decrease in ionized magnesium following exercise has been reported previously (15). In that study, 20 athletes performed a progressive treadmill ergometer test until exhaustion. Blood samples were collected immediately after finishing the test and analysed using an ion-sensitive microelectrode (AVL electrolyte analyzer 988-4). The post-exercise decrease in ionized magnesium was comparable to that found in our study. Unfortunately, these authors did not measure at more time points following exercise.

Decreases in *total* magnesium were also reported before, for example, immediately after finishing a marathon (21, 22, 29). Unfortunately, not all of these studies measured total magnesium concentrations immediately before the marathon as baseline, but 1 week (22) or 2 weeks (21) prior to the marathon. By doing so one cannot be sure that the lower magnesium concentrations are caused by the exercise, or whether these concentrations were already low at the start of the marathon.

Contradicting to our results was the observation of Duma et al., who found an increase in magnesium concentrations after a bicycle ergometer test (initial power 150W increasing with 50W/min until exhaustion) (20). A possible explanation could be a decrease in plasma volume, although specific information about fluid intake and plasma volume to confirm this theory was not mentioned. The possibility that changes in plasma volume due to exercise may have influenced our results were also considered in the present study. We measured haematocrit values and noticed only a

slight increase after exercise. This could indicate a decrease in plasma volume (haemoconcentration). Opposite to our findings, this would have contributed to an increase in magnesium concentration. To maintain total body water stable participants were allowed to drink water, and the lack of a change in body weight after exercise indicates that dehydration did not occur. In another study, increases in total magnesium were found 24 hours after an intensive basketball training (23). This might be caused by the timing of blood withdrawal, as concentrations were measured 24 hours after the training and not directly after finishing the training (23). We measured immediately after exercise and at different time points later that day. If we had measured only at 2.5 hours or later after exercise, we would not have observed the decrease in ionized and total magnesium concentration. On the other hand, we measured total and ionized magnesium up to 6 hours after exercise, so we do not know whether a putative increase may have occurred after 6 hours. Additionally, individual data showed that only 10 of our participants were back at their pre-exercise *ionized* magnesium concentrations at 2.5 hours after exercise, while 15 participants were back at their pre-exercise *total* magnesium concentrations. Even 6 hours after exercise, there were still 2 participants who were not back at their pre-exercise ionized magnesium concentrations. These results indicate that recovery differs per individual and that individual timing of blood withdrawal is crucial. It also seems that recovery of ionized magnesium concentrations after exercise is somewhat slower in comparison to recovery of total magnesium concentrations.

It is interesting to speculate about possible causes for our observed decrease in plasma magnesium concentration following exercise. One explanation might be that this is caused by an increase of catecholamines during strenuous exercise, as catecholamines, like epinephrine and norepinephrine induce $Mg2+$ uptake into muscle cells and regulate the magnesium dependent Na/K ATPase pumps in skeletal muscle (30).

Another possible explanation for a decrease of plasma magnesium could be uptake of magnesium into adipocytes due to an increased rate of lipolysis (31). Lipolysis increases during exercise, in particular when muscle glycogen levels decrease, as is the case in more prolonged exercise. As a consequence, free fatty acids are transported into the plasma. Fatty acids that remain in the adipocytes bind to magnesium, causing a decrease of the free ionized magnesium in the cells, which results in a net uptake of magnesium from the plasma into the adipocytes (29). Thus, both uptake by muscle cells and adipose tissue might explain a post-exercise drop in circulating ionized and total Mg levels. Our study does not provide data to support

these hypotheses. Additional studies, for example taking muscle and adipose tissue biopsies should be performed to evaluate this.

Higher magnesium concentrations at the end of the day

In our study ionized and total magnesium concentrations were slightly increased at the end of the day. This might be caused by innate diurnal fluctuations, by excretion, or by dietary factors. Here again, opposite findings regarding innate diurnal fluctuations have been reported in the literature, with maximum values in the morning and minimum values later that day (19). It has also been shown that magnesium excretion fluctuates throughout the day, with maximal excretion occurring at night (32). Regarding iMg, it has been shown that concentrations are variable in healthy subjects over the course of one day (33). However, more research is needed to estimate the possible diurnal fluctuations of ionized and total magnesium.

Correlation between ionized and total magnesium

We found a cross-correlation between ionized magnesium and total magnesium at both the exercise day and control day. The difference in cross-correlation values between these two conditions would suggest that the equilibrium between tMg and iMg might differ after exercise and during a resting condition. However, data supporting this possibility are lacking in the present study.

A moderate correlation between ionized and total magnesium was also found by other researchers (r = 0.585) (34). Strong correlations were found (r = .903) in 34 critically ill and injured patients (35) as well as in patients with intestinal or liver disease and healthy controls (r = .87 in 106 patients, and r = .75 in 75 healthy controls, $p < .001$) (36). Our correlation between ionized and total magnesium at control day was lower than previously found. This might be caused by the small sample size of our study. We only included 18 athletes, whereas the other studies included more individuals. We found that ionized and total magnesium correlated better at an exercise day in comparison to a rest day.

Conclusion

In conclusion, this study showed a significant decrease in both ionized and total magnesium immediately after a single bout of exercise. Total and ionized Mg concentrations were, on average, back to pre-exercise levels 2.5 hours after exercise. However, we don't know whether a similar pattern is observed after another form of exercise. This implicates that the timing of blood sampling for assessment of

magnesium status is important. More research is needed to estimate the recovery time after various types of exercise in order to facilitate a sports physician in interpreting magnesium status. Both ionized and total magnesium, showed almost similar decrease and recovery patterns, indicating that both can be used to evaluate physiological changes after exercise. Whether ionized magnesium should be the preferable parameter to evaluate Mg status in deficient participants should be studied in future.

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Magnesium
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Chapter 3

Ionized and total magnesium levels change during repeated exercise in older adults

Exercise effects on Mg levels in elderly

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ABSTRACT

Background: Magnesium is essential for health and performance. Sub-optimal levels have been reported for older persons. In addition, physical exercise is known to temporally decrease magnesium blood concentrations. **Objective:** To investigate these observations in conjunction we assessed total (tMg) and ionized magnesium (iMg) concentrations in plasma and whole blood, respectively, during 4 consecutive days of exercise in very old vital adults. **Design:** 68 participants (age 83.7±1.9 years) were monitored on 4 consecutive days at which they walked 30-40km (average ~8 hours) per day at a self-determined pace. Blood samples were collected one or two days prior to the start of exercise (baseline) and every walking day immediately post-exercise. Samples were analysed for tMg and iMg levels. **Results:** Baseline tMg and iMg levels were 0.85±0.07 and 0.47±0.07 mmol/L, respectively. iMg decreased after the first walking day (-0.10±0.09 mmol/L, $p<.001$), increased after the second (+0.11±0.07 mmol/L, $p<.001$), was unchanged after the third and decreased on the final walking day, all compared to the previous day. tMg was only higher after the third walking day compared to the second walking day ($p=.012$). In 88% of the participants, iMg levels reached values considered to be sub-optimal at day 1, in 16% of the participants values were sub-optimal for tMg at day 2. **Conclusion:** Prolonged moderate intensity exercise caused acute effects on iMg levels in a degree comparable to that after a bout of intensive exercise. These effects were not associated with drop-out or health problems. After the second consecutive day of exercise, levels were returned to baseline values, suggesting rapid adaptation/resilience in this population.

KEY WORDS: older adults, consecutive exercise days, micronutrients, reference values

INTRODUCTION

Magnesium (Mg) is an essential micronutrient for general health and physical performance (1). Research has shown that magnesium deficiency can amongst others lead to muscle weakness, neuromuscular dysfunction, and muscle cramping (2-7).
Magnesium deficiency may be more prevalent in older adults because it is known that the age- related reduction in bone mass results in a reduction of the magnesium body reserves (8). Besides, magnesium absorption decreases with age (9). Additionally, use of medicines, including the widely used proton pomp inhibitors, may increase the risk of hypomagnesemia (10).
It should be noted that circulating Mg represents < 1% of total body reservoirs and therefore is not a perfect predictor of the body magnesium status. Nevertheless, total serum magnesium is the mostly used clinical representative of the magnesium status (11).
Previous studies observed a transient decrease in blood magnesium concentrations, during and immediately after exercise in trained young adults (12-15), indicating redistribution between Mg pools in the body. In the case of elderly, where baseline magnesium levels may already be low, a post-exercise drop could result in in plasma hypomagnesemia (< 0.70 mmol/L for tMg and < 0.46 mmol/L for iMg) (16, 17). However, whether magnesium blood concentrations in older adults decrease after exercise is unknown. In addition, the effect of consecutive days of exercise on magnesium blood levels in older adults is unknown.
Earlier studies that investigated the relationship between magnesium blood levels and exercise were mainly focussed on healthy young and middle aged adults, while studies in the older adults are lacking. Therefore, we included a large unique group of active older adults, with an age above 80 years old.
The aim of this study was to assess blood magnesium levels in a vital group of older adults aged > 80 years old and to investigate the effect of (repeated) exercise on ionized (whole blood) *and* total magnesium (plasma) levels. Moreover, we explored factors that could predict low magnesium levels and the exercise induced decrease in magnesium. We hypothesized that the older adults were low in total and ionized magnesium levels and showed a decrease in magnesium levels after prolonged moderate intensity exercise.

MATERIALS AND METHODS

Study population

We selected 72 male and 22 female walkers who participated in the 2016 edition of the Nijmegen Four Days Marches, a large annual four-day walking event taking place in the Netherlands (http://www.4daagse.nl/en/). Exclusion criteria were known diabetes and/or renal dysfunction. The study was approved by the Medical Ethical Committee of the Radboud university medical center (CMO registration number: 2007/148), and all participants gave written informed consent prior to participation. This study was conducted in accordance with the Declaration of Helsinki.

Study procedure

The study took place in the summer season, i.e. July. Actual climatological conditions are specified in the results section. Measurements were performed before the start of the event ('baseline'), and at four consecutive walking days. Baseline measurements, including registering participant characteristics and collection of a blood sample, were performed in our field laboratory at the event location one or two days prior to the first walking day, between 09:30 AM and 04:00 PM. A 24 hour recall for dietary intake was done a few weeks before the start of the event.

Every walking day, immediately before the start of the march, participants' body weight was measured. Thereafter, participants walked 30 or 40 km, at a self-selected pace, starting between 4:00 and 8:00 AM. Every day, participants registered their fluid intake using a diary. Directly after finishing, post-exercise body weight was determined, a blood sample was taken (see below for details) and a set of questionnaires was completed. Heart rate was measured during the first walking day using a 2-channel ECG chest band system (Polar Electro Oy, Kempele, Finland). Mean heart rate during exercise was calculated as the average heart rate, excluding the values derived directly before the start and after the finish. Heart rate was used to estimate exercise intensity as percentage of (expected) maximal heart rate: Intensity = (Measured HR / Expected maximal HR) * 100%, with Expected max HR = 208 - (0.7 * Age) (18).

Baseline measurements

At baseline, body weight (Seca 888 scale, Hamburg, Germany) and body height were determined and body mass index (BMI) was calculated. Thereafter, resting heart rate and blood pressure were measured using an automated sphygmomanometer (M5-1

intellisense, Omron Healthcare, Hoofddorp, The Netherlands) after 5 minute supine rest.

Blood samples

Blood samples were taken at baseline and post-exercise at the four consecutive walking days. Participants were seated for 5 min after which a venous blood sample was taken from the cephalic vein. Blood was collected in lithium-heparin (LH) tubes (3.5 ml LH PST™ II and 4.0 ml LH 17 IU/mL, Becton-Dickinson, Vianen, The Netherlands). The LH PST™ II samples were centrifuged at 3000G for 8 minutes at 22 degrees and plasma was stored at -20 °C. Plasma samples were analysed for their total magnesium concentrations (Dimension Vista 1500, Siemens Healthcare, Erlangen, Germany).

For measurement of ionized magnesium status, a fraction of the whole blood sample collected in the LH 17 IU/mL vacutainer was immediately analysed using the Stat Profile pHOx Plus M analyzer (Nova Biomedical, Waltham, MA, USA) according to the manufacturers' recommendations.

Furthermore, a part of the whole blood sample collected in the LH 17 IU/ml vacutainer was used for direct analyses of plasma haemoglobin and haematocrit concentrations (Rapidpoint 400, Siemens Healthcare, Erlangen, Germany). Relative changes in plasma volume were calculated from blood haematocrit and haemoglobin concentrations using Dill and Costill's equation (19).

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences 22.0 (IBM SPSS version 22.0, Armonk, New York, USA), and the level of significance was set at $p < 0.05$. Data are presented as mean ± SD unless indicated otherwise. The Kolmogorov-Smirnov test was used to examine the normality of the data distribution. Data was normally distributed. Parametric tests were used for analysis.

Total and ionized magnesium data were analysed using repeated measures ANOVA for the effect of consecutive days (5 levels; baseline, day 1 till day 4), with a post hoc Bonferroni correction when there was a main effect for consecutive days.

A backward linear regression was used to analyse which variables contributed significantly to the observed baseline total and ionized magnesium levels. In addition, a backward linear regression was also used to analyse which variables contributed significantly to the observed decrease ionized magnesium after the first day of exercise.

RESULTS

Participant characteristics

Twenty-six of our participants did not finish the 4 Day Marches due to various reasons (e.g. knee problems, back problems, time consuming and the heat). The characteristics of the remaining 68 participants who completed all 4 days are shown in table 1.

Table 1. Participant characteristics

	Participants (n = 68)
Characteristics	
Age (years)	83.7 ±1.9
Body composition	
Height (cm)	168.9 ± 7.3
Weight (kg)	71.0 ± 10.0
BMI (kg/m^2)	24.8 ± 2.7
Cardio characteristics	
Resting heart rate (bpm)	66.6 ± 13.5
Systolic pressure (mmHg)	147.2 ± 14.0
Diastolic pressure (mmHg)	81.8 ± 10.3
Dietary intake	
Energy intake (kCal)	1990 ± 477
Total protein (En%)	16.6 ± 4.3
Total fat (En%)	33.8 ± 7.7
Total carbohydrates (En%)	43.8 ± 7.6
Fibre (g)	22.2 ± 6.7
Calcium (mg)	964 ± 368
Magnesium (mg)	336 ± 99
Walking distances per day	
Walking 30 km	n = 65
Walking 40 km	n = 3

Means ± SD are shown. BMI: Body mass index; Dietary intake values are estimated with a 24 hour recall; En%: Energy percentage of that macronutrient of total energy intake.

Exercise characteristics

Exercise was performed under warm ambient conditions, with a significant increase in humidity ($p < .001$) and with significant varying wet bulb globe temperature (WBGT) between a minimum of 15 °C (in the early morning) and a maximum of 29 °C during the four days ($p < .001$) (table 2).

Table 2. Ambient conditions at day 1 to day 4.

	Day 1	Day 2	Day 3	Day 4	Asymp. Sign.
Min WBGT (°C)	15	18	17	18	
Max WBGT (°C)	27	29	26	25	
Mean WBGT (°C)	22.2 ± 4.9	24.4 ± 4.0	22.5 ± 3.4	21.4 ± 2.8	
Friedman Test Mean Rank WBGT	2.25	3.89	2.25	1.61	< .001
Min humidity (%)	45	35	55	65	
Max humidity (%)	90	80	95	95	
Mean Humidity (%)	62.5 ± 18.6	56.1 ± 18.3	73.9 ± 14.6	85.4 ± 11.2	
Friedman Test Mean Rank Humidity	1.93	1.18	3.11	3.79	< .001

WBGT: wet bulb globe temperature. Asymp. Sign P values refer to an Intra-group Friedman ANOVA test for the effect of days.

Walking exercise intensity assessed as percentage of estimated maximal heart rate, for all participants was 76 ± 11 %, with an average heart rate of 108 ± 15 bpm (table 3). Based on this criterion, the exercise was classified as moderate intensity (20). Plasma volume decreased after the first day of walking with -2.2 ± 10.1% ($p = .035$) and increased during the other exercise days ($p = .206$, $p < .001$ and $p < .001$, respectively). Fluid intake varied with 3.9 ± 1.5 L on the first day, 3.8 ± 1.4 L on the second day, 3.8 ± 2.6 L on the third day and 2.0 ± 1.1 L on the last exercise day (table 3).

Table 3. Exercise characteristics presented for all participants at day 1 to day 4

	Day 1	Day 2	Day 3	Day 4	Asymp. Sign.
Walking					
Exercise duration (hrs:min)	8:11 ± 1:06	8:28 ± 1:05	7:55 ± 1:13	7:53 ± 1:25	< .001
Speed (km/h)	3.8 ± 0.5	3.7 ± 0.5	3.9 ± 0.6	4.0 ± 0.7	< .001
Fluid intake (L)	3.9 ± 1.5	3.8 ± 1.4	3.8 ± 2.6	2.0 ± 1.1	< .001
Physical parameters					
Weight change (kg)	-1.02 ± 0.88	-0.68 ± 0.74	-0.38 ± 0.68	0.08 ± 0.58	< .001
Plasma volume change (%)	-2.17 ± 10.10	1.36 ± 7.41	4.07 ± 7.06	7.82 ± 8.16	< .001
Mean heart rate (bpm)	108.1 ± 15.4				
Max heart rate (bpm)	122.6 ± 17.5				
Exercise intensity (%HRmax)	75.7 ± 10.9				

Values are mean values for the 4 walking days. Asymp. Sign P values refer to a repeated measures ANOVA for the effect of days. Weight difference is calculated as post-exercise - pre-exercise, a negative value means weight loss. Plasma volume change is calculated as day # - baseline, plasma volume is calculated with Dill and Costill calculation 1974 (19).

Total and ionized magnesium at baseline

Average baseline levels of tMg (0.85 ± 0.07 mmol/L) and iMg (0.47 ± 0.07 mmol/L) were above the reference value (figure 1). None of the participants had tMg levels below reference value, while 23 participants (34%) had iMg levels below reference value (table 4). The average ratio between ionized and total magnesium was 0.55.

A multiple backward regression was run to predict baseline tMg and iMg values from gender, age, body composition, resting heart rate, energy intake and dietary intake. None of these variables statistically significantly predicted baseline tMg or iMg levels ($p > .05$).

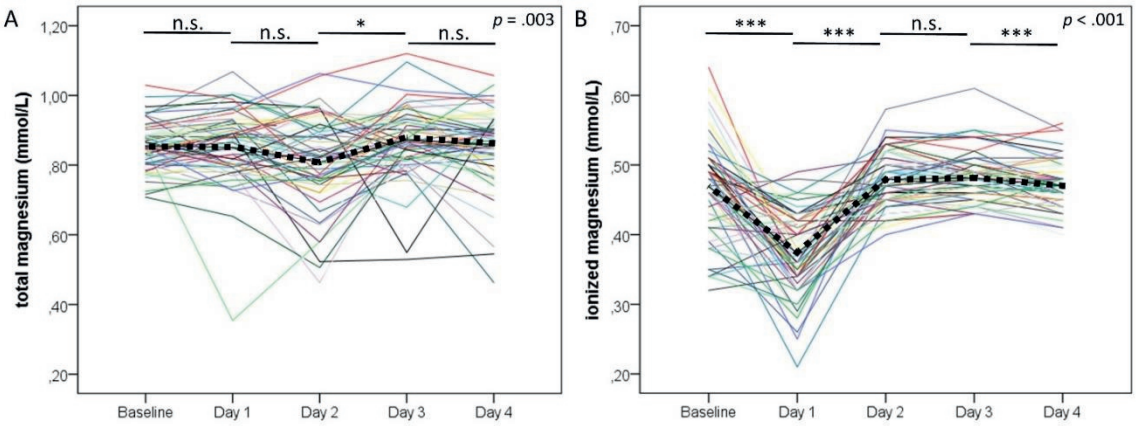

Figure 1. Total (A) and ionized (B) magnesium concentrations (mmol/L) for all 68 participants. Lines represent every single participant, dotted line represents the average magnesium concentration. P values represent a repeated measures ANOVA for the effect of days. Significant differences between days are presented with horizontal lines, with n.s. non-significant, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Table 4. Participants below reference values

	Baseline	Day 1	Day 2	Day 3	Day 4
tMg	(0) 0%	(2) 3%	(11) 16%	(3) 4%	(4) 6%
iMg	(23) 34%	(60) 88%	(18) 26%	(15) 22%	(17) 25%

Amount of participants below reference values of tMg and iMg, presented as (n) %.

Exercise response

Both tMg and iMg levels changed significantly during the four consecutive walking days ($p = .003$ and $p < .001$, respectively) (figure 1).

tMg did not change after the first and second walking day compared to baseline and the previous day ($p = 1.00$ and $p = .086$ respectively). After that, a significant increase in tMg (+ 0.07 ± 0.15 mmol/L, $p = .012$) was measured at day 3 compared to day 2. tMg remained stable after the fourth day of exercise, compared to the third day of exercise ($p = 1.00$).
A significant lower iMg was measured after the first walking day compared to baseline (- 0.10 ± 0.09 mmol/L, $p < .001$). This decline was measured in 78% of the participants.

Furthermore, a significant higher iMg concentration was measured at day 2 compared to day 1 (+ 0.11 ± 0.07 mmol/L, $p < .001$). iMg was stable between day 2 and day 3 ($p = 1.00$), and declined again at day 4 (– 0.01 ± 0.02 mmol/L, $p < .001$). The average ratio between ionized and total magnesium was lowest after the first day of exercise: 0.44. Correcting tMg and iMg levels for plasma volume changes did not affect the observed significant findings during the four exercise days.
After the first day of walking, only 2 participants (3%) had tMg levels below the reference value, while 60 participants (88%) had iMg levels below the reference value. The number of participants below the reference value at walking day 2 recovered for iMg, while it increased stepwise for tMg (table 4).

Variables contributing to iMg decrease after first day of exercise
Mean heart rate during exercise predicted significantly whether iMg decreased after the first day of exercise or not ($F (1, 46) = 7.18$, $p = .010$, $R^2 = .135$). Participants who showed a decrease in iMg after the first day of exercise had a significantly higher mean heart rate (111 ± 15 bpm) compared to the participants who showed no decrease in iMg (97 ± 14 bpm). Independent variables such as sex, age, height, weight, resting heart rate, walking duration and speed, weight and plasma change from baseline till day 1, dietary magnesium intake and total magnesium levels at baseline did not contribute significantly to the decrease in iMg.

DISCUSSION

Although lower Mg blood concentrations and (or) clinically relevant hypomagnesemia have been reported in elderly, our study population of 80+ vital years old showed average tMg and iMg levels that were within the normal range. Remarkably, their blood level patterns of iMg during and after exercise showed similarities with those found earlier in much younger adults undergoing an acute physical exercise protocol (15). The decrease in iMg after the first exercise day which restored to baseline levels during consecutive exercise days suggests a rapid adaptation and points towards redistribution between different Mg body pools.

Total and ionized magnesium at baseline

Older adults are suggested to be more prone to magnesium deficiencies due to the age related reduction in bone mass (8), a decreased magnesium absorption (9), and the frequent use of drugs by this group (10), but studies on Mg levels in older adults are scarce. For the assessment of magnesium status, the measurement of total serum or plasma magnesium (tMg) is most commonly used (11), but its reliability is subject of debate. It is suggested that iMg should be the preferable parameter to evaluate Mg status (14, 21), as iMg is the free active form, involved in cellular processes. Reference values for iMg are lacking, however, suggested is that iMg comprises 60% of the total magnesium amount (17). Therefore, we used a minimum reference value of 0.46 mmol/L for iMg.

Our finding that tMg plasma levels were in the range considered as normal may be due to the fact that our participants are very vital for their age-group and regularly exercising, also because finishing this walking event requires frequent training (22). Average iMg levels were generally within normal range as well, although they were below the assumed reference value in one third of the participants. This led to a ratio (tMg : iMg) of 0.55, while 0.60 – 0.65 is provisionally proposed (16, 17). Whether these reference values for iMg are meaningful for this age group is questionable though. Based on our ratio (0.55) the reference value might be 0.39 mmol/L. Literature about iMg levels in older adults is lacking. Therefore, we are not sure whether this reference value is applicable in this population. More research in older adults is needed to assess what a healthy iMg level is for this age group.

Exercise response

We found a clear decrease in iMg after the first day of exercise for almost all participants, while we did not find this decrease for tMg. This is in contrast to most

studies in athletes, where decreases in both tMg and iMg were reported after one bout of exercise (12-15).

An explanation for the decrease in iMg levels following exercise is an increased uptake in muscle cells and adipose tissue. Catecholamines, like epinephrine and norepinephrine, are produced during exercise and induce iMg uptake into muscle cells and regulate the magnesium dependent Na/K ATPase pumps in skeletal muscle (23). In addition, increased rates of lipolysis, which probably occurs during this type of prolonged moderate intense exercise, increases the uptake of magnesium into adipocytes (24, 25). On consecutive days, catecholamine levels might be lower, explaining the observed recovery in iMg at the second, third and final day of exercise. Another explanation for the recovery in iMg might be resilience and/or fast adaptation to the demanded exercise.

We expected plasma volume changes to cause the different patterns we observed between tMg and iMg. Bound magnesium is not able to migrate passively across the membrane and is therefore influenced by plasma volume changes, while iMg can passively diffuse and is therefore not influenced by plasma volume changes. We indeed found a decrease in calculated plasma volume (from blood haematocrit and haemoglobin concentrations) after the first day of walking and an increase after subsequent days of walking. This is in line with literature, showing that one bout of exercise causes haemoconcentration (26) and repeated endurance exercise causes long term expansion of plasma volume (27). Our data suggest that correcting tMg and iMg values for changes in plasma volume did not alter the observed difference between tMg and iMg. Therefore, something else might have influenced tMg levels as well, which causes it to react differently in this study compared to previous studies. Previous studies used shorter exercise bouts (varying from ~20 minutes to 4 hours) (12-15) compared to our ~8 hour exercise bouts. Whether this explains the decrease in tMg in those studies and the unchanged tMg in our study is questionable though. Nevertheless, a shift between bound and unbound magnesium clearly influenced the levels of tMg and iMg in this study, as ratios at baseline (0.55) decrease after one day of exercise (0.44). Possibly, changed conditions due to prolonged exercise (e.g. blood pH) might have a more profound effect on the equilibrium between bound and unbound magnesium in older adults.

The exercise induced decrease in iMg resulted in levels below the reference value (0.46 mmol/L) in as much as 88% of our participants. tMg levels were lowest at the second walking day, resulting in levels below the reference value in 16% of our participants. It is suggested that symptoms of magnesium deficit may not be manifested until plasma total magnesium levels decrease below 0.50 mmol/L (28).

This would translate to iMg levels below 0.3 mmol/L (60% of tMg). We only had 2 participants on the second walking day with tMg levels below 0.50 mmol/L and they reported no health problems. A total of 88% of our participants had iMg levels below reference value and even 6 of them had iMg levels below 0.30 mmol/L, none of them reported any health problems and/or magnesium deficiency symptoms.

Variables contributing to iMg decrease after first day of exercise

An association between mean heart rate during exercise and the decrease in iMg was found. Previous studies reporting an exercise-induced decrease in magnesium did not investigate which variables contributed to that decrease (12-15). It may be expected that a higher heart rate relates to a higher production of catecholamines, resulting in increased magnesium transport to muscle (23). It would be interesting to study the effects of different exercise intensities on magnesium levels. Unfortunately, studies comparing different exercise intensities and magnesium changes are lacking. However, studies with high intensity exercise, like a progressive treadmill ergometer test until exhaustion (14) and a heavy 90 minutes bicycle ergometer test at 70% VO2max (15) show almost comparable results with iMg decreases of 0.05 mmol/L and 0.06 mmol/L, respectively.

Limitations

A strength of the present study was the inclusion of a large group of participants. This large population enabled to establish rapid adaptation occurring to this type of exercise. Furthermore, our study population, characterised as generally healthy, regularly exercising older-aged persons is very unique and apparently underrepresented in the field of exercise physiology.

The present study had some practical limitations. First, baseline blood samples were not collected at the same time of day across participants, which might introduce some variability in the measurement, caused by a possible circadian rhythm of both ionized and total magnesium (29, 30). However, the within-day variation is much smaller than the decreases we observed between days, suggesting that timing did not affect our results. Secondly, we did not assess magnesium excretion via urine, which could have given us information about magnesium handling of the kidneys during this type of exercise.

In conclusion, these results indicate that in an older adult population, prolonged walking exercise causes an acute decrease in ionized magnesium levels while levels restore after consecutive days of exercise, suggesting rapid adaptation or resilience

in this population. Furthermore, iMg and tMg show different responses to (repeated) exercise.

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Iron
Fe
Ferritin
Transferrin

Chapter 4

Changes in iron metabolism during prolonged repeated walking exercise in middle aged men and women

Changes in iron during repeated exercise

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ABSTRACT

Purpose: the aim of the present study was to assess the effect of prolonged and repeated exercise on iron metabolism in middle-aged adults and to compare differences between sexes. **Methods:** 50 male (58.9±9.9 yr.) and 48 female (50.9±11.2 yr.) individuals were monitored on 4 consecutive days at which they walked on average 8 hours and 44 minutes per day at a self-determined pace. Blood samples were collected one or two days prior to the start of the exercise (baseline) and every day immediately post-exercise. Samples were analysed for iron, ferritin, haemoglobin and haptoglobin concentrations. **Results:** Plasma iron decreased across days, while ferritin increased across days (both $p < .001$). Haptoglobin showed a decrease ($p < .001$) after the first day and increased over subsequent days ($p < .001$). Haemoglobin did not change after the first day but increased during subsequent days ($p < .05$). At baseline, 8% of the participants had iron concentrations below minimum reference value (10 µmol/L), this increased to 43% at day 4. There was an interaction between sex and exercise days on iron ($p = .028$), ferritin ($p < .001$) and haemoglobin levels ($p = .004$), but not on haptoglobin levels. **Conclusion:** This study showed decreases in iron, increases in ferritin, a decrease followed by increases in haptoglobin and no change followed by increases in haemoglobin. This is most likely explained by (foot strike) haemolysis, inflammation, and sweat and urine losses. These processes resulted in iron levels below minimum reference value in a large number of our participants.

KEY WORDS: Hb, Fe, Hp, repetitive exercise

INTRODUCTION

Iron is involved in several processes related to exercise (1-3). Iron deficiency can lead to acute and chronic health problems, and to underperformance (3, 4). *Vice versa*, exercise can affect iron status via various mechanisms, including exercise-induced inflammation (2, 5), foot strike haemolysis (5-7), and losses through sweat or urine (8, 9).

Exercise-induced inflammation may lead to increased ferritin and hepcidin plasma levels (5, 10), which are both categorized as acute phase proteins. The rise in hepcidin levels causes a reduced influx of iron in the circulation through increased degradation of ferroportin (11), which might result in iron deficiency in the long run (12, 13). Next to this, certain types of exercise can lead to foot strike haemolysis. This causes destruction of red blood cells, resulting in the release of haemoglobin and as a consequence a decrease in haptoglobin. Haptoglobin binds free haemoglobin resulting in lower haptoglobin levels due to clearance of this complex. Foot strike haemolysis has for example been reported following 1 hour of running exercise (6), repeated exercise (10 times 1km running) (5) and football-specific training (7). Sweating is another cause of iron loss during exercise (8). And finally, blood loss through urine, i.e. hematuria, and blood loss through the breakdown of muscle, i.e. myoglobinuria, can occur after exercise as well, for example after a 400 m sprint at maximal effort (9). The vast majority of studies on exercise-induced changes in iron status have focused on well-trained athletes, during short and relatively intensive forms of exercise. However, far less is known about the effects of repeated moderate intensity endurance exercise in other populations, including middle-aged recreational sportsmen/women.

Therefore, the aim of the present study was to assess the effect of prolonged and repeated walking exercise on iron parameters in middle-aged men and women.

We hypothesised that iron, ferritin, haemoglobin and haptoglobin levels would change during prolonged and repeated walking exercise. We expected ferritin to increase and iron to decrease. In addition, we expected a decrease in haptoglobin after the first day and an increase at consecutive days, due to (foot strike) haemolysis and inflammation, respectively. Finally, we expected an increase in haemoglobin after the first day and a decrease at consecutive days, due to haemoconcentration and haemodilution, respectively.

MATERIALS AND METHODS

Study population

We selected 50 male and 50 female walkers who participated in the 2015 edition of the Nijmegen Four Days Marches, a large annual walking event taking place in the Netherlands (http://www.4daagse.nl/en/). Exclusion criteria were known diabetes and/or renal dysfunction. The study was approved by the Medical Ethical Committee of the Radboud university medical center (CMO registration number: 2007/148), and all participants gave written informed consent prior to participation. This study was conducted in accordance with the Declaration of Helsinki.

Study procedure

Measurements were performed before the start of the event ('baseline'), and at the four consecutive walking days. Baseline measurements, including recording participant characteristics, a blood sample, and questionnaires, were performed in our field laboratory at the event location one or two days prior to the first walking day, between 09:30 AM and 04:00 PM.

Every walking day, immediately before the start of the march, participants' body weight was determined. Thereafter, participants walked 30, 40 or 50 km, at a self-determined pace, starting between 4:00 and 8:00 AM. Every day, participants registered their fluid intake using a diary. Directly after finishing, post-exercise body weight was determined, a blood sample was taken and questionnaires were completed. Heart rate was measured every 5 km and at the finish during the first walking day using a 2-channel ECG chest band system (Polar Electro Oy, Kempele, Finland). Mean heart rate during exercise was calculated as the average heart rate, excluding the values derived directly before the start and after the finish. Heart rate (HR) was used to estimate exercise intensity: Intensity = (Measured HR / estimated maximal HR) * 100%, with estimated max HR = 208 - (0.7 * Age) (14).

Baseline measurements

At baseline, body weight (Seca 888 scale, Hamburg, Germany) and body height were determined and body mass index (BMI) was calculated. Thereafter, resting heart rate was measured using an automated sphygmomanometer (M5-1 intellisense, Omron Healthcare, Hoofddorp, The Netherlands) after 5 minute supine rest.

Blood samples

Participants were seated for 5 min after which a venous blood sample was taken from the cephalic vein. Blood was collected in a 4 ml Lithium Heparin (LH) gel vacutainer (Becton-Dickinson, New Jersey, America). The vacutainer was centrifuged at 3000G (3755 rpm) for 8 minutes at 22 degrees and plasma was stored at -80 degrees Celsius. Samples were analysed for their iron, ferritin and haptoglobin concentrations in October 2015 (Siemens Dimension Vista 1500, Siemens Healthcare, Erlangen, Germany).

An additional blood sample was collected in a 2 ml LH vacutainer (Becton-Dickinson, New Jersey, USA) and used for direct analyses of plasma haemoglobin and haematocrit concentrations (Rapidpoint 400, Siemens Healthcare Diagnostics Inc., Tarrytown, New York, USA). Relative changes in plasma volume were calculated from blood haematocrit and haemoglobin concentrations using Dill and Costill's equation (15). Iron, ferritin, haptoglobin and haemoglobin levels were corrected for plasma volume changes.

Urine samples

Urine samples were collected after exercise and with the use of a urinary dipstick (Clinitek Status® analyzer, Siemens Healthcare diagnostics Inc., Tarrytown, New York) tested for the presence of erythrocytes, haemoglobin and/or myoglobin.

Questionnaires

All participants completed a questionnaire about the use of supplements to check whether iron concentrations could have been influenced by iron supplement use and for female participants we collected data about their menstrual status. All participants filled out a food frequency questionnaire (FFQ) before the start of the event to estimate dietary intake.

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences 22.0 (IBM SPSS version 22.0, Armonk, New York, USA), with the level of significance set at $p < 0.05$. Data were presented as mean ± SD unless indicated otherwise. A post-hoc power analysis was conducted in GPower (version 3.0.10, Dusseldorf, Germany), revealing a power > .95 for all four parameters.

The Kolmogorov-Smirnov test was used to examine the normality of the data distribution. Participant and exercise characteristics were analysed with an independent Student t-test to examine sex differences. A backward linear regression

was used to analyse which variables contributed significantly to the observed changes in iron, ferritin, haptoglobin and haemoglobin.
Normally distributed data was analysed using ANOVA repeated measures for the effect of consecutive exercise days, with a Bonferroni post hoc correction. A two-way repeated-measures ANOVA was applied to analyse sex differences (sex x days). Non-parametric tests were used for analysing not-normally distributed data. This included a Friedman's test, to evaluate the effect of consecutive exercise days on iron parameters. When significant differences were found, a Wilcoxon signed-rank test was used to determine which consecutive days significantly differed from one another.

RESULTS

Characteristics of the study population

Two female participants did not finish the first day, due to back problems, and were excluded from further analysis. The characteristics of the remaining 98 participants who completed all 4 days are shown in Table 1. Men had a significantly higher age, height, weight, BMI, macronutrient intake, fibre intake, iron intake (total, haem and non-haem), vitamin B6 and B12 intake ($p < .05$) compared to women. There were no differences in resting heart rate, average training distance in the year prior to the marches and folic acid intake between men and women ($p > .05$).

Twenty of our 48 female participants were post-menopausal, 5 women were not sure. We did not collect data about the stage of menstruation for those female participants who were pre-menopausal. There were no significant differences in iron, ferritin, hemoglobin and haptoglobin levels between post-menopausal women and pre-menopausal women at baseline and during walking days. Therefore we treat them as one group for all following data.

Nutritional iron intake and use of supplements

Supplements of any kind were used by 33.6% (n= 37) of our participants. Iron supplements were used by only 1 female participant. Total iron intake for men was significantly higher (13.2 ± 3.0 mg per day) compared to iron intake for women (10.9 ± 3.2 mg per day) ($p < .001$) (RDA is 8 mg/day and 18 mg/day for men and women, respectively (16)) (Table 1). The dietary variables did not significantly contribute to the observed changes in iron, ferritin, haptoglobin or haemoglobin.

Table 1. Participants characteristics

	Men (n = 50)	Women (n = 48)	P value
Characteristics			
Age (years)	58.9 ± 9.9	50.9 ± 11.2	< .001
Body composition			
Height (cm)	180 ± 6	167 ± 6	< .001
Weight (kg)	88.9 ± 13.3	65.6 ± 8.1	< .001
BMI (kg/m^2)	27.3 ± 3.3	23.5 ± 3.0	< .001
Cardio characteristics			
Resting heart rate (bpm)	63 ± 9.9	64 ± 6.9	0.758
Dietary intake			
Energy intake (*10^3 kJ)	10.7 ± 2.8	8.0 ± 2.5	< .001
Total protein (g)	95 ± 25	75 ± 25	< .001
Total fat (g)	104± 35	75 ± 27	< .001
Total carbohydrates (g)	268 ± 78	204 ± 72	< .001
Fibre (g)	27 ± 8	22 ± 8	0.005
Iron intake (via diet)			
Total iron (mg)	13 ± 3	11 ± 3	< .001
Haem iron (mg)	1.2 ± 0. 6	0.9 ± 0.5	0.006
Non haem iron (mg)	12 ± 3	10 ± 3	0.001
Vitamin B6 (mg)	2.1 ± 0.8	1.8 ± 0.7	0.022
Vitamin B12 (µg)	6.0 ± 2.6	4.7 ± 2.4	0.014
Folic acid total (µg)	289 ± 78	257 ± 86	0.059
Walking characteristics			
Training distance (km)	812 ± 1072	753 ± 921	0.77
30 km/day	n = 17	n = 5	
40 km/day	n = 20	n = 39	
50 km/day	n = 13	n = 4	

Means ± SD are shown. BMI: Body mass index; Dietary intake values are estimated with a FFQ questionnaire; Training distance: specific walking distance in the year prior to the 4 Days Marches; *P* Value refers to an unpaired Students *t* test between men and women

Exercise characteristics

Walking exercise intensity assessed as percentage of estimated maximal heart rate, for all participants was 67 ± 9 %, with an average heart rate of 114 ± 17 bpm (Table 2). Exercise intensity, was not different between sexes (p = .679). Male participants walked 30 km (n=17), 40 km (n=20) and 50 km (n=13). Female participants walked the same distances (n=5, n=39 and n=4, respectively).

Table 2. Exercise characteristics presented for men and women

		Day 1	Day 2	Day 3	Day 4	Asymp. Sign.
Walking						
Exercise duration	Men	7:52 ± 2:32	8:32 ± 1:31	8:23 ± 1:45	8:51 ± 2:03	0.001
(hrs:mns)	Women	8:43 ± 1:04	9:05 ± 1:16	8:41 ± 2:15	9:45 ± 1:32	< .001
	P value	0.032	0.054	0.459	0.017	
Speed (km/h)	Men	4.8 ± 0.8	4.6 ± 0.8	4.7 ± 0.7	4.5 ± 0.8	0.003
	Women	4.6 ± 0.6	4.4 ± 0.7	4.5 ± 0.6	4.2 ± 0.6	< .001
	P value	0.235	0.186	0.046	0.009	
Fluid intake (L)	Men	4.4 ± 1.6	4.4 ± 1.5	4.3 ± 1.3	2.8 ± 1.1	< .001
	Women	4.1 ± 1.7	3.8 ± 1.5	3.6 ± 1.6	2.6 ± 1.0	< .001
	P value	0.367	0.034	0.015	0.397	
Physical parameters						
Weight difference	Men	- 1.4 ± 1.0	- 0.8 ± 0.7	-0.5 ± 0.6	-0.9 ± 0.8	< .001
(kg)	Women	- 0.3 ± 0.7	-0.3 ± 0.5	-0.3 ± 0.5	-0.5 ± 0.5	0.009
	P value	0	0	0.158	0.016	
Plasma volume	Men	-4.29 ± 12.21	-1.93 ± 8.34	3.62 ± 10.37	6.05 ± 8.98	< .001
change	Women	0.00 ± 5.91	3.40 ± 6.25	7.82 ± 7.45	8.67 ± 7.08	< .001
	P value	0.03	0.001	0.024	0.113	
Mean heart rate	Men	113 ± 18				
(bpm)	Women	115 ± 15				
	P value	0.512				
Max heart rate	Men	124 ± 27				
(bpm)	Women	129 ± 18				
	P value	0.26				
Exercise intensity	Men	67 ± 11				
(%HR_{max})	Women	67 ± 8				
	P value	0.679				

Values are mean values for the 4 walking days. P Values refers to an unpaired Students t test between men and women. Asymp. Sign P values refer to an Intra-group Friedman ANOVA test for the effect of days. Weight difference is calculated as post-exercise - pre-exercise, a negative value means weight loss. Plasma volume change is calculated as day # - baseline, plasma volume is calculated with Dill and Costill calculation 1974 (15).

Exercise duration and speed were different between men and women, as well as plasma volume changes (Table 2). Exercise intensity significantly contributed to the observed change in ferritin. Exercise intensity and duration did not significantly contribute to the observed changes in iron, haptoglobin or haemoglobin.

Laboratory parameters

Baseline iron concentrations were significantly higher in men compared to women (Table 3), while 11% of the study population had a plasma iron concentration below the minimum reference value of 10 µmol/L. A significant decrease in iron concentrations was observed across days (Figure 1A) ($p < .001$), with a larger decrease in men compared to women after the first walking day (Interaction = .028). Iron concentrations for men fluctuated, with a significant decrease after the first day of exercise, and no significant changes after that, while women showed a continuous decrease over days, although this was not significant between days. The percentage of participants below reference value increased significantly from 1% to 28% in men and from 10% to 52% in women, from baseline to the last exercise day.

Ferritin concentrations were significantly higher in men compared to women (Table 3). A significant increase in ferritin concentrations was observed across days (Figure 1B) ($p < .001$), with varying changes in men and women across days (Interaction < .001). Ferritin concentrations increased all walking days in men, while it decreased on the final walking day in women.

Haptoglobin concentrations at baseline were not significantly different between men and women (Table 3). A significant decrease in haptoglobin concentrations was observed after the first day of exercise, followed by an increase over subsequent days (Figure 1C) ($p < .001$), with no differences in change between men and women (Interaction = .249).

Haemoglobin concentrations at baseline were significantly higher in men compared to women (Table 3). A significant change in haemoglobin concentrations was observed across days (Figure 1D) ($p < .001$), with no change after the first day of walking, and increases over subsequent days. Increases in men were larger compared to women (Interaction = .004).

Table 3. Mean laboratory parameter concentrations for men and women separately, at baseline, and day 1 until day 4. n = 98.

		Baseline	Day 1	Day 2	Day 3	Day 4	Asymp. Sign.
Iron (µmol/L)	men	18.8 ± 8.1	14.1 ± 5.1	14.5 ± 6.1	13.4 ± 5.9	13.7 ± 5.8	< .001
	women	15.9 ± 5.9	14.5 ± 4.9	12.5 ± 5.0	11.2 ± 4.1	10.0 ± 4.1	< .001
	p-value	0.045	0.708	0.092	0.041	0.001	
Ferritin (µg/L)	men	137.7 ± 87.0	143.2 ± 99.9	157.3 ± 100.2	169.0 ± 107.0	173.6 ± 113.5	< .001
	women	64.0 ± 48.2	64.9 ± 48.4	73.7 ± 50.8	77.8 ± 54.0	75.6 ± 54.0	< .001
	p-value	0	0	0	0	0	
Haptoglobin (g/L)	men	1.18 ± 0.47	0.93 ± 0.48	1.03 ± 0.54	1.20 ± 0.57	1.30 ± 0.62	< .001
	women	1.06 ± 0.35	0.87 ± 0.34	0.97 ± 0.40	1.11 ± 0.44	1.17 ± 0.47	< .001
	p-value	0.197	0.516	0.575	0.378	0.237	
Haemoglobin (g/dL)	men	15.9 ± 1.1	15.5 ± 1.5	15.8 ± 1.5	16.2 ± 1.7	16.4 ± 1.6	0.022
	women	14.2 ± 0.9	14.2 ± 1.0	14.4 ± 1.1	14.7 ± 1.2	14.7 ± 1.2	< .001
	p-value	0	0	0	0	0	

Values are mean ± SD for baseline and the four walking days. Values are corrected for plasma volume changes. P values refer to an unpaired Students t test between male and female participants. Asymp. Sign P values refer to an Intra-group Friedman ANOVA test for the effect of days for non-parametric data and a repeated measures ANOVA for the effect of days on parametric data.

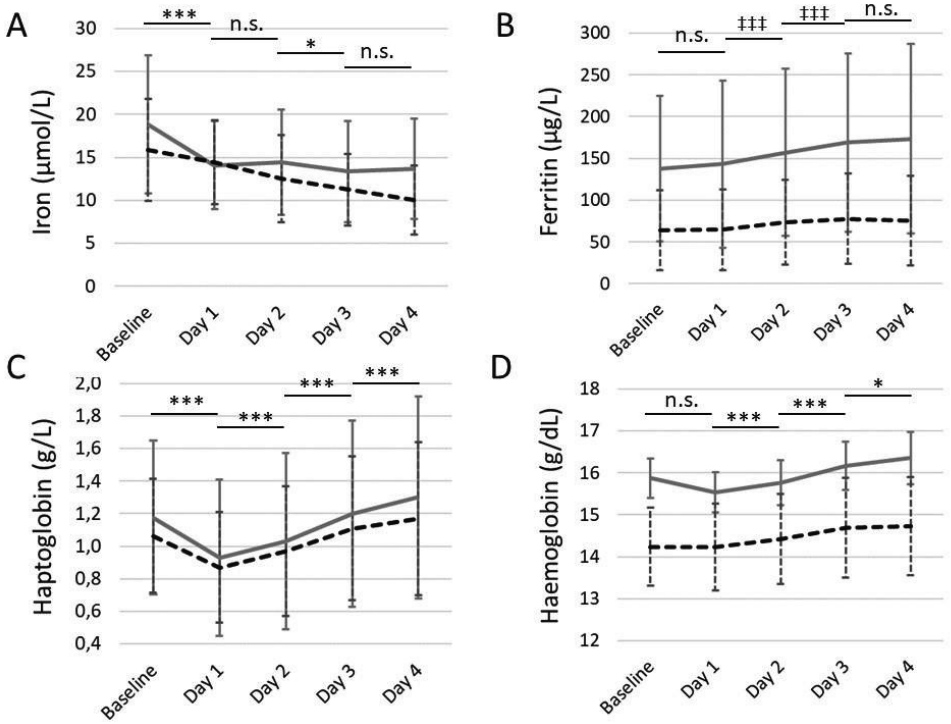

Figure 1. Iron (A), ferritin (B), haptoglobin (C) and haemoglobin levels (D) for men (grey continuous line) and women (black dotted line). Means ± SD are shown. Significant differences between days for all participants together are presented with horizontal lines, with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for repeated measures ANOVA, with ‡ $p < 0.05$, ‡‡ $p < 0.01$, and ‡‡‡ $p < 0.001$ for Friedman test.

Iron losses via urine

The number of participants with iron loss in urine was 26% at baseline, which increased during the 4 walking days (Table 4). At the final walking day, 54% of the participants had iron loss through urine.

Table 4. Iron loss via urine.

Parameter	Baseline	Day 1	Day 2	Day 3	Day 4
Negative, n (%)	72 (74)	50 (51)	54 (55)	52 (53)	45 (46)
Positive, n (%)	26 (26)	48 (49)	44 (45)	46 (47)	53 (54)

DISCUSSION

The changes in biochemical parameters found in our study population are consistent with the occurrence of an inflammatory response, haemolysis and iron losses during this prolonged and repeated exercise. We found a cumulative decrease in iron, an increase in ferritin, a decrease in haptoglobin after one day of walking followed by an increase on subsequent days and an increase in haemoglobin from walking day 2 until 4. The implications of these results are discussed below.

Iron

Our finding that iron levels decreased after one day of exercise are in line with results reported after a marathon (17) and a 100 km ultramarathon (18). On the other hand, opposite findings with increased iron levels after a triathlon (19), and a marathon (20) have been reported as well. These increased iron levels are probably caused by decreased plasma volumes. In contrast to previous studies, we corrected our results for changes in plasma volumes, and observed a decrease in iron levels after the first day.

Changes in iron levels during this type of exercise are probably related to inflammation and loss via urine. In a previous study, using the same study population, we showed that inflammation was evident by elevated cytokine levels after the first day of walking (not yet published work). This included IL-6 which has been shown to enhance hepcidin levels (21). Hepcidin is also known as acute phase protein, reducing iron influx into the circulation by binding to ferroportin and inducing its internalization (11). This could partly explain our observed decrease in iron.

In addition, we found an increase in iron loss through urine, which might also contribute to decreased iron levels. We could not distinguish between iron losses through urine by haemoglobinuria and/or myoglobinuria since the dipsticks used gave a positive test result for both. Nevertheless, we showed that this type of exercise caused iron losses through urine.

We observed a further decrease in iron after repeated days of walking exercise, which was in contrast to previous studies (22, 23). These studies were of longer duration (weeks), while we assessed the effect of four days of exercise on iron levels.

The continuous decrease in iron may be related to urinary iron loss, which continued over the four days, while inflammation may still play a role, as mentioned above.

Ferritin

We found no significant increase in ferritin after the first day of exercise, which was in contrast to results reported after a 100 km ultramarathon (18), a triathlon race (24) and a 2000 m rowing ergometer test (25). Exercise intensity differed a lot between these studies and our walking exercise. Previous studies were all-out efforts, while our walking exercise was at a moderate intensity. This could explain the observed differences.

We found a significant increase in ferritin after the second and third day of walking exercise, which was in line with previous studies (26, 27). Ferritin also belongs to the acute phase proteins, showing increased levels during inflammation (2), which was found to occur during this walking exercise (unpublished work). In addition, ferritin synthesis is increased in response to oxidative stress (28), and ferritin levels increase after leakage from damaged cells (29), like peripheral (and intestinal) cells (30). Both mechanisms possibly occurred during this event. Previous studies have shown that, for example, gastrointestinal blood loss may occur during exercise (31).

Haptoglobin and haemoglobin

We found a decrease in haptoglobin after the first day of walking, which was in line with previous studies concerning running exercise (5, 6). Haptoglobin decrease has been linked to haemolysis. Haemoglobin from damaged red blood cells is released into plasma, causing oxidative stress. This is limited by haptoglobin, which forms a complex with haemoglobin (32). The haptoglobin-haemoglobin complex is cleared from the circulation by binding to CD163 haemoglobin scavenger receptors on the surface of macrophages. After that, the complex is taken up by endocytosis in the liver (32).

Interestingly, we found larger decreases in plasma haptoglobin compared to previous studies (5, 6). Probably, the long distance and duration of the exercise in our study (~9 hours) caused a higher degree of foot strike haemolysis compared to these shorter exercises (10 km or 1 hour, respectively) (5, 6). Our measured decrease in haptoglobin levels was comparable with depressed levels reported directly after marathon running (33) and a 160 km ultramarathon (34).

We found increased levels of haptoglobin after repeated walking days. This can be explained by the ongoing inflammation causing haptoglobin, which is a positive acute phase protein (35), to increase. To our knowledge, studies reporting changes in haptoglobin after repeated exercise are scarce. One study showed an opposite result, with a cumulative effect of consecutive running sessions on haemolysis shown by a greater decrease in haptoglobin during a second running session (5). In

that specific study, inflammation was not affected by the second running session, resulting in further decreases in haptoglobin (5).
In contrast to previous studies on prolonged walking exercise (Gilligan et al. 1950; Hornbostel et al. 1970) we found no significant change in haemoglobin after the first day of walking exercise and significant increases on subsequent days. Decreases in haemoglobin levels are often mistaken for anaemia, while most of these changes are probably caused by changes in plasma volume. An acute bout of exercise mostly results in temporary haemoconcentration (36), while repeated exercise results in expansion of plasma volume (37).
Indeed, our data prior to correction for plasma volume changes showed an increase in haemoglobin after the first day of walking and a decrease after subsequent days of walking. This is in line with our observed plasma volume changes, which showed haemoconcentration after the first day of walking exercise and haemodilution after subsequent days of walking exercise. Therefore, it is important to take plasma volume changes into account during these types of exercise studies.

Differences between men and women

We found comparable haptoglobin changes between men and women, which is in line with results from a marathon race (38), suggesting that foot strike haemolysis occurred in both groups. On the other hand, ferritin, haemoglobin and iron changes were different between men and women, which has not been not reported in detail before. The magnitude of the increases and decreases differed between men and women, however, the overall trend in all parameters was roughly comparable. Therefore, we suggest that mechanisms, like inflammation, haemolysis and blood losses were present in both male and female walkers. Why the magnitude of changes differs between men and women is unknown, but it could be related to differences in exercise intensity, sweat rate and body composition between men and women.
Interestingly, iron levels and changes in our middle aged women were comparable with female athletes, who are studied more often in relation to iron and exercise (39-41), as they are at risk for an iron deficiency with and without anaemia (42). This is mainly caused by iron losses during menstruation. A large number of our female participants were already postmenopausal. Therefore, one might expect smaller differences in the iron levels between our male and female participants. However, that was not the case, suggesting that (older) female adults participating in sports should also be aware of this decrease in iron levels during exercise.

Strengths and limitations

The present study had some limitations which are mainly due to practical reasons. First, we did not include a pre-exercise blood withdrawal in the early morning. Instead we had blood drawings at baseline.

A strength of the present study was the large number of participants in which we were able to study the effect of repeated exercise. Furthermore, our study population, characterised as generally healthy, regularly exercising middle-aged persons is quite unique and apparently underrepresented in the field of exercise physiology.

Conclusion

The present study shows decreases in iron, increases in ferritin, a decrease followed by increases in haptoglobin and no change followed by increases in haemoglobin. These changes are most likely the result of (foot strike) haemolysis, inflammation and sweat and urine losses occurring during this type of exercise. These processes during exercise resulted in iron levels below minimum reference value in a large number of our participants.

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DE 4 DAAGSE
Cytokines

Chapter 5

Changes in cytokine levels after prolonged and repeated moderate intensity exercise in middle-aged men and women

Exercise induced changes in cytokine levels

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ABSTRACT

Previous studies have shown that exercise-induced changes in cytokine profiles depend on exercise duration and intensity. Studies are generally limited to a single day and insight in the time-course during multiple days of exercise is lacking. Therefore, this study assessed cytokine responses during multiple days of moderate intensity exercise in men and women. Fifty males (58.9±9.9 years) and fifty females (50.9±11.2 years) were monitored on 4 consecutive days at which they walked on average ~9 hours per day at a self-determined pace. Blood samples were collected one or two days prior to the start of the exercise (baseline) and every walking day immediately post-exercise. Blood samples were analysed for IL-6, IL-8, IL-10, IL-1β and TNF-α concentrations. All cytokine concentrations increased from baseline to post-exercise at day 1 ($p < 0.001$). Thereafter, concentrations decreased from day 1 to day 2 ($p < 0.01$), remaining rather stable during the next days. IL-1β and TNF-α were higher in men at baseline and during all days. In conclusion, exercise-induced cytokine increases attenuated on subsequent days, although daily workload remained constant. Men and women showed different baseline levels but similar exercise responses. These results suggest that individuals adapt rapidly to this type of repeated exercise.

KEY WORDS: Myokines, Interleukins, IL-1beta, TNF-alpha, repetitive exercise

INTRODUCTION

In response to exercise, various signalling proteins are released by the immune and the musculoskeletal systems (1). Many of these molecules are typically classified as cytokines and mainly known for their immune-regulatory roles. Those which are also, or exclusively, produced by skeletal muscle are often referred to as 'myokines' (2). The best studied cytokine in relation to exercise is interleukin-6 (IL-6), which can act as both anti- and pro-inflammatory cytokine. After exercise, IL-6 concentrations in blood can increase up to 100-fold (3). IL-6 has a positive effect on glucose uptake and fat oxidation (4, 5), and, when it is considered as an anti-inflammatory cytokine, IL-6 attenuates the production of TNF-α and IL-1β (5). Pro-inflammatory cytokines that show an increase after long and/or heavy exercise include TNF-α and IL-1β (3), which are known to be involved in the acute phase reaction and cell proliferation, respectively. Other cytokines that may increase upon exercise are IL-8 and IL-10 (6).

Previous studies demonstrated that cytokine concentrations after a single bout of exercise are influenced by the intensity and duration of exercise (7). For example, a 6-hour run or a marathon increased IL-6 and IL-8 plasma concentrations (7-9), while these elevations were not found after a 5-km run (10). In addition, high intensity interval exercise induced significant increases in IL-8 and IL-10 concentrations, whereas moderate intensity interval exercise had no effect on both cytokines (6).

Almost all previous studies investigated the effect of a single bout of exercise, while health- and performance-enhancing effects of exercise are predominantly apparent after repeated bouts of exercise. Recently in our lab, we showed that repeating exactly the same bout of cycling exercise after one week resulted in an attenuated cytokine response in well-trained young men, suggesting an adapted response (11). Whether such an adaptation also occurs when exercise is performed on consecutive days, or whether cytokines accumulate over time, is not clear from the literature. Suzuki et al. showed that 3 consecutive days of 90-min bicycling exercise per day increased levels of IL-6 after the first exercise bout, while these levels remained elevated until day 3 (12). A study investigating the effects of an Ironman marathon showed that IL-6 levels remained elevated (+345%; $p < .001$) for more than 24 hours after finishing, before returning to baseline (13), suggesting that accumulation may occur when a second bout of exercise would have been performed within this time frame.

Furthermore, it is suggested that the cytokine response to exercise may differ according to sex (14). As contracting skeletal muscles are an important source of IL-

6, a higher muscle mass in men could result in higher concentrations of this cytokine compared to women (15). This effect of muscle mass is clearly illustrated in exercise studies considering small muscle groups, e.g. the muscles of the upper extremities, where IL-6 concentrations were not detected immediately after exercise (16). This would suggest that the immune response to exercise may differ between men and women. However, direct comparisons between men and women in this respect are scarce in literature.

Therefore, the aim of the present study was to assess changes in circulating cytokine levels after long-distance walking (30-50 km) at moderate intensity on four consecutive exercise days in middle-aged men and women. In addition, the differences in responses between men and women were explored. We hypothesized that (at least some) cytokines would accumulate over four days of repeated prolonged moderate intensity exercise, due to the short recovery period. Secondly, we expected higher cytokine levels in men compared to women.

MATERIALS AND METHODS

Study population

We selected 50 male and 50 female walkers who participated in the 2015 edition of the Nijmegen Four Days Marches, a large annual walking event taking place in the Netherlands (http://www.4daagse.nl/en/). Exclusion criteria were known diabetes and/or renal dysfunction. The study was approved by the Medical Ethical Committee of the Radboud university medical center (CMO registration number: 2007/148), and all participants gave written informed consent prior to participation. This study was conducted in accordance with the Declaration of Helsinki.

Study procedure

The study took place in the summer season, i.e. July. Actual climatological conditions are specified in the results section. Measurements were performed before the start of the event ('baseline'), and at the four consecutive walking days. Baseline measurements, including registering participants' characteristics, collection of a blood sample and taking questionnaires, were performed in our field laboratory at the event location one or two days prior to the first walking day, between 09:30 AM and 04:00 PM.

Every walking day, immediately before the start of the march, participants' body weight was determined. Thereafter, participants walked 30, 40 or 50km, at a self-determined pace, starting between 4:00 and 8:00 AM. The walking track was almost completely flat, typical for the Dutch landscape with only small height differences for example for crossing bridges. Details about the routes can be found at https://www.4daagse.nl/en/routes/route-distance.html. Every day, participants registered their fluid intake using a diary. Directly after finishing, post-exercise body weight was determined, a blood sample was taken and a set of questionnaires was completed. Heart rate was measured during the first walking day every 5 km and at the finish. Mean heart rate during exercise was calculated as the average heart rate, excluding the values derived directly before the start and after the finish. Heart rate was used to estimate exercise intensity as percentage of HRmax = (Measured HR / Expected maximal HR) * 100%, with Expected max HR = 208 - (0.7 * Age) (17).

Baseline measurements

At baseline, body weight (Seca 888 scale, Hamburg, Germany) and body height were determined and body mass index (BMI) was calculated. Thereafter, resting heart rate and blood pressure were measured using an automated sphygmomanometer (M5-1

intellisense, Omron Healthcare, Hoofddorp, The Netherlands) after 5 minute supine rest.

Blood samples

Blood samples were taken at baseline and post-exercise at the four consecutive walking days. Participants were seated for 5 min after which a venous blood sample was taken from the cephalic vein. Blood was collected in a 4 ml EDTA vacutainer (Becton-Dickinson, New Jersey, USA). The vacutainer was immediately put on melting ice water (0 – 4 degrees Celsius) and centrifuged at 1200G for 15 minutes at 4 degrees Celsius. Plasma was transferred to polypropylene tubes and stored at -80 degrees Celsius until analysis. We measured IL-6, IL-8, IL-10, IL-1β, and TNF-α concentrations using the ultrasensitive MesoScale Discovery (MSD) QuickPlex SQ 120 Instrument with Multi-spot assay (Human Proinflammatory Panel 1, K15049D, MSD) according to the manufacturers' instructions. The lower detection limit varies per plate and was 0.029–0.159, 0.025–0.051, 0.021–0.042, 0.008–0.061, and 0.034–0.079 pg/ml for IL-6, IL-8, IL-10, IL-1β, and TNF-α, respectively. For cytokine concentrations below the lower detection limit, this lower detection limit was divided by two, assuming that data below the detection limit were normally distributed (18). Sixty out of 490 samples (<15%) for IL-1β were below the lower detection limit. The other cytokines were all above detection limit.

Precision of these validated kits was as follows: The intra-run % CV for the high-low controls were 3.6-4.5%, 2.7-3.0%, 2.6-3.7%, 3.3-4.1% and 2.7-3.4% for IL-6, IL-8, IL-10, IL-1β and TNF-α, respectively. The Inter-run % CV for the high-low controls were 5.2-7.3%, 5.0-7.1%, 5.7-10.1%, 5.5-7.7% and 6.1-10.1 % for IL-6, IL-8, IL-10, IL-1β and TNF-α, respectively. With the high-low concentrations being as follows: 239-18.4 pg/ml, 166-12.5 pg/ml, 107-7.18 pg/ml, 152-11.2 pg/ml, and 75.5-4.45 pg/ml, for IL-6, IL-8, IL-10, IL-1β and TNF-α, respectively.

Furthermore, an additional blood sample was collected in a 2 ml Lithium Heparin vacutainer (Becton-Dickinson, New Jersey, USA) and used for direct analyses of plasma haemoglobin and haematocrit concentrations (Rapidpoint 400, Siemens Healthcare Diagnostics Inc., Tarrytown, New York, USA). Relative changes in plasma volume were calculated from blood haematocrit and haemoglobin concentrations using Dill and Costill's equation (19).

Questionnaires

All participants completed an online questionnaire before the event, which included a food intake questionnaire (Food Frequency Questionnaire, FFQ), a validated Short

Questionnaire to Assess Health enhancing physical activity (SQUASH) and four questionnaires at baseline and the same four questionnaires at the end of every walking day. Relevant to the present study was a questionnaire about the use of painkilling drugs to check whether measured inflammatory markers were influenced by non-steroidal anti-inflammatory drugs (NSAID) use. The other 3 questionnaires were related to other ongoing studies and involved questions about mood states, symptoms related to upper respiratory tract infections and use of supplements. The total amount of physical activity level (PAL) in Metabolic Equivalent (MET) hours per week (MET-hr/wk) was calculated by multiplying the exercise time in hours with the accompanying MET score of the activity intensity. We incorporated commuting activities, leisure time activities and sports to assess activities of daily living (*i.e.*, total physical activity).

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences 22.0 (IBM SPSS version 22.0, Armonk, New York, USA), with the level of significance set at $p < 0.05$. Data are presented as mean ± SD unless indicated otherwise.

The Kolmogorov-Smirnov test was used to examine the normality of the data distribution. As cytokine data were not normally distributed, non-parametric tests were used for analysis. A Friedman's test was used to evaluate the effect of consecutive exercise days on cytokine concentrations. Wilcoxon signed-rank test was used to determine whether data from consecutive days significantly differed from one another. A Mann Whitney test was used to assess differences in cytokine response between men and women. Participant and exercise characteristics were analysed with an independent Students t-test to examine sex differences.

RESULTS

Participant characteristics

Two of our female participants did not finish the first day, due to back problems, and were excluded from further analysis. The characteristics of the remaining 98 participants who completed all 4 days are shown in Table 1. Significant differences between men and women were found for age, height, weight, BMI, blood pressure, physical activity level, energy, fibre, vitamin A and E intake (estimated with FFQ). There were no differences in resting heart rate, macronutrient intake and average training distance in the year prior to the Marches and vitamin C intake.

Exercise characteristics

The Four Days Marches took place under mild ambient conditions, with temperatures varying from 13 degrees Celsius wet bulb globe temperature (WBTG) at the start of the exercise (i.e., 4:00 AM) to 24 degrees Celsius at finish time. Exercise intensity determined at the first walking day, was found to be 67 ± 9% of the expected maximal heart rate, with an average of 114 ± 17 bpm (Table 2). No difference was found between male and female participants (p=.679). Based on this criteria, the exercise was classified as moderate intensity (20).

Male participants walked 30km (n=17), 40km (n=20) or 50km (n=13). Female participants walked the same distances, but these were differently divided among persons (n=5, n=39 and n=4, respectively). Mean exercise duration was longer for women compared to men during the first and last day ($p = .032$ and $p = .017$, respectively). Speed reduced significantly during the four days of walking for both men and women ($p = 0.003$ and $p > .001$, respectively). On average, the highest speed was measured on the first walking day, for both men (4.8 ± 0.8 km/h) and women (4.6 ± 0.6 km/h), followed by the third day and thereafter the second day. The slowest speed was measured at the final walking day for both men (4.5 ± 0.8 km/h) and women (4.2 ± 0.6 km/h). Speed was significantly different between men and women (4.7 ± 0.7 vs 4.5 ± 06, and 4.5 ± 0.8 vs. 4.2 ± 0.6 km/h) at day 3 and 4 ($p = .046$ and $p = .009$, respectively, Table 2).

Table 1. Participants characteristics

	Men (n = 50)	Women (n = 48)	P value
Characteristics			
Age (years)	58.9 ± 9.9	50.9 ± 11.2	< .001
Body composition			
Height (cm)	180 ± 6	167 ± 6	< .001
Weight (kg)	88.9 ± 13.3	65.6 ± 8.1	< .001
BMI (kg/m^2)	27.3 ± 3.3	23.45 ± 3.0	< .001
Cardio characteristics			
Resting heart rate (bpm)	63 ± 9.9	64 ± 6.9	0.758
Systolic pressure (mmHg)	142 ± 18	133 ± 21	0.036
Diastolic pressure (mmHg)	88 ± 10	82 ± 11	0.014
PAL score (MET-hr/wk)	1016 ± 548	1272 ± 609	0.033
Dietary intake			
Energy intake (kJ)	10730 ± 2811	8024 ± 2502	< .001
Protein (En%)	14.9 ± 2.4	15.8 ± 2.5	0.09
Fat (En%)	36.2 ± 6.0	35.0 ± 5.5	0.308
Carbohydrates (En%)	41.8 ± 6.7	42.3 ± 5.3	0.716
Fibre (g)	27 ± 7.8	22.3 ± 8.1	0.005
Anti-oxidant intake (via diet)			
Retinol (µg)	791 ± 437	491 ± 285	< .001
Vitamin E (mg)	16.9 ± 5.4	13.6 ± 5.9	0.005
Vitamin C (mg)	112 ± 56	114 ± 54	0.871
Walking distances			
Training distance (km)	812 ± 1072	753 ± 921	0.77
Walking 30 km	n = 17	n = 5	
Walking 40 km	n = 20	n = 39	
Walking 50 km	n = 13	n = 4	

Means ± SD are shown. BMI: Body mass index; PAL score: Physical Activity level in MET-hours per week; Dietary intake values are estimated with a FFQ questionnaire, included in the online questionnaire; kJ: kilo joule; En%: percentage of energy delivered by that macronutrient; Training distance: specific walking distance in the year prior to the 4 Days Marches; *P* Value refers to an unpaired Students *t* test between male and female participants

Table 2. Exercise characteristics presented for men and women at day 1 to day 4, for men and women separately

		Day 1	Day 2	Day 3	Day 4	Asymp. Sign.
Walking						
Exercise duration	Men	7:52 ± 2:32	8:32 ± 1:31	8:23 ± 1:45	8:51 ± 2:03	0.001
(hr:mn)	Women	8:43 ± 1:04	9:05 ± 1:16	8:41 ± 2:15	9:45 ± 1:32	< .001
	P value	0.032	0.054	0.459	0.017	
Speed	Men	4.8 ± 0.8	4.6 ± 0.8	4.7 ± 0.7	4.5 ± 0.8	0.003
(km/h)	Women	4.6 ± 0.6	4.4 ± 0.7	4.5 ± 0.6	4.2 ± 0.6	< .001
	P value	0.235	0.186	0.046	0.009	
Fluid intake	Men	4.4 ± 1.6	4.4 ± 1.5	4.3 ± 1.3	2.8 ± 1.1	< .001
(L)	Women	4.1 ± 1.7	3.8 ± 1.5	3.6 ± 1.6	2.6 ± 1.0	< .001
	P value	0.367	0.034	0.015	0.397	
Physical parameters						
Weight change	Men	- 1.4 ± 1.0	- 0.8 ± 0.7	-0.5 ± 0.6	-0.9 ± 0.8	< .001
(kg)	Women	- 0.3 ± 0.7	-0.3 ± 0.5	-0.3 ± 0.5	-0.5 ± 0.5	0.009
	P value	< .001	< .001	0.158	0.016	
Plasma volume	Men	-4.29 ± 12.21	-1.93 ± 8.34	3.62 ± 10.37	6.05 ± 8.98	< .001
change (%)	Women	0.001 ± 5.91	3.40 ± 6.25	7.82 ± 7.45	8.67 ± 7.08	< .001
	P value	0.03	0.001	0.024	0.113	
Mean heart rate	Men	113 ± 18				
(bpm)	Women	115 ± 15				
	P value	0.512				
Max heart rate	Men	124 ± 27				
(bpm)	Women	129 ± 18				
	P value	0.26				
Exercise intensity	Men	67 ± 11				
(%)	Women	67 ± 8				
	P value	0.679				

Values are mean values for the 4 walking days. P Values refers to an unpaired Students t test between male and female participants. Asymp. Sign P values refer to an Intra-group Friedman ANOVA test for the effect of days. Weight difference is calculated as post-exercise - pre-exercise, a negative value means weight loss. Plasma volume change is calculated as day # - baseline, plasma volume is calculated with Dill and Costill calculation 1974.

Cytokines

Baseline

Baseline IL-1β and TNF-α concentrations were significantly higher in men compared to women ($p < .01$, Table 3). Baseline IL-6, IL-8 and IL-10 concentrations were not different between men and women ($p = .146$, .963 and .134, respectively, Table 3).

Table 3. Mean cytokine concentrations (pg/ml) at baseline and day 1 to day 4, for men and women separately.

		baseline	day 1	day 2	day 3	day 4	Asymp. Sign.
IL-6	Men	0.60 ± 0.34	7.42 ± 4.74	3.94 ± 1.98	2.94 ± 1.75	3.54 ± 2.93	< .001
	Women	0.51 ± 0.32	7.58 ± 7.62	3.16 ± 2.07	2.88 ± 2.33	3.97 ± 4.19	< .001
	P value	0.146	0.268	0.015	0.227	0.877	
IL-8	Men	8.00 ± 2.81	12.14 ± 4.38	9.55 ± 3.56	8.19 ± 3.70	7.85 ± 3.31	< .001
	Women	7.78 ± 2.50	10.66 ± 3.18	8.28 ± 2.25	6.92 ± 2.01	7.18 ± 1.74	< .001
	P value	0.963	0.064	0.105	0.121	0.729	
IL-10	Men	0.23 ± 0.22	0.66 ± 0.84	0.29 ± 0.26	0.28 ± 0.27	0.33 ± 0.44	< .001
	Women	0.23 ± 0.38	0.38 ± 0.40	0.23 ± 0.14	0.30 ± 0.48	0.24 ± 0.21	< .001
	P value	0.134	0.032	0.117	0.771	0.486	
IL-1β	Men	0.24 ± 0.37	0.30 ± 0.39	0.32 ± 0.39	0.37 ± 0.74	0.18 ± 0.08	< .001
	Women	0.14 ± 0.13	0.19 ± 0.20	0.15 ± 0.16	0.18 ± 0.31	0.11 ± 0.12	< .001
	P value	0.002	< .001	< .001	< .001	< .001	
TNF-α	Men	1.76 ± 0.49	1.92 ± 0.65	1.80 ± 0.48	1.78 ± 0.63	1.81 ± 0.61	0.001
	Women	1.51 ± 0.34	1.58 ± 0.30	1.51 ± 0.30	1.46 ± 0.31	1.51 ± 0.31	< .001
	P value	0.006	0.002	0.001	0.001	0.004	

P-value represents Mann-Whitney test for differences between men and women. Asymp. Sign P values refer to an Intra-group Friedman ANOVA test for the effect of days.

Consecutive exercise days

A significant change was seen for all cytokines during the four consecutive walking days (all p-values < .001). After the first day of exercise, IL-6, IL-8, IL-10, IL-1β and TNF-α were all increased compared to baseline cytokine concentrations (all p-values < .001). A 13-fold increase was observed for IL-6, and a 1.4-fold increase for IL8, 2.3-fold for IL-10, 1.3-fold for IL-1β and 1.1-fold for TNF-α (Figure 1). After the second walking day, IL-6, IL-8, IL-10, IL-1β and TNF-α concentrations were significantly lower compared to post-exercise concentrations at day 1 ($p < .01$). However, these values were still significantly higher compared to baseline ($p < .05$).

Compared to the second walking day, IL-6 showed a further decrease at day 3 ($p < .001$), with again a slight increase at day 4 ($p < .01$) (Figure 1A). IL-8 decreased further until day 3 ($p < .001$), and did not change at day 4 compared to day 3 ($p = .682$) (Figure 1B). IL-10 did not change significantly between day 2 and day 3 ($p = .319$) and between day 3 and day 4 ($p = .829$) (Figure 1C). IL-1β did not change from day 2 till day 3 ($p = .166$), but declined significantly from day 3 until day 4 ($p < .001$) (Figure 1D). TNF-α slightly decreased on the third walking day compared to the second walking day ($p < .001$) and thereafter increased at the last walking day ($p < .05$) (Figure 1E).

Cytokine IL-6 concentrations were significantly higher in men compared to women only after the second day of exercise (3.94 ± 1.98 vs. 3.16 ± 2.07 pg/ml; $p = .015$), while there were no differences in IL-6 on other days. Cytokine IL-10 concentrations were significantly higher in men compared to women after the first day of exercise (0.66 ± 0.84 vs. 0.38 ± 0.40 pg/ml; $p = .032$), while there were no differences on the subsequent days. Just as at baseline, IL-1β and TNF-α concentrations were higher in men compared to women, during all days of exercise ($p < .01$). IL-8 concentrations were not different between men and women ($p > .05$) (Table 3).

In total, 29 participants used NSAIDs at one or more days during the four days marches (baseline included). NSAIDs that were used by our participants included (name (dosage)): Ibuprofen (200mg, 400mg, and 600mg), Diclofenac (50 mg), Advil/Naproxen (200mg), and Maxalt (5 mg). NSAID-use was higher in women (NSAIDs were used 41 times spread over all days) compared to men (20 times in total). Re-evaluating cytokine responses by excluding those participants who used NSAIDs at baseline and/or during the exercise days, resulted in comparable results. Trends in cytokine responses during the walking days did not differ between users and non-users.

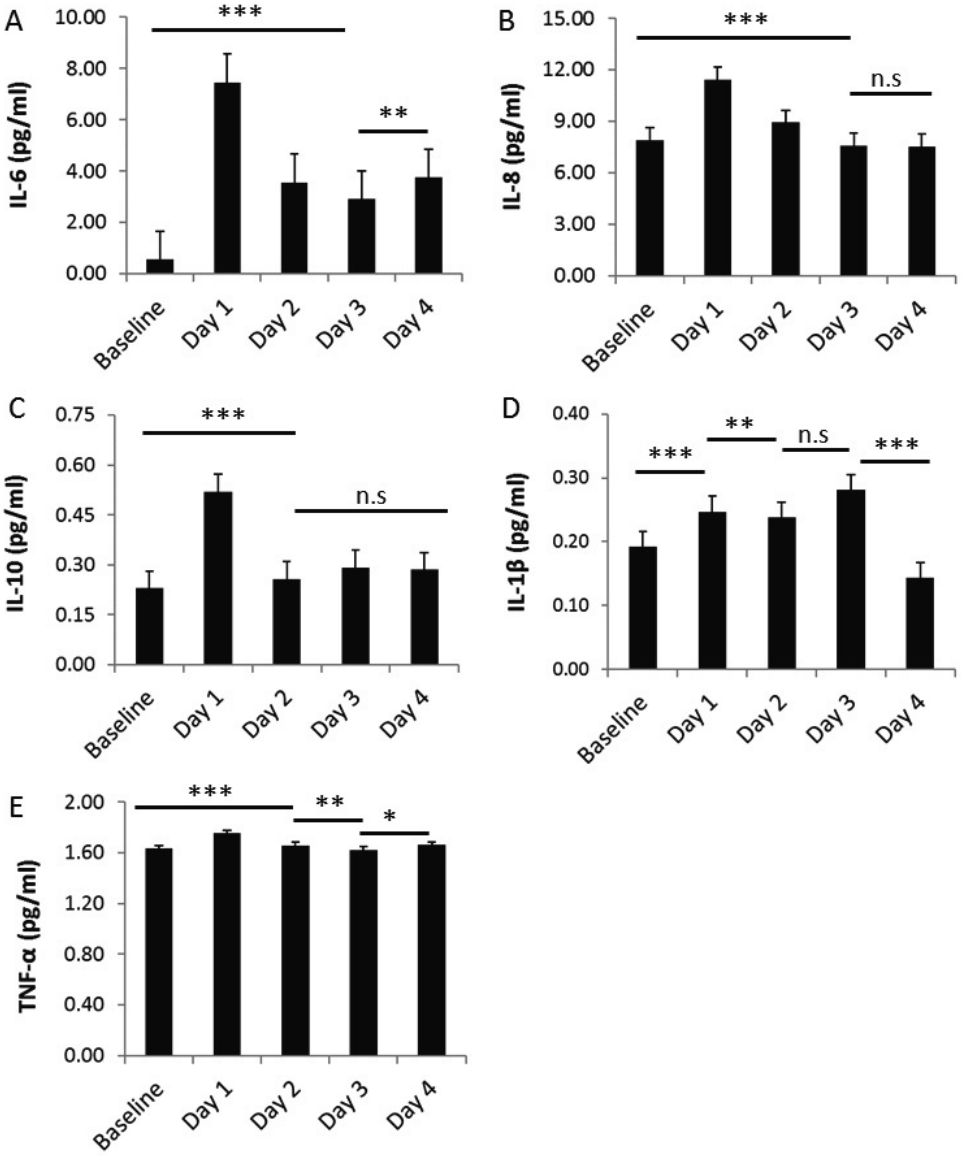

Figure 1. Cytokine concentrations for all participants together. Means ± SE are shown. Significant differences between days are presented with horizontal lines, with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Plasma Volume

Plasma volume changed significantly over time, be it with a different pattern in men and women ($p > 0.001$). Male participants showed a decline in plasma volume during the first (-4.3 ± 12.2%), and second walking day and an increase during the third and last walking day, while female participants showed no change during the first walking day (0.0 ± 5.9%), and an increase on the successive walking days, (see Table 2). Change in body mass was significantly larger for men (-1.4 ± 1.0 kg) after the first day of exercise compared to women (-0.3 ± 0.7 kg) ($p < .001$), while no difference between sexes was observed in mean fluid intake at day 1 ($p = .367$, Table 2). Change in body mass and fluid intake were both significantly different between men and women at day 2 ($p < .001$ and $p = .034$, respectively), weight change was higher in men, while fluid intake was higher in men as well. Finally, weight change was significantly higher in men (-0.9 ± 0.8 kg) compared to women (-0.5 ± 0.5 kg) at day 4 ($p = .016$), while there was no significant difference in fluid intake that day ($p = .397$).

DISCUSSION

The aim of this study was to assess changes in plasma cytokine levels during four consecutive days of long-distance walking (~9 hours each day) at moderate intensity in a group of middle-aged men and women. Our main finding was that most of the measured cytokines peaked after the first day, while showing no further increase, and even a decline during the subsequent days. This was in contrast to our hypothesis that cytokines would be likely to accumulate considering the duration, intensity as well as the relatively short recovery periods between exercise bouts. These results suggest a rapid adaptation to this type of exercise. We also observed that men showed higher baseline IL-1β and TNF-α concentrations and that these levels remained higher in men compared to women during all four exercise days. This was in line with our hypothesis, based on previous studies (15). To the best of our knowledge, studies comparable to the present study are scarce if not absent in the literature.

Cytokine concentrations after one day of exercise.

Our data showed a clear increase of IL-6, IL-8, IL-10, IL-1β and TNF-α plasma levels after the first walking day, which is in accordance with previous studies considering different types of exercise (2, 5, 7-9, 15, 21). This indicates that in our participants, a walking exercise with an average duration of 8h 44 minutes (range 4h 45min - 12h 50min) at 67 ± 9% of HRmax (range 40 – 88%) induces a cytokine response more or less comparable to responses seen after a marathon (9), or an ultramarathon (21). In comparison, a moderate-intensity walk of 30 minutes at 50% of maximal oxygen uptake does not cause an increase in circulating cytokines (22). The exercise-induced increase in IL-6 is known to be related to the duration and the intensity of the exercise (15). Based on previous studies it is plausible that the increase in plasma IL-6 after the first day of exercise is triggered by a reduced glucose availability, i.e. a decrease in muscle glycogen concentration during exercise (5). Indeed, it has been shown that pre-exercise carbohydrate (CHO) status can influence the plasma IL-6 response, with higher responses after low CHO intake and lower responses after high CHO intake (23). Whether this solely explains our observations is unlikely. For example, an increase in cytokine levels can also be related to physical tissue stress and micro damage (3, 24), which is most likely present during multiple hours of walking exercise. Moreover, participants were able to eat at any moment during the study including during the exercise. The increased levels of IL-6 may contribute to the increased levels of IL-10, as IL-6 activates monocytes to secrete IL-10. However, it is questionable whether the increases in IL-

6 are high enough to induce this IL-10 increase (25). An increase in IL-10 in turn serves as a feed-back to inhibit the synthesis of pro-inflammatory cytokine TNF- α (26).

Mild hypoxia might play a role in the increase in IL-8. Such an increase has been associated with beneficial training adaptations, as IL-8 promotes angiogenesis (27). Like IL-6, IL-8 can be released by skeletal muscle, which makes it a 'myokine'. It is suggested that reactive oxygen species (ROS) stimulate the production of myokines in skeletal muscle in response to exercise (28).Some human intervention studies support this idea (29, 30).

Although the interpretation of levels of individual cytokines in terms of their effects remains difficult, IL-6 and IL-8 are at least partly associated with an anti-inflammatory component. By contrast, the increase of IL-1β and TNF-α might indicate a more pro-inflammatory stimulus. These cytokines are known to be induced by endotoxemia. A study with athletes performing an 89.4 km race showed that 81% of the participants had plasma endotoxin concentrations above 0.1 ng/ml (31). In addition, increased plasma levels of lipopolysaccharides (LPS), indicative of endotoxemia, in athletes who took part in an ultra-distance triathlon have been recorded (32). When endotoxins cross the gut epithelial barrier and enter into the circulation, this triggers a cascade involving TNF-α, IL-1β and IL-6.

Consecutive exercise days

Our observation that cytokine concentrations did not further increase after the first day of walking is remarkable and might suggest rapid adaptation. Unfortunately, other studies concerning repeated exercise bouts mainly focussed on eccentric exercise (33, 34) and not on prolonged repeated moderate intensity exercise. In a previous study in our lab (11), be it with a different design (bicycle exercise tests), we also found marked attenuated cytokine responses after repeating the same exercise with one week in between. In contrast, Suzuki et al. studied the effect of 3 consecutive days of 90-min (at 90 Watts) bicycling exercise on cytokine concentrations and found increased levels of IL-6 after the first exercise bout, but these levels remained elevated until day 3 (12). Why they found no attenuated response is not clear, as their exercise protocol seems less challenging compared to 4 days of prolonged walking.

If some form of muscle damage occurred, an explanation could also be found in the degree of muscle injury and the potential adaptations in muscle to become more resistant to subsequent injury. Studies with repeated bouts of eccentric exercise, applying intervals between subsequent exercise bouts varying between 5 days until

10 weeks (33, 34), show that changes in variables like muscle soreness and serum creatine kinase (33) were significantly smaller after a second bout of exercise. These results suggest that an adaptation response took place following the initial eccentric exercise bout. It is plausible that similar adaptation mechanisms are induced following endurance exercise in the current study.

Additionally, the decrease in plasma volume was highest after the first day of exercise, which could result in a high peak of cytokines. However, the increase in plasma cytokine levels ranged between 1.1-fold and 13-fold. Therefore, the change in plasma volume could not solely be responsible for the increase in cytokines.

Finally, a decrease in exercise intensity on day 2 till 4 could also have played a role in the attenuated cytokine responses. Exercise intensity as percentage of maximal heart rate was only measured on the first day of walking due to practical reasons. However, speed was highest on the first day of exercise, which might suggest that exercise intensity was also the highest on the first day of exercise. Minor muscle damage after the first walking day might cause speed to drop on successive days, which could result in lower intensity. It has been shown that exercise intensity determines the cytokine change after exercise (7). Therefore, this change in speed during the four walking days, could attribute to the attenuated cytokine response on the second, third and final walking day.

Differences in Cytokine between men and women

Prior to the study we expected higher cytokine levels in men compared to women for all cytokines, during all days. As contracting skeletal muscles are an important source of IL-6, a higher average muscle mass in men may result in higher IL-6 concentrations in men compared to women (15). Remarkably, we found only higher baseline IL-1β and TNF-α concentrations in men compared to women. This has not been reported before. At the same time, IL-6, IL-8 and IL-10 baseline concentrations were comparable between men and women. This was in agreement with a study from Larsson et al. who found no differences in IL-6, IL-8 and IL-10 levels between men and women (35) and with Edwards et al. (2006) who found comparable baseline IL-6 levels in men and women (14). Notwithstanding some differences at baseline, we did not find significant sex differences in exercise-induced responses in cytokines. An exception to this appeared to be IL-10 showing a significantly higher peak after the first day of walking in men compared to women. Opposite findings regarding IL-10 response between men and women, were reported immediately and 1.5 hours after completing a marathon, where levels were comparable between men and women (36). Unfortunately, we did not ask our female participants at which point in their

menstrual cycle they were. It has been shown that the luteal phase of the menstrual cycle is associated with a greater capacity of immune cells to produce cytokines compared to the follicular phase (37, 38).

Use of Non-steroidal anti-inflammatory drug (NSAID)

NSAIDs are able to suppress cytokine production (39). As NSAID use was quite common in our population, this could have affected the responses. However, we found no differences when NSAID users were excluded from statistical analysis. This might suggest that the cytokine production induced by this type of exercise outweighs the impact of anti-inflammatory medication on cytokine responses.
Other use of medicines (corticosteroids, beta-blockers, ACE-inhibitors, and statins) with potential (mild-strong) anti-inflammatory effects, were not investigated.

Limitations, strengths and conclusion

The present study had some practical limitations. First, we did not include a pre-exercise blood withdrawal every day. Therefore, we don't know whether cytokine concentrations were already lowered in the morning of the second walking day or whether cytokine concentrations decreased during exercise that day. Secondly, we did not investigate the phase of the menstrual cycle of our female participants, which could have influenced cytokine levels. Thirdly, baseline blood draws were not collected at the same time of day across participants, which introduces some variability into the measurement. This was for practical reasons, as participants arrived in the city of Nijmegen at a different time of the day prior to the event. And finally, we did not measure markers for muscle damage, which could be related to cytokine changes.
A strength of the present study was the inclusion of a large group of participants, with only 2 drop-outs. This large population not only enabled to determine baseline differences in cytokine levels between men and women, but also to establish rapid adaptation occurring to this type of exercise. Furthermore, our study population, characterised as generally healthy, regularly exercising middle-aged persons is quite unique and apparently underrepresented in the field of exercise physiology.
In conclusion, these results indicate that in this population, prolonged exercise at moderate intensity causes acute effects on cytokine levels in a degree comparable to that seen after running a marathon. However, this effect is attenuated when performing the same exercise on consecutive days, suggesting rapid adaptation. Furthermore, gender differences resulted in higher baseline IL-1β and TNF-α

cytokine concentrations in men, and higher IL-10 and IL-6 concentrations in men compared to women after the first and second day of walking, respectively.

Perspectives

The finding that this form of exercise causes a profound cytokine response which gradually phases out during consecutive exercise days, suggests an adaptive response to prolonged repeated exercise. The exercise load is rather heavy for this group of adults. Nowadays regular exercise such as walking and cycling is practised by many middle-age adults with the aim to promote health. More insight in the associations between exercise load, repeats and health effects for this specific group is desirable. In further studies, cytokine levels could be used as biomarkers or to increase our understanding of underlying mechanisms.

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Chapter 6

Salivary cortisol and testosterone are responsive during competition, but not during a training block in elite swimming athletes

Cortisol and testosterone in swimmers

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ABSTRACT

Background: Cortisol and testosterone have been proposed as biomarkers of exercise stress in athletes. Their concentrations can be measured in saliva and hair, which is less invasive compared to blood. However, their usefulness as indicators of training load during a training periodization remains unclear. **Hypothesis:** a period of increased training load, will increase cortisol and decrease testosterone levels. In addition, mood state would decline. **Study design:** Observational training study. **Level of evidence:** Level 3. **Methods**: Ten male swimmers (age 19.9±2.3years) were monitored during 10 consecutive weeks in which they followed a specific training block ending with a competition. Saliva samples were collected every week on a set day and time before the afternoon training. Hair samples were collected in week 2, 8 and 10, as a long-term measure. Samples were analysed for cortisol and testosterone levels. Profile of mood state (POMS) questionnaires were filled out every week for mood state. **Results:** Training load decreased over the 10 weeks of training ($p<0.001$). However, both salivary cortisol and testosterone levels remained unchanged. Hair testosterone was higher in week2 compared to week10 ($p=0.013$), while hair cortisol was unchanged. POMS fatigue scores were lower ($p=0.017$) during the taper and competition phase compared to the first training phase with higher training volumes. During competition, both salivary cortisol and testosterone increased directly after the first race ($p=0.028$ and $p=0.018$, respectively) and returned to baseline levels within 2h after the last race. **Conclusions:** Both, salivary cortisol and testosterone showed a rapid response to acute exercise stress during competition. However, during training periodization no changes were observed despite significant changes in training load and profile of mood states. **Clinical relevance:** salivary cortisol and testosterone are not (yet) useful in the assessment of training load during a training block, but only in the assessment of acute exercise stress.

KEY WORDS: monitoring training load, saliva, hair, cortisol, testosterone

INTRODUCTION

Athletes generally use specific training periodization plans comprising of varying training loads and scheduled recovery in preparation for competition (1). Most periodization plans share a common set-up, consisting of general preparation, specific preparation, taper, competition and transition. From general to specific preparation to taper, training volume decreases, while intensity increases, aiming at peak performance during final competition. During the preparation phases, athletes undergo periods of functional overreaching which are necessary to elicit adaptations and improve performance (2). However, there is a potential risk to progress to non-functional overreaching or overtraining syndrome and maladaptive outcomes (3). This demands for careful monitoring of training load in relation to athletes' performance, general health and well-being.

A potentially interesting approach to assess training load could be the assessment of cortisol and testosterone levels. Cortisol and testosterone levels have been proposed as indicators of the balance between catabolic and anabolic processes and the physiological stress of training (4). Levels of both hormones are known to increase immediately after exercise (4). It has also been shown that cortisol correlates with perceived stress and training load (5). In addition, chronically decreased testosterone and increased cortisol levels are suggested to indicate a disturbance in the anabolic-catabolic balance (6). Combining these hormone levels with mood state can be a useful parameter to monitor training load and fatigue (7).

Cortisol and testosterone levels can be measured in blood. However, this is perceived as invasive for athletes and not always preferable. Both hormones can also be measured in saliva, which is less invasive and has been suggested a reliable alternative (8). Another non-invasive way to analyse cortisol and testosterone is in hair samples (9). An additional advantage of using hair samples instead of blood or saliva samples, is that a hair sample provides information about cortisol and testosterone levels over a prolonged, retrospective period (10).

Currently, the impact of training load on salivary markers receives growing interest. However, measuring hormone levels in hair samples is rather new in the field of sports. Therefore, the aim of the present study was to analyse the effect of a training block and competition on the cortisol and testosterone levels in saliva and hair. This was combined with assessment of mood states via a profile of mood states questionnaire (POMS), as it is known that mood state worsens in response to increased training load (11). We hypothesized that during a period of increased training load, cortisol levels would increase, testosterone levels decrease, and that

mood state would decline. *Vice versa,* we expected opposite effects during a period of decreased training load ('taper'). In addition, we expected that the acute effect of intense exercise during competition on saliva cortisol and testosterone were more evident compared to the effects during a training periodization.

MATERIALS AND METHODS

Study design

This observational field study had a repeated measures design and was performed over a 10-week training block in preparation for a final peak competition, which included multiple swimming races. See Figure 1 for a schematic overview of our study design.

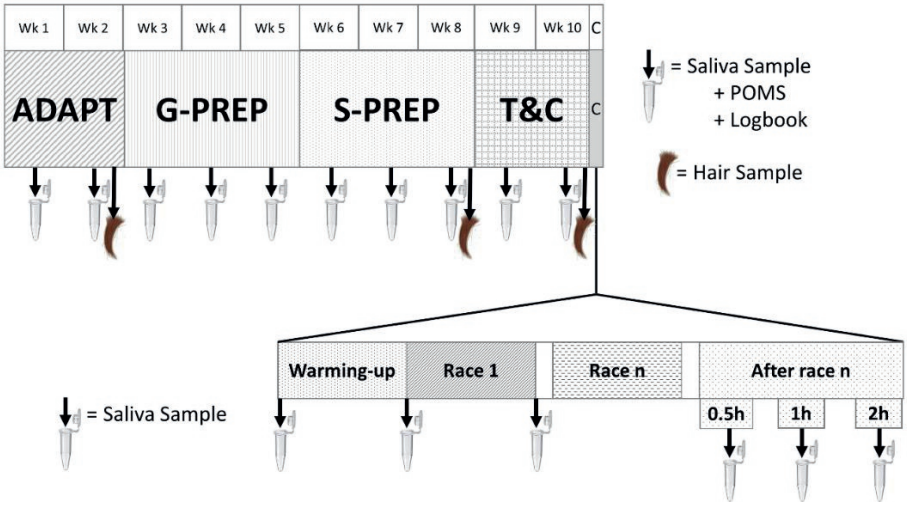

Figure 1. Schematic overview of sampling points and study design. ADAPT: Adaptation phase; G-PREP: General preparation phase; S-PREP: Specific preparation phase; T&C: Taper and competition phase; C: Competition.

Training sessions lasted either 90 or 120 minutes. Participants trained together on various mornings and afternoons, between 06:00AM and 08:00AM and between 17:00PM and 19:00PM, respectively. Types of exercise sets performed during training were categorized according to 6 goals: aerobic capacity (AC), aerobic power (AV), anaerobic capacity (ANC), lactate production (LC), Ultra short race pace training (USRPT) and sprint training (Sprint). The 10 weeks training block included 4 main phases (Figure 1): 1) two adaptation weeks (ADAPT), characterized by an average training volume with mostly AC sets, some USRPT, some sprinting and some AV sets, followed by 2) a general preparation phase of 3 weeks (G-PREP), characterized by a high training volume with AC sets, some AV, ANC, LC, USRPT and sprint sets, and 3) a specific preparation phase of 3 weeks (S-PREP), characterized by a lower training volume with high intensity training and ANC, USRPT and sprint sets, and finally, 4) a taper & competition phase of 2 weeks (T&C), including less volume, some high-intensity, ANC, LC, USRPT and sprint

sets and time for recovery and one competition. Training data, including the number of training sessions, type of training, training distance and duration were provided by the coach after the training sessions. The competition day took place at the end of week 10 of the training period.

During the 10 weeks of training, saliva samples were collected every week and hair samples were collected in week 2, 8 and 10, at the swimming pool, before the start of the training session (~16.45h). Questionnaires were taken directly with the saliva and hair sampling.

During the competition day, participants donated saliva at 6 occasions: before warming-up, after warming-up, directly after the first race and half an hour, one hour and two hours after the last race. On the day of the competition, before warm-up, participants graded their nervousness on a 10-point scale from 0 (not nervous at all) till 10 (very the most nervous possible). After their last race, participants were asked whether they were satisfied with their performance, on a scale from 0 (not satisfied at all) till 10 (very satisfied). All participants joined different types of races at different times of the day. Therefore, the timing of saliva collection was noted per individual. All races took place in the afternoon between 14:00 and 17:30.

Subjects

Ten male elite swimmers participated in this study (age 19.9 ± 2.3 years, BMI 23.2 ± 0.9 kg/m^2, VO2max 62.2 ± 3.2 ml/min/kg). They all competed at Dutch national level and were either sprinter (50m and 100m) or middle-distance (200m and 400m) swimmers. All training conditions were the same for them: one swimming club, technical support, coach, swimming pool and training hours. Exclusion criteria for this study were smoking, severe oral health problems, boldness, autoimmune diseases and endocrine disorders.

The Medical Ethical Committee of Wageningen University and Research considered the study a non-invasive observational scientific study and therefore no formal ethical approval was needed. All participants gave written informed consent prior to participation. This study was conducted in accordance with the Declaration of Helsinki. Participant characteristics can be seen in table 1.

Table 1. Participant characteristics

	Participants (n = 10)
Age (years)	19.9 ± 2.3
Body composition	
Height (cm)	185.8 ± 5.2
Weight (kg)	80 ± 5
BMI (kg/m^2)	23.2 ± 0.9
Body fat (%)	10.9 ± 1.5
Physical characteristics	
VO_2max (ml/kg/min)	62.2 ± 3.2
Max heart rate (bpm)	196.7 ± 6.8
Max Power (Watt)	378 ± 26
Power / kg body weight	4.80 ± 0.24

Means ± SD are shown. BMI: Body mass index; Physical characteristics are determined during a VO_{2max} test; bpm: beats per minute

Participants' characteristics were measured in the exercise lab at the University. This included a maximal exercise test on a bicycle ergometer (Ergoline GmbH, Bitz, Germany) to establish maximal aerobic capacity (VO_{2max}). After an initial workload of 80Watt for 5minutes, workload was subsequently increased by 20W/min until the participant could not maintain the required pedalling frequency of at least 60rpm. Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Hoechberg, Germany). VO_{2max} was defined as the maximal oxygen uptake measured in millilitres of oxygen per minute per kilogram of body weight (mL/kg/min). Heart rate was monitored by using a heart rate monitor (Polar T31-coded, Oulu, Finland) and connected exercise tracker (Polar FT1). In addition, body length (Seca213 portable stadiometer, Hamburg, Germany), weight (Seca761 scale), and four-point skinfolds thickness (Holtain Tanner/Whitehouse Skinfold Caliper, UK) to estimate body fat percentage were measured.

Data collection

Saliva Collection and analysis

Saliva was collected on a weekly basis, just before the afternoon training in the swimming pool at 16.45h. Pilot testing in our lab showed that cortisol levels were not significantly variable between 12:00 and 17:00, assuring us that any changes

measured at this time point therefore could be considered as indicative for stress and not reflecting the diurnal cycle of cortisol. For each swimmer, this collection moment was on the same day every week. Due to competition scheduling or other individual activities, some exceptions were made but saliva was collected at most 2 days before or after the planned day of collection.

In order to collect whole saliva from the mouth, unstimulated, passive drool was performed. At least ten minutes before sample collection participants rinsed their mouth with water for 5-10 seconds. Participants were asked to bend their head slightly downwards and first collect some saliva in their mouth before drooling into the saliva collection aid (Salimetrics, LLC, State College, USA). At least 0.5ml of saliva was collected in 2-ml collection tubes (Wheaton, Millville, USA) per time point, per participant. Samples were temporarily stored in a precooled box and transferred to -20°C within two hours. The next day samples were stored at -80°C for long-term storage until analysis.

Saliva samples were thawed and vortexed on the day of analysis. Saliva was analysed for cortisol and testosterone levels with commercially available, enzyme-linked immunosorbent assay kits (Salimetrics, State college, PA, USA), according to the manufacturers' instructions. The sensitivity of the kits was 0.193 nmol/L for cortisol and 0.0035 nmol/L for testosterone. The mean intra assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. Kits were of the same batch, for each swimmer, all samples were analysed on the same plate to eliminate inter-assay variance.

Hair collection and analysis

Hair locks were obtained in week 2, 8 and 10, resulting in 3 hair samples during the 10-week training period. Samples were collected according to the description of Noppe et al. (9). In brief, approximately 100–200 hairs were cut from the posterior vertex, close to the scalp, using small scissors. Hair locks were attached to a paper and send to Erasmus Medical Centre for analyses. One cm of every hair sample closest to the skull (reflecting 4 weeks) was used for analysis, samples were analysed for their cortisol and testosterone levels with a LC-MS/MS-based method, as described in the standardized protocol (9). Precision of these analyses, described by the coefficients of variation, were 14.8% for cortisol and 16.0% for testosterone. The LLoQ was <1.3 pg/mg hair for cortisol, and 2.3 pg/mg hair for testosterone.

Questionnaires

All participants filled out three questionnaires at the time of saliva sample collection: 1) a short profile of mood state (s-POMS), 2) a logbook with questions about sleep quality and stress, and 3) a questionnaire regarding factors that could influence the quality of the hair samples, *e.g.* shampoo use, hair dying, corticosteroids, etc.

The s-POMS consisted of five sub emotions: depression, anger, fatigue, strength and tension. To calculate the overall POMS score, strength was multiplied by +1 and the other sub emotions were multiplied by -1. This resulted in an overall POMS value range of -16 to +4. The logbook included questions about sleeping hours, sleep quality (scale 1=bad, till 10=good), exhaustion level (scale 1=tired, 10=well rested), and stress level (scale 1=low, 10=high stress level) during the past week.

Statistical analyses

Statistical analyses were performed using Statistical Package for Social Sciences 22.0 (IBM SPSS version 23.0, Armonk, New York, USA), with the level of significance set at $p<0.05$. Data are presented as mean ± SD unless indicated otherwise. Data were analysed per training phase and per training week. For training phases, the average was calculated from the weeks that represented the specific phases.

The Kolmogorov-Smirnov test was used to examine the normality of the data distribution. As saliva, hair and POMS data were not normally distributed, non-parametric tests were used for analysis. A Friedman's test was used to evaluate the effect of consecutive training phases and consecutive training weeks on salivary and hair cortisol and testosterone levels and POMS scores. When a significant time difference was found, a Wilcoxon signed-rank test was used to determine where the significant differences occurred.

Correlations between saliva and hair cortisol and testosterone levels were analysed with a Spearman correlation. As 1cm hair sample represents one preceding month, the saliva samples of the 4 weeks before that hair sample were used for an average value of that month. For example, the cortisol value in the hair sample taken in week 10, was correlated with the average cortisol value in the saliva samples of week 7, 8, 9 and 10. And the hair sample in week 8 was correlated with the average value in the saliva samples of week 5, 6, 7 and 8.

RESULTS

Training data

Swimmers performed on average 13.2 ± 3.3 hours of swimming per week. The average distance covered during a training session was 4401 ± 878m. Average training distance per single training session was significantly different between phases ($p<0.001$). Average distance per training was significantly higher during ADAPT (5367 ± 454m) compared to S-Prep (3591 ± 542m, $p=0.002$) and compared to T&C (3850 ± 237m, $p=0.005$). Average training distance was also significantly higher during G-Prep compared to S-Prep ($p<0.001$) and compared to T&C ($p=0.005$). Average training distance was not significantly different between ADAPT and G-Prep (4913 ± 572m, $p=0.223$) and not between S-Prep and T&C ($p=0.765$). The total training distance per week was highest in the G-Prep weeks (37.67 km/week) and lowest during the two weeks of T&C (19.25 km/week) (Figure 2).

Training hours per week decreased consecutively, with on average 15.3 hours of swimming per week in ADAPT, 14.67 hours in G-Prep, 13.5 hours in S-Prep and 8.5 hours in T&C.

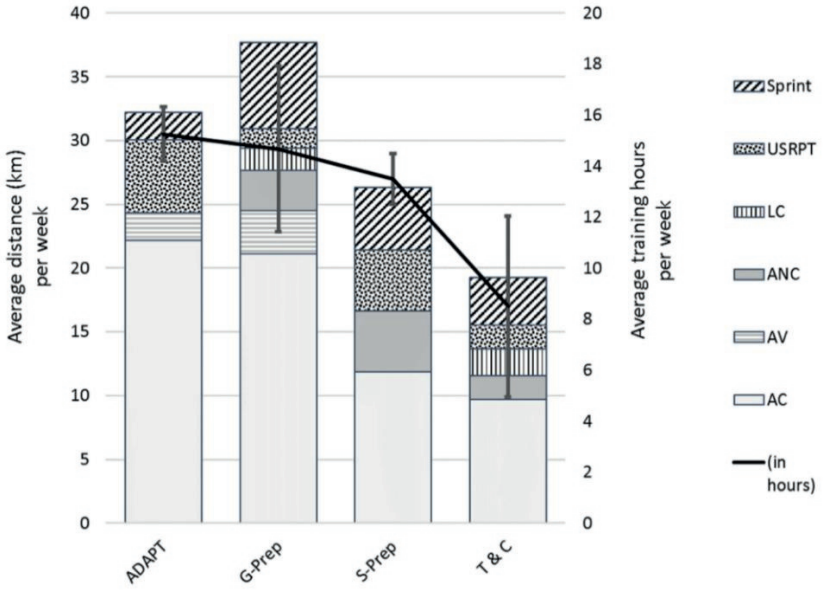

Figure 2. Training overview. USRPT: ultra-short race pace training; LC: lactate production; ANC: anaerobic capacity; AV: aerobic power; AC: aerobic capacity; ADAPT: Adaptation phase; G-PREP: General preparation phase; S-PREP: Specific preparation phase; T&C: Taper and competition phase; C: competition.

Salivary cortisol and testosterone levels during training

Salivary cortisol and testosterone levels were not significantly different between training phases (p=0.339 and p=0.948, respectively). Levels were also not significantly different between training weeks (p=0.167 for cortisol and p=0.373 for testosterone; Table 2). Both hormone levels varied considerably among individuals, with salivary cortisol levels varying between 1.10 and 13.54 nmol/L and testosterone levels varying between 0.14 and 0.82 nmol/L (Figure 3). The ratios between cortisol and testosterone (C:T) were also not significantly different between weeks or between training phases (p=0.287 and p=0.241, respectively).

Table 2. Salivary cortisol and testosterone levels during the 10 weeks training block

		Cortisol (nmol/L)		Testosterone (nmol/L)		Ratio C : T	
week 1	ADAPT	5.24 ± 1.79	5.47 ± 1.59	0.34 ± 0.07	0.36 ± 0.10	15.4 ± 3.5	15.5 ± 4.2
week 2		5.69 ± 2.69		0.38 ± 0.19		16.3 ± 8.2	
week 3	G-Prep	5.43 ± 2.33	5.47 ± 2.05	0.35 ± 0.08	0.33 ± 0.06	15.1 ± 4.6	16.8 ± 6.3
week 4		5.60 ± 2.15		0.32 ± 0.04		18.3 ± 9.0	
week 5		5.37 ± 2.85		0.32 ± 0.08		17.5 ± 10.6	
week 6	S-Prep	4.61 ± 2.57	5.45 ± 2.65	0.32 ± 0.08	0.34 ± 0.08	14.5 ± 8.3	15.9 ± 6.4
week 7		5.90 ± 4.01		0.36 ± 0.11		15.4 ± 7.9	
week 8		5.85 ± 2.38		0.33 ± 0.09		17.8 ± 5.4	
week 9	T & C	4.35 ± 1.63	4.24 ± 1.93	0.37 ± 0.09	0.32 ± 0.09	11.7 ± 5.4	13.4 ± 5.0
week 10		4.58 ± 2.36		0.31 ± 0.09		11.7 ± 2.8	
P value		0.167	0.339	0.373	0.948	0.287	0.241

Means ± SD are shown per week and per training phase. P values refer to an Intra-group Friedman ANOVA test for the effect of different training weeks and training phases. ADAPT: adaptation phase; G-Prep: general preparation phase; S-Prep: specific preparation phase; T & C: Taper and competition phase; C:T ratio: Cortisol:testosterone ratio

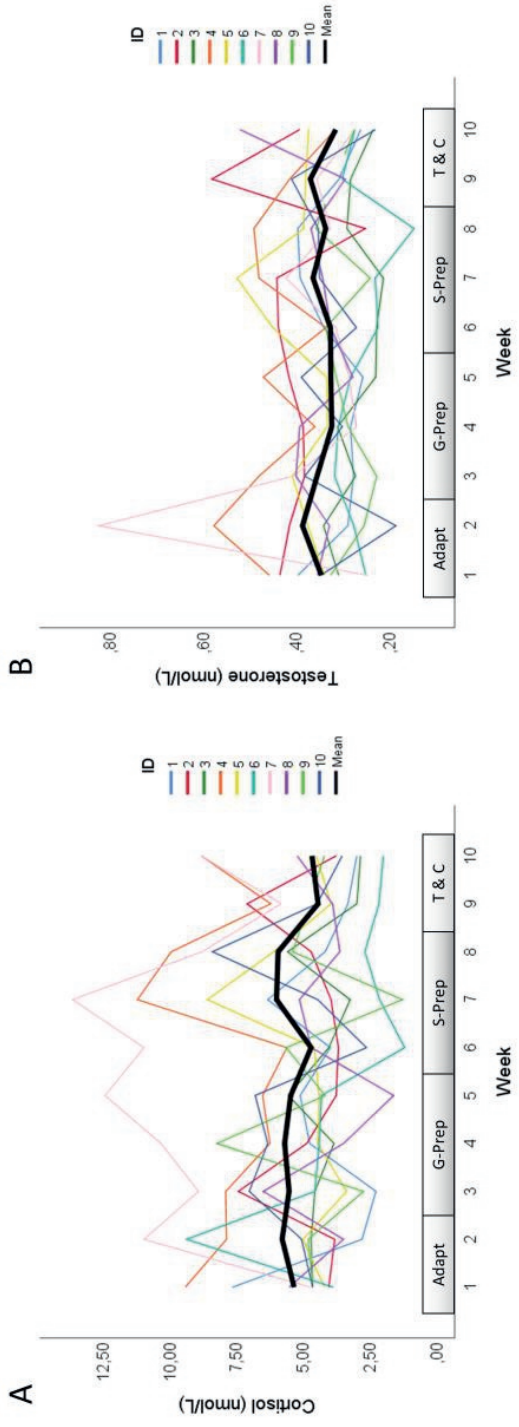

Figure 3. Salivary cortisol (A) and testosterone levels (B) during the training period.

Salivary cortisol and testosterone levels during competition

Salivary cortisol and testosterone levels were significantly different between the six time points of saliva collection during the final competition (p=0.003 and p=0.027, respectively; Figure 4).

Cortisol levels were highest directly after the first race (13.32 ± 7.52 nmol/L) and 0.5 hours after the last race (12.94 ± 6.07 nmol/L). Levels were significantly different between the time point directly after warm-up (4.21 ± 1.98 nmol/L) and race 1 (13.32 ± 7.52 nmol/L, p=0.028), between 0.5 hours after the last race (12.94 ± 6.07 nmol/L) and one hour after the last race (7.72 ± 3.36 nmol/L, p=0.018) and between 1 hour and 2 hours after the last race (4.15 ± 1.63 nmol/L, p=0.018). Cortisol levels were not different between pre-warm up (5.45 ± 2.46 nmol/L) and after warm-up (4.21 ± 1.98 nmol/L, p=0.237) and between after the first race and 0.5 hours after the last race (p=0.612).

Testosterone levels were highest after the first race (0.40 ± 0.13 nmol/L). Levels were significantly different between pre (0.32 ± 0.09 nmol/L) and after warm-up (0.26 ± 0.07 nmol/L, p=0.028) and between directly after warm-up and after the first race (0.40 ± 0.13 nmol/L, p=0.018). There were no differences in testosterone levels after the first race compared with 0.5 hours after the last race (0.34 ± 0.16 nmol/L, p=0.176), or between 0.5 hours and 1 hours after the last race (0.32 ± 0.11 nmol/L, p=0.612), or between 1 hour and 2 hours after the last race (0.25 ± 0.09 nmol/L, p=0.063).

Participants mean satisfaction with their performance was 7.3 ± 1.1 on a 10-point scale. On beforehand, participants graded their nervousness with a 5.9 ± 2.0 on a 10-point scale, with 0 being not nervous at all and 10 being the most nervous possible. This did not correlate to cortisol levels before warm-up (p=0.187, r= -0.564).

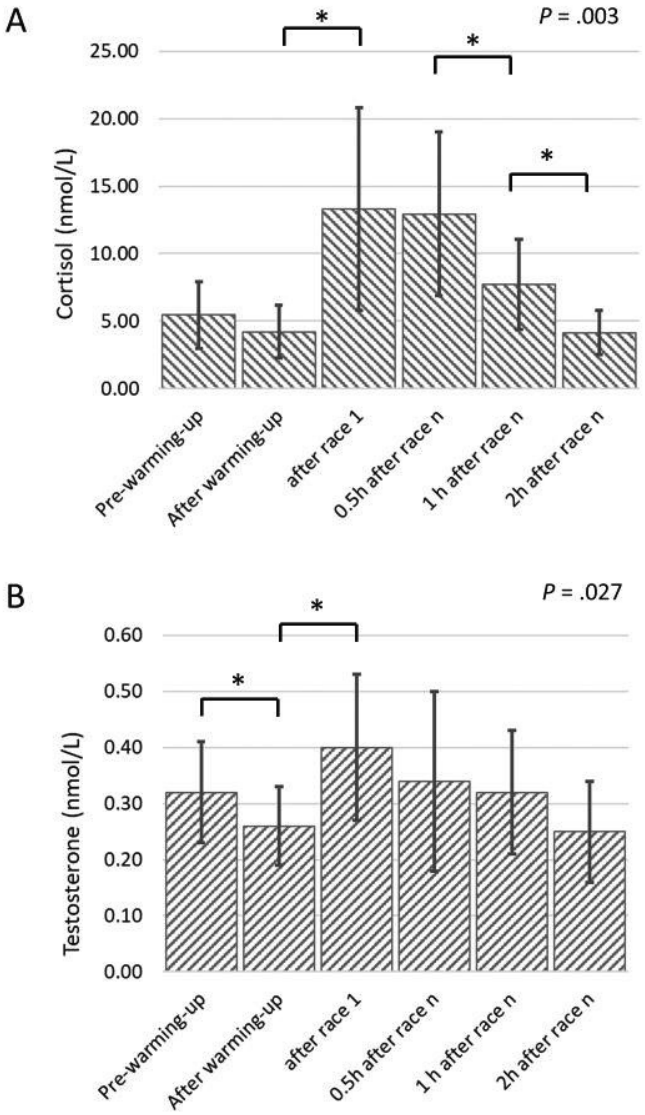

Figure 4. Salivary cortisol (A) and testosterone levels (B) during competition. P values in the right upper corner represent a Friedman Anova test for the effect of time. * meaning $p < 0.05$ with a Wilcoxon Signed Rank test.

Hair cortisol and testosterone during training

Cortisol levels in hair did not change significantly during the 10 weeks of training (p=0.882). Testosterone levels did change significantly during the 10 weeks of training (p=0.025), with significantly higher testosterone levels in the hair sample collected during the second week of training (0.68 ± 0.37 pg/mg) compared to the hair sample collected in the last (10^{th}) week of training (0.53 ± 0.28 pg/mg, p=0.013). There were no significant differences between samples taken at the second and the eighth week (p=0.074) and the eighth and tenth week (p=0.959) (Table 3). There was no correlation between cortisol and testosterone levels in hair and saliva (p>0.05).

Table 3. Hair cortisol and testosterone levels during the 10 weeks training block

Week	2	8	10	P value
Cortisol (pg/mg)	0.64 ± 0.53	0.77 ± 0.85	0.75 ± 0.56	0.882
Testosterone (pg/mg)	0.68 ± 0.37 *	0.59 ± 0.46	0.53 ± 0.28	0.025

Means ± SD are shown. P values refer to an Intra-group Friedman ANOVA test for the effect of time. * Significantly higher compared to the hair sample collected in week 10 (p = 0.013)

Questionnaires

There was a significant difference between the training phases in the 'fatigue' POMS scores (p=0.016) (Table 4). Participants reported significantly higher 'fatigue' scores during ADAPT (1.0 ± 0.4) compared to T&C (0.6 ± 0.6, p=0.017). There was no significant difference between the 'fatigue' scores of the other training phases and there were no significant differences in any other sub emotion between training phases (p>0.05).

There were no significant differences in sleeping hours (average: 7.7 ± 0.7 hours, p=0.986), sleep quality (average: 6.9 ± 1.0, p=0.228), exhaustion level (average: 6.1 ± 1.4, p=0.620), or stress level (average: 3.5 ± 1.9, p=0.441) between the different training phases (Table 4).

Table 4. POMS and Logbook data

	Training phase	ADAPT	G-PREP	S-PREP	T & C	P value
	week	1 & 2	3 & 4 & 5	6 & 7 & 8	9 & 10	
POMS subscale score	depression	0.3 ± 0.3	0.4 ± 0.4	0.2 ± 0.2	0.3 ± 0.4	0.141
	anger	0.3 ± 0.4	0.3 ± 0.3	0.2 ± 0.2	0.2 ± 0.3	0.081
	fatigue	1 ± 0.4	1 ± 0.8	0.7 ± 0.7	0.6 ± 0.6	0.016
	strength	2.3 ± 0.7	2.2 ± 0.5	2.1 ± 0.7	2.1 ± 1.1	0.801
	tension	0.3 ± 0.2	0.4 ± 0.4	0.3 ± 0.3	0.2 ± 0.3	0.243
Sleep, tired, stress questionnaire	sleep hours	7.6 ± 0.8	7.8 ± 0.5	7.9 ± 0.4	7.5 ± 1.0	0.986
	sleep quality	6.7 ± 1.1	6.7 ± 1.0	7.1 ± 0.8	7.1 ± 1.0	0.228
	tiredness	5.7 ± 1.7	6.1 ± 1.3	6.5 ± 1.1	6.1 ± 1.6	0.620
	stress level	3.3 ± 1.1	3.9 ± 1.8	3.7 ± 1.8	3.1 ± 2.7	0.441

Means ± SD per training phase are shown. Sleep quality was scaled from 1=bad to 10=good, tiredness was scaled from 1=tired to 10=well rested, stress was scaled from 1=no stress to 10=highly stressed. P values refer to an Intra-group Friedman ANOVA test for the effect of different training phases. ADAPT: adaptation phase; G-Prep: general preparation phase; S-Prep: specific preparation phase; T & C: Taper and competition phase; POMS: Profile of Mood States.

DISCUSSION

Salivary cortisol and testosterone levels

Our main finding was that salivary cortisol and testosterone levels were unchanged even though training load significantly changed over time. We were unable to discover a pattern in the cortisol and testosterone profiles. It has been shown that salivary cortisol correlates moderately with perceived stress and training load (5). Therefore, we expected higher cortisol levels during adaptation (ADAPT) and general preparation phase (G-Prep) compared to specific preparation (S-Prep) and taper and competition phase (T&C). However, no significant differences were seen in cortisol levels between any of those training phases. This was in contrast to findings from other researchers, for example reporting increased salivary cortisol levels during 3 weeks of intensive training and competition in elite rugby players (12) and during a period of intensive training and competition in basketball players (13). On the other hand, no changes in cortisol levels were also reported in soccer players during a longer training period (14) and a 20 day training period with 1 soccer match every 3 days (15).

Moreover, we expected cortisol levels to be lower in the T&C phase, firstly because training load was substantially lower during that phase and secondly, because the main goal of a taper phase is to increase recovery and enhance pre-competition performance (16). However, despite significantly shorter total training distances and lower scores for perceived fatigue during taper, lower cortisol levels were not observed. In our study design, an overall effect of the taper period on cortisol levels could have been diminished by competition stress. An increase in cortisol in response to competition stress (psychological stress) has been shown before (17, 18), which could have overruled the decrease in physiological stress.

Furthermore, variation in cortisol response within athletes, which can be explained by diurnal rhythm, pulsatile secretion and individual differences in HPA-axis setting, was large. This was visible by increased cortisol levels for some athletes, and decreased levels for others. An explanation for the large variation in cortisol response between athletes (during these 10 weeks) can be that some athletes become better accustomed to training stress compared to others (19). This underlines that these salivary cortisol levels cannot be used on a group level, but at best only on an individual level.

In addition to cortisol, salivary testosterone levels were also not different between training phases. This is in line with a study showing no changes in testosterone levels in elite synchronised swimmers performing a training camp, competition and

recovery (20). However, opposite findings have been reported as well, showing that a progressive decrease in training load during a pre-competitive period resulted in increased salivary testosterone levels in 24 world-class track and field athletes (21). In anticipation for the competition, athletes in this study showed no change in testosterone levels. This is in agreement with previous findings obtained during a rowing ergometer competition (22), but in contrast to the increased testosterone levels found before a judo competition (23). Others suggest that individual differences associated with testosterone in the pre-event phase are linked to interest in bonding with teammates (22).
Finally, it has been proposed that the ratio of testosterone and cortisol gives an indication of the anabolic/catabolic balance in response to training (24). But as both, testosterone and cortisol and their ratio showed no clear pattern during these 10 weeks of training, we did not have an indication for a more anabolic or catabolic state during any week or phase in this study.

During competition, a clear exercise stress response in both cortisol and testosterone was seen. This is in agreement with a recently executed meta-analysis, reporting that acute exercise results in both increased salivary cortisol and salivary testosterone levels (25). This appears to be the case for aerobic exercise, power exercise and resistance exercise. Timing of sampling before exercise significantly affects the magnitude of the testosterone and cortisol response observed (25, 26). Acute increases in salivary cortisol were found following a rugby match (26), a kickboxing match (27), a wrestling match (28), and a golf competition (29). Increased salivary testosterone levels were reported after a rugby match (12).
A true competition, like the one in this study, can induce greater hormonal responses in athletes compared to exercise carried out in a laboratory (26). It appears that the psychological component of competition is mainly responsible for the increment in cortisol rather than physiological factors. However, baseline cortisol levels at the competition day (5.45 ± 4.14 nmol/L) were not higher compared to the average cortisol levels during training (5.24 ± 2.48 nmol/L) and levels did not correlate to nervousness on competition day. Therefore, we suggest that physiological stress during competition serves as an important trigger for the increase in cortisol levels seen. However, we can neither rule out that psychological stress during the actual race has contributed to the effects, nor that psychological stress would not play a role during the training period. It has been shown that cortisol increases in response to different forms of exercise performed at an intensity above 60% of an individual's VO2max (30) or in response to exercise longer than 30 minutes with an intensity

higher than 60% of the maximal power (31, 32). The swimming distances performed during competition did not take longer than 5 minutes, but the high intensity and maximal effort clearly resulted in enough physical or psychological stress to cause both cortisol and testosterone levels to increase.
This direct and clear response in cortisol and testosterone levels might explain the interest researchers have in salivary cortisol and testosterone measurements. It appears to be a good marker for acute (competition) stress, however, it seems less useful for chronic training load as described above. It is of course also possible that chronic training load induced no endocrine reaction in these participants, because they are very well trained and used to this amount of training load.

Hair cortisol and testosterone levels
To our knowledge, studies measuring the effect of training load on cortisol and testosterone levels in hair are scarce (Skoluda et al. 2012). Measuring cortisol in hair has gained a lot of interest in stress research (33, 34), but is not yet that popular in exercise research. Cortisol and testosterone in hair are delivered from blood via passive diffusion (5, 35). As human hair at the posterior vertex grows approximately 1 cm per month, the cortisol and testosterone levels in a 1-cm hair segment reflect the mean systemic cortisol, and testosterone, exposure of 1 month. In other words, hair cortisol levels do not reflect acute stress, but long-term stress (36, 37).
In contrast to our expectations, only a significantly lower testosterone level in hair was seen during the final measurement (week 10) compared to the first measurement (week 2). No significant changes in cortisol were observed. Hair samples do reflect the average cortisol and testosterone exposure of 1 month, and therefore, week 10 for the hair samples actually reflects week 6 till 10 for the training load. Maybe, if we would have collected hair samples after the competition as well, those samples would reflect more the taper & competition phase. The timing of our hair sample collection can also explain why no significant difference between week 8 and 10 was observed, as they overlap when it comes to 1cm of hair representing one month. It would have been better to collect the hair samples at 4, 8 and 12 weeks.

Furthermore, there was no correlation between salivary and hair cortisol and testosterone levels. This is in agreement with many studies (not related to exercise) (38, 39). On the other hand, some studies (also not related to exercise) report good correlations (33, 34). In addition, a recently executed study showed that moderate correlations can be found when hair cortisol levels are correlated with salivary three-day averages of area under the curve values ($r=0.39$, $p<0.01$) (40). And another study

performed in 2016 showed that hair cortisol was strongly associated with the prior 30-day integrated cortisol production measure (as average salivary cortisol area under the curve) (r=0.61, p=0.01) (41). We did not collect saliva samples three days or 30 days in a row at the moments of hair collection, because measurement validation was not our primary goal. And, with such an approach, the advantage of saliva and hair collection (being non-invasive and practical) above blood collection, would be diminished. Therefore, it seems warranted to repeat a similar study with exactly one-month time intervals between hair collection moments, to get more insight in the usefulness of cortisol and testosterone levels in hair to monitor training load.

In conclusion, the significant changes in training load were not accompanied by significant changes in salivary cortisol and/or testosterone, while testosterone levels in hair and the POMS fatigue score did change over time. Then again, acute changes during competition were prominent in both salivary cortisol and testosterone. This implicates that salivary cortisol and testosterone are not (yet) useful in the assessment of training load during a training block, but only in the assessment of acute exercise stress.

More studies are needed to investigate whether hair samples are useful for assessing long term training load. Saliva samples are easily affected by factors like excitement before training or competition, which makes saliva levels to rise and drop rather quickly. Hair samples are less influenced by these factors, because they represent cortisol and testosterone levels over a prolonged period of time. Therefore, hair samples might be more useful for assessing long term training load and saliva samples for the assessment of acute training stress.

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Overtraining
Overreaching
Performance

Chapter 7

How to perform testing and training protocols to study overreaching and overtraining - experimental protocols and outcome measures revisited. A narrative Review

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ABSTRACT

Non-functional overreaching and overtraining syndrome (OTS) are serious risks for athletes. Scientific studies are done to get more insight in underlying mechanisms, but also for indicators allowing early detection and monitoring. To this end, different experimental procedures are proposed to simulate and study overtraining. Given the negative long-term effects of OTS, the aim of these experimental procedures is generally to induce a state of overreaching. Usually, combinations of blood biomarkers, performance, mood state and physiological parameters are compared between overreached individuals and matched controls.

It is beyond the scope of this paper to review the detailed description of (non)functional overreaching and OTS. Instead, the aim of this paper is to discuss the available information on (non)functional overreaching and OTS from the perspective of the researchers who design their study protocols to study these phenomenon's.

Therefore, this paper reviews studies that aimed to induce overreaching and OTS, including a pilot study we performed in our own lab. In terms of their designs, outcome measures and quality, considerable differences between studies are seen. Single exercise studies, measuring during a training camp or prolonged competition and study designs aiming to induce a state of overreaching are all discussed. These previous examples provide learnings and possible directions for future studies. Therefore, this review ends with some guidelines/advices for future research protocols in the field of overtraining.

Key Words: Overreaching, Overtraining, Aerobic exercise, Study designs,

INTRODUCTION

Overtraining syndrome (OTS) refers to a state in which accumulation of training and/or non-training stress results in long-term (weeks-months) decrement in performance capacity with or without related physiological and psychological signs and symptoms of maladaptation (1). In terms of duration, severity and recovery, OTS differs from overreaching, which is less severe and of shorter duration, and which is often further classified into 'functional' (F-OR) and 'non-functional' (NF-OR) overreaching (1). Notwithstanding the fact that the borders between these states in terms of their etiology and development are far from clear, it is evident that in particular OTS, but also NF-OR, are posing considerable risks to athletes and other groups involved in heavy training, like military etc (1).

Until now, no scientific consensus exists regarding the usefulness of specific physiological indicators to detect OTS in an early stage. While tracking for such markers, researchers are using varying study designs, generally aiming to induce (NF-)OR and trying to disentangle processes and biomarkers in overreached participants from those in non-overreached participants. Criteria for such a (set of) marker(s) are summarized in a joint consensus statement published by the European College of Sports Science (ECSS) and the American College of Sports Medicine (ACSM) (1).

Several experimental study designs to simulate and study overtraining related states have been used in the past. Examples of these study designs are single-bout exercise studies, retrospective/observational (field) studies, case studies and study designs aiming to induce a state of OR. Those study designs all have their advantages and disadvantages. This holds especially true when it comes to feasibility, external confounding factors and ethical considerations. However, major concerns exist about how to extrapolate their findings, and there is no consensus how study designs translate to OR and OTS in the real athletes' world. For example, common shortcomings in these studies relate to: not including performance tests, the lack of recovery periods, the choice of outcome measures and the vague definition of OR and OTS.

It is beyond the scope of this paper to review the detailed description of (non)functional overreaching and overtraining; readers are referred to several extensive reviews for this information (1, 2). Instead, the aim of this paper is to discuss the available information on (non)functional overreaching and overtraining from the perspective of the researchers who design their study protocols to study these phenomenon's. Therefore this review will only shortly repeat the definitions

of overreaching and overtraining as stated elsewhere. More importantly, study designs that have been described by various researchers will be discussed regarding the advantages and disadvantages of those designs. Then, advice regarding outcome measures will be given and finally, we will end with some guidelines/advices for future researchers in the field of overreaching and overtraining.

Definitions

In the past, different terminologies were used to describe overreaching and overtraining syndrome. Therefore, a joint consensus statement was formulated (1), which included the definition of OTS and overreaching (OR). OTS is defined as "an accumulation of training and/or non-training stress resulting in *long-term* decrement in performance capacity with or without related physiological and psychological signs and symptoms of overtraining in which restoration of performance capacity may take *several weeks or months*" (1). Overreaching is defined as "an accumulation of training and/or non-training stress resulting in *short-term* decrement in performance capacity with or without related physiological and psychological signs and symptoms of overtraining in which restoration of performance capacity may take from *several days to several weeks*" (1).

In short, F-OR is an intentional part of an athletes training schedule and leads, with sufficient rest and recovery, to desired training adaptations. NF-OR and OTS should be prevented, because they lead to underperformance and require several days-weeks and several weeks-months of recovery (for NF-OR and OTS, respectively). These definitions are important to distinguish between F-OR, NF-OR and OTS. At the same time it is clear that these conditions are highly multi-factorial and individually determined, and that there are no discrete boundaries between F-OR, NF-OR and OTS, both in terms of their (patho-)physiology and clinical diagnosis.

Study designs used in overtraining research

Acute stress model (single-exercise study design)

Single-exercise studies, or acute exercise bouts, are used to identify markers that change after exercise. It is suggested that these changes indicate load or overload (3), training stress (4), muscle damage (5, 6) or adaptations (7), and are therefore used as exercise stress markers. These studies do not lead or intent to lead to OR, but are primarily used to find exercise stress markers. An advantage of these studies is their simplicity and their controllability. Markers can be assessed directly pre- and post-exercise, which makes evaluation of individual changes easy. These single exercise

studies can be used in a 'challenge' mode to investigate the dynamic differences in response between athletes who are diagnosed with overtraining and healthy controls. An advantage of the latter approach is that it provides insight into physical or even psychological resilience of (potentially) overtrained persons. In this way, exercise stress markers collected with these single exercise studies can be used for a proper diagnosis of overtraining in future. However, this approach does not result in a set of markers for the early diagnosis of overtraining, because the (potentially) overtrained persons are already at the point of overtraining and not in the development of overtraining.

Retrospective/observational (field)/case studies

A large number of studies focussing on overtraining are case studies or studies that report about overtrained athletes and their symptoms after being diagnosed with overtraining (8-10). These reports are useful for sport physicians to draw some conclusions about symptoms and help them with diagnosing overtraining. However, results cannot or hardly be extrapolated. For example, most of the time, these reports lack baseline measurements, which makes before and after comparisons impossible. In addition, personal variation in levels of outcome measures cannot be taken into account. Some of these problems could be solved by using a large matched healthy control group for between-group comparisons. From these comparisons, multiple markers that are significantly different between overtrained athletes and healthy controls may surge, and can be further evaluated by longitudinal studies.

But preferably, other approaches should be used to induce overreaching in athletes. Monitoring athletes during a training camp, where volume and/or intensity is increased for training purposes is a popular and practical method (11-13), as it is known that those training camps can result in OR. The 6 days training camps executed in the study designs of Hedelin et al (2000) and Jürimäe et al (2004) seem promising, as both resulted in reduced performance (11, 12), however, both failed to include a recovery period, so whether those athletes were suffering from F-OR or NF-OR cannot be concluded. The 8 day training camp, used as study design by Ishigaki et al (2005) did not include performance tests, so no conclusion about OR status can be made for that design (13).

In theory, monitoring athletes during a training camp, is a good method to study OR, but there are 3 important issues that should be addressed: 1) athletes should be checked for their general health and illnesses should be excluded, 2) dietary patterns should be assessed and controlled, 3) performance tests should be included before and directly after training camp to conclude if there is underperformance

(overreaching) and another performance test after recovery is needed to distinguish between functional and non-functional overreaching.
Another approach is monitoring athletes during long term competition or training, like the Giro d'Italia (3 weeks) (14), the Tour4life (8 days) (15, 16), or an army recruit training course (17). These methods are practical, because they don't necessitate to force athletes to follow a certain training program and increasing training volume is often part of the program. However, monitoring and controlling some factors, like competition anxiety, sleep deprivation and external stress factors, is difficult and those factors could influence outcome measures. Therefore, when researchers only intend to focus on the impact of physical exercise, such study designs are not preferable. However, when researchers want to mimic a practical setting including competition anxiety, sleep deprivation, or other external factors, these study designs might be useful. In fact, factors like insufficient diet, excessive concurrent work/study, sleeping disorders and stress may also play roles in the development of OR and OTS (18), which makes it interesting to monitor these triggers as well. An advantage of monitoring athletes during training camp or long-term competition is that these designs don't force athletes to follow specific programs, which makes it ethically less challenging and therefore, in practice, more easy to organize for researchers. Researchers should keep in mind that as soon as an athlete is detected with underperformance, it should be diagnosed as OR, not OTS. Then, with a follow up after the diagnosis of OR, whenever recovery does not occur, NFOR and then OTS can be diagnosed.

Study designs aiming to induce a state of overreaching

Controlled intervention studies, employing increased training loads to induce a state of OR in athletes are also used (12, 19-25). With these experimental designs, athletes gradually increase their training volume and/or intensity with a certain percentage. Training volume and intensity can be tailored to the individual participants, prescribed and monitored during training. This allows researchers to control more factors as compared to the field studies (training camp or long-term competition). Major issues of these controlled training program studies is that it raises the ethical dilemma to what extent athletes can be compelled to change their normal (training) routines and program, and which increment is considered acceptable in view of the '*primum non nocere*' principle. Furthermore, these studies entail some practical issues, like compliance, how to monitor athletes closely and intensively, and how to keep other factors as stable as possible. Besides, the possibility of long-term adverse physical and psychological effects is present.

An overview of prescribed training programs to induce OR as found in literature and meeting our search criteria is given in Table 1 (at the end of this manuscript). The number of athletes in these studies varied from 3 (19) to 24 (20). The training volume increment varied from 10% per week (20, 21) to double volume over a certain period (12, 22-24). Training intensity increment varied from 0% to 152% (25). In short, studies varied in sample size, training design and type of sports.

Duration, volume and intensity

For the training design, it appears that increasing volume is more efficient for inducing OR than increasing intensity (26). Although it is not completely clear, it could be that athletes have to dedicate more time to the actual training to meet the criteria of increased training volume and have therefore less time available for recovery. In other words: the balance between training and recovery is more disturbed in this setting compared to a setting were training intensity is increased. In addition, there may also be a 'mechanical' explanation for this difference, as increased training volume results in a longer time frame where impact on bones and muscles takes place. But this finding might also be a coincidence and caused by the study population in the different studies. As far as we know, it has not been investigated why an increased volume resulted more often in OR compared to increased intensity. For the sake of overtraining studies and the understanding of the (patho)physiology of overtraining, it might be very interesting and helpful to study which protocol is more efficient for inducing OR and why. Not all (training) interventions used in these studies (Table 1) caused NF-OR (21, 27-29). Maybe a time frame of only one week for increased training load was too short (27), or increasing volume with only 30 or 38% was too low (21, 29). Researcher could overcome these issues by increasing the duration of the increased training period and by increasing the training volume with more than 38%. It appears that at least 2 weeks and an increment of 100 % is sufficient in causing underperformance (30).

Inclusion of performance testing and a recovery period

Many of the study designs reported did not include a recovery period (11-13, 17, 19, 20, 22, 23, 26, 27, 31-34). To determine whether an athlete suffers from F-OR or NF-OR, a recovery period is needed, as an increment in performance after this recovery period points out that the athlete was functionally overreached, whereas no increment in performance points out that the athlete was non-functionally overreached. This brings us to another important shortcoming: the lack of performance testing (13, 33, 35). Underperformance is key in OR and OTS, and

without performance testing, one cannot conclude whether an athlete is performing worse and therefore suffering OR.
Studies that included a recovery period with performance testing and resulted in NF-OR are unfortunately scare (30, 36-38). Those studies lasted from 10 days (36) till 6 weeks (30), suggesting that time frame is not the most important factor of a training program when inducing OR. Interestingly, increasing training volume with 100%, i.e. cyclists going from 7 hours/week to 14 hours/week (30) or runners going from 60 km/week to 120 km/week (38), seems a good approach as both resulted in overreached athletes. To be complete, researchers need to exclude confounding disorders before they even start a performance test, because other disorders could result in underperformance as well.

Nutrition
Another very important issue, not approached in most studies mentioned in Table 1 is the assessment and control of dietary aspects. Inadequate nutrition can increase the risk for NFOR and OTS (18). To be comprehensive, researchers should aim to assess dietary patterns before and during the training intervention. A 3 day food record can be used to collect and analyse an athletes' daily nutrition before the intervention. Prescribed diets, based on the athletes' needs, can be used to make sure that the athlete consumes a diet that is adequate. However, such a diet is of course another burden for participants.
To conclude on these prescribed training programs: an advantage is the controllability of the design, the possibility to plan pre- and post-exercise measurements and the possibility to collect data about other factors influencing the study results. However, when designing such studies, ethical considerations should be taken into account. First, there is a possibility of long-term adverse physical and psychological effects for the volunteers/athletes. Secondly, the load of these study designs is very high for the volunteers/athletes, as it involves constant monitoring of training load and time management. When those limitations are not a problem, controlled interventions that increase training volume with 100% during >10 days might be the most useful interventions for studying OR.

Experiences and learnings from our recent study

Based on the before mentioned considerations, we set out to test a 'best practices' design to induce a state of overreaching in athletes in a controlled intervention study. The study was approved by the Medical Ethical Committee of Wageningen University and Research (NL60915.081.17) and all athletes gave written informed consent before participation.

We doubled training volume in four weeks' time, without changes in intensity, and we investigated whether that increase in volume would lead to non-functional overreaching. Our starting point was to explore whether such a design could be used in a larger overreaching study we planned to do. To this end, we included 7 healthy athletes, both men and women, either triathletes or cyclists. Participant characteristics are shown in Table 2.

Table 2. Participant characteristics

Sex	5 male, 2 female
Age (years)	26.9 ± 5.5
Length (cm)	179.4 ± 7.9
Weight (kg)	76.95 ± 7.66
BMI	23.87 ± 1.23
Body fat (%)	17 ± 7
VO2max (ml / kg / min)	59.7 ± 8.7
Power max (Watt)	392 ± 73
Training hours per week	7.25 ± 1.11

Mean ± SD are shown.

Participants monitored their normal training program with a SPARK3 TomTom heart rate watch including GPS tracking (Amsterdam, the Netherlands), for two weeks. This normal training program was recorded and an adapted training program was developed, which increased in four weeks' time to double volume. In practice this meant that athletes followed one week of their normal training program again (100%), the second week the volume increased to 133%, the third week to 166% and the fourth and last week to 200%. Again participants kept track of training with their heart rate watches with GPS. Thereafter, participants followed two weeks of recovery training at 50% of their normal volume. Dietary patterns were not controlled, diet was ad libitum. Baseline measurements before the study included a VO_{2max} test, body composition tests (weight and skin folds to analyse body fat percentage) and a familiarization with the time trial test on the bicycle ergometer.

All participants performed three simulated 10 km time trials on the bicycle ergometer: one after the two weeks of monitoring their usual training load, one directly after four weeks of the increased training load and one directly after two weeks of recovery. An overview of the study design can be seen in Figure 1.

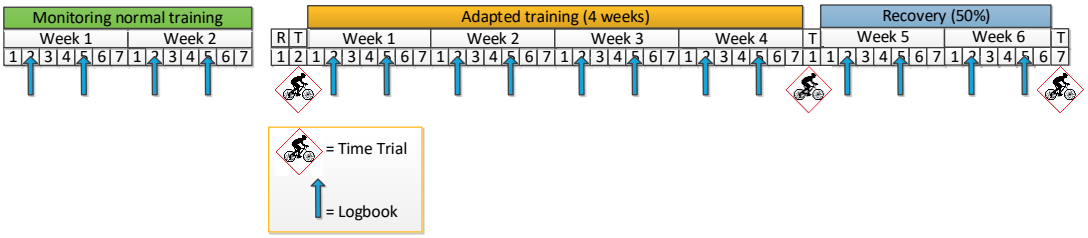

Figure 1. Study overview. Athletes monitored their normal training volume in the first two weeks. Thereafter they followed four weeks of adapted training with increased training volume, followed by two weeks of recovery training at 50% of their normal volume. They filled out a logbook and POMS questionnaire every second and fifth day of the week, during the total study.

All circumstances during time trials were kept as equal as possible. Time trials were done on the same time and day of a week, warming-up was a fixed program, see Figure 2, and music and encouragements were not allowed.

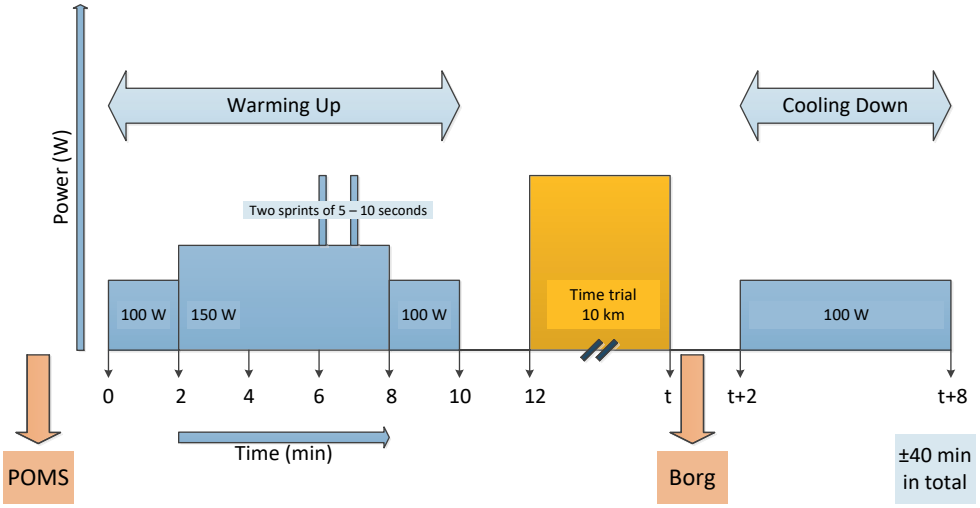

Figure 2. Time trial protocol. Before the start of the warming up, athletes filled out a POMS questionnaire, thereafter athletes followed the strict warming up protocol. Before the time trial they sat still for 2 minutes on the bike. The time trial was without encouragements of any kind. Directly after finish, a Borg scale was shown to indicate their rate of perceived exertion.

Table 3. Study results. Time trial results of all 7 participants separately.

	TT 1			TT 2			TT 3			Comparison	
PN	time (sec)	HR_{max} (bpm)	Borg score (6-20)	time (sec)	HR_{max} (bpm)	Borg score (6-20)	time (sec)	HR_{max} (bpm)	Borg score (6-20)	2^{nd}-1^{st} time trial	3^{rd}-1^{st} time trial
1	1047.7	193	19	1082.8	187	20	1064	198	19	103.4	101.6
2	773.9	178	18	738.7	176	17	743.4	179	17	95.5	96.1
3	1424.4	193	17	1573.5	191	19	1444.4	203	17	110.5	101.4
5	727.3	189	20	704.9	182	20	710.4	187	20	96.9	97.7
6	889.4	176	19	874.9	178	19	849.5	180	18	98.4	95.5
7	849.7	190	18	765.9	190	18	767.9	191	19	90.1	90.4
8	758	195	20	781.3	194	18.5	767.4	194	19	103.1	101.2

PN: Participant number; Basic: normal amount of training hours per week; Adapted: amount of training hours during adapted training; TT: time trial; HR: heart rate; 2nd-1st time trial: increase or decrease in time between the first and second time trial; 3rd-1st time trial: increase or decrease in time between the second and third time trial

Time trial results are presented in Table 3. Based on the assumption that a normal variation between time trials is approximately 2% in experienced bikers (39-41), we included the criteria that time to complete the test should be increased by >3% to be classified as underperformance. We observed in only three participants such an increase of >3%, while the other participants performed better (n=3) or the same (n=1) after four weeks of increased training load. After the recovery period performance of these three participants was comparable to their first time trial (within 2% of that time). In other words, they were overreached, but not non-functionally overreached according to the definition. In addition, POMS mood score indicated a significant increase in mental fatigue (p=0.030; one-sided, paired t-test), while time trial performance tests were not that convincing. Therefore, we concluded that this study design with these participants did not lead to non-functionally overreached athletes and was therefore not useful for a future study in which we would explore exercise stress markers to distinguish overreached athletes from healthy well-trained athletes.

Interestingly, mood changes indicated more fatigue in our athletes, but performance tests did not. This points out that only questionnaires are not sufficient in diagnosing OR and OTS. A possible drawback in our study was the training history of the included participants. A couple of years before this study, five of these athletes were performing at a higher level, training more hours per week compared to their training schedule at the time of this study. This might result in more resilience to the increased training load. Of course, the negative outcome of this study is a risk of using a design to deliberately induce NF-OR. Researchers might need to start with a large sample size to end up with enough overreached athletes after the increased training intervention. Nevertheless, an advantage of such a design is the controllability of many factors.

(Key) Outcome measures to assess in overtraining research

As research progresses, more and more outcome measures are taken into account in the search for markers that could possibly indicate or predict (NF-)OR. In the second part of this review we will discuss the essential outcome measure “performance”, into more detail. In addition, we will discuss two other interesting outcome measures: mood/behaviour/cognition and parameters for immune functionality, because these are both evident in overtraining symptoms (42, 43). Finally, other outcome measures are briefly discussed, based on the other major symptoms of overtraining as presented by Fry, Morton and Keast in 1991 (2). They divided the symptoms in subcategories (physiological performance, psychological/information

processing, immunological and biochemical changes), and per subcategory, researchers might think of certain outcome measures.

Performance

The most important outcome measures that should always be taken into account in an OR and OTS study is performance, as the definition of OR and OTS contains at least the phrase 'overtraining is an unwanted, long lasting period of underperformance'. Without (assessed) underperformance, one cannot diagnose OR or OTS. Performance depends on the type of exercise. With some sports, speed is important (cycling, running, swimming, etc.), whereas in other sports distance is important (jumping, throwing (javelin and hammer)), and other sports are focussed on power (weight lifting). However, most sports are a combination of speed, power and coordination (team sports, CrossFit, gymnastics). Therefore, it is important to assess performance or underperformance in a sport-specific manner. In addition, researchers should keep some important circumstances during those performance tests as equal as possible: 1) the warming up protocol performed before the actual performance test should be a standardized protocol that is used for every performance test included in the study design, 2) performance tests should be done on the same time and day of a week, with a standardized training session the day before the performance test day, 3) music and encouragements are not allowed during performance testing, 4) nutritional intake should be controlled.

The performance test that is used during these study designs should be well chosen and sport-specific. Time trials can be used to assess performance in sports reflecting speed. Distance can be assessed in jumping or throwing sports and 1RM (1 repetition maximal) tests can be used to assess performance in power sports. Power multiplied by speed can also be used to assess performance in power sports. Sports that include coordination and speed might use a T-test for measuring agility or the Illinois agility test, the 505 test, the L-run test and the zigzag test (44).

Which performance test is chosen in a study design depends on the type of athlete involved, however, most important is that performance is tested. Many designs that are summarized in Table 1 met this criterion (11, 12, 17, 19-23, 26-32, 34, 36-38, 45-48), while a few failed (13, 33, 35).

Mood/behaviour/cognition

A consistent finding linked to overtrained athletes is a change in mood/behaviour/cognition (49). It is even suggested that the behavioural symptoms of overtrained athletes are similar to clinical depression (49). These behavioural

symptoms generally include reduced appetite, weight loss, reduced libido, depression, loss of interest, and in case of athletes: fatigue and disinterest in training and competition. These "sickness" behaviours probably assist the athletes in recovery.

It is suggested that these changes in mood, behaviour and cognition can also be used as a marker for OR and OTS. Indeed, it has been shown that NF-OR/OTS athletes make more mistakes on a Stroop Color Word Test compared to healthy athletes (50). And F-OR cyclists had longer reaction times compared to a control group, although this difference was just not significant (51). On the other hand, no differences in choice reaction time were found between fatigue and F-OR subjects who participated in a 8-day cycling event (15). Nevertheless, integrating a psychomotor speed test into an OR study is rather easy, the tests are computerised, objective and not manipulability, and they can be performed in a quiet room on a laptop. In addition, single training sessions don't have detrimental effects on complex reaction time performance, when assessed after physical exercise, and computerised assessment techniques are affordable (52).

Possible tests and questionnaires include for example: choice reaction time tests, cued reaction time tests, questionnaires about mood, stress and recovery (e.g. profile of mood states (POMS) and recovery and stress questionnaire (REST-Q). Researchers could also measure factors involved in the brain-immune system interactions. Proteins, like 5-hydroxytryptamine (53), cytokines, like Interleukin-1 (54), Interleukin-6 and tumour necrosis factor-α and hormones including adrenocorticotropic hormone (ACTH), corticotrophin-releasing hormone (CRH), prolactin (PRL), growth hormone (GH), and cortisol, are all involved in this interaction. Both cytokines and hormones are linked to sickness behaviour and depression (55). As a consequence, these might therefore be interesting outcome measures in an OR/OTS study.

Immune system

More recently, immunological effects have been incorporated in overtraining studies. It is hypothesized that overtraining is reflected in changes in cytokine levels as an adaptation to excessive (exercise) stress (42). Interestingly, cytokines are linked to behavioural changes, as described above, but also to muscle functionality and systemic inflammation. It is suggested that excessive musculoskeletal stress, with insufficient recovery, induces a local acute inflammatory response, which may contribute to low-grade systemic inflammation. This in turn leads to the release of more substantial quantities of pro-inflammatory cytokines. These cytokines interact

with the central nervous system and induce "sickness behaviour". Next, cytokines activate the sympathetic nervous system (43) and HPA-axis (2), resulting in changed blood catecholamine, glucocorticoids and gonadal hormone levels (42). In addition, in a recently performed system-wide proteomics study in overreached subjects 13 proteins were identified that increased on the morning of day 1 and/or day 2 of recovery (56). Subsequently, STRING protein-protein interactions showed that most of these proteins were involved in the immune defence response including the acute phase response, complement activation and humoral responses mediated by circulating immunoglobulins (56). So, many mechanisms seen in overtrained athletes can be explained by immune responses, which makes it interesting to take these factors into account in overtraining studies. Although these are not key outcome measures, like performance and mood, they are potential biomarkers for the detection of early OTS.

Other outcome measures

Besides performance and mood, the first outcome measures that might come to mind are physiological markers that change during exercise. This includes markers associated with muscle damage, like Creatine kinase (5), creatinine (57), myoglobin (6)), and cardiac damage, e.g. troponin (57), or physiological and biochemical markers such as glycogen depletion (58), lactate production (4), reduced maximal heart rate (4) and change in heart rate (variability) (47). When researchers aim to focus on hormonal changes they can study cortisol, adrenocorticotrophic hormone (ACTH), growth hormone and prolactin (PRL) for example. Or maybe focus on estradiol and testosterone (59). These hormones can also be measured after a challenge with two maximal exercise tests, separated by 4 hours, as suggested by Meeusen et al (60). Noteworthy, the use of only a few biological markers for monitoring athletes is not recommended, with the upcoming techniques like metabolomics and proteomic, it is possible to link metabolism and the immune system. A more systems biology approach is therefore recommended (61).

Future research considerations and guidelines

In view of the considerations discussed above future study designs and exercise programs to study OR and OTS require careful attention. Whilst this seems obvious it is important to start from the precondition that the study approach actually enables to investigate NF-OR and not merely fatigue or something else causing discomfort in an athlete.

Whether it concerns a retrospective, observational, case study, or a controlled intervention to induce NF-OR in athletes, at least a number of criteria are important:
1) Studies should include a sport-specific performance test, because underperformance is the defining element of OR and OTS. Baseline performance needs to be measured as well, which makes case studies and retrospective studies complicated, as symptoms are often evident before a performance test was executed. Intervention studies involving a set-up to induce NF-OR in a controlled setting seem favourable. However, ethical issues and the choice for such an adapted training program remain complex factors.
2) Studies should include a recovery period, because a performance test after this recovery period shows whether athletes were F-OR or NF-OR. Without a recovery period followed by a performance test one can only conclude whether an athlete was overreached, without distinction between the type of overreaching.
3) The same definition of F-OR, NF-OR and OTS should be used in all future research (1, 62) and diagnosis should be based on this definition. Besides, researchers need to distinguish OTS from OR and exclude other causes of underperformance, like anaemia, infection, and insufficient diet.
4) Additional outcome measures, besides performance (as described in point 1) must be well selected. Many options have been investigated, including patterns that reflect processes and dynamics instead of rather isolated endpoints. Furthermore, literature data, including those from adjacent fields are frequently suggesting alternatives, for example related to immune functions or cognitive function. In addition, studies that did not find "the promising set of markers" that could distinguish well-trained from NF-OR athletes are helpful as well, because they are a stepping stone to the future solution.
In conclusion, a lot of research has been done in the field of overtraining. However, not all studies are equally useful and some suffer from methodological issues. There are certain critical aspects that should be kept in mind, like the study design and training program, performance testing and recovery periods, which outcome measures, and the definitions of (functional and non-functional) overreaching and overtraining.

Table 1. Overview OR and OT study designs used before

Author	Athletes	Period	Regular training program	Design	Performance tests included?	Recovery period included?	Results	Conclusion
Booth et al. 2006 (17)	38 male, 5 female army recruits	45 days	n.a.	45-day Army Common Recruit Training course	yes	not	Fitness testing (maximal push-ups, maximal sit-ups, vertical jump, 2.4km run) showed that power improved, but speed on run reduced.	Physical performance did not deteriorated greatly. Accumulated sleep deprivation might be a major contributor to the adverse hormonal changes. We conclude that there was some evidence of recruits being overtrained.
Bosquet et al. 2003 (38)	9 experienced male endurance athletes	4 week	Running training, ~60km/week	Training volume increased with 100% over 4 weeks' time + 14 days of recovery. (According to protocol Lehmann)	yes	yes	No significant changes in LF/HF (HRV data) at night. Maximal aerobic speed (MAS) decreased after the four weeks of increased training volume	Overtraining in 6 subjects, according to authors
Callister et al. 1990 (31)	15 elite judo athletes, 8 male, 7 female	10 weeks	Resistance training 3d.wk-1, Interval training 2d.kw-1, Judo training 5d.wk-1	Week 1-4: regular training; week 4-8: 50% increase in interval and resistance; week 9-10: baseline.	yes	not	Isokinetic strength of elbow and knee extensors and flexors increased sign from weeks 2 to 4 (3-13%), was unchanged from weeks 4 to 8, and decreased sign	6 weeks of overtraining may affect some but not all aspects of performance and that performance may be affected before

				Judo training volume was unchanged in weeks 1-8 and increased by 100% in weeks 9-10.			(6-12%) from weeks 4 to 10. Total time for 3x300 m intervals increased between week 2 and 4 and between week 4 and 8. Total time for 5x50m sprints decreased from weeks 8 to 10 (less than 2%).	symptoms of the overtraining syndrome appear.
Costill et al. 1988 (22)	12 highly trained male swimmers	10 days	1.5 h/day, 5 days/week: 4,266m per day, Intensity: 94% of their VO2max	2 training sessions per day, 1.5h per training session. Average training distance increased from 4,266 to 8,970 m/day	yes	not	Swimmers experienced local muscular fatigue and difficulty in completing the training session. performance (sprint 25 yards and endurance 400 yards) was unchanged	4 of the 12 swimmers were unable to tolerate the heavier training demands and were forced to swim at a slower speed during training. These men had sign reduced muscle glycogen levels, this was a result of their abnormally low carbohydrate intake. No overreaching in the group of swimmers
Dressendorfer et al. 1991 (27)	10 moderately fit men, joggers	7 days	16 km/week	Increasing weekly training distance 8 times to 129 ± 2 km/week.	yes	not	Treadmill test showed no underperformance or improvement	No overreaching

Filaire et al. 2004 (33)	12 national level male cyclists	8 months	During T0, the training programme consisted of non-specific training at a low intensity (60% of VO2max) (4.3 ± 1.1 h and 150 km per week).	Training load was 440.08 ± 23.8 training units (TU) during T0, 728.83 ± 43.8 TU during T1, 2026 ± 24 TU during T2 and 940.7 ± 36.4 TU during T3.	not	not	Cortisol:testosterone ratio decreased at T2. Mood State (POMS) was unchanged. Questionnaire about OT was used to evaluate OT (Legros et al. 1993)	No overtraining based on OT questionnaire.
Fry et al. 1992 (36)	5 male members of the special air services regiment of the Australian army	16 days	army training	10 days of twice daily interval training sessions on a treadmill, followed by 5 days of active recovery	yes	yes	Significant fall in performance at day 11 at run to exhaustion test (18 km/h with 1% gradient). Return to pre exercise levels on day 16.	Overreaching at day 11
Fry et al. 1994 (46)	5 well trained men from the Special air services regiment of the Australian army	10 days + 5 days recovery	army training	2x intensive interval training per day, for 10 days. Followed by 5 days active recovery	yes	yes	Performance at time to exhaustion running test (18 km/h, 1% grade), mean time to reach exhaustion depressed by 29.3%	No OT. Time to exhaustion is decreased after 10 days of intensified training, but back to baseline after 5 days of recovery.

Halson et al. 2002 (30)	8 male endurance cyclists	6 weeks	Normal training = 7 ± 2 hours/week.	2 weeks normal, 2 weeks intensified (14 ± 5h/week) and 2 weeks recovery training (3.5 ± 2.5h/week).	yes	yes	Decline in maximal power output, and decreased performance on time trial after the intensified training weeks. Performance decreased from 59.4 ± 1.9min to 65.3 ± 2.6 min. And a 29% increase in global mood disturbance.	Overreaching criteria were: reduced performance on lab test and negative mood. All 8 subjects completed the intensified training period and met the criteria for OR.
Hedelin et al. 2000 (11)	9 elite canoeists (6 men and 3 women)	6 days, training camp	Cross country skiing and strength training, during 6 day training camp	50% increase in training load, to a total of 13.0 ± 1.6 hours. 25% was high intensity/anaerobic training, 65% was endurance training, 10% was strength training.	yes	not	Time to exhaustion on incremental treadmill test decreased, VO2max and La(max) decreased.	The reduced maximal performance indicated a state of fatigue/overreaching
Hooper et al. 1993 (63)	5 male and 9 female elite swimmers	6 months	10 to 12 workouts/week and 6 days/week		?	?	Stress hormone levels at 5 points during 6 months. Plasma norepinephrine and epinephrine levels were significantly correlated with swim training volume ($r = 0.37$ and 0.33, respectively, $p < .05$)	Symptoms of OT were identified in three swimmers, based on performance decrements and high levels of fatigue.

Ishigaki et al. 2005 (13)	13 male collegiate distance runners	8 days, during training camp	Half of what was used in the study design	284.1 ± 48.2 km running (almost twice the amount of a normal training week)	not	not	Ratio testosterone:cortisol in serum dropped with 50%	No performance tests included, so no conclusions on OR or OT possible
Jeukendrup et al. 1992 (37)	7 male competitive cyclists	2 weeks		intensifying normal training program, performance tests before, during, and after two weeks of intensified training and after two weeks of recovery	yes	yes	General state of wellbeing declined, performance on time trial declined. (note: time trial was 8.5km outside)	Overreaching
Jürimäe et al. 2002 (32)	10 male junior rowers	6 days, training camp	6 training sessions per week, during 6 day training camp	100% increase in load to 21.5 ± 2.2 hr in 12 training sessions per week. 58% of training volume was low-intensity endurance training (rowing or running), 5% was high intensity anaerobic training (rowing) and 10% was resistance training.	yes	not	2000m time trial on a rowing ergometer increased (underperformance) from 406.8 ± 9.2 sec to 410.9 ± 8.8 sec.	Significant increase in fatigue scores from stress-related scales. Performance on time trial reduced. In conclusion: overreaching

Jürimäe et al. 2004 (12)	21 male competitive rowers	6 days, training camp	6 training sessions per week, during 6 day training camp	Rapidly increase training volume with 100% to 19.6 ± 3.8 hours. 58% of training volume was low-intensity endurance training (rowing or running), 5% was high intensity anaerobic training (rowing) and 10% was resistance training.	yes	not	2000m rowing ergometer performance time worsened. The RESTQ-Sport revealed an increase in somatic components of stress	Indications for a state of heavy training stress and incomplete recovery: overreached
Lehmann et al. 1991 (23)	8 middle and long distance runners	4 weeks	Average 85.9 km / week	Volume group: training volume was doubled from 85.9 km/w in week 1. To 115.1 km in week 2, 143.1 km in week 3 and to 174.6 km per week in week 4. Training 6 days a week. With nearly 30km/day in the fourth week.	yes	not	Decrease in total running distance (from 4719+912m to 4361+788m) on treadmill in 6 runners.	Overreaching

Lehmann et al. 1992 (26)	17 middle or long distance runners	3 weeks	85.9 km/week	Volume group: training volume was doubled from 85.9 km/week to 174.6 km/week in week 3. Intensity group: intensity runs increased from 9km/week in week 1 to 22.7 km/week in week 3, and distance increased from 61.6km in week 1 to 84.7 km/week in week 3.	yes	not	Higher training volume resulted in a decrease in running distance in increment test. Higher intensity produced an improvement in running velocity and distance.	Overreaching in the higher volume group. No overreaching in the higher intensity group.
Lehmann et al. 1993 (28)	6 recreational athletes (medical or dental students)	6 weeks	1 or 2 hours in total per week (6 weeks training + 3 weeks recovery)	4 days/week 31-33 min endurance training on bike ergometer, and 2 days/week interval training of 3-5 x 3-5 min interval	yes	yes	Submaximal and maximal performance increased: at 2 mmol lactate by 25%, at 4mmol lactate by 12% and maximum performance by approximately 12%.	No overreaching
Lehmann et al. 1996 (25)	17 middle or long distance runners	4 weeks	85.9 km/week	Increase in volume (103% increase per 4 weeks) or increase in intensity training (152% increase			Total running distance decreased in the incremental test during intensified volume. It increased in the intensified intensity training.	Overreaching in the increased volume group, no overreaching in the increased intensity group

				per 4 weeks in tempo-pace and interval run).				
Mackinnon et al. 1997 (20)	24 elite swimmers (8 female and 16 male 100-200m swimmers)	4 weeks	28.5 km per week swim training, and 151.2 min per week dry-land resistance training. Training 6 days per week, usually twice per day. Sunday is rest day.	Training volume increased by ± 10% each successive week. At the end of week 4, swim volume was 38.9 km per week (36.5% higher) and dry-land was 184.8 min (22.2% higher).	yes	not	200m freestyle time trial worsened	8 (6 female, 2 male) swimmers (out of 24) were overreached
Maso et al. 2004 (34)	25 young international rugby players	1 day tests (after two months of hard training)	15 hr/week plus one match a week.	Saliva cortisol, testosterone and their ratio was assessed at 8am, 11am and 5pm. Players answered an overtraining questionnaire.	yes	not	OT score on questionnaire correlated with testosterone levels at 8am (r= -0.53), mean testosterone levels and testosterone:cortisol ratio at 8am..	No OT diagnosis possible, as performance tests (that represented different aspects of rugby) were described in methods but not taken into account in results.

Parry-Billings et al. 1992 (64)	40 male international athletes participating in cycling, distance running, sprint running, race-walking, rowing, squash and swimming	3 weeks	varying					
Pichot et al. 2002 (47)	6 sedentary males	2 months	n.a.	Intensive physical training (3 days/week) for 8 successive weeks, followed by 1 transition/recovery week, followed by 4 weeks of overload training and 2 weeks of recovery.	yes	yes	HRV data changed after training. VO_{2peak} levels increased.	No OR or OT. VO2peak increased from before training to after training and further increased after overload training.

Robson-Ansley et al. 2007 (35)	8 healthy endurance trained male triathletes	4 weeks	18 hours of training per week.	Additional interval training run sessions on three successive days in week 2 and 3.	not	vague, week 4 is normal training period	Plasma IL-6 and creatine kinase activity were elevated following intensified training. DALDA questionnaire showed higher stress, tiredness, reduced effort during training, need for a rest, congestion and sore throat in week 2 and 3, compared with week 1.	No OR or OT diagnosis possible. 1 min maximum effort sprint on a 400m outdoor running track was measured but not taken into account after the intensified training weeks.
Smith et al. 2000 (21)	10 male and 8 female elite rowers	3 weeks	Rowing (9 sessions/week), weights (2 sessions/week) and running (1 session/week)	33% increase in frequency and a 30% increase in training volume + 1 week recovery (75% volume)	yes	yes	Ergometer sprint performance time at 500m did nog change significantly	No overreaching
Steinacker et al. 2000 (45)	10 rowers (in preparation for the World Championships in 1995)	18 days		High training loads of 3.2 hours a day for 18 days	yes	yes	Incremental all out rowing ergometer tests. The 2000m rowing speed was slowest after training phase, and fastest after taper and World Championships	Overreaching, and after taper recovery, so F-OR

Thiel et al. 2011 (19)	3 male ATP tennis players	30 days	Varying: conditioning training included endurance, speed and agility, as well as strength and sensorimotor training.	Following normal training period, increment of volume by 120%, 140% or 180% for the top 30, top 100 and top 1000 player	yes	not	Speed-strength assessment for performance testing. All 3 players improved in aerobic capacity and speed strength. No underperformance	No overtraining, maybe functional overreaching
Uusitalo et al. 1998 (48)	15 healthy female endurance athletes (5 runners, 4 skiers, 3 triathletes, 3 orienteers)	10 to 15 weeks	varying	6 to 9 weeks of experimental intensive training and 4 to 6 weeks of recovery. Training 7days/week. Training volume increased by 80% during the intensive training period.	yes	yes	No changes in nocturnal urine catecholamines. Plasma noradrenaline and adrenaline decreased from after 4 weeks of training to the end.	Criteria for OT were: decreased VO2max, decreased treadmill performance, unwillingness to train. 5 females were OT according to authors. However, mean VO2max did not decrease during the training period.
Verde et al. 1992 (29)	10 highly trained male distance runners	3 weeks	Normally running 70-100km per week, increasing it with 38% for 3	Normally running 70-100km per week, increasing it with 38% for 3 weeks.	yes	yes	No worse outcome at progressive treadmill run to exhaustion.	No underperformance, no overreaching

			weeks, after that 3 weeks of normal training.					

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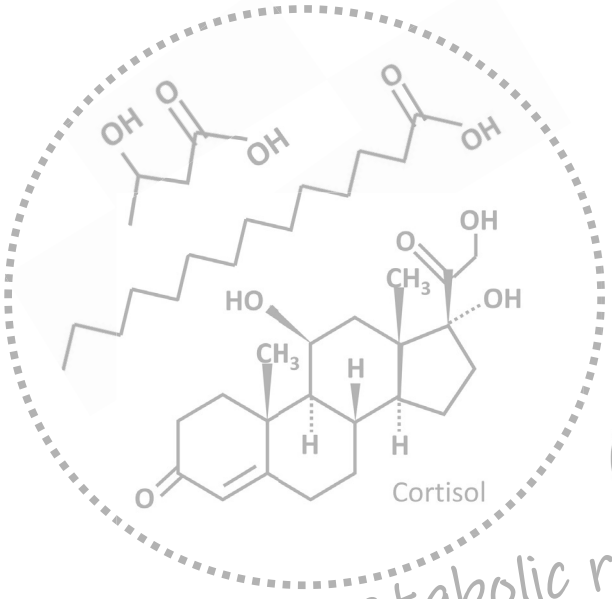

Stress & metabolic response

Low Carb

Chapter 8

Effects of a low carbohydrate diet on the exercise induced stress and metabolic response in well trained individuals

LC diet affects exercise-induced stress and metabolic response

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ABSTRACT

We investigated the effects on exercise induced stress and metabolic responses of acute (2days) and prolonged (2 weeks) adherence to a LC diet and compared this to a high carbohydrate (HC) diet. **Methods:** In this cross-over study, fourteen well-trained male athletes (32.9±8.2 years, VO2max 57.3±5.8 ml/kg/min) followed a two week LC diet (<10En% CHO) and a two week high carbohydrate (HC) diet (>50En% CHO), in random order, with a wash-out period of >2 weeks in between. After 2 days and 2 weeks on either diet, participants cycled for 90 minutes at 60% $W_{max.}$. Blood samples for cortisol, free fatty acids, glucose and ketones and saliva samples for immunoglobin A (s-IgA) were collected at different time points before and after exercise. **Results:** Short-term adherence to the LC diet already led to metabolic changes, shown by higher FFA, higher ketones and lower glucose levels compared to the HC diet ($p < 0.05$). Exercise induced cortisol response was highest after 2 days on the LC diet (822±215 nmol/L) compared to 2 weeks on the LC diet (669±243 nmol/L, $p=0.004$) and compared to both test days following the HC diet (609±208 and 555±173 nmol/L, both $p<0.001$). Workload was lower on the LC diet compared to the HC diet at both durations. A drop in s-IgA following exercise was not seen after 2 days on the LC diet, in contrast to the HC diet. **Conclusions:** Results point towards different adaptation times to the LC diet in terms of the metabolic and stress response.

KEY WORDS: Cortisol, ketones, s-IgA, exercise, low carbohydrate diet

INTRODUCTION

Although already described in 1920 (1), the use of low carbohydrate, high fat (LCHF) diets in sports has recently regained considerable interest. Such diets are also often referred to as ketogenic diets, although they are generally not comparable to the strict ketogenic diets applied for medical purposes. Following a LCHF diet is known to increase fat oxidation (1), and alleged to postpone glycogen use during prolonged exercise (2). In addition, studies have demonstrated an enhanced activation of some enzymes and mediators involved in adaptation to endurance training, like AMPK (3) and PGC-1a (4), in situations of low carbohydrate (CHO) availability (3-6). However, at the same time, improvement of exercise performance following LCHF diets has not been observed in studies (7, 8). It is proposed that long-term training with a reduced CHO availability might impair the ability to oxidise exogenous CHO during exercise (9), as pyruvate dehydrogenase activity is suppressed by a LCHF diet (10). Besides, training with low CHO availability is associated with the inability to maintain the desired training intensity (11, 12).

It has also been suggested that training with low CHO availability may lead to an increased exercise-induced stress response, reflected by higher cortisol levels, and may lead to an attenuated immune response (13). This would further argue against this use of LCHF diets in sports practice, in particular in view of an often already increased risk for upper respiratory tract infections (URTIs) in athletes (14). The incidence of URTI in athletes is, amongst others, related to low levels of salivary Immunoglobulin A (s-IgA). This s-IgA is an antibody isotype that is produced locally by B lymphocytes present in mucosal tissues and appears in mucosal secretions such as saliva, thereby protecting against bacteria and viruses entering the body (14). Human s-IgA, as a dimer, has a mostly equal representation of IgA1 and IgA2 antibody isotypes, with igA2 being more resistant to digestion because of a shorter hinge region and more compact structure. A shortage of carbohydrate as energy substrate might stimulate cortisol release, inhibiting B-cell immunoglobulin production, resulting in lower s-IgA levels (15). However, it is presently unknown whether such an effect of a low CHO availability occurs in practice and to what extent it might affect URTI incidence.

Another question that remains is whether the duration of a LCHF diet plays a modulating role in these parameters. In sports practice, several dietary strategies are used, including different 'train-low, compete-high' schedules (16) or even chronic ketogenic diets (17). In the present study, we investigated the effect on the exercise-induced stress and metabolic responses of acute (2 days) and prolonged (2 weeks)

adherence to a low carbohydrate diet, and compared this to a high carbohydrate diet. Cortisol, salivary IgA levels, URTI symptoms, respiratory exchange ratio (RER), circulating metabolites, work output and perceived exertion during exercise were measured in this randomised cross-over dietary intervention study.

MATERIALS AND METHODS

Participants

Fourteen well-trained male athletes participated in this study. They were recruited by contacting local cycling and triathlon clubs and via social media. All trained regularly, at least 4 hours per week. Additional inclusion criteria were a BMI between 18.5 and 25 kg/m^2 and age between 18 and 45 years. Exclusion criteria were presence of food allergies, chronic illnesses, use of asthma medication, anti-inflammatory- and/or immunosuppressive medicines. All participants had a hemoglobin concentration > 8.5 mmol/L, and they had not donated blood during six weeks prior to the study.

Study enrolment took place between October 2018 and January 2019. The study was conducted at the Human Nutrition Research Unit, Wageningen University & Research. The study was approved by the Medical Ethical Committee of Wageningen University (NL6540408118, ClinicalTrials.gov ID: NCT04019730) and all participants gave written informed consent prior to participation. This study was conducted in accordance with the Declaration of Helsinki.

Study design

This study had a cross-over design, with 2 times 2 weeks of dietary intervention (Figure 1).

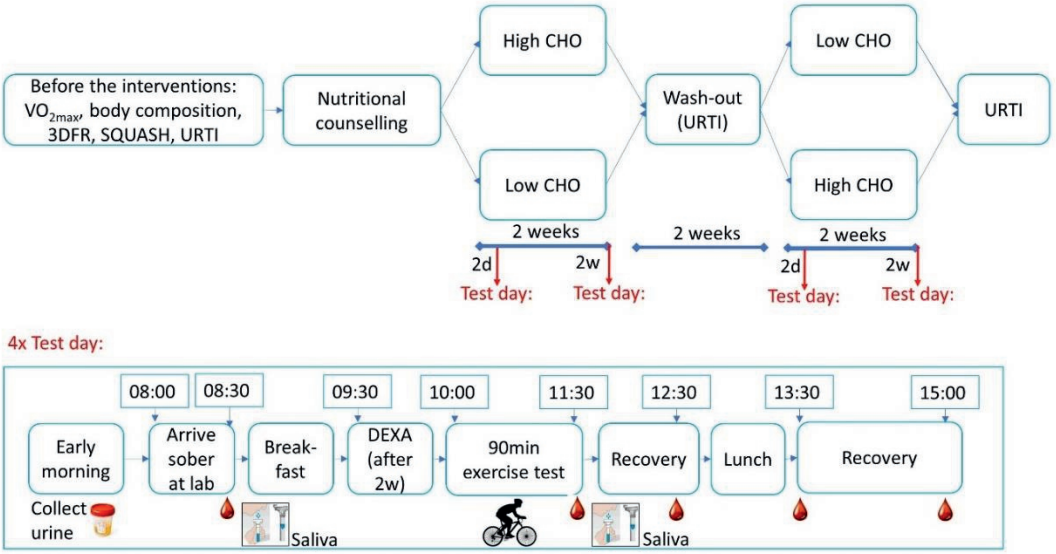

Figure 1. Study design. Schematic study design, 3DRF: 3-day food record; SQUASH: Short Questionnaire to Asses Health enhancing physical activity; URTI: Upper respiratory tract infection questionnaire; CHO: carbohydrate; 2d: after 2 days on the diet; 2w: after 2 weeks on the diet; DEXA: dual energy x-ray absorptiometry.

Subjects were randomly assigned to start with either the low- or the high carbohydrate diet. Before intervention, participants characteristics were assessed, including a physical exercise test (VO_{2max} test), body composition measurements and questionnaires. Before the intervention, dietary intake was estimated to gain insight in the participants current eating habits and to estimated energy needs. Dietary guidelines were individually explained before the start of each dietary intervention period. Both dietary interventions were followed for two weeks. Each intervention period included two test days: a first test day to investigate the short-term response was performed after 2 days on the diet, and a second test day for the long-term response was performed after 2 weeks on the diet. A wash-out period of at least two weeks was applied between both diets. An upper respiratory tract infection (URTI) questionnaire was filled out before the intervention and two weeks after each diet ended (Figure 1).

Maximal aerobic capacity and body composition

A maximal exercise test on a bicycle ergometer (Lode Excalibur, Groningen, the Netherlands) was performed to establish maximal aerobic capacity (VO_{2max}). After an initial workload of 100 Watt for 5 minutes, workload was subsequently increased by either 25W/min or 40W/2min until the participant could not maintain the required pedalling frequency of at least 60 rpm. Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Hoechberg, Germany), and VO2 peak was recorded. Heart rate was monitored by using a heart rate monitor (Polar T31-coded, Oulu, Finland) and connected exercise tracker (Polar FT1). In addition, body length (Seca 213 portable stadiometer, Hamburg, Germany) and weight (Seca 761 scale) were measured. Thereafter DEXA measurements were carried out using a Lunar Prodigy Advanced DEXA scanner (GE Health Care, Madison, WI). A quality assurance test was performed to ensure system suitability and precision of the scanner. Whole body scans were performed according to the manufacturer's protocol and identical scan protocols were used for all subjects.

Dietary intervention and physical activity

Food diaries were obtained before the intervention using a 3-day food record (3DFR) (2 week days and 1 weekend day, randomly assigned). These were analysed for the total energy intake and macronutrient distribution using Compl-eat software ™ (Department of Human Nutrition and Health, Wageningen University, www.compl-eat.nl) (18). Personalized diet plans were designed based on the participants estimated total energy needs. In total 6 energy groups were considered: from 10 to 15 MJ with increments of 1 MJ. Participants were instructed to strictly follow their personalized diets. The diets were either a low carbohydrate diet aiming for ketogenesis (<10 En% Carbohydrates and ~75 En% Fats) or a high carbohydrate diet (~50 En% Carbohydrates and ~35 En% Fats). Protein intake was equal in both diets with 15 En%.

Habitual physical activity was assessed before the start of the intervention, using a questionnaire for physical activity level (Short Questionnaire to Asses Health enhancing physical activity (SQUASH)) (19). Participants were advised to keep their physical activity level the same during both diets, although this was not tracked with a wearable.

Nutritional counselling

Each participant individually received nutritional counselling. A detailed menu for two weeks and some standard products were provided. For the HC diet these were: 30+ cheese (cheese with less fat per 100gram), sunflower oil, margarine, nuts, muesli bars, fruit juices. For the LC diet these comprised: 48+ cheese (cheese with more fat per 100 gram), olive oil, margarine, nuts, low-carb bread and beet muffins. The detailed menu consisted of a shopping list, prescribed recipes for every eating moment of every day of the week and information about drinks (water, coffee and tea without sugar or milk were allowed) and herbs. Participants received electronically weighing scales (Impuls, Inter-East B.V., Roosendaal) to precisely measure their dietary intake to ensure that the prescribed menus were followed during the two weeks of intervention. Participants had to weigh all products, except for bread, which was measured in standardized household portion sizes. Deviations from the diet were written down by the participants and leftovers were measured at the end of both intervention periods to assess compliance. Dietary intake was assessed at the end of each diet by calculating the deviations from the diet that were written down by the participants and by subtracting the leftovers from the provided foods which were weighted by distribution and return.

Exercise test days

Test days were performed after 2 days and 2 weeks on each of the diets. See Figure 1 for an overview of a test day. Participants arrived after an overnight fast whilst they already collected their morning urine at home to assess ketosis (ketostick, strips 50 A2880 B51, Bayer, Leverkusen, Germany). At 08:00 AM an intravenous cannula was inserted in an antecubital vein and a first blood sample was taken at 08:30 AM. Simultaneously, participants donated saliva via unstimulated, passive drool (20). A standardized breakfast customized to their energy needs and current diet was provided after the first blood drawing (LC breakfast: 588 kCal (average) ~74 En% fat, 17 En% protein, 6 En% carb; HC breakfast: 505 kCal (average) ~34 En% fat, 15 En% protein, 48 En% carb). Thereafter, only after 2 weeks on both diets, body composition was assessed using a DEXA scan (GE Health Care, Madison, WI). Scans were performed on the same time of the day during all sessions to minimize measurement errors.

After that, a 90 minutes bicycle ergometer test (Lode Excalibur, Groningen, the Netherlands) at 60% of the athletes' individual Wmax (~ 70% VO_{2max}) was performed from 10:00 am to 11:30 AM. If an athlete failed to maintain the prescribed workload, the workload was decreased to the level that allowed the athlete to keep

up until the end of the test. Adjustments were written down and the power multiplied by time in seconds was used to calculate total workload. Workload during the 90 minutes exercise tests was assessed as area under the curve in kilo joule (kJ).
Heart rate was measured with a heart rate belt (Polar T31-coded, Oulu, Finland), placed around the chest. Gaseous exchange was measured (Oxycon Carefusion, Hoechberg, Germany) before the start of the exercise test (while sitting still on the bike for 5 minutes) and at 60 minutes during the exercise test during a 5 min period, to assess respiratory exchange rate (RER: ratio VCO_2/VO_2).
Participants were allowed to drink plain water during the test, but were not allowed to eat. Directly after the exercise test a Borg scale was shown to ask for the rate of perceived exertion (RPE) and another blood sample and saliva sample were taken. Thereafter, participants could take a shower and relax. Another blood sample was taken 1 hour after the end of the exercise. Subsequently participants received a standardized lunch customized to their energy needs and current diet (LC lunch: 1027 kCal (average) ~78 En% fat, 16 En% protein, 4 En% carb; HC lunch: 781 kCal (average) ~31 En% fat, 14 En% protein, 52 En% carb). Two more blood samples were taken at 2 and 3.5 hours after exercise respectively. (Figure 1)

Blood sampling and analysis
Blood samples were collected in lithium-heparin, EDTA and serum tubes. Lithium-heparin tubes (4.5 ml LH PST™ II, Becton-Dickinson, New Jersey, America) were centrifuged at 1300 CRF for 10 minutes at room temperature (RT), plasma was frozen at -80ºC until it analyzed for glucose. Glucose was measured by means of an end-point technique (Siemens, the Netherlands). EDTA tubes (8 ml, Becton-Dickinson, New Jersey, America) were centrifuged at 1200 G for 15 minutes at 4 °C, and plasma was frozen at -80 degrees until it was analysed for free fatty acids concentrations. Free fatty acids were assessed using an enzymatic test kit according to the manufacturer's protocol (InstruChemie, Delfzijl, Netherlands). Serum tubes (5 ml, Becton-Dickinson, New Jersey, America) were set aside for at least 30 minutes, where after they were centrifuged at 1300 G for 10 minutes at RT, serum was frozen at -80 degrees until it was analysed for ketone content and cortisol concentration. Beta-hydroxybutyrate (βHB) was determined via a colorimetric enzymatic assay (Sigma-Aldrich; St. Louis, MO). Analysis was performed according to manufacturer's protocol. Cortisol was measured with immunometric chemiluminescence (sandwich) assay with Immulite XPi (Siemens, the Netherlands).

Saliva sampling and analysis

Saliva was collected at two time points at every test day: one before breakfast and one directly after exercise. In order to collect whole saliva from mouth, unstimulated, passive drool was performed (20). Participants were asked to bend their head slightly downwards and first collect some saliva in their mouth before drooling into the saliva collection aid (Salimetrics, LLC, State College, USA). At least 0.5 ml of saliva was collected in 2-ml collection tubes (Wheaton, Millville, USA) per time point. Samples were temporarily stored on dry ice and transferred to a refrigerator at -80°C within seven hours until analysis. IgA antibodies in saliva were determined by enzyme-linked immunosorbent assay (ELISA) as described before (21). The samples for each individual participant were run on the same assay to eliminate inter-assay variance.

URTI Questionnaires

Before the intervention, and two weeks after the final day of each dietary intervention, participants received a questionnaire about symptoms related to upper respiratory tract infections (URTI). This questionnaire was adapted from the validated WURSS-21 questionnaire (22), and translated to Dutch.

Statistical analysis

Data was analysed using IBM SPSS version 25 Statistical Package for Social Sciences (IBM SPSS version 25.0, Armonk, New York, USA). A paired t-test was performed to assess differences in diets, ketone levels, body composition, RER, RPE and work load between the two diets (at t=2 days and 2 weeks). A two-way repeated measures ANOVA (two factor, time x diet) was performed to analyse the stress and metabolic response to both diets. When an effect of condition or time or interaction was identified, a pairwise multiple comparison with Bonferroni correction was done to identify the differences. URTI data was analysed using a sign test and the correlation between URTI and IgA data was performed using a Spearman correlation test. The level of significance was set at $p < 0.05$. Data are presented as mean ± SD unless indicated otherwise.

RESULTS

Participant characteristics

Baseline characteristics of the fourteen participants are depicted in Table 1. They were active in a variety of sports (Cyclist (n = 5), Triathlete (n = 1), Climber (n = 2), Strength trainer (n = 2), Swimmer (n = 1), Volleyball player (n = 1), Football player (n = 1), Runner (n = 1)). They were 32.9 ± 8.2 years old and had a VO2max of 57.3 ± 5.8 ml/kg/min. Their habitual diet contained 2961 ± 528 kCal, 36 ± 6 En% fat, 16 ± 3 En% protein, 43 ± 5 En% carbs. Their habitual training consisted of 5.6 ± 1.1 training hours per week.

Table 1. Participant characteristics

	Participants (n = 14)
Age (years)	32.9 ± 8.2
Body composition	
Height (cm)	181.7 ± 4.7
Weight (kg)	76.4 ± 5.4
BMI (kg/m^2)	23.1 ± 1.4
Lean mass (kg)	61.9 ± 3.4
Lean mass (%)	81.2 ± 4.4
BMC (kg)	3.2 ± 0.25
BMC (%)	4.2 ± 0.32
Body fat (kg)	11.2 ± 4.0
Body fat (%)	14.5 ± 4.6
Total training (hours/week)	5.6 ± 1.1
Maximal exercise performance	
VO2max (ml/kg/min)	57.3 ± 5.8
Max heart rate (bpm)	186.9 ± 8.8
Max Power (Watt)	346 ± 46
Max Power / kg body weight	4.5 ± 0.5

Means ± SD are shown. BMI: Body mass index; BMC: Bone mineral content. Physical characteristics are determined during a VO2max test

Dietary intake and blood and urine ketone levels

Energy intake between the LC (3104 ± 297 kCal) and the HC diet (3075 ± 298 kCal) was not different ($p = 0.221$). As intended, macronutrient intake was significantly different between both diets, with significantly higher fat intake in the LC diet compared to the HC diet (73 ± 1 vs 33 ± 0 En%, for LC and HC, respectively; $p < 0.001$) and in line with the experimental design a lower carbohydrate intake in the LC diet compared to the HC diet (8 ± 0 vs 49 ± 0 En%, for LC and HC, respectively; $p < 0.001$). Protein intake was higher in the LC diet compared to the HC diet (16 ± 1 vs 15 ± 0 En%, for LC and HC, respectively; $p < 0.001$), although this was not intended. An overview of the total daily energy intake and macronutrient distribution at baseline and during both dietary intervention periods, can be seen in Table 2.

The LC diet was geared to induce nutritional ketosis. Deviations from the prescribed diets were negligible. Urine ketone levels ranged from 0 – 1.6 g/L (average: 0.16 ± 0.42 g/L) after 2 days on the LC diet and ranged from 0 – 0.8 g/L (0.26 ± 0.25 g/L) after 2 weeks on the LC diet. One out of the 14 participant had no detectable ketones in his urine after 2 weeks on the LC diet. There were no ketones present in urine samples during the HC diet. Baseline blood ketone (β-hydroxy-butyrate) levels ranged from 0.06 – 0.68 mmol/L (average: 0.31 ± 0.18 mmol/L) after 2 days and from 0.21 – 0.97 mmol/L (0.54 ± 0.26 mmol/L) after 2 weeks on the LC diet. On the HC diet, baseline ketone levels were significantly lower: after 2 days ranging from 0.06 – 0.45 mmol/L (0.14 ± 0.10 mmol/L) and after 2 weeks ranging from 0.05 – 0.32 mmol/L (0.13 ± 0.08 mmol/L) ($p < 0.001$ compared to the LC diet for both test days).

Table 2. Dietary intake and fasting serum and urine ketone levels.

	Habitual	LC diet	HC diet	p value
Energy (kCal)	2961 ± 528	3104 ± 297	3075 ± 298	0.221
Protein (g)	116 ± 22	124 ± 12	112 ± 11	< . 001
Protein (En%)	16 ± 3	16 ± 1	15 ± 0	< . 001
Carbohydrate (g)	318 ± 72	64 ± 6	373 ± 38	< . 001
Carbohydrate (En%)	43.4 ± 5.3	8 ± 0	49 ± 0	< . 001
Total Fat (g)	122 ± 29	254 ± 25	116 ± 11	< . 001
Total Fat (En%)	36 ± 6	73 ± 1	33 ± 0	< . 001
Saturated Fat (g)	43 ± 13	68 ± 6	32 ± 3	< . 001
Saturated Fat (En%)	13.1 ± 3.2	19.7 ± 0.3	9.3 ± 0.3	< . 001
Monounsaturated Fat (g)	46 ± 13	127 ± 13	35 ± 3	< . 001
Monounsaturated Fat (En%)	13.9 ± 3.3	36.8 ± 1.1	10.3 ± 0.2	< . 001
Polyunsaturated Fat (g)	22 ± 7	39 ± 5	41 ± 5	0.002
Polyunsaturated Fat (En%)	6.6 ± 1.8	11.4 ± 0.4	12.1 ± 0.2	< . 001
Cholesterol (mg)	354 ± 242	699 ± 57	165 ± 18	< . 001
Dietary Fiber (g)	31 ± 6	28 ± 3	41 ± 4	< . 001
Dietary Fiber (En%)	2 ± 0	5 ± 1	9 ± 2	< . 001
Fasting serum βHB (mmol/L)		0.27 ± 0.13	0.07 ± 0.04	< . 001
Urine ketone levels (g/L)		0.26 ± 0.25	0.00 ± 0.00	< . 001

Means ± SD are shown. p-values represent a dependent t-test between both intervention diets (LC vs HC); serum βHB and urine ketone bodies represent data after following the diets for 2 weeks

Body composition

Body mass was significantly lower after 2 weeks on each of the diets compared to baseline (73.8 ± 4.5 and 75.2 ± 4.7 kg after vs 76.4 ± 5.4 kg before start of the study, after LC and HC respectively; $p < 0.001$ and $p = 0.005$). Body mass was also significantly lower on the LC diet compared to HC ($p < 0.001$). Body fat percentage was lower after each of the diets compared to baseline (12.9 ± 4.3% and 13.5 ± 4.6% vs 14.5 ± 4.6%, for LC, HC and baseline respectively; $p < 0.001$). Body fat percentage was not different between diets ($p = 0.101$). Lean body mass percentage was higher after both diets compared to baseline (82.8 ± 4.2 and 82.2 ± 4.5% vs 81.3 ± 4.9%; $p = 0.017$ and $p = 0.011$ respectively). Bone mineral content (BMC) was 4.3

± 0.3% (3.2 ± 0.2 kg) and comparable between diets (p = 0.271). The difference in lean mass percentage between diets was also not significant (p = 0.110).

Work, Respiratory exchange ratio and perceived exertion

Exercise data can be found in Table 3. The total work in kJ that had to be performed during the 90 minutes exercise was 1120 ± 148 kJ. However, exercise intensity had to be reduced on multiple occasions. The total work output was significantly lower during the LC diet compared to the HC diet, both after 2 days as well as after 2 weeks (939 ± 163 vs 1042 ± 151 kJ after 2 days and 1003 ± 129 kJ vs 1043 ± 141 kJ after 2 weeks, for LC and HC diet respectively, p < 0.02 between diets). Total workload significantly increased on the LC diet after 2 weeks compared to 2 days (p = 0.03), while no time-effect was seen for the HC diet.

Substrate oxidation patterns at rest and during exercise were significantly different between diets. At rest, RER was significantly lower after 2 days and after 2 weeks on the LC diet (0.76 ± 0.03 and 0.77 ± 0.06) compared to the HC diet (0.86 ± 0.05 and 0.87 ± 0.05)(both p < 0.001). Also during exercise, RER was significantly lower after 2 days and 2 weeks on the LC diet (0.82 ± 0.03 and 0.82 ± 0.03) compared to the HC diet (0.90 ± 0.04 and 0.91 ± 0.04)(both p < 0.001). Within each diet group, RER at rest and during exercise did not differ between 2 days and 2 weeks (p > 0.05).

Heart rate during exercise was significantly higher after 2 weeks on the LC diet compared to the HC diet (170 ± 11 bpm vs 165 ± 114 bpm, p = 0.001). There was no significant difference in heart rate between the diets after 2 days (165 ± 13 for LC vs 163 ± 18 for HC, p = 0.652).

Participants rated their perceived exertion higher after 2 days on the LC diet compared to 2 days on the HC diet (18.0 ± 1.4 vs 15.5 ± 2.7, for LC vs HC; p = 0.001). This difference in perceived exertion diminished after 2 weeks, but still tended to be higher on the LC diet (17.3 ± 1.7 vs 16.1 ± 2.0, for LC and HC; p = 0.053).

Table 3. Work, RER, HR and Rate of Perceived exertion

	LC			HC			intervention	
	after 2 days	after 2 weeks	Time effect p-value	after 2 days	after 2 weeks	Time effect p-value	after 2d p-value	after 2w p-value
Work (AUC kJ)	939 ± 163	1003 ± 129	0.03	1042 ± 151	1043 ± 141	0.974	0.005	0.016
RER (rest)	0.76 ± 0.03	0.77 ± 0.06	0.282	0.86 ± 0.05	0.87 ± 0.05	0.564	< 0.001	< 0.001
RER (t60)	0.82 ± 0.03	0.82 ± 0.03	0.681	0.90 ± 0.04	0.91 ± 0.04	0.612	< 0.001	< 0.001
HR (t60)	165 ± 13	170 ± 11	0.014	163 ± 18	165 ± 14	0.633	0.652	0.001
RPE score	18.0 ± 1.4	17.3 ± 1.7	0.151	15.5 ± 2.7	16.1 ± 2.0	0.300	0.001	0.053

Values are mean ± SD calculated after 2 days and 2 weeks on both diets. LC: low carb; HC: high carb; AUC: area under the curve; kJ: kilo Joule; RER: respiratory exchange ratio; t60: after 60min exercise; HR: heart rate; RPE: rate of perceived exertion. P-values represent paired t-test

Blood metabolites (Free fatty acids, glucose, cortisol and ketone bodies)

Blood metabolite levels over time are depicted in Figure 2. Circulating markers of lipid metabolism indicated a significantly higher degree of ketogenesis and lipolysis with the LC diet. Serum free fatty acids (FFAs) at baseline were comparable between diets and test days ($p > 0.05$). However, peak FFAs levels at the end of the exercise were significantly higher with the LC diet (3.4 ± 0.9 and 3.7 ± 0.8 mmol/L after 2 days and 2 weeks respectively) compared to the HC diet group (2.3 ± 0.6 and 2.2 ± 0.5 mmol/L after 2 days and 2 weeks, respectively, $p < 0.001$ vs LC) (Figure 2A).

Serum beta-Hydroxy-Butyrate (β-HB) levels were significantly higher with the LC diet compared to those with the HC diet at all time points, and at both test days ($p < 0.001$) (Figure 2B).

Glucose levels were in general lower on the LC diet compared to those on the HC diet. Baseline glucose levels were not different between diets after 2 days on each of the diets (4.7 ± 0.6 vs 4.9 ± 0.4 mmol/L for LC vs HC, $p = 0.153$), but were significantly lower after 2 weeks on the LC diet (4.7 ± 0.4 vs 5.0 ± 0.4 mmol/L, for LC vs HC; $p = 0.035$). The exercise-induced decrease in glucose was large on the LC diet (-1.00 ± 0.76 mmol after 2 days and -0.76 ± 0.27 mmol/L after 2 weeks, both $p < 0.001$ compared to baseline glucose levels), and much smaller after 2 days on the HC diet (-0.26 ± 0.37 mmol/L, $p = 0.018$ compared to baseline glucose levels) or even absent after 2 weeks on the HC diet (-.018 ± 0.49 mmol/L, $p = 0.192$). After lunch (2 hours after exercise), glucose levels increased with both diets, but to a greater extent on the HC diet (Figure 2C).

The exercise induced cortisol response was highest after 2 days on the LC diet compared to 2 weeks on the LC diet (822±215 nmol/L vs 669±243 nmol/L, for 2 days vs 2 weeks; $p=0.004$) and compared to the HC diet (609±208 and 555±173 nmol/L, for 2 days and 2 weeks on the HC diet, both $p<0.001$ vs LC diet). The exercise-induced increase in cortisol levels was 83 % after 2 days on the LC diet. While after 2 weeks on the LC diet, this increase was reduced to 31%, while on the HC diet it was 28% and 19% after 2 days and 2 weeks of diet intervention, respectively. Resting plasma cortisol concentration was not affected by diet. See Figure 2D.

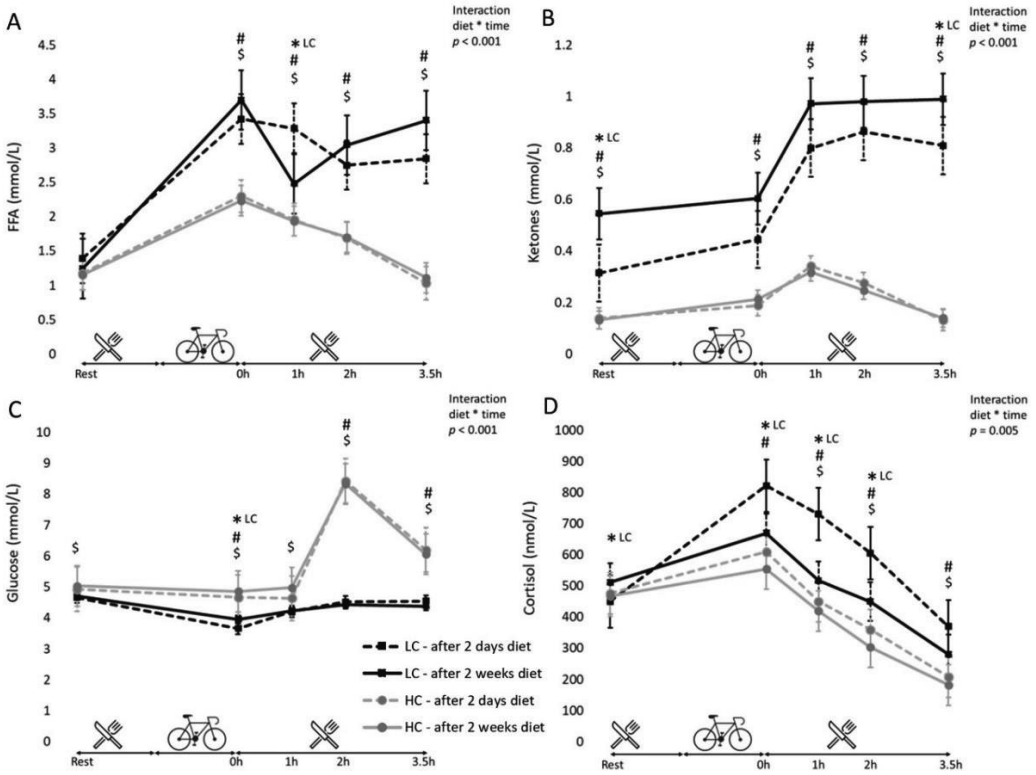

Figure 2. Metabolites. Circulating concentrations of Free fatty acids (A), Ketones (B), Glucose (C) and Cortisol (D). Means ± SE are shown. LC = low carb diet; HC = HC diet. All variables showed significant interactions (diet x time) effects. * LC indicates that this difference was between 2 days and 2 weeks on the LC diet. Within the HC diet there were no differences between concentrations after 2 days and 2 weeks on that diet. # indicates significant differences between the LC and HC diet after 2 days. $ indicates significant differences between the LC and HC diet after 2 weeks.

Salivary IgA

No clear exercise effect was seen for salivary IgA1 and IgA2, neither during the HC diet, nor the LC diet. See Figure 3. However, there was a significant interaction between diet and time point (before vs after exercise) for IgA2 after 2 weeks on both diets ($p = 0.049$). Post-exercise IgA1 and IgA2 levels were lower on the HC diet compared to the LC diet after two days adaptation (IgA1: 326 ± 143 vs 502 ± 247 µg/ml for HC and LC; $p = 0.004$; IgA2: 102 ± 96 vs 149 ± 162 µg/ml for HC and LC; $p = 0.04$).

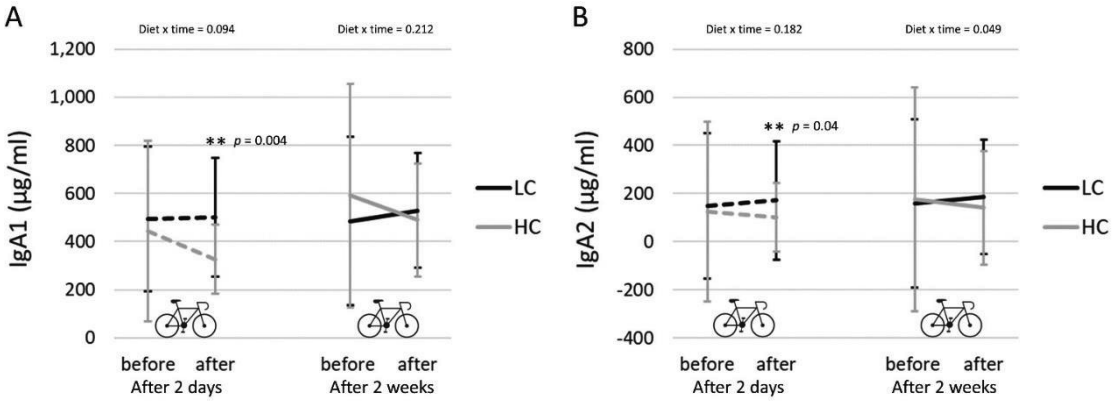

Figure 3. Salivary IgA levels. Salivary IgA1 (A) and IgA2 (B) levels (mean ± SD) before and after exercise, measured after 2 days on the LC diet (black dotted lines) and HC diet (grey dotted lines) and after 2 weeks on the LC diet (black continues line) and HC diet (grey continues line). Significant *p* values represent a paired samples t-test for the HC vs LC diet.

URTI symptoms

The URTI scores for the LC and HC diet were 1.8 ± 2.3 and 3.6 ± 5.5, respectively. A sign test did not show any statistically significant difference between the two median URTI scores ($p = 0.187$). After both diets, all participants rated the question "how ill do you feel today?" with a 0 "not ill" or a 1 "very mildly" on a scale of 0 to 7 "very ill". For the LC diet, only one participant rated this question with a 1, all other participants rated this question with a 0. For the HC diet, 3 participants rated this question with a 1, all others with a 0. In addition, there were no significant correlations between URTI symptoms and IgA levels, $p > 0.05$.

DISCUSSION

In our study we investigated short-term (2 days) and prolonged (2 weeks) adaptation to a LC diet with regard to its effect on exercise-induced metabolic and stress responses, and compared this to a HC diet. Many previous studies didn't take time into account and considered adaptation after a prolonged period of time, while in practice athletes follow low carbohydrate strategies for different durations (days till weeks), depending on their goals and planning. We already observed a difference in metabolic response after 2 days on the LC diet compared to the HC diet. Peak FFA levels after exercise were elevated on the LC diet compared to the HC diet, both directly after initiation of the diet and after 2 weeks. In line with our expectations, glucose levels remained lower on the LC diet compared to the HC diet. At the same time, work output during the exercise sessions was significantly lower with the LC diet, in particular after two days, despite equal or even higher (perceived) exertion and equal or even higher heart rate compared to the HC diet. Short-term adaptation to the LC diet led to a marked initial effect on the exercise-induced stress response as reflected by an 81% increase of plasma cortisol levels, compared to ~20-30% at the other occasions. This effect had apparently disappeared after 2 weeks and was not seen with the HC diet on both test days. Interestingly, a drop in s-IgA following exercise was not seen after 2 days on an LC diet, in contrast to the HC diet. However, this was not linked to URTI symptoms.

Our study was conducted over a 2 times 2-week period, with test days after 2 days and 2 weeks on each diet. Both diets, which included specific recipes and clear instructions, were followed fairly good by our participants. The LC diet, delivering less than 10 En% CHO was challenging as it required more preparation and planning compared to the HC diet. Nevertheless, the LC diet was received as tasteful and, as expected and intended, led to higher urine and blood ketone levels in all individuals. Noteworthy, the LC diet resulted in a significantly lower work output during the exercise session, in particular after two days. Despite this lower work load, cortisol as well as heart rate and perceived exertion showed a comparable or even higher response during the exercise session at the LC diet. Therefore, we assume that this did not interfere with our results.

The 2 weeks of adaptation might be considered a short time frame to induce detectable changes in metabolism. However, previous studies showed that already 5 days of a LCHF diet induces changes in substrate utilization (7). We observed after 2 days already higher circulating FFA and ketone levels, lower glucose levels, and a lower RER, indicating more reliance on fat oxidation. Except for a further increase

in ketone levels, these metabolic changes on the LC diet were similar after 2 days as after 2 weeks. In addition, the time required to achieve optimal adaptation to a LC diet is claimed to be ~2 weeks, with at least 1 week required before the feelings of lethargy and reduced exercise capacity decline (17), as was reflected in the increased work capacity and decreased cortisol after 2 weeks LC compared to 2 days. Furthermore, knowing that some athletes apply LC diets during a training period at the beginning of a periodized training program, two weeks is a relevant duration from an applied perspective. The experimental testing after 2 days on the diet provided us with more information about the acute stress of a LC diet, while the measurement after 2 weeks gave more information about the adapted state.

Metabolic effects - The baseline free fatty acids were not different between diets and between moments, which can be explained by a lower release of FFA from the liver during the LC diet at baseline. In addition, the conversion of carbohydrates into fat (i.e. *de novo* lipogenesis), which occurs in the liver, might also be reduced when fat intake is increased (23). Free fatty acids peaked after exercise in the LC diet. FFAs also increased after exercise in the HC diet, which is in agreement with other studies (2, 7).

It is known that a short-term use of a LC diet reduces exercise capacity by depleting liver and muscle glycogen stores, without producing a compensatory increase in fat oxidation (24, 25). This was in agreement with the observed lower workload and higher RPE after 2 days on the LC diet in our study. On the other hand, RER levels were already lower after 2 days, which would suggest that fat oxidation was already increased (26). Studies suggest that prolonged adherence to a LC diet enhances the breakdown, transport, and oxidation of fat in skeletal muscle (27), explaining the improved workload after 2 weeks on the LC diet, however this was not supported by even lower RER levels in our study. Noteworthy, the interpretation of RER, VO2 and VCO2 values for fat and glucose oxidation should be done with caution, as the oxidation of ketone bodies confounds the results (28). The higher ketone levels after 2 weeks on the LC diet might explain the slightly improved work output and lower RPE. The period required for adaptation to a non-ketogenic LC diet is around 5 days, without further enhancement thereafter (29). In our study, work output was still lower after 2 weeks on the LC diet compared to the HC diet. Whether an even longer period on the LC diet would result in equal outcomes between both diets is questionable, because no further increase in fat oxidation is expected after 5 days (29). On the other hand, others suggest that long-term consumption of a LCHF diet

results in adaptations in the homeostatic regulation of muscle glycogen and even further improved fat oxidation during exercise (2).
Baseline glucose levels were lower after two weeks on the LC diet, this was not the case after two days on both diets, signifying that a LC ketogenic diet will lower baseline glucose levels only after following it for a sufficient time. Blood glucose levels decreased after exercise in the LC diet, which was in agreement with others (7). A glucose peak was observed in the HC diet group after the standardized meal, which was expected as this meal contained ~200 grams of carbohydrates.

Stress response - The exercise induced increase in cortisol was by far the highest after 2 days on the LC diet. Since 2 days on a LC diet is likely to be too short for fat adaptation to occur, glucose remains the primary fuel source. As a consequence, strenuous exercise will therefore rapidly lead to glycogen depletion, as they likely started with already reduced values, and this may result in excess release of cortisol (24, 25). The exercise intensity in our study was fairly high, reflected by heart rates above 160 bpm (187 bpm was their max HR at the VO_{2max} test). Several studies have shown that when individuals perform exercise after several days on very low carbohydrate diets, this leads to cortisol levels that are markedly higher than with a normal or high carbohydrate diet (30, 31). Here we showed that this cortisol response is not significantly higher anymore after two weeks on the LC diet compared to two weeks on the HC diet, indicating adaptation to the LC diet.
Salivary IgA1 and IgA2 were both lower post-exercise after 2 days on the HC diet compared to levels after 2 days on the LC diet, which might suggest that a short-term LC diet attenuates exercise-induced decreases in IgA. The reduced IgA (both in IgA1 and IgA2) is often linked to an increased risk for URTI (32), despite being also debated (33), which could be interpreted as a favourable outcome of the LC diet. This protective role of a short term LC diet on the exercise induced decrease in IgA was not foreseen and in contrast to studies showing a post-exercise decrease in IgA regardless of diet (14). It also seems to be opposed to the expected effects of the higher cortisol production in the LC diet, which has been described to result in a lower immunoglobulin production by B-cells, thereby attenuating the s-IgA reservoir refilling process, producing a chronic s-IgA suppressive state (15). However, in the majority of studies no separate detection of IgA1 and IgA2 levels was performed and, given the different susceptibility of these isotypes for proteolysis, this might affect the association with exercise-induced changes in mucosal immunity. Both our findings on s-IgA1 and s-IgA2 connected to this

apparent discrepancy with stress levels merit further investigation, preferably in a long-term study.
In addition, variation in IgA levels was very large between our participants, which might reflect the health status of the oral cavity (33) and makes it hard to draw solid conclusions. Furthermore, there were no differences in post-exercise IgA changes after two weeks with both diets, which is in agreement with a 3-week trial, showing that post-exercise changes in IgA were comparable between a HC and ketogenic diet (34).
This was also reflected by finding no differences in URTI related symptoms in our athletes 2 weeks after the end of each of the diets whereby none of the participants indicated to feel ill. Although it should be noted that these URTI questionnaires are filled in by the participants and not established by an additional throat swab. Unfortunately, the data in this study does not suggest that one of the diets could protect against URTI symptoms, although the inhibition of the IgA decrease after exercise on the LC diet after 2 days seems promising. To our best knowledge, there is no scientific literature on URTI symptoms in relation to exercise and a ketogenic diet. Several articles already stated that there was no evidence of a beneficial effect of carbohydrates on URTI incidence (35, 36). But whether a LC ketogenic diet would have beneficial effects should be studied in future.

Body mass - Mean body mass was lower after following both diets compared to before the intervention. Decreases in body mass were expected after the LC diet (37, 38), as a LC diet decreases glycogen concentrations which is associated with a loss of body water and thus body weight (39). Decreases on the HC diet were not directly foreseen, but may have been caused by underreported energy intakes in the 3-day food records at intake, leading to a dietary advice with a lower energy intake then needed for the participant. This would account for both the HC diet and the LC diet, as athletes were subscribed the same energy group with both diets. This underreporting is common in the athletic population (40).

In conclusion, the results of the present study showed that short-term adherence to a LC diet already leads to metabolic changes, as reflected by lower RER, lower glucose, higher FFA and higher ketone levels. These metabolic changes were comparable between short-term and prolonged adherence to the LC diet, except for ketone levels which were further increased after 2 weeks. On the other hand, the exercise induced stress response was higher after 2 days and attenuated after 2 weeks, as shown by an 81% increase of plasma cortisol levels after 2 days on the LC

diet, compared to ~20-30% on other occasions. The exercise tolerance after adherence to the LC diet was low, with lower workload, higher or comparable HR and higher RPE compared to the HC diet. Interestingly, a drop in s-IgA following exercise was not seen after 2 days on the LC diet, in contrast to the HC diet, which might suggest some form of protective effect, although we could not relate this to URTI incidences. Our results underline that adaptation to a LC diet in terms of the metabolic and exercise-stress response have different time spans.

Perspectives

These new data showed that the duration of adherence to a LC diet affects the exercise-induced stress and metabolic response differently. Metabolic adaptations already occurred after 2 days on the LC diet, while this was not directly followed by adaptations in stress response and work output. This was shown by an increased exercise-induced stress response, shown by a higher cortisol peak, and a lower level of work output during the exercise test. Since work output was still lower after 2 weeks adherence, but stress response attenuated, we can imagine that athletes use a prolonged LC diet during a period of low intensity training, but not during peak competition.

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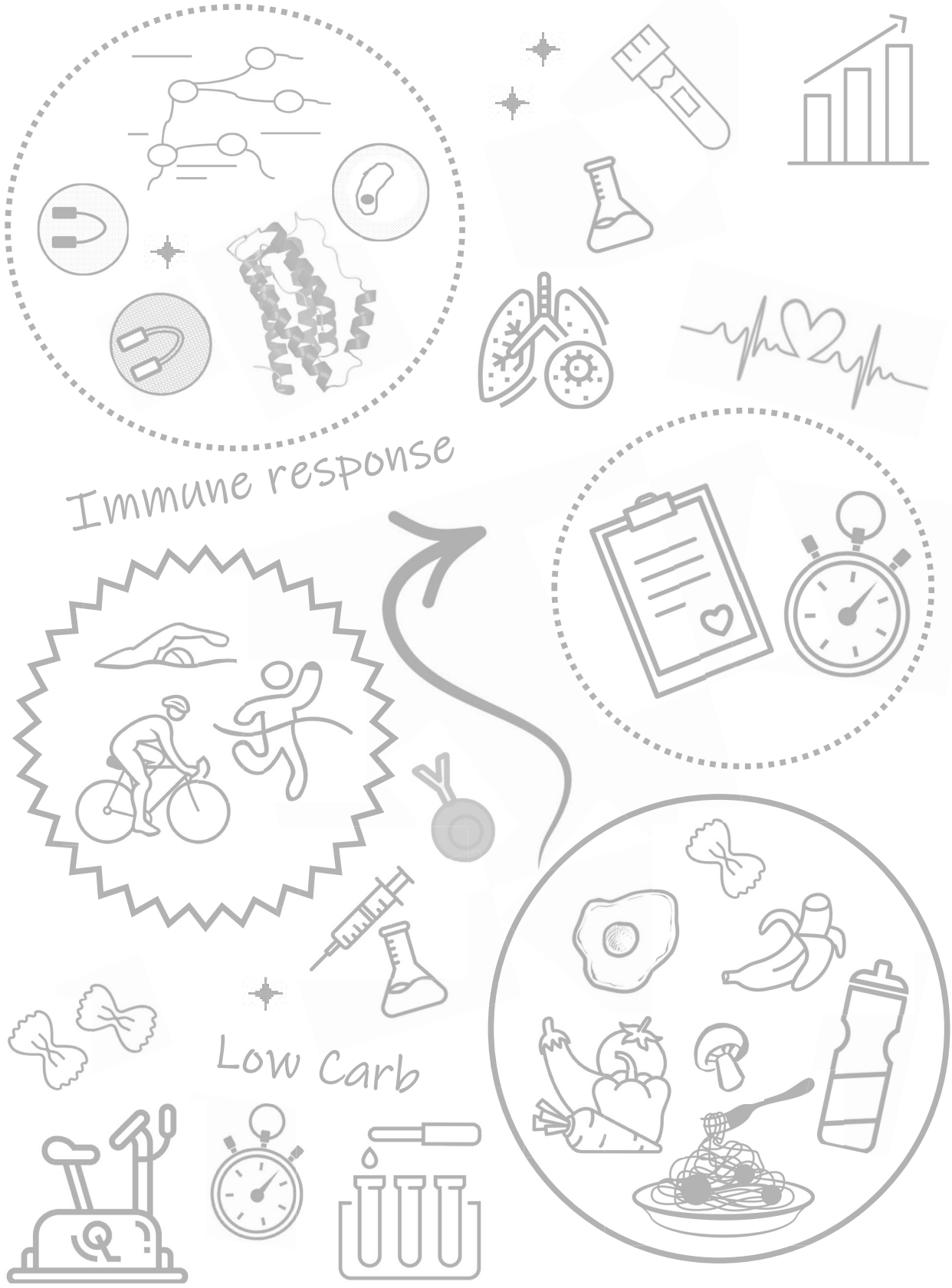
Immune response
Low Carb

Chapter 9

A short-term low carbohydrate diet affects differential count, homing and proliferation rate of immune cells following strenuous exercise: a randomised cross-over trial

LC diet affects exercise-induced immune response

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ABSTRACT

Purpose: Low carbohydrate (LC) diets gained popularity because of their supposed performance-enhancing properties. However, their immunological effects are still unclear. Therefore, we studied the effects of a LC diet on the exercise-induced immune response. **Methods:** Fourteen well-trained male athletes (32.9±8.2 years, VO_{2max} 57.3±5.8 ml/kg/min) randomly consumed a LC diet (<10En% CHO) and a high carbohydrate (HC) diet (>50En% CHO) for 2 weeks , with a wash-out period of >2 weeks in between. Two days and 2 weeks during both intervention periods , participants cycled for 90min at 60% $W_{max.}$ Blood samples for cortisol, immune cell differential count, proliferation, and homing markers were collected at different time points before and after exercise. **Results:** Two days adherence to the LC diet augmented the exercise-induced stress response as reflected by an 81% increase of serum cortisol levels, compared to ~20-30% at the other occasions. Baseline cell differential counts were comparable between diets ($p > 0.005$). After 2 days adherence to the diets, cell differential count directly after exercise differed significantly between diets, with higher T cell and Th cell counts on the HC diet ($p<0.05$). Two hours later T cell, Th cell and B cell counts were higher on the HC diet ($p<0.05$), but monocyte counts were lower, compared to the LC diet ($p=0.016$). After 2 weeks on the diets, no differences were found in cell counts at all time points ($p>0.05$). The HC diet resulted in a significant decrease in cell proliferation rate from directly post-exercise till 2 hours post-exercise ($p=0.024$ after 2 days and $p=0.015$ after 2 weeks diet), whereas the LC diet did not. Th cell airway homing was lower after 2 days adherence to the LC diet compared to the HC diet at 2 hours post-exercise ($p=0.038$), with no differences after 2 weeks. **Conclusions:** Effects on both stress and immune response to strenuous exercise were different after 2 days on a LC compared to a HC diet. However, after 2 weeks of continuing the diets, differences had disappeared. Compared to the effects of acute immune-effects of exercise itself, those of carbohydrate intake were found to be less pronounced.

KEY WORDS: exercise-induced immune response, cell differential count, homing, cell proliferation rate, low carbohydrate diet, ketogenic diet, sports

INTRODUCTION

The immune system is highly responsive to exercise, the degree and duration being determined by the level of physiological stress imposed on the individual (1). Exercise acutely increases circulating neutrophil and monocyte counts, while reducing circulating lymphocyte count during recovery (2). A classic theory in exercise immunology suggests the occurrence of an 'open window' of immunosuppression during recovery from intense exercise (3). Following exercise, lymphocytes, natural killer cell counts and antibody production decrease below pre-exercise levels. As a result of this immunosuppressive effect, an individual would be more susceptible to infection and other illness (4). Other investigators suggest that the dramatic reductions of blood lymphocyte counts and function after exercise reflect a transient and time-dependent redistribution of immune cells to peripheral tissues, resulting in a heightened state of immune surveillance and immune regulation, as opposed to immunosuppression (5, 6). This redistribution of immune cells is under the control of a network of surface ligands and receptors, often referred to as 'homing' (7).

Either way, this close link between the immune system and exercise, has stimulated interest in possible strategies, including dietary intervention and (or) the use of nutritional supplements to modulate the exercise-induced immune response (8, 9). Much research has focussed on the effects of carbohydrate intake before and/or during exercise in relation to the recovery of systemic immune functionality during the first few hours after exercise (10-12). Consumption of carbohydrates during prolonged, intense exercise attenuates exercise-induced increases in circulating cytokines (13) and the redistribution of neutrophils (14), monocytes (14), natural killer cells (12) and lymphocytes (11).

Interestingly, athletic training often involves conditions of low carbohydrate availability (15). This happens when recovery periods are short, or as part of a 'train low-compete high' regime or as part of a so-called ketogenic diet (15-17). The latter two are gaining increasing interest among athletes, because of their stimulating effect on fat oxidation capacity (18) and the reported enhanced release of proteins and signalling molecules involved in training adaptive mechanisms (e.g., AMPK (19), p38 mitogen-activated protein kinase (20), p53 (21) and PGC-1a (22)).

Scientific data on effects of low carbohydrate (LC) -ketogenic- diets on the exercise-induced immune response are scarce. This applies to their acute effects, but also to those resulting from prolonged adherence to LC diets. Nevertheless, LC diets are followed by many athletes with a variety of durations and for a diversity of goals

(e.g., body composition adaptations, training adaptations, fat oxidation). To this end, we here investigated the effects on the exercise-induced immune response following acute (2 days) and prolonged (2 weeks) adherence to a low carbohydrate diet, and compared this to a high carbohydrate diet. Taking into account the two opposing viewpoints described above, the 'open window' and 'redistribution' theories, respectively, we primarily aimed to investigate the effect of a LC diet on T cell homing, as this could make the redistribution pattern more clear. In addition, we investigated cell proliferation and differential immune cell counts in this randomised cross-over dietary intervention study.

MATERIALS AND METHODS

Participants

Fourteen well-trained male athletes participated in this study (age; 32.9 ± 8.2 years, VO2max; 57.3 ± 5.8 ml/kg/min, body weight 76.4 ± 5.4 kg). All participants had no food allergies, no chronic illnesses, and did not use asthma-, anti-inflammatory- and/or immunosuppressive medication. Study enrolment took place between October 2018 and January 2019. The study was conducted in accordance with the Declaration of Helsinki and approved by the Medical Ethical Committee of Wageningen University (NL6540408118, ClinicalTrials.gov ID: NCT04019730) and all participants gave written informed consent prior to participation.

Study design

This study had a cross-over design, with 2 times 2 weeks of dietary intervention (Figure 1). Subjects were randomly assigned to start with either the low- or the high carbohydrate diet. Before the intervention, participants characteristics were recorded, which included a physical exercise test (VO_{2max} test), body composition measurements and taking questionnaires. Furthermore, their dietary intake was estimated to gain insight in current eating habits and to estimate energy needs. Both dietary interventions were followed for two weeks. A wash-out period of at least two weeks was applied between both diets. Each intervention period included two test days: a first test day to investigate the short-term effects on the exercise-response was performed after 2 days on the diet, and a second test day to study the response following long-term intervention was performed after 2 weeks on the diet.

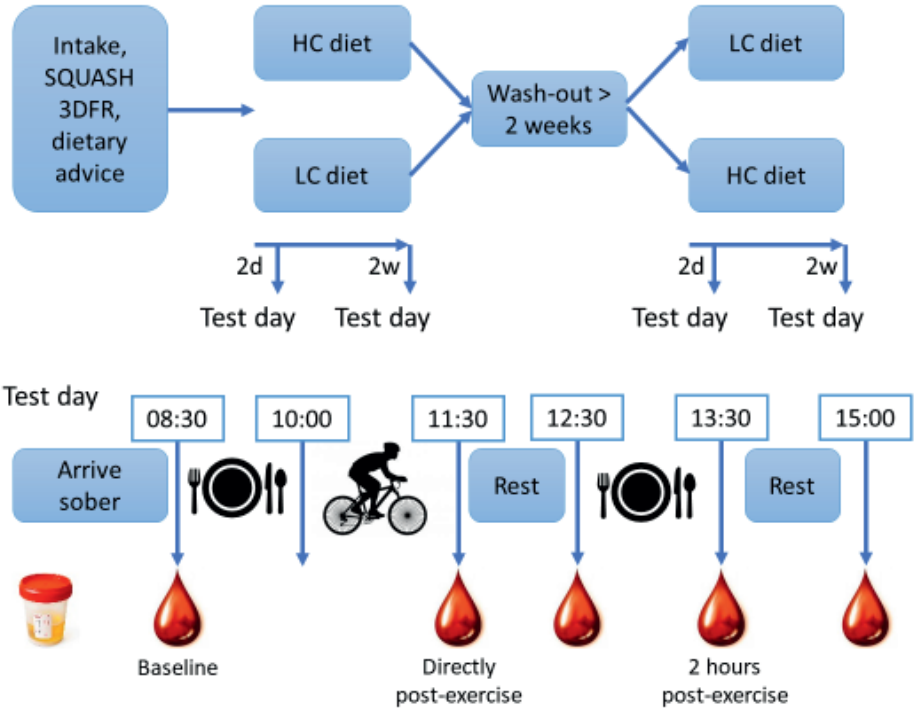


Figure 1. Schematic overview of study design. 3DRF: 3-day food record; SQUASH: Short Questionnaire to Asses Health enhancing physical activity; HC: high carbohydrate; LC: low carbohydrate; 2d: after 2 days on the diet; 2w: after 2 weeks on the diet.

Participants' characteristics at baseline

A maximal exercise test on a bicycle ergometer (Lode Excalibur, Groningen, the Netherlands) was performed to establish maximal aerobic capacity (VO_{2max}). After an initial workload of 100 Watt for 5 minutes (min), workload was subsequently increased by either 25W/min or 40W/2min until the participant could not maintain the required pedalling frequency of at least 60 rpm. Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Hoechberg, Germany), and VO_2 peak was recorded. Heart rate was monitored by using a heart rate monitor (Polar T31-coded, Oulu, Finland) and connected exercise tracker (Polar FT1). In addition, body length (Seca 213 portable stadiometer, Hamburg, Germany) and weight (Seca 761 scale) were measured.

Dietary intervention and physical activity

Food diaries were obtained before the intervention using a 3-day food record (3DFR) (2 week days and 1 weekend day, randomly assigned). These were analysed for the total energy intake and macronutrient distribution using Compl-eat software ™ (Department of Human Nutrition and Health, Wageningen University, www.compl-eat.nl) (23). Personalized diet plans were designed based on the participants' estimated total energy needs and had to be followed strictly. The diets were either a low carbohydrate diet aiming for ketogenesis (<10 En% Carbohydrates and ~75 En% Fats) or a high carbohydrate diet (~50 En% Carbohydrates and ~35 En% Fats). Protein intake was equal in both diets at 15 En%.

Each participant received nutritional counselling on an individual basis. A detailed menu with shopping list and prescribed recipes for every eating moment of every day and some standard products were provided. See Chapter 8 for the details of this dietary intervention. Participants had to weigh all products, except for bread, which was measured in standardized household portion sizes. Dietary intake was assessed at the end of each intervention period by calculating the deviations from the diet that had been written down by the participants and by subtracting the leftovers from the provided foods which were weighted by distribution and return.

Habitual physical activity was assessed before the start of the intervention, using a questionnaire for physical activity level (Short Questionnaire to Asses Health enhancing physical activity (SQUASH)) (24).

Exercise test days

Test days were performed after 2 days and 2 weeks on each of the diets, as previously described in Chapter 8. See Figure 1 for an overview of a test day. Briefly, participants arrived after an overnight fast whilst they already collected their morning urine at home to assess ketosis (Ketostick, strips 50 A2880 B51, Bayer, Leverkusen, Germany). At 08:00 AM, an intravenous cannula was inserted in an antecubital vein and a first blood sample was taken at 08:30 AM. A standardized breakfast customized to their energy needs and current diet was provided after the first blood drawing. Thereafter, a 90 min bicycle ergometer test (Lode Excalibur, Groningen, the Netherlands) at 60% of the athletes' individual Wmax (~ 70% VO_{2max}) was performed from 10:00 am to 11:30 AM. Workload during the 90 min exercise tests was assessed as area under the curve in kilo joule (kJ). Heart rate was measured with a heart rate belt (Polar T31-coded, Oulu, Finland), placed around the chest.

Participants were allowed to drink plain water during the test, but were not allowed to eat. Directly after the exercise test a Borg scale was shown to ask for the rate of perceived exertion (RPE) and a second blood sample was taken. Thereafter, participants could take a shower and relax. Subsequently, participants received a standardized lunch customized to their energy needs and current diet. Two more blood samples were taken at 2 and 3.5 hours after exercise respectively. (Figure 1)

Blood sampling and analysis for ketones and cortisol

Blood samples for ketone and cortisol analysis were collected in serum tubes (5 ml, Becton-Dickinson, New Jersey, America). Tubes were set aside for at least 30 min, where after they were centrifuged at 1300 G for 10 min at RT. Next, serum was frozen at -80 degrees until it was analysed for ketone content and cortisol concentration. Beta-hydroxybutyrate (βHB) was determined via colorimetric enzymatic assay (Sigma-Aldrich; St. Louis, MO). Analysis was performed according to manufacturer's protocol. Cortisol was measured with immunometric chemiluminescence (sandwich) assay with Immulite XPi (Siemens, the Netherlands).

PBMC isolation

Blood samples for Peripheral blood mononuclear cells (PBMCs) isolation were collected in CPT tubes (BD Diagnostics, Plymouth, United Kingdom). PBMCs were isolated by gradient centrifugation for 20 min at 1800g (without brake, 20 °C). The plasma layer was immediately aspirated and discarded. The PBMC layer was transferred to a 15 mL conical centrifuge tube and PBS was added until a final volume of 15 ml. Tubes were inverted 5 times and then centrifuged for 10 min at 300g (20 °C). The supernatant was discarded. PBMCs were washed in PBS one more time.

Cryopreservation of PBMCs

PBMCs were prepared for cryopreservation by adding 100% FCS to a total of ± 4 x 10^7 cells/ml and then put on ice. Thereafter, an equal amount of ice-cold freezing medium (20% DMSO & 80% FCS) was added drop wise (final concentration 10% DMSO). The tube was mixed gently. The resulting solution was pipetted in 1.8 ml cryovials (25). The cryovials were incubated in a pre-cooled (4 °C) isopropyl alcohol bath in 'Cryo 1 °C freezing containers' ('Mr. Frosty's'). The freezing containers were placed in a -80 °C for one night, before the cells were transferred to -140 °C until analysis.

Flow cytometric analysis for cell differentiation and homing

The frozen isolated PBMCs were thawed for 5 min in a 37 °C water bath. Thereafter, they were washed 3 times with washing medium (RPMI-1640 w/o phenol red + 20% FBS) to remove DMSO. The PBMCs were stained with antibody panels and analysed using multiparameter flow cytometry. The selection of tissue-specific homing marker profiles was based on literature research and previous experience (26), the composed antibody panel are shown in Table 1.

For this panel, 1.5 × 10^6 isolated PBMCs were stained in a 96-well plate (NUNC PP Sigma-Aldrich 7116, Zwijndrecht, The Netherlands) for 30 min on ice in the dark, washed with cold FACS buffer (500 ml PBS, 2.5 g (0,5%) BSA, 372 mg (2mM) EDTA and centrifuged at 400g for 3 min at 4 °C. Cells were analysed at medium flow (30 µl/min) for 300s using a CytoFlex LX (Indianapolis, IN, USA).

Table 1. Antibodies used to analyse PBMCs for homing and cell differentiation

Antibody	Fluorochrome	Putative cell subset
CD45	APC-H7	Leucocytes
CD3	Alexa Fluor 700	T cells
CD4	BUV496	T helper cells
CD8	BUV395	Cytotoxic T cells
CD14	PerCP-Cy5.5	Monocytes
CD19	PE-Cy7	B cells
CD16 CD56	BV605 BV711	} Natural killer cells
CCR7	PE-CF594	Homing marker
CCR9	BV421	Homing marker
CCR10	BB515	Homing marker
CLA	Alexa 647	Homing marker
α4β1	BV650	Homing marker
α4β7	PE	Homing marker

To exclude dead cells in these samples, cells were stained with fixable viability dye (FVD, eBioscience, cat.nr.: 65-0866-14) for 30 min in dark on ice. The viability of cells was measured using fixable viability dye eFluor 506 and resulted in a minimum of 81.5% alive cells (max 95.6%), which is widely considered a good score for frozen and thawed cells. Fluorescent minus one (FMO) controls were included for all antibodies except CD45.

Flow cytometry data analysis was performed by using FlowJo software (version 10 TreeStar, Inc.) and gating was performed as shown in Figure 2. Data were exported % values of the different gating steps and subsequently converted to % of CD45+ cells.

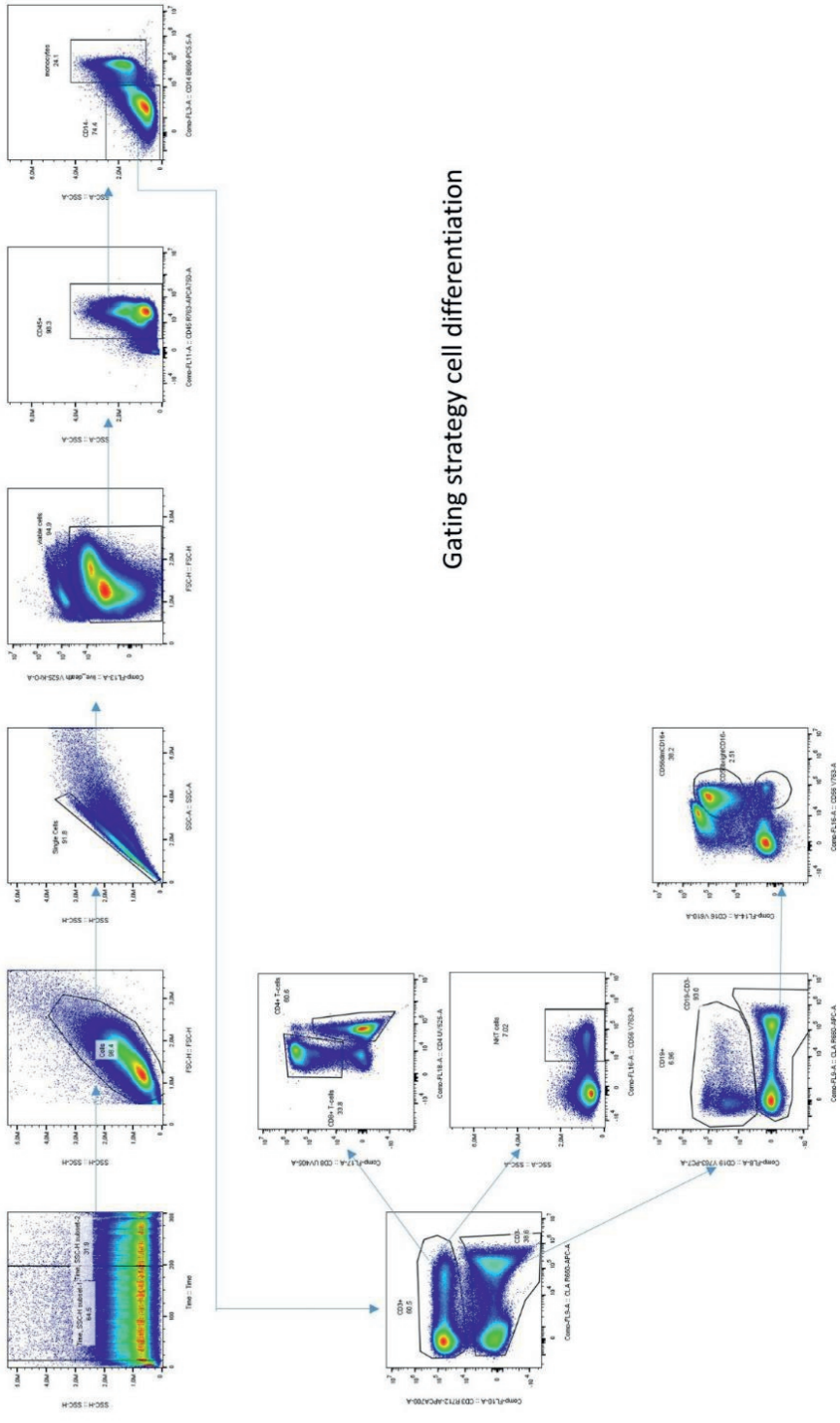


Figure 2. Gating strategy for cell differentiation.

Proliferation preparation and analysis (ki-67)

PBMCs were plated in 96-wells culture plates. Four types of stimuli were applied: medium control, LPS (1µg/ml final concentration), ConA (1µg/ml) and PWM (20 µg/ml). Cells were incubated at 37 °C at 5% CO_2.

Extracellular staining

After 4 days, the 96-wells plates were centrifuged at 300g for 4 min. Supernatants from ConA and medium control were removed and frozen at -80 °C. Cells were resuspended in 200 µL FACS buffer and brought to a 96-wells 500 µL NUNC plate. This was spun down for 3 min at 400g and supernatant was removed. Cells were resuspended in 35 µL of appropriate extracellular antibody mix in FACS buffer and incubated for 30 min on ice in dark. FACS buffer was added, plates were centrifuged for 3 min at 400g and supernatant was removed. To discriminate between live and dead cells, PBS was added, plates were centrifuged (3 min at 400g), supernatant was removed, and this step was repeated. Cells were resuspended in 35 µL 400x diluted fixable live/dead stain in PBS (freshly made) and incubated for 30 min on ice in dark.

Intracellular staining

FACS buffer was added and plates were centrifuged for 3 min at 400g. Cells were resuspended in 100 µL Fix/Perm buffer (1x) and incubated at room temperature for 30 – 60 min. Two hundred µL of Perm buffer (1x) was added and plates were centrifuged for 3 min at 400 g. Cells were washed twice with 300 µL Perm buffer (and spun down at 400g for 3 min). Cells were resuspended in 35 µL intracellular antibody mix in Perm buffer and incubated for 30 min at ice in dark. Three hundred µL of Perm buffer was added and cells were centrifuged for 3 min at 400g. Two hundred µL of FACS buffer was added and Ki-67 was measured on a Flow-cytometer (CytoFlex LX ; Indianapolis, IN, USA).

Statistical analysis

Data was analysed using a two-way repeated measures ANOVA (two factors, time x diet) using IBM SPSS version 25 Statistical Package for Social Sciences (IBM SPSS version 25.0, Armonk, New York, USA). When a main effect of condition, time or interaction was identified, a pairwise multiple comparison with Bonferroni correction was done to identify differences. A paired t-test was performed to assess differences in diets (at t=2 days and 2 weeks). The level of significance was set at $p < 0.05$. Data are presented as means ± SD unless indicated otherwise.

RESULTS

Participant characteristics

Baseline characteristics of the fourteen participants are depicted in Table 2. They were active in a variety of sports (Cyclist (n = 5), Triathlete (n = 1), Climber (n = 2), Strength training (n = 2), Swimmer (n = 1), Volleyball player (n = 1), Football player (n = 1), Runner (n = 1)). Their average habitual training consisted of 5.6 ± 1.1 training hours per week.

Table 2. Participant characteristics

	Participants (n = 14)
Age (years)	32.9 ± 8.2
Body composition	
Height (cm)	181.7 ± 4.7
Weight (kg)	76.4 ± 5.4
BMI (kg/m^2)	23.1 ± 1.4
Total training (hours/week)	5.6 ± 1.1
Physical characteristics	
VO2max (ml/kg/min)	57.3 ± 5.8
Max heart rate (bpm)	186.9 ± 8.8
Max Power (Watt)	346 ± 46
Max Power / kg body weight	4.5 ± 0.5

Means ± SD are shown. BMI: Body mass index; Physical characteristics are determined during a VO2max test.

Dietary intake and blood and urine ketone levels

Energy intake between the LC (3104 ± 297 kCal) and the HC diet (3075 ± 298 kCal) was not different (p = 0.221). As intended, macronutrient intake was significantly different between both diets, with significantly higher fat intake in the LC diet compared to the HC diet (73 ± 1 vs 33 ± 0 En%, for LC and HC, respectively; p < 0.001) and in line with the experimental design a lower carbohydrate intake in the LC diet compared to the HC diet (8 ± 0 vs 49 ± 0 En%, for LC and HC, respectively; p < 0.001). Protein intake was higher in the LC diet compared to the HC diet (16 ± 1 vs 15 ± 0 En%, for LC and HC, respectively; p < 0.001), although this was not intended. An overview of the total daily energy intake and macronutrient distribution at baseline and during both dietary intervention periods, can be seen in Table 3.

Table 3. Dietary intake and fasting serum and urine ketone levels.

	Baseline	LC diet	HC diet	p value
Energy (kCal)	2961 ± 528	3104 ± 297	3075 ± 298	0.221
Protein (g)	116 ± 22	124 ± 12	112 ± 11	< . 001
Protein (En%)	16 ± 3	16 ± 1	15 ± 0	< . 001
Carbohydrate (g)	318 ± 72	64 ± 6	373 ± 38	< . 001
Carbohydrate (En%)	43.4 ± 5.3	8 ± 0	49 ± 0	< . 001
Total Fat (g)	122 ± 29	254 ± 25	116 ± 11	< . 001
Total Fat (En%)	36 ± 6	73 ± 1	33 ± 0	< . 001
Saturated Fat (g)	43 ± 13	68 ± 6	32 ± 3	< . 001
Saturated Fat (En%)	13.1 ± 3.2	19.7 ± 0.3	9.3 ± 0.3	< . 001
Monounsaturated Fat (g)	46 ± 13	127 ± 13	35 ± 3	< . 001
Monounsaturated Fat (En%)	13.9 ± 3.3	36.8 ± 1.1	10.3 ± 0.2	< . 001
Polyunsaturated Fat (g)	22 ± 7	39 ± 5	41 ± 5	0.002
Polyunsaturated Fat (En%)	6.6 ± 1.8	11.4 ± 0.4	12.1 ± 0.2	< . 001
Cholesterol (mg)	354 ± 242	699 ± 57	165 ± 18	< . 001
Dietary Fiber (g)	31 ± 6	28 ± 3	41 ± 4	< . 001
Dietary Fiber (En%)	2 ± 0	5 ± 1	9 ± 2	< . 001
Baseline serum βHB (mmol/L)		0.27 ± 0.13	0.07 ± 0.04	< . 001
Urine ketone levels (g/L)		0.26 ± 0.25	0.00 ± 0.00	< . 001

p-values represent a dependent t-test between both intervention diets; serum βHB and urine ketone bodies represent data after two following the diets for 2 weeks

Exercise test

The total work in kJ performed during the 90 min exercise test was aimed at 1120 ± 148 kJ. The total work in kJ performed during the 90 min exercise test was significantly lower during the LC diet compared to the HC diet, both after 2 days as well as after 2 weeks (939 ± 163 vs 1003 ± 129 kJ for 2 days vs 2 weeks LC diet; 1042 ± 151 and 1043 ± 141 kJ, for 2 days and 2 weeks on the HC diet, $p < 0.02$ between diets). Heart rate during exercise was significantly higher after 2 weeks on the LC diet compared to the HC diet (170 ± 11 bpm vs 165 ± 114 bpm, $p = 0.001$), but there was no significant difference in heart rate between diets after 2 days. Participants rated their perceived exertion higher after 2 days on the LC diet compared to 2 days on the HC diet (18.0 ± 1.4 vs 15.5 ± 2.7, for LC vs HC; $p = 0.001$). This difference in perceived exertion diminished after 2 weeks, but still tended to be higher on the LC diet (17.3 ± 1.7 vs 16.1 ± 2.0, for LC and HC; p =

0.053). In conclusion, although a lower work output was reached during the LC diet, both heart rate and RPE showed that effort was comparable or even higher on the LC diet.

Blood cortisol and ketone levels

Serum cortisol levels directly after exercise were highest after 2 days on the LC diet compared to 2 weeks on the LC diet (822±215 nmol/L vs 669±243 nmol/L, for 2 days vs 2 weeks; p=0.004) and compared to both test days during the HC diet (609±208 and 555±173 nmol/L, for 2 days and 2 weeks on the HC diet, both p<0.001 vs LC diet). The exercise-induced increase in cortisol levels was 83 % after 2 days on the LC diet. After 2 weeks on the LC diet, this increase was 31%, while on the HC diet it was 28% and 19% after 2 days and 2 weeks of dietary intervention, respectively. Baseline cortisol level was not affected by diet. Serum Beta-Hydroxy-Butyrate (β-HB) levels were significantly higher with the LC diet compared to those with the HC diet, at all time points, and at both test days ($p < 0.001$). In summary, exercise-induced cortisol peak was highest after 2 days adherence to the LC diet, and ketone levels were higher at all time points during the LC diet.

Immunological parameters: cell differential count, cell proliferation and homing

Total leucocyte blood counts (CD45+ cell count) increased directly after exercise (23% increase when both diets and both test days were included) and decreased back to baseline within 2 hours following exercise (also 23% decrease). During the low carb diet, the exercise-induced increase in CD45+ cells was significant, both after 2 days on the diet (1.3-fold, $p = 0.002$) and after 2 weeks on the diet (1.3-fold, $p = 0.012$). During the high carb diet, the exercise-induced increase in CD45+ cells, was not significant, neither after 2 days on the diet (1.2-fold, $p = 0.239$) nor after 2 weeks on the diet (1.1-fold, $p = 0.058$). With both diets, after 2 days and 2 weeks, the CD45+ cell count was comparable between baseline and 2 hours post-exercise ($p > 0.05$). Baseline leucocyte count was comparable between diets and at all days, although after 2 weeks there was a trend for a slightly lower leucocyte count in the LC diet compared to the HC diet (51*10^4 vs 61*10^4 cells; $p = 0.068$). These results indicated a clear effect of exercise and the LC diet on the leukocyte counts in blood. See Figure 3A and B.

Cell differential count

When both diets and both test days were taken together, exercise alone induced a change in cell differential count (% of CD45+ cells) which can be categorized into 4 distinguishable patterns:

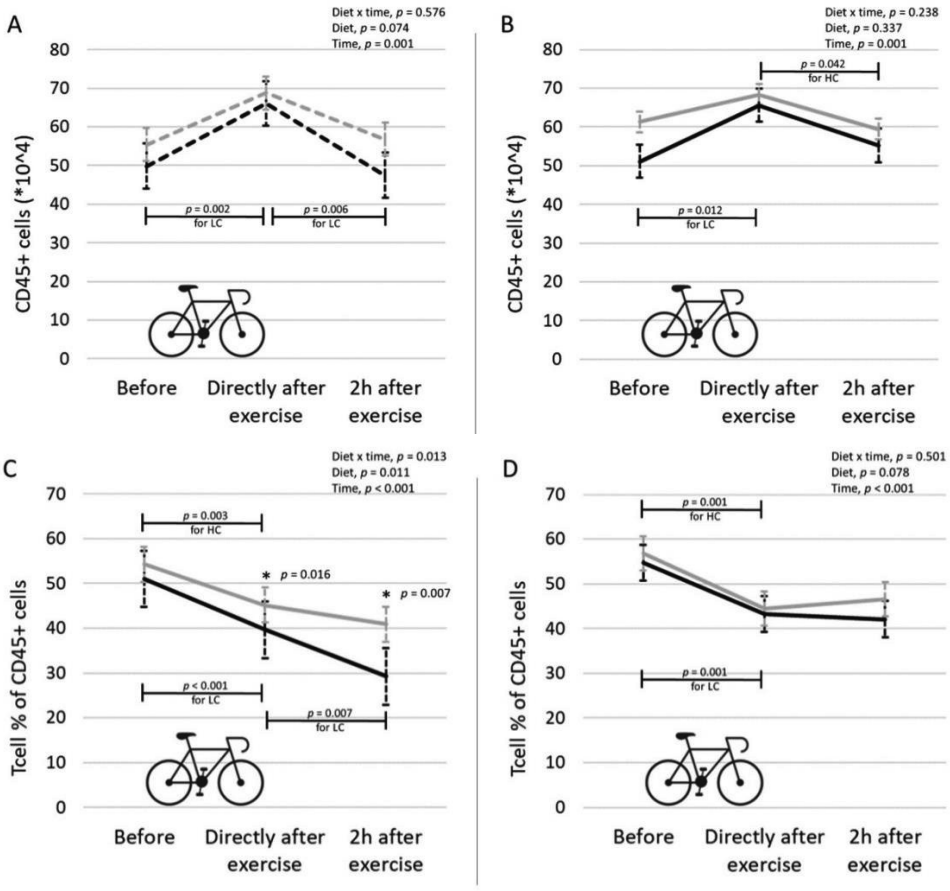
1) a decrease directly post-exercise with no significant change 2 hours later (T cells, Th cells), 2) no change post-exercise and a decrease 2 hours later (NK T cells), 3) no change post-exercise and an increase 2 hours later (monocytes), and 4) no changes at all (B cells and Tc cells). See Figure 3 and Supplementary Table 1 (end of this manuscript).

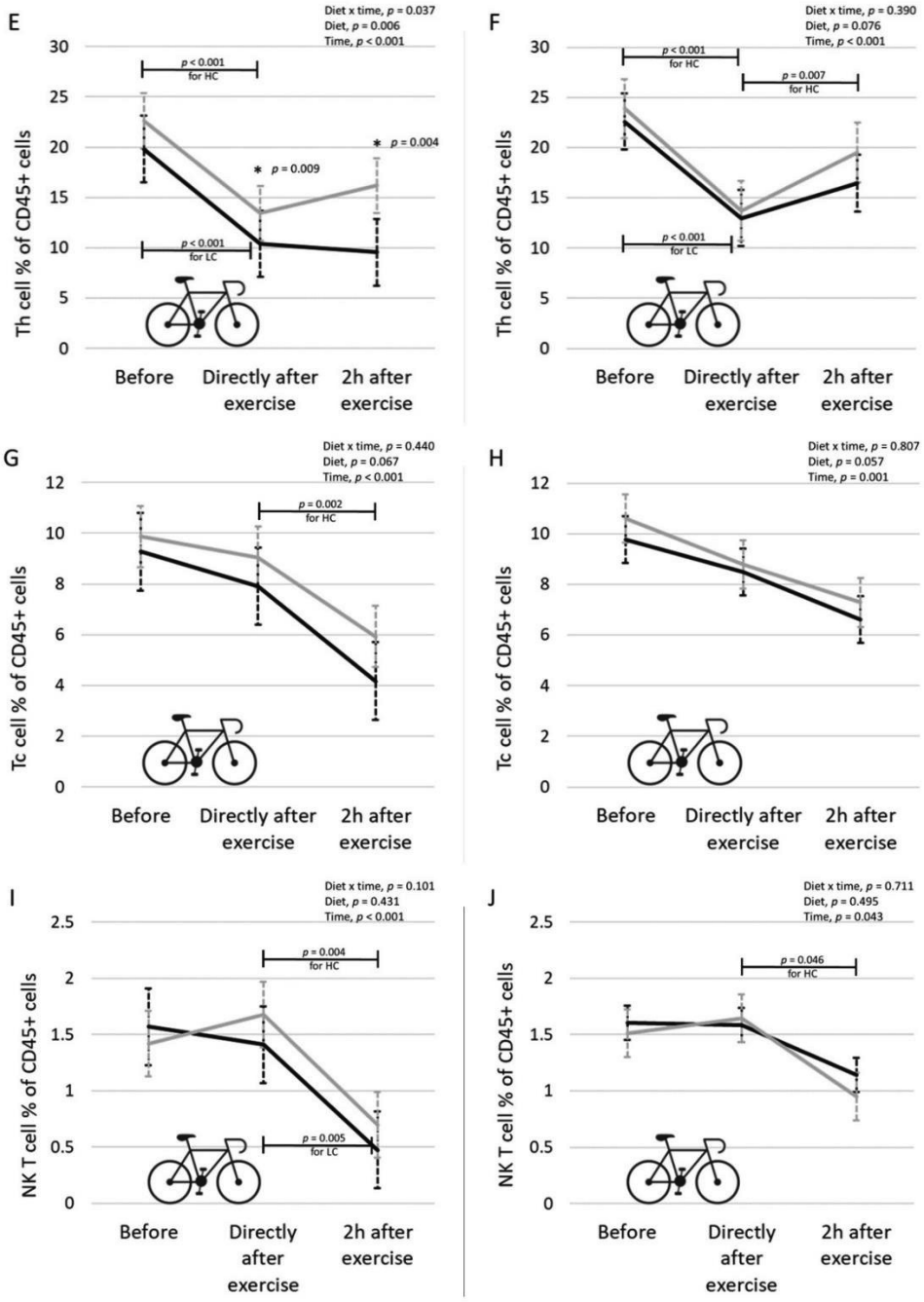
Baseline differences between diets were not observed. Diet did not affect the change in immune cell counts from baseline to those directly post-exercise, for T cells, Th cells, Tc cells, NK T cells, B cells or monocytes, See Figure 3C till 4N. The changes that were observed between these 2 time points were comparable between both diets at both test days. In other words, if a significant change was observed from baseline to directly post-exercise, the same change was observed in the LC diet after 2 days compared to the HC diet after 2 days, and in the LC diet after 2 weeks and the HC diet after 2 weeks.

Diet did affect the immune cell count from directly post-exercise to 2 hours post-exercise. For example, T cells decreased between these time points after 2 days on the LC diet ($p = 0.007$), while no difference was observed on the HC diet ($p = 0.113$). Th cells increased after 2 weeks on the HC diet ($p = 0.007$), but not on the LC diet ($p = 0.189$). Tc cells decreased after 2 days on the HC diet ($p = 0.002$) with no changes on the LC diet ($p = 0.989$). NK T cells decreased after 2 weeks on the HC diet ($p = 0.046$) and showed no changes on the LC diet ($p = 0.931$). B cells increased after 2 days on the HC diet ($p = 0.007$), with no changes when on the LC diet ($p = 1.000$). However, monocytes increased significantly from directly post-exercise till 2 hours post-exercise after 2 weeks on the LC diet ($p = 0.004$), while no changes were observed with the HC diet ($p = 0.192$).

After 2 days on the diets, differences in cell counts were observed at different time points after exercise. T cell count (% of CD45+ cells) was significantly higher in the HC diet compared to the LC diet, at the time points directly post-exercise and 2 hours post-exercise ($p = 0.016$ and 0.007, respectively)(Figure 3C). T helper cell count (% of CD45+ cells) was also significantly higher in the HC diet compared to the LC diet, at the time points directly post-exercise and 2 hours post-exercise ($p = 0.009$ and 0.004, respectively)(Figure 3E). B cell count (% of CD45+ cells) was also significantly higher in the HC diet compared to the LC diet at the time point 2 hours post-exercise ($p = 0.014$)(Figure 3K). By contrast, monocyte count (% of CD45+

cells) was significantly lower in the HC diet compared to the LC diet at the time point 2 hours post-exercise (p = 0.016)(Figure 3M). These differences were not present after 2 weeks on both diets. Using the viability staining, no evidence was found for an increase in apoptosis in any of the conditions tested (data not shown). No other significant differences between the diets were observed when the same time points were compared. In summary, differences in cell differential count between diets were observed after 2 days adherence to the diets, while these were not observed after 2 weeks adherence to the diets.




E
Diet x time, p = 0.037
Diet, p = 0.006
Time, p < 0.001
Th cell % of CD45+ cells
p < 0.001 for HC
* p = 0.009
* p = 0.004
p < 0.001 for LC
Before
Directly after exercise
2h after exercise
F
Diet x time, p = 0.390
Diet, p = 0.076
Time, p < 0.001
Th cell % of CD45+ cells
p < 0.001 for HC
p = 0.007 for HC
p < 0.001 for LC
Before
Directly after exercise
2h after exercise
G
Diet x time, p = 0.440
Diet, p = 0.067
Time, p < 0.001
Tc cell % of CD45+ cells
p = 0.002 for HC
Before
Directly after exercise
2h after exercise
H
Diet x time, p = 0.807
Diet, p = 0.057
Time, p = 0.001
Tc cell % of CD45+ cells
Before
Directly after exercise
2h after exercise
I
Diet x time, p = 0.101
Diet, p = 0.431
Time, p < 0.001
NK T cell % of CD45+ cells
p = 0.004 for HC
p = 0.005 for LC
Before
Directly after exercise
2h after exercise
J
Diet x time, p = 0.711
Diet, p = 0.495
Time, p = 0.043
NK T cell % of CD45+ cells
p = 0.046 for HC
Before
Directly after exercise
2h after exercise

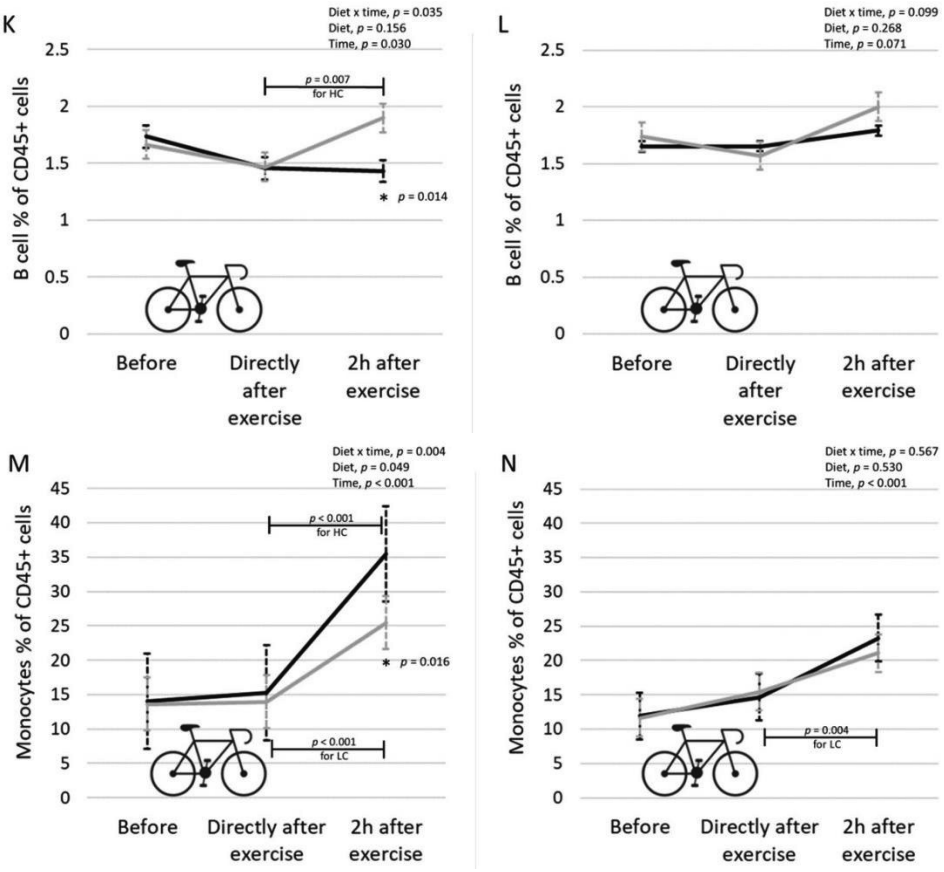


Figure 3. Cell differential counts (mean ± SE) before, directly after and 2 hours after exercise are shown, measured after 2 days on the LC diet (black dotted lines) and HC diet (grey dotted lines) and after 2 weeks on the LC diet (black continues line) and HC diet (grey continues line). * indicates significant differences between the LC and HC diet. Horizontal lines represent significant differences between subsequent time points.

Cell proliferation of T helper cells

T helper (Th) cell proliferation rate, as reflected by Ki67 activity, in unstimulated cultures tended to be higher at baseline after 2 weeks on the LC diet (1.41 ± 0.82%) compared to cell proliferation rate at baseline after 2 weeks on the HC diet (0.86 ± 0.43%) (p = 0.051). This was not seen after 2 days adherence to the diets.

Th cell proliferation rate was significantly higher at 2 hours post-exercise after 2 days on the LC diet (1.38 ± 0.88%) compared to cell proliferation rate at 2 hours post-exercise after 2 days on the HC diet (0.81 ± 0.56%) (p = 0.016).

The overall pattern was an insignificant increase in proliferation rate from baseline till directly post-exercise for both diets. When effects 2 hours post-exercise were

compared with those directly post-exercise, a significant decrease in proliferation rate was seen in the HC diet, both after 2 days and 2 weeks (-0.53 ± 0.78 and -0.37 ± 0.50%; p = 0.024 and p = 0.015 respectively), whereas no significant decrease in proliferation rate was seen with the LC diet, neither after 2 days nor 2 after weeks on the LC diet (p > 0.05). See Figure 4.

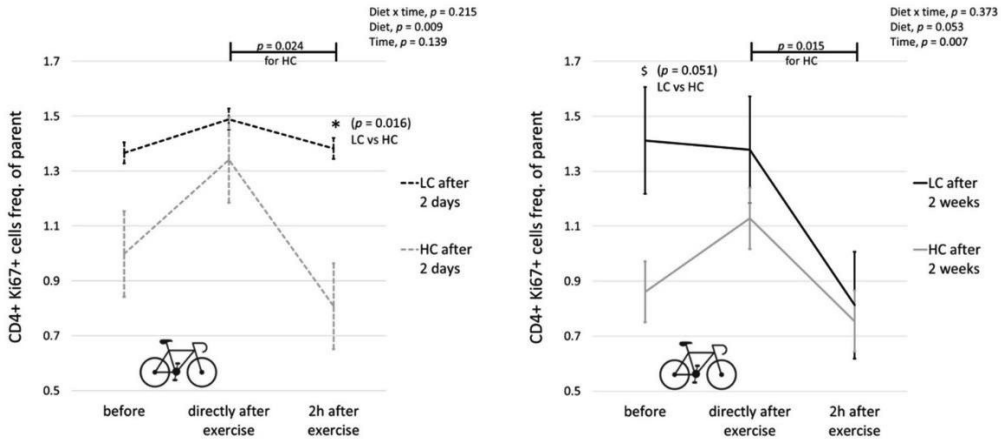


Figure 4. Cell proliferation rates. Means ± SE are shown. * indicates significant differences between the LC and HC diet. $ indicates a trend between the LC and HC diet. Horizontal lines represent significant differences between subsequent time points.

Homing of T helper and cytotoxic T cells to airway mucosa

It was evaluated whether diet induced different homing kinetics for T helper and cytotoxic T (Tc) cells to the airway mucosa (CD4+ / CD8+ ; CCR7- CLA- Integrinβ1+ β7-). The homing gating strategy is shown in Supplementary Figure S1). Exercise seemed to elicit an increase in airway homing directly post-exercise, and a decrease 2 hours post-exercise.

When baseline airway homing potential were compared to post-exercise airway homing potential, a significant increase in Tc cell airway homing was found after 2 days on the high carb diet (HC: p = 0.028), which was not found after 2 weeks or for the LC diet at both occasions. For Th cells, there was no significant change in airway homing potential from baseline till directly post-exercise (p > 0.05), neither for the LC, nor the HC diet at both occasions.

When effects directly post-exercise were compared to those 2 hours post-exercise, a significant decrease in Tc cell airway homing potential was found after 2 days on the HC diet (p = 0.001), 2 days on the LC diet (p < 0.001) and after 2 weeks on the HC diet (p = 0.004). For Th cells there was no significant change in airway homing potential from directly post-exercise till 2 hours post-exercise (p > 0.05).

Significantly lower Th cell airway homing potentials were observed in the LC diet compared to the HC diet 2 hours post-exercise after 2 days on the diet ($p = 0.038$). No other significant differences between diets were observed for Th and Tc cells at any other time points ($p > 0.05$). See Figure 5 and supplementary Table 1. Thus, homing data showed a decreased Th cell airway homing potential after 2 days 2 hours post-exercise in the LC diet, compared to the HC diet.

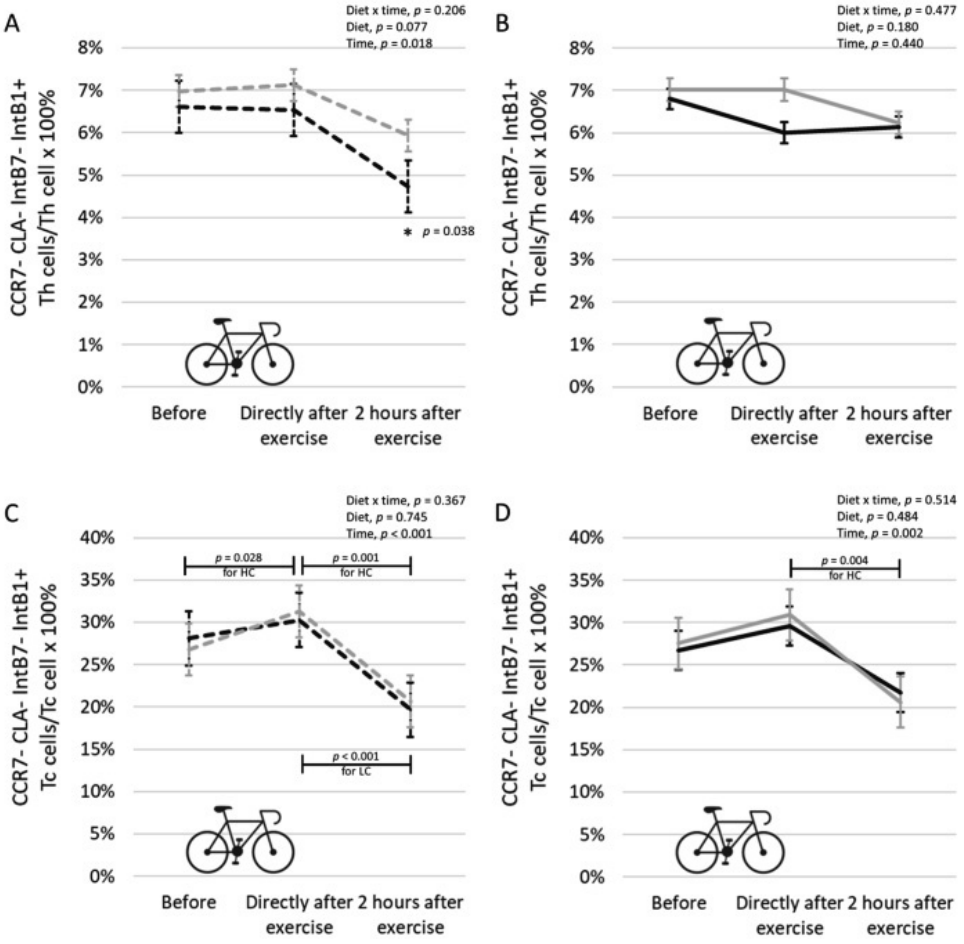


Figure 5. Homing potential of CD4+ and CD8+ cells (mean ± SE) before, directly after and 2 hours after exercise are shown, measured after 2 days on the LC diet (black dotted lines) and HC diet (grey dotted lines) and after 2 weeks on the LC diet (black continues line) and HC diet (grey continues line). * indicates significant differences between the LC and HC diet. Horizontal lines represent significant differences between subsequent time points.

DISCUSSION

Many scientific studies on LC (ketogenic) diets have focussed on body composition and/or sport performance, while their effects on the exercise-induced immune response appear to be largely underexposed. Notwithstanding this, it has been speculated that a LC diet might induce immunosuppression via increased cortisol secretion. In order to elucidate this matter, we here studied the effects of acute (2 days) and prolonged (2 weeks) adherence to a LC diet on the exercise-induced immune response and compared this to a high carbohydrate control diet. We previously reported **(chapter 8)** that this LC diet initially resulted in a markedly increased exercise-induced stress response as reflected by an 81% increase of serum cortisol levels, compared to ~20-30% at the other occasions. This effect had apparently disappeared after 2 weeks, and was not seen with the HC diet on both test days. Exercise alone caused a clear difference in immune cell counts. After 2 days on the diets, differences between the LC and HC diet were found directly post-exercise and two hours post-exercise for the cell differential counts, indicating a redistribution of immune cells with higher T, Th and B cells in the blood with a HC diet, while monocyte count was higher in the LC diet. Cell proliferation activity increased directly post-exercise while it decreased 2 hours later. In addition, this appeared to be more pronounced with the LC diet, with a significant difference between diets after 2 days at 2 hours post-exercise. Finally, exercise-induced differences in homing patterns of immune cells, and the differences between both diets were small, with only a decreased Th cell homing 2 hours post-exercise after 2 days on the LC diet. Based on these data it seems that exercise, more than diet, affects differential count and homing of immune cells.

Dietary strategy and the exercise test

Both prescribed diets, which included specific recipes and clear instructions, were well followed by our participants. The LC diet with less than 10 En% CHO led to significantly higher urine and blood ketone levels in almost all individuals. Following a (very) low carbohydrate diet will result in temporarily ketogenesis, in other words, the body will produce and use ketone bodies (acetoacetate, acetone and β-hydroxybutyrate) for the production of energy (27).

It has been speculated that athletes on a LC diet are at increased risk for the immunosuppressive effect of cortisol, including the suppression of antibody production, lymphocyte proliferation and natural killer cell cytotoxic activity (28). The exercise-induced increase in cortisol was by far the highest after 2 days on the

LC diet. Since 2 days on a LC diet is likely to be too short for fat adaptation to occur, glucose remains the primary fuel source. As a consequence, strenuous exercise will therefore rapidly lead to glycogen depletion and this may result in excess release of cortisol (29, 30). The exercise intensity in our study was fairly high, reflected by heart rates above 160 bpm (187 bpm was their max HR at the VO_{2max} test). Several studies have shown that when individuals perform exercise after several days on very low carbohydrate diets, this leads to cortisol levels that are markedly higher than with a normal or high carbohydrate diet (10, 28, 31). Here we showed that this cortisol response was not significantly higher anymore after two weeks on the LC diet compared to two weeks on the HC diet suggesting some form of adaptation to the LC diet.

Immune response

In all situations, cell differential counts clearly indicated an acute exercise-induced immune response as shown by changed lymphocyte counts and changed cell proliferation rates immediately post-exercise and/or 2 hours post-exercise. These results are in agreement with studies reporting exercise induced immune responses as well. Some show that exercise results in decreased lymphocyte counts and decreased cell proliferation rate (32, 33). While others found increased lymphocyte subsets after a one hour of exercise at 75% VO2max in a glycogen-depleted state, induced by prior exercise and 2 days on a LC diet (28). In a previous study from our own lab we found a (non-significant) trend for increased lymphocyte counts following exercise in a glycogen-depleted state (34).

In the present study, cell differential count after 2 days on the diets showed differences between the diets, with higher T, Th and B cells in the blood with a HC diet, and higher monocyte count with the LC diet. Interestingly, cell differential count after 2 weeks on the diets was comparable. Our findings are consistent with the idea that changing to a LC diet requires metabolic adaptations which are not accomplished after 2 days, and thus result in a stress-associated decreased resilience of the immune system when triggered by exercise. Interestingly, these effects were paralleled by the more pronounced rise of plasma cortisol levels. This also suggests a stress-related effect of the LC diet, which gradually disappeared after 2 weeks. Cortisol has a known immunosuppressive effect, including the suppression of antibody production, lymphocyte proliferation and natural killer cell cytotoxic activity (28). Although it would be tempting to conclude that cortisol plays a causal role in the effects found in our study this cannot be concluded with our present design. Changes in cell count could be explained by a few mechanisms. First,

exercise could lead to muscle damage, provoking an immunological response. Although the exercise tests in this study involved neither maximal, nor eccentric exercise, both known to cause more muscle damage, small muscle damages cannot be excluded in this study. Second, increased circulation and higher blood pressure induce higher shear forces that may promote a passive mobilization of immune cells from the lymphatic system into peripheral blood flow (3). Third, catecholamine levels increase during exercise, these induce mobilizing effects on leukocyte subsets by β-adrenergic signalling (2).
Recent evidence suggests an enhanced immune activation following acute exercise (6). Therefore, decreased cell counts directly post-exercise, are rather interpreted as a redeployment of immune cells to target tissues than an actual loss of cells. Nevertheless, solely data showing a post-exercise decrease in immune cells, does not provide information on immune cell function. Therefore, we also analysed cell proliferation rates and homing patterns.

Cell proliferation rates
Exercise resulted in an insignificant increase in cell proliferation rate, as reflected by an increase in Ki67 activity, directly post-exercise for both diets. Two hours post-exercise a significant decrease in proliferation rate was seen on the HC diet and not on the LC diet. This resulted in significantly lower proliferation rates on the HC diet 2 hours post-exercise after 2 days. This down-regulation of Ki67 activity after exercise could be caused by the presence of anti-proliferative cytokines, like TGF-β and IL-10 (35, 36). The observed significant increased frequency in monocytes from post-exercise till 2 hours post-exercise and particularly after 2 weeks on the HC diet might suggest that these are the main producers of these cytokines. This, however, needs to be analysed further.
Most mature lymphocytes, and in particular CD4+ T cells, which is the largest fraction of T-cells, recirculate continuously, from blood to tissue and back to blood again. This process is not random, as it targets cells to sites where they are most likely to encounter their specific antigen or are best adapted to function. As upper respiratory tract infections (URTIs) or its related symptoms are of high prevalence in athletes (37), we were especially interested in airway-specific homing properties. Stressors such as intense exercise can change migration patterns by decreasing the expression of homing receptors and adhesion molecules. This mechanism was shown to induce a substantial redistribution of lymphocytes, including T cells (38, 39). For example, a decreased T lymphocyte migration to the lungs of exercising mice has been reported before (40).

To our knowledge, this is the first study that documents the homing preference of Th and Tc cells following a LC diet compared to a HC diet, with the extra stressor of a single bout of exercise. As the changes observed in cell counts were probably caused by a redeployment of immune cells to target tissues, we were interested in differences in Th (CD4+) and Tc cell (CD8+) airway homing kinetics (CD4+ / CD8+ ; CCR7- CLA- Integrinβ1+β7-) caused by exercise and between the diets. Overall, exercise caused an increase in airway homing directly after exercise, and a decrease in airway homing 2 hours post-exercise. Th cell homing showed no differences between baseline and directly post-exercise or 2 hours post-exercise for the HC diet, but only for the LC diet (from baseline till 2 hours post-exercise) after 2 days adherence. Tc cell airway homing was increased from baseline till directly post-exercise after two days with the HC diet. This was followed by a decrease in airway homing 2 hours post-exercise. Exercise clearly caused different homing patterns, while the difference between diets were less pronounced. Only two hours post-exercise after 2 days on the diets a difference was observed, with lower Th cell airway homing in the HC diet compared to the LC diet. Taken these observations together, this suggests that exercise has a regulatory role for the dynamics of Th and Tc cells, while diet has less influence.

Based on the number of live cells we found, no evidence for the induction of apoptosis after exercise and therefore the differential cell counts of immune cells in the blood is reflective of a redistribution induced by exercise in individuals on both diets. The most pronounced effects were observed with exercise, and this finding was verified by the expression of homing markers on CD8+ and CD4+ immunocompetent cells as these could be stimulated in vitro to proliferation. The expression of CCR9, CCR10 and α4β1 together with the lack of CLA expression is reflective of an exercise-induced phenotype of T-cell subset homing to the upper airways.

The exercise-induced changes in homing properties could contribute positively to immune defence against URTIs. When exercising, breathing frequency increases, increasing the exposure to foreign pathogens in the respiratory tract. To be able to fight these pathogens, an increased number of Th cells is required that are fully immunocompetent and can be rapidly activated (32, 41). As a consequence, a delayed influx of Tc cells will occur to destroy virus-infected cells. Thus, at different time points the total number of CD3+ T-cells in blood might remain stable. Further studies are needed to more precisely study the kinetics of the homing process of T-cells.

'LC diet and the immune response'

The main diet followed in this study was a very low carbohydrate diet, which resulted in temporarily ketogenesis, as detected by the production of β-hydroxybutyrate. There are indications that ketones can have a positive effect on the immune response. For example, β-hydroxybutyrate inhibits the activity of the NLRP3 inflammasome, causing it to release less cytokines (42). A ketogenic diet is known to have positive effects on persons with certain skin disorders and epileptic attacks (43). Furthermore, the LC diet contained more polyunsaturated fatty acids, and n-3 PUFAs can directly inhibit TLR4 signalling and the subsequent pro-inflammatory response (44). Furthermore n-3 PUFAs can activate the anti-inflammatory transcription factor PPAR-γ and inhibit NF-κβ and the subsequent pro-inflammatory cytokine production (45). Finally, previous studies from our own group have shown that endogenously formed amine-conjugates of n-3 PUFAs are anti-inflammatory (46, 47).

On the other hand, negative effects of a high fat diet on the immune response are also reported. A high fat diet is known to increase the intestinal permeability (48) which induces a state of metabolic endotoxemia (49). Furthermore, saturated fatty acids increase the activation of TLR4 (50). Saturated fatty acids act as non-microbial TLR4 agonists or indirectly promote the TLR4 activation, triggering its inflammatory response. Increased expression of TLRs in circulating macrophages will results in an increased proinflammatory cytokine production (51, 52), especially an increased expression of interleukin-1β, TNF-α, IL-6 and necrosis factor- κB (NF-κB) in the colon (51, 53). Besides, a high intake of saturated fatty acids leads to altered gut microbiota, increasing the amount of Gram-negative bacteria and thereby the natural ligand for TLR4, namely LPS (49). These effects on inflammasomes, cytokines and TLR expression and activities all point to the important role of monocytes, which underscores the increased frequency of blood monocytes shown in this study as well.

In conclusion, the results of the present study showed a clear exercise-induced immune response that paralleled with the exercise-induced stress response shown by the increased cortisol levels. Cell differential count after 2 days on the diets showed differences between the diets, while cell differential count after 2 weeks on the diets was comparable. Also, airway homing and cell proliferation rate showed a difference between diets after 2 days and not after 2 weeks of dietary adherence. Exercise, more than diet, affected cytotoxic T cell airway homing by an insignificant increase directly post-exercise and a significant decrease 2 hours post-exercise. This would

support the redistribution theory, stating that immune cells home to tissues where they are mostly needed. Cell differential count, airway homing and cell proliferation rate were all clearly affected by exercise, while the dietary affects were less pronounced.

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Supplementary table 1. Cell differential count

			CD45+ cells (count *10^4)	T cell (% of 45+ cells)	Th cell (% of 45+ cells)	Tc cell (% of 45+ cells)	NK T cell (% of 45+ cells)	B cell (% of 45+ cells)	Monocytes (% of 45+ cells)
After 2 days on diet	LC	Baseline	49.9 ± 8.3	51.0 ± 10.8	19.8 ± 6.4	9.3 ± 4.0	1.6 ± 0.9	1.7 ± 0.7	14.1 ± 7.7
	HC	Baseline	55.4 ± 16.3	54.2 ± 9.8	22.6 ± 6.6	9.9 ± 3.7	1.4 ± 0.9	1.7 ± 0.6	13.6 ± 6.7
		p value	0.17	0.195	0.106	0.288	0.486	0.646	0.805
	LC	directly after exercise	66.1 ± 16.4	39.7 ± 12.9	10.4 ± 5.0	7.9 ± 4.9	1.4 ± 1.1	1.5 ± 0.6	15.3 ± 7.0
	HC	directly after exercise	68.8 ± 19.6	45.2 ± 9.6	13.4 ± 4.8	9.1 ± 4.2	1.7 ± 1.2	1.5 ± 0.6	14.0 ± 7.6
		p value	0.513	0.016	0.009	0.103	0.219	0.945	0.304
	LC	2 hours after exercise	47.5 ± 14.9	29.2 ± 14.8	9.6 ± 6.3	4.2 ± 4.1	0.5 ± 0.4	1.4 ± 0.6	35.5 ± 15.2
	HC	2 hours after exercise	56.8 ± 17.3	40.9 ± 10.1	16.2 ± 6.4	5.9 ± 2.7	0.7 ± 0.5	1.9 ± 0.6	25.5 ± 8.2
		p value	0.138	0.007	0.004	0.117	0.081	0.014	0.016
After 2 weeks on diet	LC	Baseline	51.1 ± 14.2	54.7 ± 9.1	22.6 ± 6.8	9.8 ± 3.5	1.6 ± 0.8	1.7 ± 0.5	11.9 ± 5.0
	HC	Baseline	61.3 ± 20.3	56.7 ± 5.1	23.9 ± 2.8	10.6 ± 3.4	1.5 ± 0.8	1.7 ± 0.6	11.7 ± 4.8
		p value	0.068	0.248	0.382	0.127	0.295	0.366	0.736
	LC	directly after exercise	65.6 ± 13.4	43.2 ± 13.1	13.0 ± 6.1	8.5 ± 4.9	1.6 ± 1.1	1.7 ± 0.5	14.7 ± 8.2
	HC	directly after exercise	68.4 ± 19.9	44.4 ± 13.4	13.7 ± 6.4	8.8 ± 4.8	1.6 ± 1.3	1.6 ± 0.6	15.5 ± 11.4
		p value	0.678	0.367	0.315	0.607	0.804	0.347	0.64
	LC	2 hours after exercise	55.2 ± 15.0	42.1 ± 13.0	16.4 ± 7.5	6.6 ± 3.2	1.1 ± 1.5	1.8 ± 0.7	23.3 ± 11.2
	HC	2 hours after exercise	59.5 ± 25.7	46.6 ± 8.4	19.5 ± 6.5	7.3 ± 2.1	1.0 ± 0.5	2.0 ± 0.9	21.1 ± 6.8
		p value	0.567	0.16	0.094	0.291	0.589	0.082	0.421

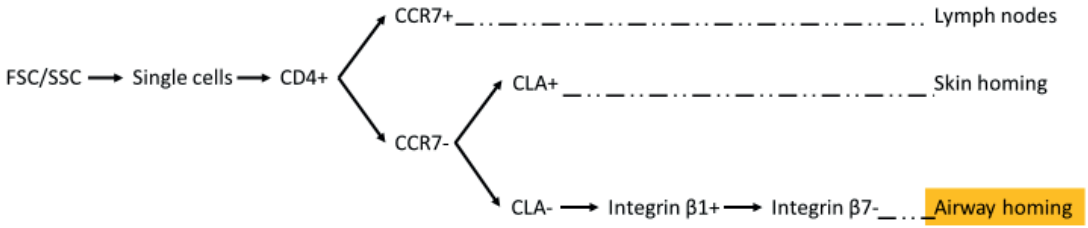
Cell differential counts are presented as percentage of total CD45+ cells (% of CD45+ cells). Means ± SD are shown. P-values represent a dependent samples t-test.

Supplementary table 2. Homing data

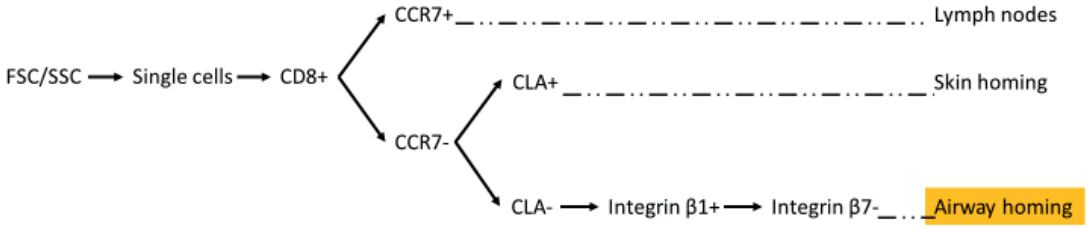
		CD4+ CCR7- CLA- IntB7- IntB1+		CD8+ CCR7- CLA- IntB7- IntB1+	
		After 2 days	After 2 weeks	After 2 days	After 2 weeks
Baseline	LC	6.6 ± 3.0	6.8 ± 3.6	28.1 ± 16.1	26.7 ± 16.0
	HC	7.0 ± 4.1	7.0 ± 3.6	26.8 ± 15.6	27.5 ± 16.2
	p-value	0.138	0.673	0.319	0.292
directly after exercise	LC	6.5 ± 4.2	6.0 ± 3.3	30.3 ± 18.0	29.6 ± 18.4
	HC	7.1 ± 4.1	7.0 ± 4.8	31.3 ± 17.7	30.9 ± 18.6
	p-value	0.314	0.222	0.445	0.432
2 hours post-exercise	LC	4.7 ± 1.9	6.1 ± 3.4	19.7 ± 15.1	21.7 ± 14.5
	HC	5.9 ± 2.5	6.2 ± 2.5	20.7 ± 13.1	20.6 ± 13.2
	p-value	0.038	0.838	0.463	0.561

means ± SD are shown. P-values refer to a dependent samples t-test.

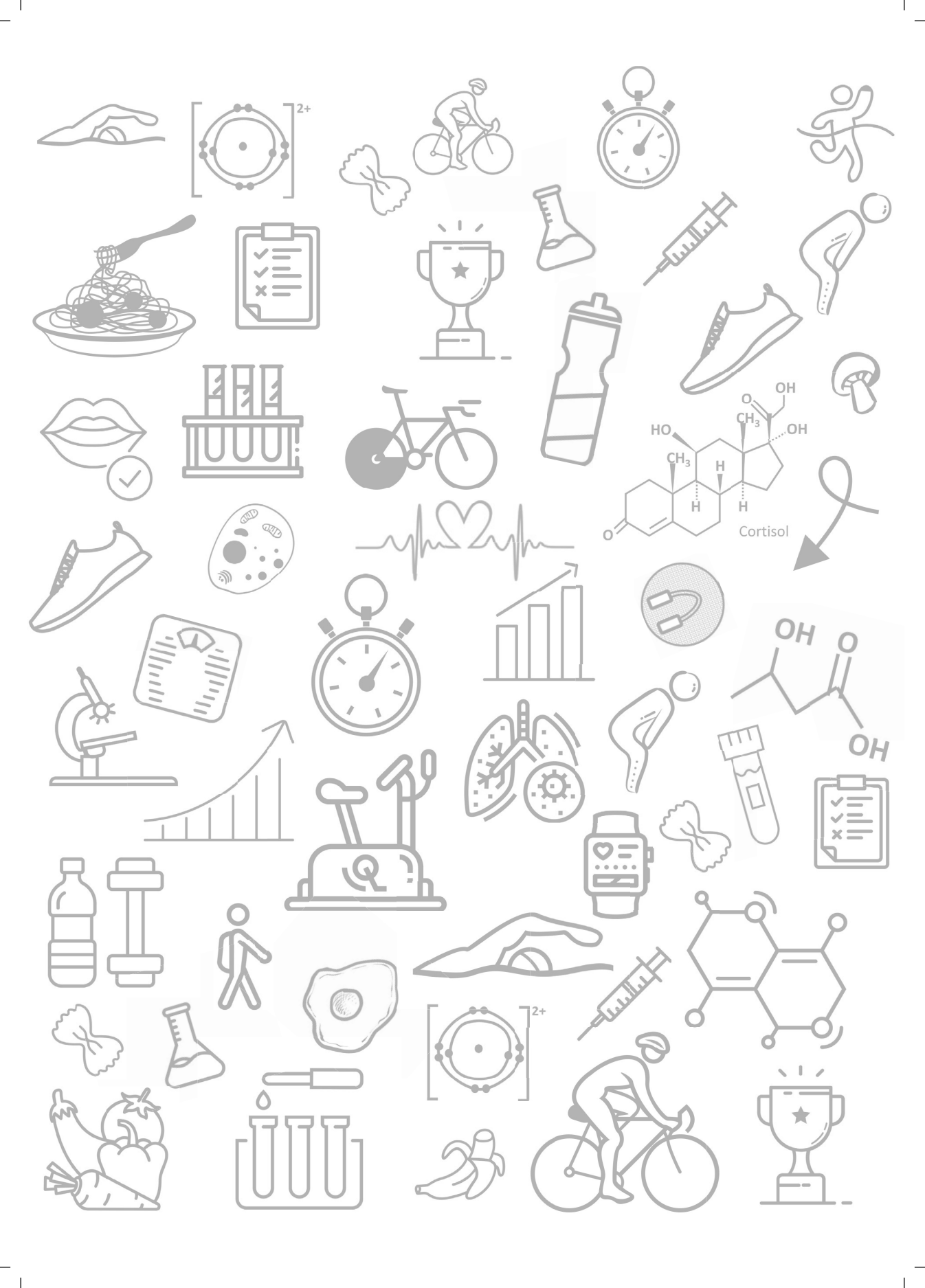
Supplementary Figure S1: Gating strategy CD4+ T cells and homing potential towards airway



Supplementary Figure S1: Gating strategy CD8+ T cells and homing potential towards airway



Supplementary Figure 1. Homing gating strategy


2+
OH
O
CH3
HO
CH3
H
H
H
O
Cortisol
OH
OH
2+

Chapter 10

General discussion

The central aim of this thesis was to further investigate the exercise responses during acute and repeated exercise and during periods of exercise training. More specifically, this aim was translated into the following sub-questions: 1) how does micronutrient status change during acute and repeated exercise, and 2) how is the stress- and immune response affected during repeated exercise and periods of exercise training? Subsequently, these two sub-questions were combined together in a dietary intervention study which aimed to study how an exercise-induced stress and immune response can be modulated by nutrition.

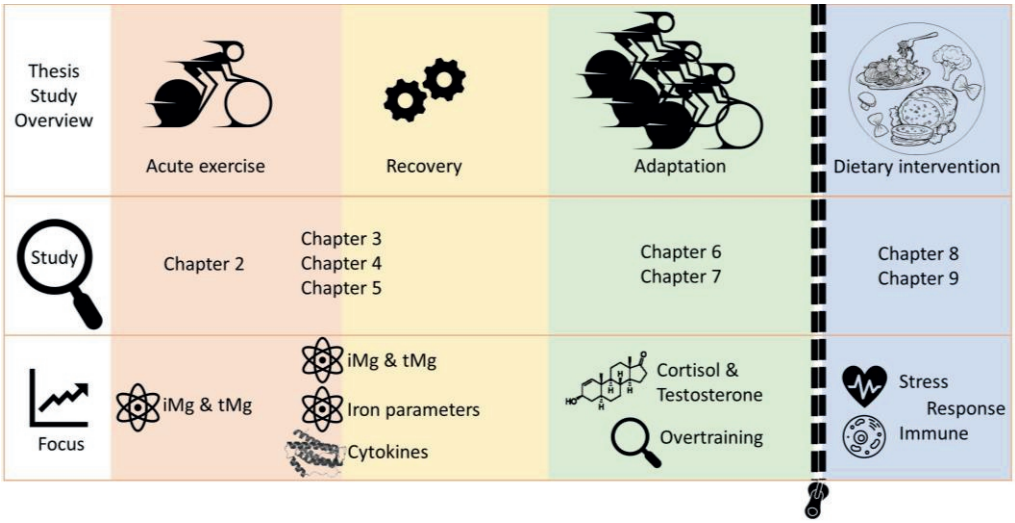


Figure 1. Schematic overview of the different chapters in this PhD thesis

Figure 1 provides an overview of the different chapters in this thesis. To start with, we analysed the exercise-induced changes in ionized and total magnesium in **chapter 2**. We were able to demonstrate that blood levels of ionized (iMg) and total magnesium (tMg) decreased directly after exercise and returned to baseline a few hours later. Next, we followed different groups of participants of the Nijmegen 4-days marches to study the effects of four consecutive days of long distance walking on the course of Magnesium (**chapter 3**), markers of iron metabolism (**chapter 4**), and a set of cytokines (**chapter 5**) in blood and plasma of middle-aged and older recreationally active persons. Interestingly, the impact of the exercise as shown by decreased magnesium concentration levels and increased levels of cytokines appeared to be more pronounced after day 1, while gradually diminishing after the next 3 days of exercise. In addition, we showed that ionized magnesium is possibly a more sensitive marker for changes in magnesium status compared to total magnesium. In **chapter 6,** we demonstrated that salivary cortisol and testosterone

are responsive to acute exercise stress, but not to prolonged training load. And finally, in **chapter 8** and **9** we analysed the effect of a nutritional intervention on the exercise-induced stress and immune response. We found a more pronounced stress response when the exercise was performed after 2 days on a low carbohydrate diet compared to that after 2 weeks of following such a diet, or compared to a high carbohydrate diet. Differences between exercise- induced immune responses following the low- and high carb diet were more pronounced after 2 days compared to 2 weeks on the diets, indicating some form of adaptation. In this final chapter, results are summarized and the consequences of the findings will be discussed. Moreover, suggestions for future research will be made.

Micronutrient status (during and directly after exercise)

Micronutrients are important for sport performance and health. A deficiency can lead to underperformance and health problems. For example, iron deficiency can lead to anaemia and fatigue (1), magnesium deficiency can lead to muscle weakness (2), and vitamin D deficiency can lead to increased risk for bone fractures (3). Athletes might be at increased risk for a deficiency, because of increased losses and decreased intakes. Increased losses can be a consequence of an increased sweat rate associated with exercise (affects Mg, Ca, Zn, Fe etc.) (4), and/or exercise induced inflammation (affecting iron status) (5). A decreased intake can occur as a consequence of an imbalanced diet, the necessity to lose weight, aesthetic reasons or certain nutritional believes. In addition, exercise itself affects micronutrient levels in athletes. For example, decreased blood magnesium levels are measured directly after exercise (6), probably reflecting a redistribution to skeletal muscle, and increased plasma pyridoxal 5'-phosphate (PLP) levels are measured directly after the start of exercise (7). This is probably due to the transfer of B6 vitamers from liver to skeletal muscle. Therefore, micronutrient status is of major importance for anyone performing exercise. However, the majority of research so far only focussed on athletes solely or only on one acute bout of exercise. This merits collection of data in other groups and following other forms of exercise as well. Collectively, this information should complete the guidelines on supplementation and nutritional advice during and following exercise. To contribute to filling these knowledge gaps, we investigated the effects of exercise on the magnesium and iron status in athletes and exercising adults, during acute, as well as repeated prolonged exercise.

In **chapter 2** we showed that exercise directly affected ionized and total magnesium status. Levels were decreased immediately following exercise and returned back to

baseline levels within 3.5 hours. This is important to know, as monitoring magnesium levels in athletes is done very often. When plasma levels (instead of balance studies based on 24h urine) are used, this could lead to misinterpretation. Finding a decreased level generally leads to the advice to improve diet or to use supplements. However, it should be taken into account whether a training took place before plasma analysis, because this affects the status at least up to 3.5 hours. Interestingly, 6 hours after exercise there were still 2 participants who were not back at their pre-exercise ionized magnesium levels. Which factors interfere with this 'return to homeostasis' remains of interest to further investigate.

Factors like exercise type, intensity and duration will also affect the magnitude of the physiological response. During exercise, magnesium is needed in skeletal muscle for contraction, relaxation and ATP production (2). In addition, during strenuous exercise an increase of catecholamines, like epinephrine and norepinephrine, induces $Mg2+$ uptake into muscle cells and regulates the magnesium dependent Na/K ATPase pumps in skeletal muscle (8). Furthermore, an increase in lipolysis probably increases the uptake of magnesium into adipocytes (9). Therefore, the decrease in plasma magnesium levels probably reflects a redistribution of the magnesium pool. As our study did not address this issue the underlying mechanisms of our findings remain unclear. Nevertheless, the results of this study indicated that return to baseline differs per individual and that timing of blood withdrawal is crucial.

It has been reported that magnesium status of most athletes is sufficient and that deficiencies are not common when athletes consume a normal healthy diet (10). Still, athletes participating in sports requiring weight control (e.g., wrestling, gymnastics) often consume less magnesium than advised (11). Sub-optimal magnesium levels are, however, often reported for older persons (12, 13). One of the causes is frequent use of specific medicines, in particular proton pump inhibitors, in this group (14).

The gathered knowledge from our acute study, showing that exercise causes a temporary decrease in magnesium made us wonder whether this decrease could lead to sub-optimal levels in older people who are already at a higher risk for magnesium deficiencies. In **chapter 3,** we therefore examined magnesium levels during four consecutive days of exercise in vital adults above 80 years of age. The main finding in this study was that plasma ionized magnesium level was more responsive to exercise compared to total magnesium. This was shown by a pronounced dip in blood ionized magnesium levels after the first day of walking exercise, while this was not observed for total magnesium levels. Ionized magnesium levels restored back to baseline levels on the second day of walking. These observations were not

caused by changes in plasma volume, but most likely by a shift between bound and unbound magnesium. This could indicate that exercise has an acute effect on the equilibrium between bound and unbound magnesium, next to the effect of exercise on magnesium distribution. Both phenomena are probably a healthy/normal reflection of an acute response to exercise, and followed by a return to homeostasis. Since total and ionized magnesium levels in blood are responsive to exercise, it is questionable if the assessment of the magnesium status should be done in blood. The assessment of total magnesium in serum or plasma is most commonly used to determine magnesium status (15), but its reliability is subject of debate (16). Since free ionized magnesium is the active, directly available form involved in cellular processes, it is suggested that ionized magnesium should be the preferable parameter to evaluate magnesium status (17). However, only ~0.3% of our body magnesium is present in blood, while most of it is stored in muscles and bones, see Figure 2. Now that we showed that one bout of exercise affects both total and ionized magnesium, while consecutive bouts of exercise affect ionized magnesium, it becomes questionable whether (ionized) magnesium in blood is a good reference values to assess magnesium status at all. The alternative, a balance study involving measurement of 24hour urine magnesium output is more laborious though. This creates a demand for methodologies to assess magnesium status, for example using nuclear magnetic resonance (NMR) or by administering and measuring of stable isotopes.

Interesting to notice was that these very old vital adults had good baseline magnesium levels. Sub-optimal levels are reported for older adults, especially when suffering from bone loss, or, as mentioned above, for older adults who regularly use medicines, including proton pump inhibitors (14, 18). We realize that we analysed a very vital group of older people. Not all men and women above 80 years of age are able to exercise 4 consecutive days at which they walk 30-40 km (average ~ 8 hours) per day. Clearly these individuals walked on a regular basis or were training themselves in other ways to be fit enough to complete such a challenge.

As we didn't measure magnesium levels in the early morning before the start of exercise, we don't know whether (ionized) magnesium levels were already back to baseline levels at the start of the second walking day, or whether magnesium levels increased during the second exercise day. In addition, this research does not provide insight in the magnesium pattern that would occur in healthy athletes who are exercising during consecutive days. More research is needed to investigate these magnesium distribution patterns, and to find out how our observations in this group

of older adults compares to an exercise-induced magnesium response in younger adults or athletes.

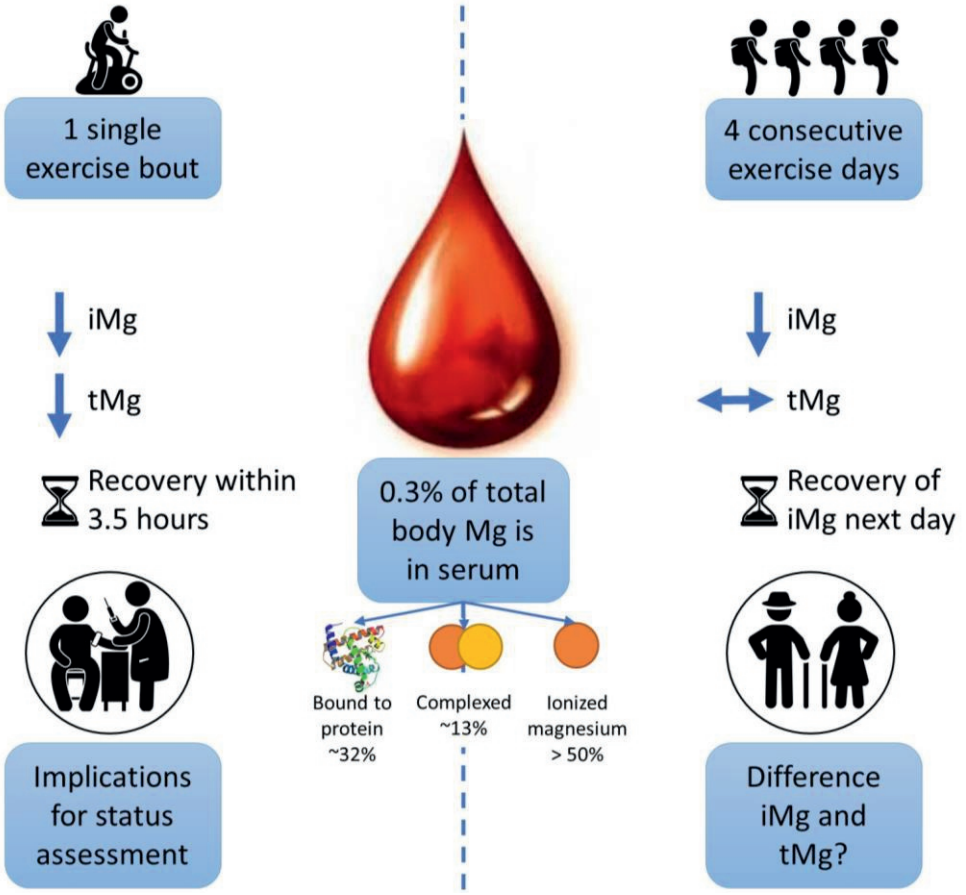


Figure 2. Magnesium response to one single bout of exercise (left side) and 4 consecutive exercise days (right side). One single bout of exercise resulted in a decrease in iMg and tMg directly after exercise in a group of athletes. Recovery to baseline levels occurred within 3.5 hours. Four consecutive days of walking exercise resulted in a decrease in iMg directly after the first day of walking, while no changes were observed in tMg levels in a group of older adults. These findings have implications for status assessment and show a difference in exercise response for iMg and tMg in different forms of exercise and different research populations.

Iron metabolism

In **chapter 4**, we showed that iron, ferritin, haptoglobin and haemoglobin all respond to repeated bouts of exercise. Most previous studies only investigated iron levels in athletes before and after an entire training season or its response to exercise solely after one single bout of work (19-21). Therefore, we investigated the effect of four consecutive days of exercise on iron levels. During this period, we found that blood

iron levels decreased, while ferritin levels increased across days. Haptoglobin levels initially decreased, which was followed by an increase, while haemoglobin levels were not changed after the first day but increased during subsequent days. These responses are most likely explained by (foot strike) haemolysis, inflammation, and losses via sweat and urine. These events resulted in iron levels below minimum reference value in a large percentage of our participants.

The observations in this study can be explained by an exercise-induced inflammatory response. During such a process, IL-6 is released, which in turn increases the release of the 'iron hormone' hepcidin by the liver (22). Hepcidin is a hormone that inhibits intestinal iron absorption and the release of iron from macrophages, which results in the decrease of iron delivery to maturing erythrocytes in the bone marrow (23). This results in decreased iron levels in blood and increased iron trapped in ferritin. The increase in haptoglobin can also be explained by inflammation, as haptoglobin is a positive acute phase protein, which increases with inflammation (24). Whether these results lead to an increased risk for anaemia in exercising people is questionable though. In theory, female athletes or athletes who follow an inadequate diet and exercise regularly might be at greater risk for a deficiency compared to sedentary age-matched adults, however, this should be studied in a real life setting. Such a study should investigate the difference between athletes on different diets containing different amounts of iron with sedentary age-matched sedentary persons. Ideally, such a study results in specific guidelines regarding iron intake in athletes.

Exercise-induced immune response

An exercise-induced immune response belongs to the protective mechanisms that help the body to adapt and to prepare for subsequent bouts of exercise. However, this immune response is also accompanied by a time-frame in which the body could be more susceptible to infections (25). Therefore, it is proposed that a second bout of exercise should be planned carefully, to enable adequate recovery of the immune system. Nevertheless, every bout of exercise induces small or larger changes in the immune system and these changes affect other body systems as well (26).

Cytokines are small molecules that are released by immune cells and other cell types. When released by muscles, they are usually referred to as myokines, and these small molecules function as signalling molecules, enhancing glucose metabolism, angiogenesis, muscle adaptations, etc. (27, 28). These molecules are essential for training adaptations. However, some researchers suggest that excessive levels may contribute to inflammatory reactions and have detrimental effects (29). A long-held view was that the exercise-induced inflammatory response was harmful and that it

was generally relevant to dampen its arise and (or) effects. Interestingly, studies with non-steroidal anti-inflammatory drugs (NSAIDs) have shown that suppressing these post-exercise inflammatory responses led to decreased training adaptations (30). Therefore, it became clear that these reactions are of crucial importance to induce training adaptations, notwithstanding the increased risk of developing a (viral) infection, due to the temporary reduced immune surveillance after intense exercise (26).

Cytokine responses after exercise have been extensively investigated in acute exercise studies, but not that much during- or following repeated bouts of exercise. Additionally, inflammatory responses after one acute bout of exercise raised the question whether repeated bouts of exercise would result in cumulation of inflammation or to an attenuated response. To this end, we first investigated the effects of four consecutive days of walking exercise on exercise-induced cytokine production. In **chapter 5** we showed that cytokine levels significantly increased after the first day, while levels were much lower at the end of the next 3 days of walking exercise. Especially IL-6, IL-8 and IL-10 showed a clear pattern, suggesting an adaptation of the response. A smaller response to a second bout of exercise is a known phenomenon from previous studies and it has been reported that even with a time interval of 2 weeks, it may still be detectable (31). Our results suggested that participants apparently adapted quickly to this type of repeated exercise. When these observations indeed reflect rapid adaptation, it is of interest to note that this would be in line with the known principle from training practice that the same type of exercise training, day in day out, is not the most effective way to achieve optimal training results and improve performance. At the same time, adaptations in cytokine response could be an essential part of the overall positive effect of training, and beneficial for increasing resilience and resistance to inflammatory and other potentially harmful stimuli during future physical challenges.

Taken together, these observations merit further studies to optimize training type and load in relation to adaptive processes of the immune system and other physiological processes. Ultimately, this should lead to better strategies, supported by measurements, that are tailored to the individual, taking into account factors like periodisation, personal goals etc.

The effect of nutritional status on the exercise induced stress/immune response

Methods to enhance recovery and training adaptations in athletes are investigated extensively. One way to optimise these processes includes modulation of nutritional status. Currently, low carbohydrate diets, often (but not correctly in a formal sense) referred to as ketogenic diets, are very popular amongst athletes, even though their underlying mechanisms and consequences are not fully understood (32). Most of the studies performed with low carbohydrate diets focussed on weight loss (33), athletic performance (34, 35) or investigated effects of clinical 'true' ketogenic diets, for example on epileptic seizures (36). Thus far, effects of low carbohydrate diets on exercise-induced stress and immune responses were understudied, even though this is very important for athletes who train regularly and put themselves under high stress levels and increased risk for infections. In principle, a low carbohydrate diet could well increase stress levels and risks for infection. As mentioned above, the exercise-induced immune response is important, as it should probably be strong enough to induce training adaptations, while at the same time an excessive response could contribute to injuries, illness or even overtraining. Therefore, **in chapter 8**, we investigated whether a very low carbohydrate diet could influence the exercise-induced stress response and symptoms of upper respiratory tract infections. In **chapter 9**, we further investigated this exercise-induced immune response after the very low carbohydrate diet, specifically focussing on immune cell differentiation and homing patterns.

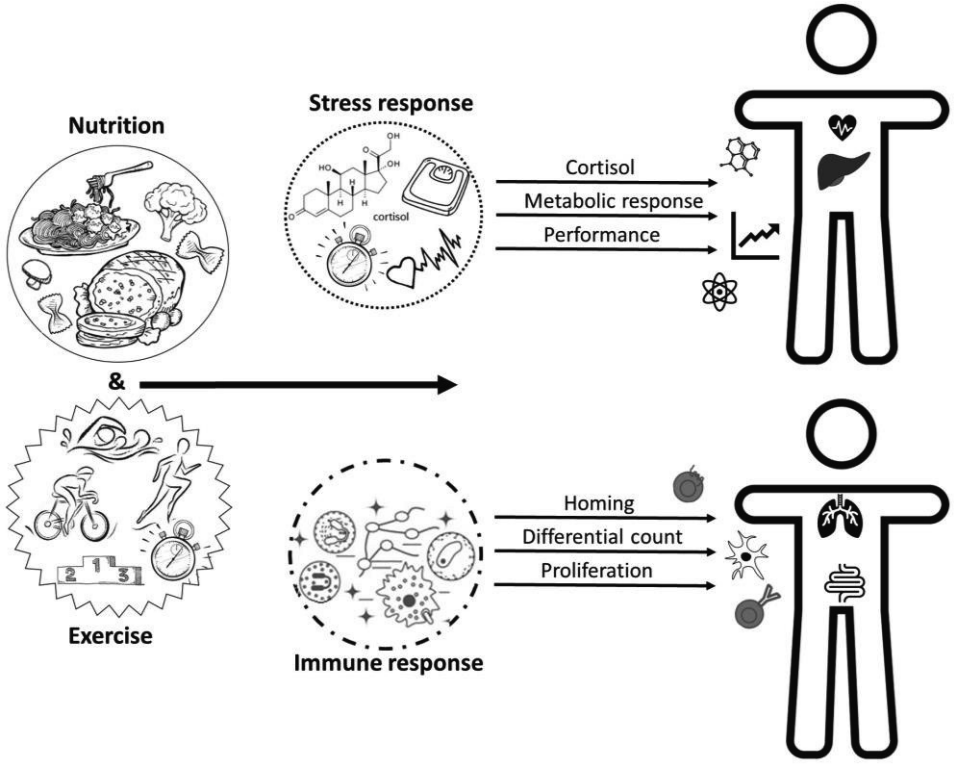


Figure 3. Conceptual model of the studies performed in chapter 8 and 9 in this thesis. Nutrition can have a modulating effect on the the exercise-induced stress and immune response. More specifically: the modulating effect of a low carbohydrate diet on the exercise-induced stress and immune response was compared to the responses after a high carbohydrate diet. In the study described in chapter 8, the stress response was investigated by measuring cortisol levels, metabolic markers (glucose, ketones, free fatty acids), and performance during an exercise test. In the study described in chapter 8, the immune response was investigated by measuring immune cell homing, differential count and proliferation rate.

Because many previous studies presumably didn't take the time to adapt to a low carbohydrate (LC) diet into account, we investigated the effects on the exercise-induced metabolic and stress responses after a short-term (2 days) and prolonged (2 weeks) adaptation to the LC diet, and compared this to a high carbohydrate (HC) diet. In practice, athletes follow low carbohydrate strategies for different durations (days till weeks till months), depending on their goals and planning. With the two time points in our study, we were able to detect metabolic changes already after 2 days on the LC diet, while adaptations were not yet observed when their exercise-induced stress response was measured. Metabolic changes were reflected by lower respiratory exchange ratios (RER), lower glucose, higher free fatty acids and higher ketone levels after 2 days on the LC diet. In contrast, the stress response was much

higher after 2 days, but attenuated after 2 weeks on the LC diet, shown by an 81% exercise-induced increase of plasma cortisol levels after 2 days on the LC diet, compared to a modest ~20-30% increase after 2 weeks on a LC diet or on the HC diet after 2 days as well as 2 weeks. Additionally, the exercise tolerance after 2 days adherence to the LC diet was much lower compared to the HC diet, as reflected by a lower workload, higher heart rate and a higher rate of perceived exertion. Apparently, metabolic changes are not directly followed by adaptations that are needed for a lower stress response or adapted exercise tolerance. The lower RER levels after 2 days of adherence to the LC diet suggested that fat oxidation was already increased. Noteworthy, the interpretation of RER, VO2 and VCO2 values for fat and glucose oxidation should be done with caution, as the oxidation of ketone bodies confounds the results (37). Studies suggest that prolonged adherence to a LC diet enhances the breakdown, transport, and oxidation of fat in skeletal muscle (38), which would result in even lower RER levels and improved exercise tolerance. We found better exercise tolerance after 2 weeks on the LC diet compared to 2 days on the LC diet. However, this was not supported by even lower RER levels in our study. Others suggest that glycogen levels are optimised after prolonged adherence to a LC diet (39), while a short-term LC diet simply depletes liver and muscle glycogen stores, without improving fat oxidation (40, 41). Unfortunately, we did not check for glycogen content in our study.
In addition, it would have been interesting to investigate the expression of genes involved in fat oxidation or transport of fatty acids. It has been shown that a 5 day LC diet (65 En% fat) resulted in increased gene expression of fatty acid translocase (FAT/CD36) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) (42). Another study showed increased carnitine acyltransferase (CAT) activity after 5 days on a high fat diet (43). Whether these effects can also be expected after 2 days adherence to a low carbohydrate diet remains unknown.

The difference in cortisol response between the acute (2 days) and the prolonged adherence (2 weeks) was also visible in the exercise-induced immune response (**chapter 9**). Cell differential count after 2 days on the diets showed differences between the diets, while cell differential count after 2 weeks on the diets was comparable. Cortisol has a known immunosuppressive effect, including the suppression of antibody production, lymphocyte proliferation and natural killer cell cytotoxic activity (44). After 2 days on the diets, T cell, T helper cell and B cell count were lower after exercise in the low carbohydrate condition compared to the high carbohydrate condition. Monocyte count was higher in the low carbohydrate

condition. Comparable responses were reported before, with changes caused by exercise and diets. However, whether these changes should be interpreted as beneficial or unfavourable is not clear. One theory, the "open window theory", suggests that decreased cell counts point towards immune-suppression and that a person is more susceptible to infection and illnesses during that period of time (45). Others suggest that the reduction in cell counts after exercise reflects a transient and time-dependent redistribution of immune cells to peripheral tissues, resulting in an elevated state of immune surveillance and improved immune regulation (46, 47). As both theories seemed plausible and worth investigating, we took off to study the effects of our LC diet on redistribution of immune cells. This process is under control of a network of surface ligands and receptors, often referred to as 'homing' (48). Our main finding regarding homing, was a decrease in T helper cell homing potential to the airways, 2 hours post-exercise after 2 days adherence to the LC diet, compared to the HC condition. Exercise itself affected cytotoxic T cell airway homing by an insignificant increase directly after exercise and a significant decrease 2 hours post-exercise. This would support the redistribution theory, stating that immune cells move to tissues where they are mostly needed.

When exercising, breathing frequency increases, thereby increasing the exposure to foreign pathogens in the respiratory tract. To effectively fight these pathogens, an increased number of CD4+ T-cells is required that are fully immunocompetent and can be rapidly activated (29, 49). As a consequence, a delayed influx of CD8+ T-cells will occur to destroy virus-infected cells. Thus, at different time points the total number of CD3+ T-cells in blood might remain stable. Further studies are needed to more precisely study the kinetics of the homing process of T-cells.

How the immune system reacts to exercise is of great interest to athletes who want to improve their performance by staying healthy and continue training. However, this issue is also of relevance for other groups, including patients, and for the development of new therapeutic strategies. It has become clear that nutritional status affects the stress and immune response, and it is therefore very important to incorporate nutrition and/or the nutritional status in scientific studies in these fields.

Future study directions

The work described in this thesis is rather diverse, but the overarching research question underlying the studies described in this thesis is whether gaining more insight into exercise-induced stress will make it possible to adjust these processes in a tailor-made manner to improve training and recovery at various levels. We

specifically focused on nutritional factors and the involvement of the immune-system. This is a relatively unknown field in which a lot remains to be investigated.

Much more has been studied when it comes to effects of exercise from a wider perspective, in relation to both general health and athletic performance. Several studies underline that exercise can decrease the risk for an early death, obesity and even mental problems. On the other hand, exercise also puts homeostatic mechanisms to the test, for example via the production of reactive oxygen species, and induction of an inflammatory response. It is clear that too much exercise can result in a state of overtraining, probably not only in athletes that regularly perform exercise, but also in those people that use exercise to improve their health or are recovering from disease. Evidence suggests that there is a thin line between too little and too much exercise, and that this is individually determined and depending on other, external factors.
Still, it is very difficult to determine how much is too little and how much is too much exercise. Studies focussing on overreaching and overtraining in athletes aim to find that thin line and identify markers that can distinguish functional overreaching from non-functional overreaching and overtraining. So far, no studies have found (a set of) biomarkers that reliably and reproducibly detect and (or) predict overtraining. However, as we already pointed out in our review in **chapter 7**, many of these studies contain methodological flaws. Ideally, a strictly controlled training study should be performed taking into account the considerations and recommendations described in our review. These include that such a study should assess exercise performance, include a recovery period, discriminate between fatigue, functional overreaching, non-functional overreaching and overtraining, and that all other causes for underperformance should be excluded. A study designed according to these recommendations could help to answer the question whether there are biomarkers that are useful to discriminate between too much and too little exercise.

For athletes, current dietary guidelines mainly focus on macronutrient intake and timing, even though micronutrients are essential for health and performance. As shown in chapter 2 and chapter 4, exercise affects micronutrients status. Moreover, micronutrient status also affects exercise, probably leading to decreased performance or trainability when deficiencies develop. Therefore, athletes should be advised to consume a healthy diet with enough vitamins and minerals. When micronutrient status is assessed, the training background and diurnal cycle should be taken into

account, as both affect the outcome. As exercise-induced changes in plasma levels can also relate to redistribution, care should be taken in their interpretation. While this is a normal physiological response, it is very interesting to investigate the differences in these responses between persons and within persons. For example, we found that 2 athletes were not back at their baseline magnesium levels 6 hours after exercise. This raises several questions regarding the underlying causes, including possible associations with fitness, diet etc. In the older population walking for four consecutive days, we also observed people who had a decrease in ionized magnesium and people who didn't. Like in the athletes, an interesting question remains whether this effect is related to general health or resilience. These observations can be used as outcome measures in an overtraining study.

The intervention study described in chapter 9 could be extended with the measurement of cytokine levels in stimulated PBMC cells. Especially cytokines that are produced by monocytes can give more insight to explain the patterns that were observed in chapter 9. Exercise clearly increased the monocyte cell count in blood, leaving the question whether these changes are associated with functional changes as reflected by shifts in the secretory patterns of cytokines, especially anti-proliferative cytokines, like TGF-beta and IL-10.

Clinical and social implications

This thesis showed that exercise causes changes in micronutrient status, cortisol and testosterone levels, and the immune response. The nature and magnitude of these changes depend on factors including exercise type, intensity and duration on the one hand, and the individual's functional capacity on the other hand. The primary goal of these changes is to enable exercise itself and to restore homeostasis. On the longer term, adaptive responses are initiated, again depending on the type of exercise, training level and other internal and external factors.

We demonstrated that these changes occur after acute, repeated and prolonged (training) exercise and that they can be influenced by nutrition. Which responses are more beneficial and how to adjust these exercise induced responses with diet or other factors is a topic for future research. The results of this thesis could have implications for nutritional and/or exercise guidelines for older adults, guidelines for micronutrient status assessment in athletes and nutritional plans for athletes to enhance or attenuate exercise-induced changes to enhance recovery and training adaptation.

For the non-athletic population, exercise can be important because of its health benefits. Even for diseased persons, performing exercise can be relevant and beneficial. For example, there is growing evidence that regular exercise not only prevents development of cancer but can also be beneficial for improving treatment outcome and survival rate. For instance, a study in humans showed that natural killer cells with a highly mature effector phenotype were better redistributed after exercise, and that these cells had the capacity to exert increased cytotoxicity against myeloma and lymphoma cells in vitro (50, 51). Another example is that more and more studies are conducted to link the beneficial impact of acute exercise on lymphocyte kinetics for the purposes of cancer immunotherapy (52).

People are getting older and remain more mobile during aging. It is well-known that nutritional deficiencies, including that of micronutrients, are common in this group. At present it is unknown to what extent these are interrelated with the ability to perform exercise. Much research in older adults has focussed on protein intake to maintain muscle mass and functionality. However, more research is warranted in the older population regarding the interaction between micronutrient status and optimal exercise, recovery and trainability.

The increasing attention for nutrition and health during the last decade has also generated numerous nutrition hypes, which are particularly communicated via social media and other easily accessible media. Athletes and other physically active persons are interesting target groups because of their health consciousness and focus on performance. This also applies to low carbohydrate, 'ketogenic', diets which have received increasing attention from athletic population in the past few years. Although their benefit-risk balance is still not clear, in particular in the longer term, many athletes have adopted such dietary strategies. Research that investigated the effects of ketogenic diets on athletes has mainly focussed on body composition and exercise performance thus far. However, health outcomes, like the immune response and long-term health effects are understudied. In this thesis, we showed that stress response and immune response are affected. Other studies show that peak performance is diminished on a ketogenic diet, therefore, athletes should be cautious when it comes to following a ketogenic diet during their competition phase. Taken together, more research is needed in this field before athletes can be advised to follow a low carb/ketogenic diet.

Overall conclusion

In this thesis I studied the effect of exercise on the micronutrient status, stress- and immune response. From previous research it was already known that one acute bout of exercise provokes a clear response of all these processes, which was confirmed by my studies. Far less has been studied on their interaction and relevance for adaptation after consecutive bouts of exercise and prolonged training. My studies have shown that dietary intervention, in terms of reducing the relative amount of carbohydrates, provokes a clear stress and immune response after a few days, while adapting to this diet results an extinction of these effects. It can be concluded that a healthy person, whether it is an athlete, an adult or a vital older adult, is highly resilient to exercise and nutrition. Taken together, nutritional status evidently affects the exercise stress and immune response, while exercise in turn affects the nutritional status. Therefore, nutrition requires considerable more scientific attention when it comes to studies on exercise, adaptation and trainability.

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Chapter 11

Summary
Dankwoord
List of publications
About the author
Portfolio

Summary

Exercise induces a range of physiological responses involving different organs, tissues and systems, including the circulatory, endocrine, immune, muscular and nervous system. The acute exercise response refers to the metabolic and mechanical effects directly following exercise, while the recovery after exercise concerns mechanisms to repair, refuel, replenish and return to homeostasis. Longer-term adaptations are important for growth and supercompensation. The role of nutrition or specific nutrients in these processes is substantial and demands further investigation. In this thesis, we investigated the exercise response during acute and repeated exercise and exercise training. Specifically, micronutrient status during acute and repeated exercise and the stress and immune response during repeated and training exercise was investigated. In addition, a dietary intervention was conducted to investigate whether nutritional status, i.e. high vs low carbohydrate intake, modulated of the exercise induced stress and immune response.

In **chapter 2** we assessed the impact of exercise on the variation in blood magnesium levels before exercise and during 6 hours post-exercise recovery in well-trained cyclists and triathletes. We showed that both ionized and total magnesium decreased directly after exercise and returned to pre-exercise levels within 3.5 hours after exercise. Furthermore, we observed an increase in both ionized and total magnesium at the end of the control day without exercise, suggesting a diurnal cycle. The decrease in blood magnesium levels after exercise and subsequent increase a few hours later likely reflects re-distribution to muscles and to blood respectively. We concluded that exercise affects magnesium levels and timing of blood sampling to analyse magnesium status is important.

These observations highlighted the impact of an acute bout of exercise, but what would happen when exercise is performed for multiple days in a row remained unclear. In addition, most research regarding exercise and micronutrient status is performed in younger athletes, even though older persons might be at increased risks for micronutrient deficiencies. Therefore, we examined changes in ionized and total magnesium levels during four consecutive days of prolonged walking exercise (~8 hours) in a group of very old adults (> 80 years) in **chapter 3.** Blood samples were collected at baseline (1 or 2 days before the first walking day) and every walking day directly after finishing. Our results showed that ionized magnesium levels significantly dropped directly after the first day of walking, while total magnesium showed no clear pattern. During subsequent days, ionized magnesium levels did not drop after exercise. After exercise, sub-optimal ionized and total magnesium levels

were found in 88% and 16% of the participants, respectively. These sub-optimal levels were not associated with drop-out or health problems, suggesting that these magnesium changes probably reflect re-distribution of magnesium through the body. In addition, this population was apparently healthy and vital, being 80+ years old and being able to walk 30-40km every day for four consecutive days.

In **chapter 4** we measured iron parameters during four consecutive days of walking exercise in a group of healthy adults. We showed that plasma iron decreased across days, while ferritin increased across days. Haptoglobin showed a decrease after the first day and increased over subsequent days. Haemoglobin did not change after the first day but increased during subsequent days. These observations probably reflect increased iron losses via foot strike haemolysis, increased losses via sweat and urine, but also the impact of exercise-induced inflammation on hepcidin and iron status.

The exercise-induced inflammatory response was the topic of **chapter 5**. In this chapter the cytokine response during four consecutive days of walking exercise was examined. Again, blood samples were taken at baseline and every walking day after the finish. Samples were analysed for IL-6, IL-8, IL-10, TNF-α and IL-1β levels. The first day of walking exercise caused an increase in cytokine levels, thereafter, levels decreased from day 1 to day 2 and remained rather stable during the following days, even though daily workload remained constant. These results suggest that an acute inflammatory response occurred after the first day of walking and that individuals adapt rapidly to this type of repeated exercise.

Shifting our focus to prolonged training instead of acute exercise, we first assessed whether salivary and hair cortisol and testosterone could be used to assess training load in a group of elite swimmers in **chapter 6.** Previous research showed that cortisol and testosterone can be used to assess acute exercise stress in athletes, while their usefulness as indicators of long-term training load during a training periodization remained unclear. In our study, ten male elite swimmers were monitored during 10 consecutive weeks of training, ending with a competition. Although the training load decreased over the 10 weeks, both salivary cortisol and testosterone levels remained unchanged. Hair testosterone levels were increased in the second week of training. During competition, both salivary cortisol and testosterone increased directly after the first race and returned back to baseline levels within 2 hours after the last race. This let us conclude that salivary cortisol and testosterone can be used to assess acute exercise stress but not prolonged training load over several weeks.

In **chapter 7** we reviewed the existing literature regarding various research designs that were used to study overreaching and overtraining. We had noticed how the field

is struggling with the question on how to stimulate, measure and/or follow up overtraining in athletes. Therefore, we discussed the available information on (non)functional overreaching and overtraining from the perspective of the researcher. This review can be seen as a kind of guideline on 'how to perform an overreaching/overtraining study' and what are the critical issues that a researcher should keep in mind. Previous examples let us conclude that a good overreaching/overtraining study should at least include performance tests, a recovery period and exclusion of other causes for underperformance like illness, anaemia, infection or insufficient diet. A pilot study performed in our lab to test an increased training load protocol did lead to fatigue, however, not to non-functional overreaching or overtraining as all participants performed comparable or even better after a short recovery period.

In **chapter 8 and 9** we investigated the effects of a short-term (2 days) and prolonged (2 weeks) low carbohydrate diet (< 10 En% carbohydrates) on the exercise induced stress and immune response in a cross-over study design with a high carbohydrate diet as a control diet. Low carbohydrate ketogenic diets gain a lot of interest in athletes even though the consequences for health are not always known. Therefore we first focussed on the metabolic and exercise induced stress response in **Chapter 8.** The results of this study showed that short-term adherence to a LC diet already led to metabolic changes, as reflected by lower respiratory exchange rates (RER), lower glucose, higher free fatty acids and higher ketone levels and that these metabolic changes were comparable between short-term and prolonged adherence to the LC diet, except for ketone levels which were further increased after 2 weeks. The exercise induced stress response was higher after 2 days on the LC diet, and attenuated after 2 weeks on the LC diet, shown by an 81% increase in plasma cortisol after 2 days on the LC diet compared to ~20-30% increase after 2 weeks on the LC diet as well as on both timepoints on the HC diet.

In **chapter 9** our focus shifted to the exercise induced immune response, which showed that 2 days adherence to the LC diet resulted in different immune cell counts after exercise compared 2 days adherence to the HC diet, resulting in lower T cell, Th cell and B cell counts in the LC diet. These differences were diminished and not significant anymore after 2 weeks adherence to both diets. T cell homing kinetics to the airways also showed differences between the LC and HC diet after 2 days adherence, but not after 2 weeks adherence to the diets, with lower Th cell airway homing on the LC diet 2 hours after exercise. At baseline, immune cell count and homing were not different between diets, while differences between diets were detected directly after exercise and 2 hours after exercise. A clear exercise response

was evident for immune cell differential count and cell proliferation rate. We concluded that adaptation to a LC diet in terms of metabolic and stress response occurs within two weeks, but not after 2 days; and that exercise, more than diet, differentially affects homing of immune cells in our study.

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Yes, het dankwoord. Dit is misschien wel het leukste om te schrijven van deze gehele thesis. Want ik ben nogal wat mensen dankbaar. Mensen die mij afgelopen jaren hebben geholpen, die er voor mij waren, waar ik wat van heb mogen leren, waar ik mee kon discussiëren, lachen, kletsen, rennen, fietsen, zwemmen, lezen, praten, analyseren, samenwerken, etc.

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Collega's / Dames van de begane grond

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PhD genoten

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Studenten die mij hielpen bij onderzoeken

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List of publications

Terink R, Balvers MGJ, Hopman MTE, Witkamp RF, Mensink M, Klein Gunnewiek JMT. Decrease in ionized and total magnesium blood concentrations in endurance athletes following an exercise bout restores within hours – potential consequences for monitoring and supplementation. *IJSNEM, 2017*

Terink R, Bongers CCWG, Witkamp RF, Mensink M, Eijsvogels TM, Klein Gunnewiek JMT, Hopman MTE. Changes in cytokine levels after prolonged and repeated moderate intensity exercise in middle-aged men and women. *Translational Sports Medicine (TSM), 2018*

Terink R, ten Haaf D, Bongers CCWG, Balvers MGJ, Witkamp RF, Mensink M, Eijsvogels TM, Klein Gunnewiek JMT, Hopman MTE. Changes in iron metabolism during prolonged repeated walking exercise in middle aged men and women. *European Journal Applied Physiology, 2018*

Terink R, Balvers MGJ, Bongers CCWG, Eijsvogels TM, Witkamp RF, Mensink M, Hopman MTE Klein Gunnewiek JMT. Ionized and total magnesium levels change during repeated exercise in older adults. *The journal of nutrition, health and aging, 2019*

Terink R, Witkamp RF, Hopman MTE, Mensink M. How to perform testing and training protocols to study overreaching and overtraining - experimental protocols and outcome measures revisited. A narrative Review. *(under review)*

Terink R, Witkamp RF, van Rossum EFC, Mensink M. Salivary cortisol and testosterone are responsive during competition, but not during a training block in elite swimming athletes. *(under review*)

Terink R, Witkamp RF, Hopman MTE, Siebelink E, Savelkoul H, Mensink M. Effects of a low carbohydrate diet on the exercise induced stress and metabolic response in well trained individuals. *(under review)*

Terink R, Mensink M, Meijer B, Witkamp RF, Savelkoul H. A short-term low carbohydrate diet affects differential count, homing and proliferation rate of immune cells following strenuous exercise: a randomised cross-over trial. *(under construction)*

About the author

Rieneke Terink was born on March 13, 1984 in Doetinchem, the Netherlands. After completing secondary school at the "Ulenhof College' in Doetinchem, she started the Bachelor's programme 'Biology' at Wageningen University in 2002. After having received her BSc in 2006, she enrolled in the Master's programme 'Cell Biology'. During her Master she did two additional minors outside the biology programme, namely 'Nutrition' and 'Immunology'. Her Internship was entitled: 'Blood pressure in relation to sports background', this was done at Sport Centre Papendal under supervision of a sports cardiologist. Her thesis was entitled: 'Heart rate variability (HRV) and overtraining' this was done at UMC Utrecht in the department of Sports medicine. This was all combined with more than 25 hours of swimming practice per week with the goal to make the Olympics.

After finishing her master's degree, she worked 2 years as a project leader at the Radboud University to develop guidelines on how to treat patients with a rare cognitive iron disorder. In 2011 she performed this job from Spain, as she moved there with her boyfriend Geert for swimming. The guidelines were delivered in 2012 and the accessory peer-reviewed article has been cited much since then. Between 2012 and 2015 Rieneke choose to put all her time and effort in swimming, which unfortunately didn't result in the Olympics (0.09 sec short), but it did result in several Dutch and European championships, world cups and one world championships.

In March 2015 Rieneke was appointed as a PhD candidate at the Division of Human Nutrition at Wageningen University and Research. The main topic of her PhD research was: to study the micronutrient state and exercise stress markers to monitor training load in athletes. This research was initiated in the Eat2Move consortium, which is a collaboration between Wageningen University and Research and other Universities and companies. Her supervisors were Dr Marco Mensink, Prof Renger Witkamp, Prof Maria Hopman and Dr Jacqueline Klein Gunnewiek. During her PhD, Rieneke attended several national and international courses and conferences and she was involved in teaching activities. Furthermore, she attended the tv and internet show 'Universiteit van Nederland' where she spoke about her research and she helped with the development of the online course (MOOC) 'Nutrition, Exercise and Sports' for Edx. Rieneke completed her PhD in February 2020.

Overview of completed training activities

Discipline specific activities

Name	Organiser	Year
Nutriscience	VLAG	2015
European congress Sport Science	ECSS, Sweden	2015
Nutritional Science days	NWO / WUR	2015-2019
Physiology days	DPD, Maastricht	2016
Symposium Publish with impact	VLAG	2017
Webinar: Overtraining	BASES	2017
International symposium Exercise and Immunology	ISEI, Portugal	2017
NOC*NSF LPC – Nutritional conference	NOC*NSF	2017-2019
RAAK symposium	RAAK, Utrecht	2017
Topsport Community	Jumbo, Veghel	2018
NL'se vereniging voor zwemsport trainers	NVVZT	2018
Energy metabolism&body composition in nutrition&health	VLAG	2018
Exp and theoretical approaches in immunology	WIAS / VLAG	2019
Working at an international organization	WUR / NCIF	2019
International symposium Exercise and Immunology	ISEI, China	2019

General courses

Name	Organiser	Year
VLAG PhD week	VLAG	2015
Scientific writing	VLAG	2015
Good Clinical Practice Course	Human Nutrition	2016
Data management planning	WUR-library	2017
Efficient writing strategies	WGS	2017
Masterclass Mixed Models	Leiden University	2017
MOOC NUTR107x "Nutrition, Exercise and Sports"	WUR	2019

Optional activities

Name	Organiser	Year
Adjusting PhD proposal	WUR	2015
Research group meetings	WUR	2015-2019
Eat2Move 'kantine'	Eat2Move	2015-2019
Pitch Goodbye party Frans Kok	Human Nutrition	2015
Pitch Health Valley	Nijmegen	2016
Poster presentation iMg validation	Clinical Chemistry	2016
Pitch Sport Conference	HAN, Arnhem	2016
College "Universiteit van Nederland"	Universiteit van NL	2016
Presentation Nova Biomedical	Utrecht	2017
Muscle meetings	WUR	2016-2018

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