Effect of exercise on micronutrient status and stress and immune response

Rieneke Terink
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Thesis committee

Promotors

Prof. Dr. R. F. Wijtkamp
Professor of Nutritional Biology
Wageningen University & Research

Prof. Dr. M. T. E. Hopman
Professor of Integrative Physiology
Radboud University Medical Centre, Nijmegen

Co-promotors

Dr. M. R. Mensink
Assistant professor, Division of Human Nutrition and Health
Wageningen University & Research

Dr. J. M. T. Klein Gunnewiek,
Specialist in laboratory medicine, Cluster manager ZorgSlingel and Ziekenhuis, Doetinchem

Other members

Prof. Dr. E. J. M Feskens
Wageningen University & Research

Prof. Dr. K Krüger,
Institute of Sports Science, Giessen, Germany

Dr. J. W van Dijk
HAN University of Applied Sciences, Nijmegen

Dr. M. K. C. Hesseling
Maastricht University.

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Chapter 1

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

Directly following exercise are often referred to as “acute exercise response”. While
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 enable high performance with the next exercise session. Or the other way around, insufficient recovery can lead to 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.

Modulation of exercise response and recovery is crucial for optimal performance. During acute exercise, the sympathetic nervous system is activated, stimulating the heart rate and blood flow to muscles. Once exercise begins, the sympathetic nervous system is activated, stimulating the heart rate and blood flow to muscles. When training results in temporary fatigue with subsequent improvements after appropriate recovery, the temporary fatigue is considered functional overreaching. When training overload occurs without improvements, it is described as non-functional overreaching. 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 longer than expected, it is considered as overreaching. 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 longer than expected, it is considered as overreaching. 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 longer than expected, it is considered as overreaching.

Regular exercise training is needed for training adaptations and these adaptations enable high performance with the next exercise session. Or the other way around, insufficient recovery can lead to 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.

Figure 1: Presentation of the different phases of training, overreaching and overtraining syndrome (adapted and adjusted from Meeusen et al. 2013).

Figure 2.

Acute exercise

Further more, minerals including magnesium (4), potassium (5) and calcium (6) are essential nutrients for energy systems depending on the duration and intensity of the exercise. They stimulate alertness and block pain signals. The body will use different energy systems depending on the duration and intensity of the exercise. Diurnal processes allow exercising more efficiently, as they influence the functioning of the digestive system, diverted blood flow away from the gastrointestinal (GI) tract and skin. These processes allow exercising more efficiently, as they influence the functioning of the digestive system, diverted blood flow away from the gastrointestinal (GI) tract and skin. At the same time, energy is mobilized via neuronal and hormonal pathways [7]. At the same time, energy is 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), and accumulated heat in the brain (9) and depleted energy sources.

The sympathetic nervous system is activated, stimulating heart rate and blood flow to muscles. This allows exercising more efficiently, as it influences the functioning of the digestive system, diverted blood flow away from the gastrointestinal (GI) tract and skin. These processes allow exercising more efficiently, as they influence the functioning of the digestive system, diverted blood flow away from the gastrointestinal (GI) tract and skin. At the same time, energy is mobilized via neuronal and hormonal pathways [7]. At the same time, energy is 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), and accumulated heat in the brain (9) and depleted energy sources.
Recovery
Directly after terminating exercise, the main goals of the body are recovery and return to homeostasis. In the end, athletes aim for specific training adaptations that could enhance their performance. Readers are referred to reviews (21, 26).

Adaptation
Longer term, adaptive responses to exercise are called training adaptations. Training adaptation eventually supercompensation. Possible training adaptations are tightly coupled to the mode, frequency, intensity and duration of the exercise regimen, which is called training specificity (22). Possible training adaptations occur only in those muscles and muscle fibers that have been recruited during the exercise performed (21). Furthermore, the majority of training-induced adaptations are tightly coupled to the mode, frequency, intensity and duration of the exercise performed (21). In the trained muscle. It is beyond the scope of this introduction and thesis to go into more detail about adaptive responses to training. Micronutrient (11, 12) and hormone levels (13), as well as immune cell invasion of inflammatory cells (macrophages and T-cells), and secretion of growth factors and cytokines (18). Exercise causes a (short-term) disturbance in cellular function and 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 supercompensation.

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 an influx of extracellular calcium (17), an increase in 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. Oxidative capacity of the endurance-trained muscle and power of the resistance-trained muscle are enhanced. The improved delivery of oxygen and fuels to the muscle facilitated by changes in the enzymes, increased capillary density (24), increased mitochondrial density (25) and adaptations include muscle hypertrophy (23), increased activity of oxidative enzymes, increased capillary density and increased mitochondrial density.
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 achieving long-term goals. Therefore, exercise training concerns the process of multiple bouts of physical activity performed within a certain timeframe, with the aim of increasing functional capacity. This becomes clear when chronic intensive training and inadequate recovery lead to performance decrement and ultimately the overtraining syndrome.

The underperformance period is called ‘overtraining syndrome’. 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.

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, general introduction.
Directly after terminating exercise, the main goals of the body are recovery and return to homeostasis. In the end, athletes aim for specific training adaptations that could enhance their performance. To this end, athletes aim for specific training adaptations that could enhance their performance.

Chapter 1

Training adaptations are tightly coupled to the mode, frequency, intensity and duration of exercise performed. Furthermore, the majority of training-induced adaptations occur only in those muscles and muscle fibers that have been recruited during the exercise performed. Longer term, adaptive responses to exercise are called training adaptations. Training adaptation eventually supercompensation.

Muscle damage can occur in the sarcolemma, contractile proteins and connective tissue. 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).

Training adaptations include muscle hypertrophy (23), increased activity of oxidative enzymes, increased capillary density (24), increased mitochondrial density (25) and adaptations induced by training response via a personalized 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.

Modulating the acute exercise stress response can be a way to enhance training and performance. While acute exercise stress results in higher cortisol levels and suppressed immune function (39), optimal nutritional strategies are not only relevant when it comes to the recovery process (33). But also increased risk for disease or psychological discomfort is probable (28, 30).

A one size fits all approach is not always the best option. For example, studies examining the effects of protein supplementation and manipulation of macronutrient profiles have revealed that the effects of exercise performed under low CHO availability may increase exercise-induced stress response, resulting in higher cortisol levels and suppressed immune function. Examples of such proteins and signaling molecules involved in training adaptation may be induced to a greater extent when exercise is performed under low CHO availability. Another example is that studies have demonstrated that proteins and signaling mechanisms are increased when endurance training adaptations in healthy young males are undertaken (34).

These processes are characterized by an influx of extracellular calcium (17), an increased invasion of inflammatory cells (macrophages and T-cells), and secretion of growth factors and cytokines (18). Exercise causes a (short-term) disturbance in cellular processes (glucose uptake and fatty acid oxidation), myogenesis, and mitochondrial function (20) to allow return to homeostasis and for adaptation and repair (32). At the molecular level, exercise affects gene expression, enhances nutrient uptake and transport, and modulates signaling pathways. This results in the synthesis of specific proteins (20) to allow return to homeostasis and for adaptation and repair (32). Nutritional manipulation on this response requires more investigation.

However, how to monitor this acute exercise stress response, and the impact of nutritional manipulation on this response requires more investigation. In some cases, the exercise response should be enhanced to gain greater training adaptations, while in other scenarios, the response should be reduced. 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 increased risk for disease or psychological discomfort is probable (28, 30).

Therapeutically, ways to enhance the recovery process is of high scientific interest. Therefore, ways to enhance the recovery process is of high scientific interest. Therefore, ways to enhance the recovery process is of high scientific interest.
Topic of this thesis

- Understanding the acute exercise response and its impact on subsequent adaptation.
- Investigating the role of nutrition and training overload in performance enhancement.
- Analyzing the interplay between exercise response, adaptation, endurance, and performance.

Figure 3: Presentation of the different phases of training, overreaching, and overtraining syndrome.

- Presentation of the different phases of training, overreaching, and overtraining syndrome.
Recovery

Directly after terminating exercise, the main goals of the body are recovery and return to homeostasis. In the end, athletes aim for specific training adaptations that could enhance their performance. These processes are characterized by an influx of extracellular calcium (17), an increase in intracellular calcium, an increase in intracellular ionized calcium, and an increase in intracellular magnesium (18). The influx of extracellular calcium leads to a (short-term) disturbance in cellular functions. The intracellular calcium level is tightly regulated and can be modulated by nutrition.

Muscle damage can occur in the sarcolemma, contractile proteins and connective tissue. Various exercise models are used to study overtraining, in these models changes in exercise load without sufficient recovery can lead to overtraining syndrome. This demands for biomarkers that indicate early signs of overtraining. Therefore, in chapter 6, we first study which biomarkers are suitable to indicate early signs of overtraining in middle-aged adults. We also wanted to study what happens with ionized magnesium levels in healthy well-trained athletes during exercise as well as throughout the day and insight in the time course during multiple days of exercise was lacking. Therefore, in chapter 5, the effect of an acute bout of exercise on 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.

Outline of the thesis

Chapter 2 investigated the usefulness of saliva and hair cortisol and testosterone levels to study which method could provide a stable indicator of long-term effects. In contrast, measuring blood cortisol and testosterone has not yet been published for athletes to our knowledge, while such methods could provide a stable indicator of long-term effects. By contrast, measuring blood cortisol and testosterone has not yet been published for athletes to our knowledge, while such methods could provide a stable indicator of long-term effects. 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.

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Chapter 4 investigated the usefulness of saliva and hair cortisol and testosterone levels to study which method could provide a stable indicator of long-term effects. In contrast, measuring blood cortisol and testosterone has not yet been published for athletes to our knowledge, while such methods could provide a stable indicator of long-term effects. By contrast, measuring blood cortisol and testosterone has not yet been published for athletes to our knowledge, while such methods could provide a stable indicator of long-term effects. 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.

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Chapter 6, we first
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 enables high performance with the next exercise session. Or the other way around, possible without the detrimental effects of overtraining. So, adequate recovery lead to performance decrement and ultimately the 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 proposed. If training results in temporary fatigue with subsequent 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.

Presentation of the different phases of training, overreaching and overtraining syndrome

Figure 2.
Directly after terminating exercise, the main goals of the body are recovery and return to homeostasis. ... the end, athletes aim for specific training adaptations that could enhance their performance. 

Chapter 1


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

Regular training enables high performance with the next exercise session. Or the other way around, general introduction

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General introduction

11


Chapter 1

Directly after terminating exercise, the main goals of the body are recovery and return to homeostasis. 

In the end, athletes aim for specific training adaptations that could enhance their performance. 

Adaptation eventually supercompensation.

Investigating the mechanism for AMP activation of the AMP-activated protein kinase activity and substrate.

Knstein AJ, Litte.JP

Rodriguez NR, Di Marco NM, Langley S. America


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.
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

Rieneke Terink
Michael Balvers
Maria Hopman
Renger Witkamp
Marco Mensink
Jacqueline Klein Gunnewiek

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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.; VO2max, 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% VO2max) 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
Decrease in ionized and total magnesium blood concentrations in endurance athletes following an exercise bout restores within hours – potential consequences for monitoring and supplementation

INTRODUCTION

Decrease in ionized and total magnesium status. A decrease affects both ionized and total magnesium. Previous studies showed inconclusive results for the effect of exercise on ionized and total magnesium status. A decrease in tMg was found after a marathon (21, 22) and a stepwise treadmill test (15). Decreases in tMg were found after marathon testing (21, 22) and a stepwise treadmill test (15). A significant increase in iMg was observed after a bicycle ergometer test (20). Decreases in tMg were found after a marathon (21, 22) and a stepwise treadmill test (15).

Another problem with monitoring magnesium status is that exercise itself seems to influence magnesium concentrations. Decreases in tMg were found after a marathon (21, 22) and a stepwise treadmill test (15). Significant increases in iMg were observed after a bicycle ergometer test (20). Decreases in tMg were found after a marathon (21, 22) and a stepwise treadmill test (15).

Account for approximately 60 – 70% of those for tMg, giving reference ranges for iMg. 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. Since free ionized magnesium (iMg) is the active, directly available form involved in numerous metabolic processes (2), its evaluation is important for athletes.

Cur r ent l y, measurement of total serum or plasma magnesium (tMg) is most frequently used by athletes (13). 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.

Since free ionized magnesium (iMg) is the active, directly available form involved in numerous metabolic processes (2), its evaluation is important for athletes. 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.

Muscular dysfunction. At the same time, studies suggest that Mg supplements are frequently used by athletes (13). Generating a demand to monitor it in athletes to prevent magnesium-related problems is expected. Since free ionized magnesium (iMg) is the active, directly available form involved in numerous metabolic processes (2), its evaluation is important for athletes. 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.

Muscular contraction (4), with ATP and calcium both depending on Mg. Magnesium is for example crucial for glycolysis (1, 3), protein synthesis and metabolic processes (2). In terms of exercise capacity, muscular contraction (4), with ATP and calcium both depending on Mg. Magnesium is for example crucial for glycolysis (1, 3), protein synthesis and metabolic processes (2). In terms of exercise capacity,
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.; VO2max, 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% VO2max) 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

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

**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.

**Experimental design**

At 70% of the athletes’ individual VO\(_{2}\text{max}\) for women and 100 W for men, participants performed an initial workload of 75 W for 25 minutes on a cycle ergometer (Ergoline GmbH, Bize, Germany) to establish maximal aerobic capacity (VO\(_{2}\text{max}\)).

Questionnaires about supplement use, sports background, and food intake (Food (Holstein Tanner/Whitehouse Skinfold, Crosswell, UK) to estimate body fat.

Hamburg, Germany) were taken at 11:00 am, 12:30 pm, and 13:30 pm.

In addition, body length (Seca 213 portable stadiometer, Hoechberg, Germany). Measurements were taken at least two weeks between both measuring days. Prior to the study, participants underwent the first preliminary measurement. The study was approved by the Ethics Committee.

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 participants did not donate blood during the last six weeks prior to the study.

Pariticipants did not use supplements and did not take medications. All athletes trained regularly, for at least 5 hours per week.

Since free ionized magnesium (iMg) is the active, directly available form involved in numerous metabolic processes (2). In terms of exercise capacity, magnesium is for example crucial for glycolysis (1, 3), protein synthesis and muscular dysfunction. At the same time, studies suggest that Mg supplements are generating a demand to monitor its status in athletes to prevent magnesium-related muscle weakness, neuromuscular dysfunction, and muscle cramping (2, 6-11). As a common used to determine magnesium status (14), but its reliability is subject to frequent use by athletes (13).
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.; VO2max, 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% VO2max) between 11:00 and 12:30. Whole blood samples were analyzed 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

Blood sampling

Blood samples were taken through a cannula from the cephalic vein and collected in lithium-heparin tubes (3.5 ml LH PSTTM II and 4.0 ml 17 I. U./mL, Becton-Dickinson, New Jersey, America). LH PSTTM 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, Wal t ham, 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 VO2max) 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.

Across-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 dietary 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 ± stand 2

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>23.0 ± 2.0</td>
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<td>185.7 ± 5.9</td>
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<td>87.4 ± 11.3</td>
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<td>Body Mass Index</td>
<td>26.3 ± 3.9</td>
<td>25.0 ± 4.2</td>
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<tr>
<td>VO2max (ml/kg/min)</td>
<td>54.9 ± 6.2</td>
<td>46.9 ± 6.8</td>
</tr>
</tbody>
</table>

Data is expressed as mean ± SD.

Table 1. Characteristics of the study population

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

Decrease in ionized and total magnesium blood concentrations in endurance athletes following an exercise bout restores within hours – potential consequences for monitoring and supplementation.
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.; VO2max, 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% VO2max) 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

Chapter 2

<table>
<thead>
<tr>
<th>Time</th>
<th>Control day</th>
<th>Exercise day</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:30</td>
<td>0.53 ± 0.04</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td>11:00</td>
<td>0.53 ± 0.04</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>12:30</td>
<td>0.53 ± 0.04</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>13:30</td>
<td>0.52 ± 0.03</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>15:00</td>
<td>0.52 ± 0.03</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>16:00</td>
<td>0.52 ± 0.03</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>18:30</td>
<td>0.54 ± 0.03</td>
<td>0.54 ± 0.04</td>
</tr>
</tbody>
</table>
Magnesium (Mg) is an essential micronutrient for health and performance (1). It is involved in numerous metabolic processes (2). In terms of exercise capacity, it is suggested that ionized magnesium (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 total magnesium, 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. Decreases in tMg were found after a marathon (21, 22) and a stepwise treadmill exercise bout restores within hours – potential consequences for monitoring and supplementation (23).

Professional athletes are at a higher risk for magnesium deficiency, as increased Mg losses are observed after intense exercise. Professional soccer players consuming Mg-supplemented food (19 mmol Mg) and exercising at an exercise intensity ≥ 5 mmol/L had higher Mg losses compared with the control group (7.3 mmol Mg) (20). Similarly, increases in ionized and total magnesium blood concentrations in endurance athletes following an incremental running exercise has been reported (15). Contrarily, a significant increase in iMg was observed after a bicycle ergometer test (20).

Moreover, previous studies did not account for a possibly underlying diurnal rhythm (21, 22). Further, participants (one man; one woman) were still below their pre-exercise total magnesium concentration (Δ Post-exercise ≥ 0 mmol/L), while 15 participants were back at their pre-exercise total magnesium concentration, however, 3 out of 18 participants were still below their pre-exercise ionized magnesium concentration (Δ Post-exercise ≥ 0 mmol/L) (23).

### Table 1: Mean Ionized (A) and Total (B) Magnesium Concentrations, During Exercise (Dotted Line) and Post-exercise (Solid Line)

<table>
<thead>
<tr>
<th>Time</th>
<th>Control day</th>
<th>Exercise day</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:30</td>
<td>0.83 ± 0.06</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>11:00</td>
<td>0.81 ± 0.05</td>
<td>0.81 ± 0.07</td>
</tr>
<tr>
<td>12:30</td>
<td>0.83 ± 0.04</td>
<td>0.73 ± 0.06</td>
</tr>
<tr>
<td>13:30</td>
<td>0.83 ± 0.05</td>
<td>0.75 ± 0.05</td>
</tr>
<tr>
<td>15:00</td>
<td>0.84 ± 0.05</td>
<td>0.85 ± 0.06</td>
</tr>
<tr>
<td>16:00</td>
<td>0.85 ± 0.06</td>
<td>0.85 ± 0.09</td>
</tr>
<tr>
<td>18:30</td>
<td>0.87 ± 0.06</td>
<td>0.90 ± 0.08</td>
</tr>
</tbody>
</table>

* * 

### Recovery of magnesium concentrations after exercise

- At 2.5 hours after exercise (15:00 PM), concentrations seemed to be recovered, as concentrations were significantly higher compared with all earlier time points (p < 0.05).
- However, when individual data were analyzed, only 10 participants (out of the 18) – exercise ≥ 0 mmol/L, while 15 participants were back at their pre-exercise concentration, however, 3 out of 18 participants were still below their pre-exercise ionized magnesium concentration (Δ Post-exercise ≥ 0 mmol/L) (23).

![Graph showing recovery of magnesium concentrations after exercise](image)
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.; VO2max, 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% VO2max) 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

Table 2: Recovery of ionized and total magnesium after exercise

<table>
<thead>
<tr>
<th>Time after exercise</th>
<th>Number</th>
<th>Recovered</th>
<th>Mean ± SD</th>
<th>Number</th>
<th>Recovered</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Directly after exercise</td>
<td>0 / 18</td>
<td>-</td>
<td>0.06 ± 0.03*</td>
<td>0 / 18</td>
<td>-</td>
<td>0.08 ± 0.04*</td>
</tr>
<tr>
<td>1 hour after exercise</td>
<td>1 / 18</td>
<td>-</td>
<td>0.05 ± 0.02*</td>
<td>1 / 18</td>
<td>-</td>
<td>0.06 ± 0.04*</td>
</tr>
<tr>
<td>2.5 hours after exercise</td>
<td>10 / 18</td>
<td>-</td>
<td>0.007 ± 0.02</td>
<td>15 / 18</td>
<td>0.04 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>3.5 hours after exercise</td>
<td>13 / 18</td>
<td>0.00 ± 0.02</td>
<td>15 / 18</td>
<td>0.04 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 hours after exercise</td>
<td>16 / 18</td>
<td>0.03 ± 0.03</td>
<td>18 / 18</td>
<td>0.09 ± 0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Differences between pre-exercise and different time points after exercise. Concentrations are recovered when post-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

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

DISCUSSION

Decreased total and ionized magnesium concentrations after exercise
Chapter 2

Magnesium is essential for optimal sport performance, generating an interest to monitor its status in practice. Therefore, we measured the effect of an acute bout of exercise on ionized (iMg) and total plasma magnesium (tMg) in blood obtained from participants.

Our study does not provide data to support the uptake by muscle cells and adipose tissue might explain a post-exercise drop in magnesium, causing a decrease of the free ionized magnesium in cells, which is caused by an increase of catecholamines during strenuous exercise, as is the case in more prolonged exercise. As a consequence, free fatty acids are increased during exercise, in particular when muscle glycogen levels decrease, as is also suggested. Thus, another possible explanation for a decrease of plasma magnesium could be uptake into adipocytes due to an increased rate of lipolysis (31). Lipolysis might be caused by the timing of blood withdrawal, as concentrations were measured immediately after exercise and at different time points later that day. If we had measured only at 2.5 hours after exercise, we would not have observed the decrease in ionized and total magnesium concentrations. On the other hand, we measured up to 6 hours after exercise, so we do not know whether a putative increase may have occurred after 6 hours. Additionally, we measured total and ionized magnesium up to 24 hours after an intensive basketball training (23). This is caused by an increase of catecholamines during training and not directly after finishing training (23). We measured total and ionized magnesium concentrations up to 6 hours after exercise, so we do not know whether a putative increase may have occurred after 6 hours. Additionally, we measured total and ionized magnesium concentrations up to 6 hours after exercise, so we do not know whether a putative increase may have occurred after 6 hours. Additionally, we measured total and ionized magnesium concentrations up to 6 hours after exercise, so we do not know whether a putative increase may have occurred after 6 hours.
Decrease in ionized and total magnesium blood concentrations in endurance athletes following an exercise bout restores within hours – potential consequences for monitoring and supplementation

Higher magnesium concentrations at the end of the day

Correlation between ionized and total magnesium

Conclusion

However, we don’t know whether a similar pattern is observed after another form of exercise.
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.; VO2max, 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% VO2max) 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
Decrease in ionized and total magnesium blood concentrations in endurance athletes following an exercise bout restores within hours – potential consequences for monitoring and supplementation

REFERENCES
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.; VO₂max, 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% VO₂max) 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

Chapter 2

References:
Magnesium (Mg) is an essential micronutrient for health and performance (1). It is involved in numerous metabolic processes (2). In terms of exercise capacity, 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 consequence, performance is highly dependent on adequate magnesium levels (12), 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).

Moreover, previous studies did not account for a possibly underlying diurnal- or circadian-related fluctuation in iMg and tMg. In the present study, we aimed to determine approximate reference ranges for iMg and tMg in 20 healthy subjects. The simultaneous measurement of ionized and total calcium and magnesium in intensive care unit patients. Journal of critical care. 2002;17(3):203-8.

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).

Free ionized magnesium concentrations in serum/plasma are commonly used to determine magnesium status (14), but its reliability is subject to stressor states. The American journal of the medical sciences. 2013;345(5):401-4.

In the present study, we aimed to determine approximate reference ranges for ionized and total magnesium in a group of healthy individuals.


The present study included 20 healthy volunteers who were monitored for their magnesium status. The simultaneous measurement of ionized and total calcium and magnesium in intensive care unit patients. Journal of critical care. 2002;17(3):203-8.

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In the present study, we aimed to determine approximate reference ranges for ionized and total magnesium in a group of healthy individuals.

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

Exercise effects on Mg levels in elderly

Rieneke Terink
Michael Balvers
Coen Bongers
Thijs Eijsvogels
Renger Witkamp
Marco Mensink
Maria Hopman
Jacqueline Klein Gunnewiek

The journal of nutrition, health & aging, 2019
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).

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 combination with assessed total (tMg) and ionized magnesium (iMg) concentrations in plasma and whole blood, respectively, during 4 consecutive days of exercise in very old adults.

Design: 68 participants (age 83.7±1.9 years) were monitored on 4 consecutive days at which they walked 30-40 km (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 bout of intense 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: very old 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 among others lead to muscle weakness, neuromuscular dysfunction, and muscle cramping (2-7). In addition, a backward linear regression was also used to analyse which variables significantly contributed to the observed baseline total and ionized magnesium levels. In this study, we assessed blood magnesium levels in a vital group of older adults. The aim of this study was to assess blood magnesium levels in a vital group of older adults. Moreover, we explored the effect of (repeated) exercise on blood magnesium levels in older adults aged > 80 years and to investigate the effect of (repeated) exercise on blood magnesium levels in older adults aged > 80 years. Therefore, we included a large unique group and exercise were mainly focused on healthy young and middle-aged adults, while earlier studies in the older adults are lacking. Therefore, we included a large unique group and exercise were mainly focused on healthy young and middle-aged adults, while earlier studies in the older adults are lacking. The blood magnesium levels and showed a decrease in magnesium levels after prolonged exercise (8). Besides, magnesium absorption decreases with age (9). Additionally, the age-related reduction in bone mass results in a reduction of the magnesium concentration in the body (10). Furthermore, a part 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. 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. 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 plasma magnesium levels. 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 magnesium (plasma) levels. Moreover, we explored changes in plasma volume were calculated from blood haematocrit and haemoglobin concentrations using Dill and Costill's equation (19). Furthermore, a part 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. 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. 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 plasma magnesium levels. 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 magnesium (plasma) levels. Moreover, we explored changes in plasma volume were calculated from blood haematocrit and haemoglobin concentrations using Dill and Costill's equation (19). In the case of elderly, where baseline magnesium levels may already be low, a post-exercise drop could result in plasma magnesium levels. 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 magnesium (plasma) levels. Moreover, we explored changes in plasma volume were calculated from blood haematocrit and haemoglobin concentrations using Dill and Costill's equation (19).
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.

Study procedure
Immediately before the start of the march, participants’ body weight (Seca 888 scale, Hamburg, Germany) and body height were 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).

Baseline measurements
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. Baseline 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 after the first walking day.

Intensity = (Measured HR / Expected maximal HR) * 100%, with Expected max HR calculated from: $\text{BMI} = \sqrt{\text{Age} / 208 - \text{Age}}$ (18).
Ionized and total magnesium levels change during repeated exercise in older adults.

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 collected in the LH 17 IU/mL vacutainer. Blood was collected in lithium-heparin (LH) tubes (3.5 ml LH PSTTM II and 4.0 ml LH 17 IU/mL, Becton-Dickinson, Vianen, The Netherlands). The LH PSTTM 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 using Dill and Costill’s equation (19).

Furthermore, a part of the whole blood sample collected in the LH 17 IU/mL vacutainer was immediately analysed using the Statistel 3 (Erlangen, Germany). For measurement of ionized magnesium status, a fraction of the whole blood sample collected in the LH 17 IU/mL vacutainer was used for direct analyses of plasma haemoglobin and haematocrit concentrations using Dill and Costill’s equation (19).

Statistical analysis

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

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.
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.

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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: 

\[ \text{Intensity} = \left( \frac{\text{Measured HR}}{\text{Expected maximal HR}} \right) \times 100\% \]

with Expected max HR = 208 - (0.7 \times \text{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).

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>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>83.7 ± 1.9</td>
</tr>
<tr>
<td>Body composition</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168.9 ± 7.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.0 ± 10.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.8 ± 2.7</td>
</tr>
<tr>
<td>Cardio characteristics</td>
<td></td>
</tr>
<tr>
<td>Resting heart rate (bpm)</td>
<td>66.6 ± 13.5</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>147.2 ± 14.0</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>81.8 ± 10.3</td>
</tr>
<tr>
<td>Dietary intake</td>
<td></td>
</tr>
<tr>
<td>Energy intake (kCal)</td>
<td>1990 ± 477</td>
</tr>
<tr>
<td>Total protein (En%)</td>
<td>16.6 ± 4.3</td>
</tr>
<tr>
<td>Total fat (En%)</td>
<td>33.8 ± 7.7</td>
</tr>
<tr>
<td>Total carbohydrates (En%)</td>
<td>43.8 ± 7.6</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>22.2 ± 6.7</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>964 ± 368</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>336 ± 99</td>
</tr>
<tr>
<td>Walking distances per day</td>
<td></td>
</tr>
<tr>
<td>Walking 30 km</td>
<td>n = 65</td>
</tr>
<tr>
<td>Walking 40 km</td>
<td>n = 3</td>
</tr>
</tbody>
</table>

Table 1. Participant characteristics

Means ± SD are shown. BMI: Body mass index. Dietary intake values are estimated with a 24 hour recall. En%: Energy percentage.
Ionized and total magnesium levels change during repeated exercise in older adults

Exercise characteristics

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 TM II and 4.0 ml LH 17 IU/mL, Becton -Dickinson, Vianen, The Netherlands). The LH PSTTM 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 concentrations using Dill and Costill's equation (19).

Total and ionized magnesium data were analysed using repeated measures ANOVA analysis. Statistical analysis was performed on all participants. Data distribution. Data was normally distributed. Parametric tests were used for significance was set at

A backward linear regression was used to analyse which variables contributed significantly to the observed baseline total and ionized magnesium levels. In hoc Bonferroni correction when there was a main effect for consecutive days.

Table 2. Effect of the environmental conditions on the physical load.

<table>
<thead>
<tr>
<th>Friedman Test Mean Rank WBGT</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min WBGT (°C)</td>
<td>15</td>
<td>18</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Max WBGT (°C)</td>
<td>27</td>
<td>29</td>
<td>26</td>
<td>25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Friedman Test Mean Rank Humidity</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min Humidity (%)</td>
<td>45</td>
<td>35</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Max Humidity (%)</td>
<td>62.5</td>
<td>73.9</td>
<td>43</td>
<td>43</td>
</tr>
</tbody>
</table>

Asym. Gr. 22.0 (IBM SPSS version 22.0, Armonk, New York, USA), and the level of significance was set at 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. Furthermore, a part 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. For measurement of ionized magnesium status, a fraction of the whole blood sample collected in the LH 17 IU/mL vacutainer was used for direct analyses of plasma haemoglobin and haematocrit concentrations using Dill and Costill’s equation (19). Changes in plasma volume were calculated from blood haematocrit and haemoglobin in vacutainer was used for direct analyses of plasma haemoglobin and haematocrit.
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: 

\[ \text{Intensity} = \left( \frac{\text{Measured HR}}{\text{Expected maximal HR}} \right) \times 100\% \]

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).

Table 3
<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration (hr: min)</td>
<td>8:11 ± 1:06</td>
<td>8:28 ± 1:05</td>
<td>7:55 ± 1:13</td>
<td>7:53 ± 1:25</td>
</tr>
<tr>
<td>Speed (km/h)</td>
<td>3.8 ± 0.5</td>
<td>3.7 ± 0.5</td>
<td>3.9 ± 0.6</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>Fluid intake (L)</td>
<td>3.9 ± 1.5</td>
<td>3.8 ± 1.4</td>
<td>3.8 ± 2.6</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>P values</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td></td>
</tr>
<tr>
<td>Asymptotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sign</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight change (kg)</td>
<td>-1.02 ± 0.88</td>
<td>-0.68 ± 0.74</td>
<td>-0.38 ± 0.68</td>
<td>0.08 ± 0.58</td>
</tr>
<tr>
<td>P values</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td></td>
</tr>
<tr>
<td>Asymptotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight change (kg)</td>
<td>-1.02 ± 0.88</td>
<td>-0.68 ± 0.74</td>
<td>-0.38 ± 0.68</td>
<td>0.08 ± 0.58</td>
</tr>
<tr>
<td>P values</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td></td>
</tr>
<tr>
<td>Asymptotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max heart rate (bpm)</td>
<td>108.1 ± 15.4</td>
<td>122.6 ± 17.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P values</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean values for the 4 walking days. Asym. = Asymptotic. 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 Dil and Costill calculation 1974 (19).
Total and ionized magnesium at baseline

Figure 1

Table 4

Exercise response
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:

\[ \text{Intensity} = \left( \frac{\text{Measured HR}}{\text{Expected maximal HR}} \right) \times 100\% \]

\[ \text{Expected max HR} = 208 - (0.7 \times \text{Age}) \]

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). Variables contributing to iMg decrease after first day of exercise

As 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. Further more, 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 lower after the first day of exercise: 0.44. Correcting iMg 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).
Ionized and total magnesium levels change during repeated exercise in older adults

DISCUSSION

Total and ionized magnesium at baseline

Exercise response
and blood pressure were measured using an automated sphygmomanometer (M5).

Baseline measurements

Intensity = (Measured HR / Expected maximal HR) * 100%, with Expected max HR
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

We selected 72 male and 22 female walkers who participated in the 2016 edition of

Study population

We expect 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

Materials and Methods

Study population

We selected 72 male and 22 female walkers who participated in the 2016 edition of

Study procedure

The study took place in the summer season, i.e. July. Actual climatological

Results

The exercise induced decrease in iMg resulted in levels below the reference value
in as much as 88% of our participants. Mg levels were low at the

Discussion

The exercise induced decrease in iMg resulted in levels below the reference value
Ionized and total magnesium levels change during repeated exercise in older adults

Variables contributing to iMg decrease after first day of exercise

In conclusion,
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: 

$$\text{Intensity} = \left( \frac{\text{Measured HR}}{\text{Expected maximal HR}} \right) \times 100\%$$

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...
REFERENCES

and blood pressure were measured using an automated sphygmomanometer (M5).

Thereafter, resting heart rate and body mass index (BMI) was calculated. Thereafter, resting heart rate and blood pressure were measured using an automated sphygmomanometer (M5).

Baseline measurements:

- Body weight (Seca 888 scale, Hamburg, Germany)
- Body height

Intensity = (Measured HR / Expected maximal HR) * 100%, with Expected max HR calculated using the formula:

\[ \text{Age} - 80 \times \text{Age}^{0.2} \]

Mean heart rate during exercise was calculated as the average heart rate, excluding the first 10 seconds of exercise.

Nutrition and exercise metabolism. 2016:1


Centers for Disease Control. CDC Health Information for International Travel. 2016

The study took place in the summer season, i.e., July. Actual climatological conditions are specified in the results section. Measurements were performed before and during 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.

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: 2016/663).

The study was conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent prior to participation.

For the calculation of expected maximal heart rate, the formula used was:

\[ \text{Age} - 208 - 0.7 \times \text{Age} \]

Intensity = (Measured HR / Expected max HR) * 100%, with Expected max HR determined using the formula:

\[ \text{Age} - 208 - 0.7 \times \text{Age} \]


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

Contributions significantly to the observed decrease in ionized magnesium after the first day of exercise.

A backward linear regression was used to analyze which variables contributed significantly to the observed baseline total and ionized magnesium levels. In addition, a backward linear regression was also used to analyze which variables contributed significantly to the observed baseline total and ionized magnesium levels. Classification of magnesium status was performed using the normal distribution. Data was normally distributed. Parametric tests were used for otherwise. The Kolmogorov-Smirnov test was used to examine the normality of the data distribution. Data was normally distributed. Parametric tests were used for otherwise. The Kolmogorov-Smirnov test was used to examine the normality of the data distribution. Data was normally distributed. Parametric tests were used for otherwise.
Iron

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

Rieneke Terink
Dominique ten Haaf
Coen Bongers
Michael Balvers
Renger Witkamp
Marco Mensink
Thijs Eijsvogels
Jacqueline Klein Gunnewiek
Maria Hopman

European Journal of Applied Physiology, 2018
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. Afterwards, 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.

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, hemoglobin 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.

Other processes resulted in iron levels below minimum reference value in a large number of our participants.

 KEYWORDS: Hb, Fe, Hp, repetitive exercise
INTRODUCTION

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS). Statistical analysis was used to estimate dietary intake. Participants filled out a food frequency questionnaire (FFQ) before the start of the study. For female participants, we collected data about their menstrual status. All participants completed a questionnaire about the use of supplements to check whether iron concentrations could have been influenced by iron supplement use and other factors.

Questionnaires were tested for the presence of erythrocytes, haemoglobin and/or myoglobin using the Clinitek Status® analyzer (Siemens Healthcare diagnostics Inc., Tarrytown, New York). Urine samples were collected after exercise and with the use of a urinary dipstick (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. An additional blood sample was collected in a 2 ml LH vacutainer (Becton-Dickinson, New Jersey, America). The vacutainer was centrifuged at 3000G (12,000 rpm) for 8 minutes at 22 degrees and plasma was stored at -80 degrees Celsius.

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 for 10 minutes at 22 degrees and plasma was stored at -80 degrees Celsius. Blood samples were analysed for their iron, ferritin and haptoglobin concentrations in the Rapidpoint 400 (Siemens Healthcare Diagnostics Inc., Dusseldorf, Germany), revealing a power > .95 for all four parameters.

The Kolmogorov-Smirnov test was used to examine the normality of the data. Participant and exercise characteristics were analysed with an independent Student t-test to examine sex differences. A backward linear regression model was used to determine the influence of dietary intake on iron concentrations.

The most common exercise-induced muscular injury is muscle soreness, which can be caused by repeated moderate intensity endurance exercise in other populations, including middle aged men and women. In addition, we expected a decrease in haptoglobin.

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

**p < 0.05.** Data were presented as mean ± SD unless indicated.
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 samples were analysed for their iron, ferritin and haptoglobin concentrations in 1500 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). The vacutainer was centrifuged at 3000G and used for direct analyses of plasma haemoglobin concentrations. 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 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.

Urine samples

Blood samples

Questionnaires

Statistical analysis

The Kolmogorov-Smirnov test was used to examine the normality of the data otherwise. A post-hoc power analysis was conducted in GPower (version 3.0.10, Dusseldorf, Germany), revealing a power > .95 for all four parameters. Partici pant and exercise characteristics were analysed with an independent Student t-test to examine sex differences. A backward linear regression analysis was performed using Statistical Package for Social Sciences (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.

Iron, ferritin, haptoglobin and haemoglobin levels were corrected for plasma volume changes. Partici pant and exercise characteristics were analysed with an independent Student t-test to examine sex differences. A backward linear regression analysis was performed using Statistical Package for Social Sciences (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.

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 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 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.

Statistical analysis

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

Costill’s equation

Urine samples

Blood samples

Questionnaires

Statistical analysis

Changes in iron metabolism during prolonged repeated walking exercise in middle aged men and women
Chapter 4

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.

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

Statistics were performed using Statistical Package for Social Sciences (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.

Participants filled out a food frequency questionnaire (FFQ) before the start of the study. Whether iron concentrations could have been influenced by iron supplement use and whether participants filled out a FFQ before the start of the study were analyzed with an independent Student t-test to examine sex differences. A backward linear regression analysis was performed to examine the influence of age, smoking intensity, and exercise characteristics on iron, ferritin, and haptoglobin levels.

Twenty of our 48 female participants were post-menopausal, 5 women were not sure. There were no significant differences in iron, ferritin, haptoglobin, or hemoglobin.

Nutritional iron intake and use of supplements

Iron, ferritin, haptoglobin, and hemoglobin levels were measured. Iron supplements were used by only 1 female participant. Total iron intake for men was higher than for women, as was the intake of vitamin B6 and B12 compared to women. There were no significant differences in resting heart rate, average training distance in the year prior to the study, height, weight, BMI, macronutrient intake, fiber intake, iron intake (total, hem and non-hem), and exercise characteristics of the remaining 98 participants.

Blood samples

An additional blood sample was collected in a 2 ml LH 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.

An additional blood sample was collected for female participants. We did not collect data about the stage of menopause for those female participants. Twenty of our 48 female participants were post-menopausal, 5 women were not sure.

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

Questionnaires

Samples were analyzed for their iron, ferritin, and haptoglobin concentrations in October 2015 (Siemens Dimension Vista 1500, Siemens Healthcare, Erlangen, Germany). Relative changes in plasma volume were calculated from blood hematocrit and hemoglobin concentrations using Dill and Costill’s equation (15). Relative changes in plasma volume were analyzed with an independent Student t-test to examine sex differences. A backward linear regression analysis was performed to examine the influence of age, smoking intensity, and exercise characteristics on iron, ferritin, and haptoglobin levels.

Iron, ferritin, haptoglobin, and hemoglobin levels were measured. Iron supplements were used by only 1 female participant. Total iron intake for men was higher than for women, as was the intake of vitamin B6 and B12 compared to women. There were no significant differences in resting heart rate, average training distance in the year prior to the study, height, weight, BMI, macronutrient intake, fiber intake, iron intake (total, hem and non-hem), and exercise characteristics of the remaining 98 participants.

We did not collect data about the stage of menopause for those female participants. Twenty of our 48 female participants were post-menopausal, 5 women were not sure.
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.

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Table 1. Participant characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Men (n = 50)</th>
<th>Women (n = 48)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.9 ± 9.9</td>
<td>50.9 ± 11.2</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Body composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>180 ± 6</td>
<td>167 ± 6</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>88.9 ± 13.3</td>
<td>65.6 ± 8.1</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.3 ± 3.3</td>
<td>23.5 ± 3.0</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Cardiac characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting heart rate (bpm)</td>
<td>63 ± 9.9</td>
<td>64 ± 6.9</td>
<td>0.758</td>
</tr>
<tr>
<td>Dietary intake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy intake (*10³kJ)</td>
<td>10.7 ± 2.8</td>
<td>8.0 ± 2.5</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Total protein (g)</td>
<td>95 ± 25</td>
<td>75 ± 25</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>104± 35</td>
<td>75 ± 27</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Total carbohydrates (g)</td>
<td>268 ± 78</td>
<td>204 ± 72</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>27 ± 8</td>
<td>22 ± 8</td>
<td>0.005</td>
</tr>
<tr>
<td>Iron intake (via diet)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total iron (mg)</td>
<td>13 ± 3</td>
<td>11 ± 3</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Hem iron (mg)</td>
<td>1.2 ± 0.6</td>
<td>0.9 ± 0.5</td>
<td>0.006</td>
</tr>
<tr>
<td>Non hem iron (mg)</td>
<td>12 ± 3</td>
<td>10 ± 3</td>
<td>0.001</td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>2.1 ± 0.8</td>
<td>1.8 ± 0.7</td>
<td>0.022</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>6.0 ± 2.6</td>
<td>4.7 ± 2.4</td>
<td>0.014</td>
</tr>
<tr>
<td>Protein (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walking characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Training distance (km)</td>
<td>812 ± 1072</td>
<td>753 ± 921</td>
<td>0.77</td>
</tr>
<tr>
<td>30 km/day</td>
<td>17</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>40 km/day</td>
<td>20</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>50 km/day</td>
<td>13</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

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 refers to an unpaired Student t test between men and women.

Exercise characteristics

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 refers to an unpaired Student t test between men and women.
Changes in iron metabolism during prolonged repeated walking exercise in middle aged men and women

Table 2. Changes in iron metabolism during prolonged repeated walking exercise in middle aged men and women

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
<th>Men</th>
<th>Women</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>4.29 ± 12.21</td>
<td>0.03 ± 0.03</td>
<td>61 ± 62</td>
<td>0.26 ± 0.02</td>
<td>61 ± 62</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>After</td>
<td>4.4 ± 0.8</td>
<td>0.00 ± 0.00</td>
<td>61 ± 62</td>
<td>0.23 ± 0.01</td>
<td>61 ± 62</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Significance</td>
<td>&lt;0.001</td>
<td>0.016</td>
<td>0.015</td>
<td>0.016</td>
<td>0.015</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Variables: Mean ± SD
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. The reafter, 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.

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 (Interaction = .028). Iron concentrations 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. A significant decrease in ferritin concentrations was observed after the first day of exercise, followed by an increase over subsequent days (Interaction = .249).

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 (Interaction = .302).

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

<table>
<thead>
<tr>
<th>Table 3. Mean laboratory parameters for men and women separately, at baseline, and day 1 until day 4. n = 98.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Iron</td>
</tr>
<tr>
<td>Ferritin</td>
</tr>
<tr>
<td>Haptoglobin</td>
</tr>
<tr>
<td>Hemoglobin</td>
</tr>
</tbody>
</table>

Iron, ferritin, haptoglobin and hemoglobin levels were calculated from blood hematocrit and hemoglobin concentrations using Dill and Dusseldorf, Germany), revealing a power > .95 for all four parameters. Otherwise, a post-hoc power analysis was conducted in GPower (version 3.0.10, Germany).
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.

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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.

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.

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative, n (%)</td>
<td>72 (74)</td>
<td>50 (51)</td>
<td>54 (55)</td>
<td>52 (53)</td>
<td>45 (46)</td>
</tr>
<tr>
<td>Positive, n (%)</td>
<td>26 (26)</td>
<td>48 (49)</td>
<td>44 (45)</td>
<td>46 (47)</td>
<td>53 (54)</td>
</tr>
</tbody>
</table>
DISCUSSION

Iron

We observed a further decrease in iron after repeated days of walking exercise, which is in contrast to previous studies (22, 23). These studies were of longer duration. However, we showed that this type of exercise contributes to decreased iron levels. We could not distinguish between iron losses that have been shown to enhance hepcidin levels (21). Hepcidin is also known as acute phase protein. Changes in iron metabolism during prolonged repeated walking exercise in middle aged men and women.
Ferritin

Haptoglobin and haemoglobin
Differences between men and women

In contrast to previous studies on prolonged walking exercise (Gilligan et al. 1950; Horbostel et al. 1970) we found no significant change in haemoglobin after the first day of walking. This is in line with our observed plasma volume changes, which showed increases and decreases between men and women, which has not been reported in detail 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 model was used to test for the presence of erythrocytes, haemoglobin and/or myoglobin. All participants filled out a food frequency questionnaire (FFQ) before the start of the event to estimate dietary intake. All participants completed a questionnaire about the use of supplements to check for female participants we collected data about their menstrual status. The magnitude of the increases and decreases differed between men and women, which has not been reported in detail otherwise. A post-hoc power analysis was conducted in GPower (version 3.0.10, Dusseldorf, Germany), revealing a power > .95 for all four parameters. However, we suggest that mechanisms, like inflammation, haemolysis and blood loss, should also be aware of this decrease in iron levels during exercise. 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. Therefore, we suggest that mechanisms, like inflammation, haemolysis and blood loss, should also be aware of this decrease in iron levels during exercise. Therefore, we suggest that mechanisms, like inflammation, haemolysis and blood loss, should also be aware of this decrease in iron levels during exercise. Therefore, we suggest that mechanisms, like inflammation, haemolysis and blood loss, should also be aware of this decrease in iron levels during exercise.
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Chapter 4

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. As 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 hemoglobin. These changes are most likely the result of (foot strike) hemolysis, 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.
Changes in iron metabolism during prolonged repeated walking exercise in middle aged men and women

REFERENCES


4. Costill's equation (15)

5. Samples were analysed for their iron, ferritin and haptoglobin concentrations in (3755 rpm) for 8 minutes at 22 degrees and plasma was stored at -80 degrees Celsius.

6. An additional blood sample was collected in a 2 ml LH vacutainer (Becton - Dusseldorf, Germany), revealing a power > .95 for all four parameters.

7. The cephalic vein. Blood was collected in a 4 ml Lithium Heparin (LH) gel vacutainer.

8. Blood samples were collected after exercise and with the use of a urinary dipstick.

9. Urine samples were collected after exercise and with the use of a urinary dipstick.

10. Costill's equation (15)


14. Interleukin-

15. Interleukin-


Chapter 4

MATERIALS AND METHODS

Study population
We selected 50 male and 50 female walkers who participated in the 2015 edition of the 4-daagse, a 100-km marathon, which was conducted in the Netherlands. Exclusion criteria were known diabetes mellitus, hypertension, cardiovascular disease, asthma, or intake of medication that could influence haematology. Participants were recruited from the event organization. Overweight or obesity was defined as a body mass index (BMI) ≥ 25 kg/m² calculated at baseline (16).

Baseline measurements

At baseline, body weight (Seca 888 scale, Hamburg, Germany) and body height were determined, a blood sample was taken and questionnaires were registered. 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. Directly after finishing, post-exercise body weight was determined. The reafter, participants walked 30, 40 or 50 km, at a self-determined pace, starting between 4:00 and 8:00 AM. Every day, participants 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).

At baseline and after each walking day, a blood sample was taken. The sample was centrifuged immediately after blood collection at 3,000 rpm for 10 min to separate serum and plasma. The plasma sample was stored at −80°C until analysis. Plasma free ferritin was determined using a commercial assay (Ferritin sandwich immunoassay test, DiaSorin, Stillwater, MN) with an inter-assay coefficient of variation (CV) of 4.5% and a sensitivity of 1.8 ng/L. Serum albumin concentration was determined using an automated colorimetric assay (Cobas 6000, Roche Diagnostics, Rotterdam, The Netherlands) with an inter-assay CV of 3.1% and a precision of 1%. Hb was measured using an automated sphygmomanometer (M5-1 intellisense, Omron Healthcare, Hoofddorp, The Netherlands) after 5 minute supine rest. 

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM, Armonk, New York). Data are presented as mean ± standard deviation (SD) or median and interquartile range (IQR). Differences between baseline and walking days were calculated using paired t-tests. Comparison between groups was performed using unpaired t-tests and ANOVA with post-hoc tests. A p-value < 0.05 was considered significant. Differences in the percentage change in Hb and ferritin between groups were calculated using the a priori determined alpha level of 0.05.
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marker, as it is mainly a leakage product from damaged cells. Metallomics: biometal science. 2014;6(4):748-73.


The American journal of the medical sciences. 2007;334(1):72-4
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. The reafter, 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.
Changes in iron metabolism during prolonged repeated walking exercise in middle aged men and women

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). 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
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

Rieneke Terink
Coen Bongers
Renger Witkamp
Marco Mensink
Thijs Eijsvogels
Jacqueline Klein Gunnewiek
Maria Hopman
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 into the 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-1β, TNF-α, repetitive exercise
INTRODUCTION

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

also, or exclusively, produced by skeletal muscle are often referred to as ‘myokines’

...
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 was conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent prior to participation.

Study procedure

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 12:00 AM. Directly after finishing, post-exercise 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. The walking track was almost completely flat, typical for the Dutch landscape with only small height differences.

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 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 12:00 AM. Directly after finishing, post-exercise 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. The walking track was almost completely flat, typical for the Dutch landscape with only small height differences.

Baseline measurements

Baseline measurements included body weight (Seca 888 scale, Hamburg, Germany) and body height. Expected maximal heart rate (HRmax) was used to estimate exercise intensity as percentage of HRmax = (Measured HR / Expected max HR) * 100%, with Expected max HR = 208 - (0.7 * Age) (17).

Baseline measurements were performed before the start of the event (‘baseline’), and at the four consecutive walking days. Baseline 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 conditions are specified in the results section. 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 conditions are specified in the results section.
Chapter 5

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 min 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 TNFα concentrations using the ultrasensitive Mesoscale Discovery (MSD) QuickPlex SQ 120 Instrument with Multiplex 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 TNFα, 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 TNFα, 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 TNFα, 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 TNFα, respectively.

Further more, 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 hematocrit concentrations (Rapidpoint 400, Siemens Healthcare Diagnostics Inc., Tarrytown, New York, USA). Relative changes in plasma volume were calculated from blood hematocrit and hemoglobin concentrations using Dill and Costill’s equation.

Questionnaires

All participants completed an online questionnaire before the event, which included a food intake questionnaire (Food Frequency Questionnaire, FFQ), a validated Short
Changes in cytokine levels after prolonged and repeated moderate intensity exercise in middle-aged men and women

Study population
We selected 50 male and 50 female walkers who participated in the 2015 Nijmegen Four Days Marches, a large annual walking event taking place in the summer season, i.e. July. Actual climatological conditions are specified in the results section. Measurements were performed before the event location one or two days prior to the first walking day, between 09:30 AM and 04:00 PM.

Statistical analysis
Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 22.0, with the level of significance set at 0.05. Data are presented as mean ± SD unless indicated otherwise. Changes in cytokine levels after prolonged and repeated moderate intensity exercise were used for analysis. A Friedman’s test was used to evaluate the effect of exercise intensity as percentage of HRmax = (Measured HR / HRmax) * 100 and other-wise.

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 responses between men and women were compared using an independent Student's t-test to examine sex differences. Participant and exercise characteristics were assessed with an ANOVA. The relationship between exercise intensity and cytokine concentrations was examined with a Pearson’s correlation test. A Wilcoxon signed-rank test was used to determine whether data from consecutive days significantly differed. The Mann-Whitney U-test was used to compare cytokine concentrations between groups, and a Chi-squared test was used to determine associations between cytokine concentrations and exercise characteristics. Significant results were further analyzed with a Bonferroni correction. All statistical tests were two-sided.
RESULTS

Participant characteristics

Exercise characteristics
and blood pressure were measured using an automated sphygmomanometer (M5 -1

determined and body mass index (BMI) was calculated. Thereafter, resting heart rate

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Study population

Study population

Table 1

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Body mass index (kg/m2)</th>
<th>Waist circumference (cm)</th>
<th>Waist-to-hip ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (n = 50)</td>
<td>58.9 ± 9.9</td>
<td>88.9 ± 13.3</td>
<td>8.9 ± 1.6</td>
<td>88 ± 10</td>
<td>0.758</td>
</tr>
<tr>
<td>Women (n = 48)</td>
<td>50.9 ± 11.2</td>
<td>65.6 ± 8.1</td>
<td>23.45 ± 3.0</td>
<td>64 ± 6.9</td>
<td>0.758</td>
</tr>
</tbody>
</table>

Means ± SD are shown. BMI: Body mass index; PAL score: Physical Activity level in

hours per week; Dietary intake values are estimated with the FFQ questionnaire; En%: percentage of energy delivered

that macronutrient; Training distance: specific walking distance in the year prior to the 4 Days Marches; P Value refers to an unpaired Stu

between male and female

men.
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Table 2

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Exercise characteristics presented for men and women at day 1 to day 4, for men and women separately</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>Asympt.</td>
<td>Sign.</td>
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<td></td>
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<tr>
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<tr>
<td>P value</td>
<td>0.032</td>
</tr>
</tbody>
</table>

| Speed  | Men     | 4.8 ± 0.8 |
|        | Women   | 4.6 ± 0.6 |
| P value | 0.235   | 0.186   |

| Fluid intake | Men     | 4.4 ± 1.6 |
|             | Women   | 4.1 ± 1.7 |
| P value     | 0.367   | 0.034   |

<table>
<thead>
<tr>
<th>Physical parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight change</td>
</tr>
<tr>
<td>Men</td>
</tr>
<tr>
<td>Women</td>
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<tr>
<td>P value</td>
</tr>
</tbody>
</table>

| Plasma volume change (%) | Men         | -4.29 ± 12.21 |
|                         | Women       | 0.001 ± 5.91  |
| P value                | 0.03       |

| Mean heart rate (bpm) | Men         | 113 ± 18 |
|                       | Women       | 115 ± 15 |
| P value               | 0.512      |

| Max heart rate (bpm) | Men         | 124 ± 27 |
|                      | 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 refer to an unpaired Student's t test between male and female participants. Asympt. Sign P values refer to an Intragroup 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 Dil and Costil calculation 1974.
Changes in cytokine levels after prolonged and repeated moderate intensity exercise in middle-aged men and women

Cytokines

Baseline

Table 3.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Men baseline</th>
<th>Men day 1</th>
<th>Men day 2</th>
<th>Men day 3</th>
<th>Men day 4</th>
<th>Women baseline</th>
<th>Women day 1</th>
<th>Women day 2</th>
<th>Women day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.24 ± 0.37</td>
<td>0.30 ± 0.39</td>
<td>0.32 ± 0.39</td>
<td>0.37 ± 0.74</td>
<td>0.18 ± 0.08</td>
<td>&lt; .001</td>
<td></td>
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<tr>
<td>IL-10</td>
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<tr>
<td>IL-6</td>
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<td></td>
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<tr>
<td>TNF-α</td>
<td>1.80 ± 0.48</td>
<td>7.58 ± 7.62</td>
<td>3.16 ± 2.07</td>
<td>2.88 ± 2.33</td>
<td>3.97 ± 4.19</td>
<td>&lt; .001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Consecutive exercise days

IL-1β and TNF-α concentrations were significantly higher in men compared to women (all p-values < .001). Asymptomatic symptoms were higher in women than in men (p-values: 0.134, 0.032, 0.117, 0.064, 0.121). Changes in cytokine levels were significant for IL-1β and IL-6 (p < .001), but not for IL-10 and TNF-α (p-values: 0.227, 0.877). After the first day of exercise, IL-6, IL-8, IL-10, IL-1β, and TNF-α were all increased compared to baseline (all p-values < .001). After the first day of exercise, IL-6, IL-8, IL-10, IL-1β, and TNF-α concentrations were significantly higher in men compared to women (p-values: 0.064, 0.018, 0.051, 0.064, 0.064). After the first day of exercise, asymptomatic symptoms were significantly higher in women than in men (p-values: 0.032, 0.117, 0.064, 0.121, 0.064).
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.

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, 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 and 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 NSAI Ds at one or more days during the four days marches (baseline included). NSAI Ds that were used by our participants included (name (dose): ibuprofen (200mg, 400mg, and 600mg), diclofenac (50mg), Advil / Naproxen (200mg), and Maxalt (5mg). NSAI D-use was higher in women (NSAI Ds 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 NSAI Ds 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.
Changes in cytokine levels after prolonged and repeated moderate intensity exercise in middle-aged men and women

Figure 1

A

IL-6 (pg/ml)

Baseline  Day 1  Day 2  Day 3  Day 4

***

B

IL-8 (pg/ml)

Baseline  Day 1  Day 2  Day 3  Day 4

***

C

IL-10 (pg/ml)

Baseline  Day 1  Day 2  Day 3  Day 4

****

D

IL-1β (pg/ml)

Baseline  Day 1  Day 2  Day 3  Day 4

***

E

TNF-α (pg/ml)

Baseline  Day 1  Day 2  Day 3  Day 4

***

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**Plasma Volume**

Plasma volume changed significantly over time, but 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

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

Cytokine concentrations after one day of exercise.
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.

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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 focused on eccentric exercise (33, 34) and not on prolonged repeated moderate intensity exercise. In a previous study in our lab (11), albeit 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.
Changes in cytokine levels after prolonged and repeated moderate intensity exercise in middle-aged men and women

Differences in Cytokine between men and women
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.

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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.

As 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, characterized 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.

Further more, gender differences resulted in higher baseline IL-1β and TNF-α.
and blood pressure were measured using an automated sphygmomanometer (M5 -1
determined and body mass index (BMI) was calculated. Thereafter, resting heart rate
At baseline, body weight (Seca 888 scale, Hamburg, Germany) and body height were
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Baseline measurements
was used to estimate exercise intensity as percen tage of HRmax = (Measured HR /
the finish. Mean heart rate during exercise was calculated as the average heart rate,
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Every walking day, immediately before the start of the march, participants' body
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and 04:00 PM.
the event location one or two days prior to the first walking day, between 09:30 AM
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measurements, including registering participants' characteristics, collection of a
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Study population
Study population

MATERIALS AND METHODS

Perspectives
Chapter 5

REFERENCES


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

Baseline measurements.

For Data Analysis EPA QA/G-9, expected maximal HR = (208 - (0.7 * Age)) * 100%, with Expected max HR = 208 - (0.7 * Age).

Heart rate was used to estimate exercise intensity as percentage of HRmax = (Measured HR / Expected max HR) * 100%, with Expected max HR = 208 - (0.7 * Age).

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.

Every walking day, immediately before the start of the march, participants' body weight was determined, a blood sample was taken and a set of questionnaires was 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 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.

Blood sample and taking questionnaires, were performed in our field laboratory at the start of the event ('baseline'), and at the four consecutive walking days. Baseline conditions are specified in the results section. Measurements were performed before conditions were specified in the results section. Measurements were performed before

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Study procedure.
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.


physiology. 1990;60(1):26

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the finish. Details about the routes can be found at

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Fertility and sterility. 2000;74(5):1008

al. Non-steroidal anti-inflammatory drugs inhibit the expression of cytokines and

37. Faas M, Bouman A, Moesa H, Heineman MJ, de Leij L, Schuling G. The


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

Cortisol and testosterone in swimmers

Rieneke Terink
Renger Witkamp
Elisabeth van Rossum
Marco Mensink

Under review in: Sports Health, 2020
ABSTRACT

Background: Salivary and hair 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 period 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 ± 2.3 years) 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 analyzed for cortisol and testosterone levels. Profile of mood states (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 week 2 compared to week 10 (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 period 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

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

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<tr>
<th>Participants (n = 10)</th>
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<tbody>
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<td>Weight (kg)</td>
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<td>Body fat (%)</td>
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<td>BMI (kg/m²)</td>
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<tr>
<td>VO2max (ml/kg/min)</td>
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<tr>
<td>Max Power (Watt)</td>
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<td>Max heart rate (bpm)</td>
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</table>

Table 1. Participant characteristics
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 (~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/m², 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.
MATERIALS AND METHODS

Study design

Table 1. Participant characteristics

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<td>Body composition</td>
<td>Height (cm)</td>
<td>185.8 ± 5.2</td>
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<tr>
<td></td>
<td>BMI (kg/m²)</td>
<td>23.2 ± 0.9</td>
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<tr>
<td></td>
<td>Weight (kg)</td>
<td>80 ± 5</td>
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<tr>
<td></td>
<td>VO₂max (ml/kg/min)</td>
<td>378 ± 26</td>
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<tr>
<td></td>
<td>Max Power (Watt)</td>
<td>109 ± 1.5</td>
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<tr>
<td></td>
<td>Max heart rate (bpm)</td>
<td>196.7 ± 6.8</td>
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</tbody>
</table>

Figure 1. The 10 weeks training block included multiple swimming races. See Figure 1 for a schematic overview of our study design. ADAPT: Adaptation phase; G-PREP: General preparation phase; S-PREP: Specific preparation phase; T&C: Taper and competition phase; C: Competition.

Figure 1 characterized by a lower training volume with high intensity training (AC), aerobic power (AV), anaerobic capacity (ANC), lactate production (LC), Ultra short race pace training (USRPT) and sprint training (Sprint). 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 finally, 4) a taper & competition phase of 2 weeks (T&C), including less volume, some high intensity, ANC, LC, USRPT and sprint.
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.

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Ten male elite swimmers participated in this study (age 19.9 ± 2.3 years, BMI 23.2 ± 0.9 kg/m², 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.

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Table 1

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<tr>
<th>Physical characteristics</th>
<th>Means ± SD</th>
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<tbody>
<tr>
<td>Power / kg body weight</td>
<td>4.80 ± 0.24</td>
</tr>
<tr>
<td>Max heart rate (bpm)</td>
<td>196.7 ± 6.8</td>
</tr>
<tr>
<td>VO2max (ml/kg/min)</td>
<td>185.8 ± 5.2</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>10.9 ± 1.5</td>
</tr>
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<td>BMI (kg/m²)</td>
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</tbody>
</table>

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

Participants’ characteristics were not significantly variable between 12:00 and 17:00, assuring us that any changes do not significantly influence cortisol levels.

Data collection

Saliva Collection and analysis

Swimming pool at 16:45 h. Pilot testing in our lab showed that cortisol levels were not significantly variable between 12:00 and 17:00, assuring us that any changes do not significantly influence cortisol levels.

Participants (n = 10) Salivary cortisol and testosterone are responsive during competition, but not during a training block in elite swimming athletes.
Ten male elite swimmers participated in this study (age 19.9 ± 2.3 years, BMI 23.2 ± 0.9 kg/m², VO₂max 62.2 ± 3.2 ml/min/kg). They all competed at Dutch national competitions or international events.

Subjects

Individual. All races took place in the afternoon between 14:00 and 17:30. Therefore, the timing of saliva collection was noted per participant, with the collection of saliva samples performed before warming-up, after warming-up, directly after the first race and half an hour, one hour, two hours, and 24 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) to 10 (very nervous), and whether they were satisfied with their performance on a scale from 0 (not satisfied at all) to 10 (very satisfied). All participants joined different types of races at different times of the day. This study was conducted in accordance with the Declaration of Helsinki. Participant characteristics can be seen in Table 1.

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Hair collection and analysis

Samples were collected according to the description of Noppe et al. (9). In brief, approximately 100 hairs per swimmer, all samples were analyzed on the same plate to eliminate inter-assay variability. The sensitivity of the kits was 0.193 nmol/L for cortisol and 0.0035 nmol/L for testosterone. Kits were of the same batch, for each hormone concentration, the intra-assay coefficient of variation, were 14.8% for cortisol and 16.0% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone. The mean intra-assay coefficients of variation were 7% for cortisol and 6.7% for testosterone.

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Hoechberg, Germany). VO2max was defined as the maximal oxygen uptake measured.

Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Germany) to establish maximal aerobic capacity (VO2max). After an initial workload of 80Watt for 5 minutes, workload was subsequently increased by 20W/min until the participant could not maintain the required pedalling frequency of at least 60rpm. Pilot testing in our lab showed that cortisol levels were not significantly variable between 12:00 and 17:00, assuring us that any changes are not influenced by differences in cortisol level caused by time of day.

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 different between saliva samples collected on different days of the week. Saliva was ampliﬁed of week 5, 6, 7 and 8. And the hair sample in week 8 was correlated with the average value in the saliva samples of week 7, 8, 9 and 10. And the hair sample in week 8 was correlated with the average cortisol value in the saliva samples of week 7, 8, 9 and 10. Significant differences occur.

Correlations between saliva and hair cortisol and testosterone levels were analyzed. When a significant time effect of consecutive training phases and consecutive training weeks on saliva vary and 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

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Statistical analyses

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1. a short profile of mood state (s-POMS),
2. a logbook with questions about sleep,
3. three questionnaires at the time of saliva sample collection:

Questionnaires

- 1) a short profile of mood state (s-POMS),
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Statistical analyses

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS, IBM, 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.

1. a short profile of mood state (s-POMS),
2. a logbook with questions about sleep,
3. three questionnaires at the time of saliva sample collection:
Chapter 6

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

Figure 2

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 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).
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/m², 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>
<thead>
<tr>
<th>Week</th>
<th>Cortisol (nmol/L)</th>
<th>Testosterone (nmol/L)</th>
<th>Ratio C:T</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.24 ± 1.79</td>
<td>5.47 ± 1.59</td>
<td>0.34 ± 0.07</td>
<td>0.167</td>
</tr>
<tr>
<td>2</td>
<td>5.69 ± 2.69</td>
<td>0.38 ± 0.19</td>
<td>15.4 ± 3.5</td>
<td>0.339</td>
</tr>
<tr>
<td>3</td>
<td>5.43 ± 2.33</td>
<td>5.47 ± 2.05</td>
<td>0.35 ± 0.08</td>
<td>0.373</td>
</tr>
<tr>
<td>4</td>
<td>5.60 ± 2.15</td>
<td>0.32 ± 0.04</td>
<td>18.3 ± 9.0</td>
<td>0.948</td>
</tr>
<tr>
<td>5</td>
<td>5.37 ± 2.85</td>
<td>0.32 ± 0.08</td>
<td>17.5 ± 10.6</td>
<td>0.287</td>
</tr>
<tr>
<td>6</td>
<td>4.61 ± 2.57</td>
<td>4.24 ± 1.93</td>
<td>0.37 ± 0.09</td>
<td>0.241</td>
</tr>
<tr>
<td>7</td>
<td>5.90 ± 4.01</td>
<td>0.36 ± 0.10</td>
<td>15.4 ± 7.9</td>
<td>0.287</td>
</tr>
<tr>
<td>8</td>
<td>5.85 ± 2.38</td>
<td>0.33 ± 0.09</td>
<td>17.8 ± 5.4</td>
<td>0.287</td>
</tr>
<tr>
<td>9</td>
<td>4.35 ± 1.63</td>
<td>4.24 ± 1.93</td>
<td>0.37 ± 0.09</td>
<td>0.241</td>
</tr>
<tr>
<td>10</td>
<td>4.58 ± 2.36</td>
<td>0.31 ± 0.09</td>
<td>11.7 ± 2.8</td>
<td>0.287</td>
</tr>
</tbody>
</table>

Means ± SD are shown per week and per training phase. P values refer to an Intraclass Correlation Coefficient (ICC) analysis.
Table 1. Participant characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>19.9 ± 2.3</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>185.8 ± 5.2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2 ± 0.9</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>10.9 ± 1.5</td>
</tr>
<tr>
<td>VO₂ max (ml/kg/min)</td>
<td>62.2 ± 3.2</td>
</tr>
<tr>
<td>Max heart rate (bpm)</td>
<td>196.7 ± 6.8</td>
</tr>
<tr>
<td>Max Power (Watt)</td>
<td>378 ± 26</td>
</tr>
<tr>
<td>Power / kg body weight</td>
<td>4.80 ± 0.24</td>
</tr>
</tbody>
</table>

Means ± SD are shown. BMI: Body mass index; Physical characteristics are determined during a VO₂ max 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 (VO₂ max). After an initial workload of 80 Watt for 5 minutes, workload was subsequently increased by 20 Watt/min 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). VO₂ max 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.

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

Figure 3. Salivary cortisol (A) and testosterone levels (B) during the training period.
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 (~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/m², 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.

**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 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 0.26 ± 0.07 nmol/L, p=0.028) and between directly after warm-up and after the last race (0.40 ± 0.13 nmol/L, p=0.018). There were no differences in testosterone levels 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).

There was a significant correlation between the level of anxiolysis after the race and the level of testosterone after warm-up (p=0.187, r= 0.564).
Salivary cortisol and testosterone are responsive during competition, but not during a training block in elite swimming athletes.

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

Correlation coefficients between training parameters and cortisol and testosterone levels were not significant (r>0.05).

Table 3. Hair cortisol and testosterone during training

<table>
<thead>
<tr>
<th>Week</th>
<th>2</th>
<th>8</th>
<th>10</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol (pg/mg)</td>
<td>0.64 ± 0.53</td>
<td>0.77 ± 0.85</td>
<td>0.75 ± 0.56</td>
<td>0.882</td>
</tr>
<tr>
<td>Testosterone (pg/mg)</td>
<td>0.68 ± 0.37</td>
<td>0.59 ± 0.46</td>
<td>0.53 ± 0.28</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Means ± SD are shown. P values refer to an Intraclass Correlation Coefficient 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 subemotion between training phases (p>0.05).
Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Hoechberg, Germany). VO2max was defined as the maximal oxygen uptake measured.

Participants could not maintain the required pedalling frequency of at least 60rpm. Of 80Watt for 5 minutes, workload was subsequently increased by 20W/min until the participant could not maintain the required pedalling frequency of at least 60rpm.

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 were not influenced by this factor.

Data collection included 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 and connected exercise tracker (Polar FT1). In addition, body length (Seca 213) rate was monitored by using a heart rate monitor (Polar T31 -coded, Oulu, Finland).

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) to establish maximal aerobic capacity (VO2max). After an initial workload included a maximal exercise test on a bicycle ergometer (Ergoline GmbH, Bitz, Germany) to establish maximal aerobic capacity (VO2max).

Physical characteristics are determined during a VO2max test; bpm: beats per minute

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ADAPT</th>
<th>G</th>
<th>PREP</th>
<th>S Pre</th>
<th>T &amp; C</th>
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<tbody>
<tr>
<td>Power / kg body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Power (Watt)</td>
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<td></td>
</tr>
<tr>
<td>Max heart rate (bpm)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO2max (ml/kg/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body fat (%)</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>BMI (kg/m2)</td>
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<tr>
<td>Weight (kg)</td>
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<td>Height (cm)</td>
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<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
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</table>

Table 1. Participant characteristics
Participants (n = 10)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Week</th>
<th>1 &amp; 2</th>
<th>3 &amp; 4 &amp; 5</th>
<th>6 &amp; 7 &amp; 8</th>
<th>9 &amp; 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>3.3</td>
<td>1.1</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>6.7</td>
<td>1.1</td>
<td>1.0</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>3.3</td>
<td>1.1</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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

Salivary cortisol and testosterone levels

Our main finding was that salivary cortisol and testosterone levels were unchanged during the taper and competition phase (T&C). However, no significant differences were seen between the general preparation phase (G-Prep) compared to specific preparation (S-Prep) and matching every 3 days (15). Moreover, we expected cortisol levels to be lower in the T&C phase, first because cortisol levels during 3 weeks of intensive training and competition in elite rugby players foundings from other researchers, for example reporting increased salivary cortisol. This was in contrast to the cortisol level in the taper and competition phase. Therefore, we expected higher cortisol levels during adaptation (ADAPT) and 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, warming-up, after warming-up, directly after the first race and half an hour, one hour and two hours after the last race. 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. 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 training sessions (~16.45h). Questionnaires were taken directly with the saliva samples. During the study a non-invasive observational scientific study and therefore no formal ethical approval was needed. All participants gave written informed consent prior to the study. This study was conducted in accordance with the Declaration of Helsinki. Participant characteristics can be seen in table 1.

More over, we expected cortisol levels to be lower in the T&C phase, first because cortisol levels during 3 weeks of intensive training and competition in elite rugby players foundings from other researchers, for example reporting increased salivary cortisol. This was in contrast to the cortisol level in the taper and competition phase. Therefore, we expected higher cortisol levels during adaptation (ADAPT) and 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, warming-up, after warming-up, directly after the first race and half an hour, one hour and two hours after the last race. 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. 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 training sessions (~16.45h). Questionnaires were taken directly with the saliva samples. During the study a non-invasive observational scientific study and therefore no formal ethical approval was needed. All participants gave written informed consent prior to the study. This study was conducted in accordance with the Declaration of Helsinki. Participant characteristics can be seen in table 1.
Salivary cortisol and testosterone are responsive during competition, but not during a training block in elite swimming athletes.

Table 1. Participant characteristics

<table>
<thead>
<tr>
<th>Participants (n = 10)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>19.9 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>185.8 ± 5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>10.9 ± 1.5</td>
<td></td>
<td></td>
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<tr>
<td>BMI (kg/m²)</td>
<td>23.2 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO₂max</td>
<td>62.2 ± 3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max Power (Watt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max heart rate (bpm)</td>
<td>4.80 ± 0.24</td>
<td></td>
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</tr>
<tr>
<td>Max Power / kg body weight</td>
<td></td>
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</tr>
<tr>
<td>Race characteristics</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Power / kg body weight</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different forms of exercise performed at an intensity above 60% of an individual’s
Ten male elite swimmers participated in this study (age 19.9 ± 2.3 years, BMI 23.2 ± 0.9 kg/m², 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, level and were either sprinter (50m and 100m) or middle-distance (200m and 400m) swimmers. All 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 sampling for the hair cortisol and testosterone levels.

To our knowledge, studies measuring the effect of training load on cortisol and testosterone are very well trained and used to this amount of training load. It is of course also possible that this direct and clear response in cortisol and testosterone levels might explain the higher than 60% of the maximal power(31, 32). The swimming distances performed appeared to be a good marker for acute (competition) stress, however, it seems less interesting researchers have in saliva cortisol and testosterone measurements. It seems that there are very well training cortisol and testosterone correlations can be found when hair cortisol levels are correlated with saliva every three-hours (33, 34). In addition, a recently conducted study showed that moderate(38, 39). On the other hand, some studies (also not related to exercise) report good correlations (36, 37). However, it seems that there is no correlation between saliva and hair cortisol and testosterone levels. This is in agreement with many studies (not related to exercise) who have found that cortisol and testosterone levels are similar to those found in saliva. Furthermore, there was no correlation between saliva and hair cortisol and testosterone levels. This is in agreement with many studies (not related to exercise) who have found that cortisol and testosterone levels are similar to those found in saliva.
Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Germany) to establish maximal aerobic capacity (VO2max). After an initial workload of 80Watt for 5 minutes, workload was subsequently increased by 20W/min until the participant could not maintain the required pedalling frequency of at least 60rpm.

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). Mean ± SD are shown. BMI: Body mass index; Physical characteristics are determined during a VO2max test; bpm: beats per minute.

### Table 1. Participant characteristics

<table>
<thead>
<tr>
<th>Participants (n = 10)</th>
<th>Age (years) 19.9 ± 2.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body composition</strong></td>
<td><strong>Height (cm) 185.8 ± 26</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Body fat (%) 10.9 ± 1.5</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Weight (kg) 80 ± 5</strong></td>
</tr>
<tr>
<td></td>
<td><strong>BMI (kg/m2) 23.2 ± 0.9</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Max Power (Watt) 378 ± 26</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Max heart rate (bpm) 185.8 ± 5.2</strong></td>
</tr>
<tr>
<td></td>
<td><strong>VO2max (ml/kg/min) 4.80 ± 0.24</strong></td>
</tr>
</tbody>
</table>

**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 quickly.

**In conclusion,** hair samples might be more useful for assessing long-term training load and saliva samples are easily affected by factors like excitement and food intake. Therefore, hair samples are less influenced by these factors, because they represent a snapshot of the stress of the past weeks. Therefore, it seems warranted to repeat a similar study with saliva and hair collection (being non-invasive and practical) above blood collection, as cortisol and testosterone levels were responsive during competition, but not during a training block in elite swimming athletes.

Salivary cortisol and testosterone are responsive during competition, but not during a training block in elite swimming athletes. This might be more useful for assessing long-term training load and saliva samples are easily affected by factors like excitement and food intake. Therefore, hair samples are less influenced by these factors, because they represent a snapshot of the stress of the past weeks. Therefore, it seems warranted to repeat a similar study with saliva and hair collection (being non-invasive and practical) above blood collection, as cortisol and testosterone levels were responsive during competition, but not during a training block in elite swimming athletes.

In conclusion, hair samples might be more useful for assessing long-term training load and saliva samples are easily affected by factors like excitement and food intake. Therefore, hair samples are less influenced by these factors, because they represent a snapshot of the stress of the past weeks. Therefore, it seems warranted to repeat a similar study with saliva and hair collection (being non-invasive and practical) above blood collection, as cortisol and testosterone levels were responsive during competition, but not during a training block in elite swimming athletes.
Chapter 6

REFERENCES


VO2max 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). Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Germany). VO2max was not significantly variable between 12:00 and 17:00, assuring us that any changes in salivary stress hormones and cortisol were not due to time of day. Pilot testing in our lab showed that cortisol levels were not significantly variable between 12:00 and 17:00, assuring us that any changes in salivary stress hormones and cortisol were not due to time of day. Pilot testing in our lab showed that cortisol levels were not significantly variable between 12:00 and 17:00, assuring us that any changes in salivary stress hormones and cortisol were not due to time of day. Pilot testing in our lab showed that cortisol levels were not significantly variable between 12:00 and 17:00, assuring us that any changes in salivary stress hormones and cortisol were not due to time of day.

Table 1. Participant characteristics

<table>
<thead>
<tr>
<th>Participants (n = 10)</th>
<th>Age (years)</th>
<th>19.9 ± 2.3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body fat (%)</td>
<td>10.9 ± 1.5</td>
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<tr>
<td></td>
<td>BMI (kg/m2)</td>
<td>23.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Weight (kg)</td>
<td>80 ± 5</td>
</tr>
<tr>
<td></td>
<td>Height (cm)</td>
<td>185.8 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>VO2max (ml/kg/min)</td>
<td>62.2 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Max Power (Watt)</td>
<td>4.80 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>Max heart rate (bpm)</td>
<td>196.7 ± 6.8</td>
</tr>
</tbody>
</table>


I n t e r n a l o n g-t e r m p h y s i o l o g i c a l  s t r e s s  e f f e c t s  b y c o r t i s o l  r e a c t i v i t y i n s a l i v a and h a i r .

S a l i v a v a r y c o r t i s o l  p r o f i l e s  a n d a r e i n c r e a s e d i n o b e s e p r e p u b e r t a l  g i r l s .

P a p a s o t i r i o u I ,  e t  a l .  H a i r  c o r t i s o l  c o n c e n t r a t i o n s  e x h i b i t  a p o s i t i v e a s s o c i a t i o n w i t h
d u r i n g a n e n d u r a n c e c o m p e t i t i o n a n d r e c o v e r y .  T h e J o u r n a l  o f  s p o r t s  m e d i c i n e a n d

E u r o p e a n  j o u r n a l  o f  a p p l i e d  p h y s i o l o g y a n d  o c c u p a t i o n a l  p h y s i o l o g y .

r e l a t i v e  s i g n i f i c a n c e  o f  e x e r c i s e  i n t e n s i t y a n d d u r a t i o n a n d p e r f o r m a n c e  l e v e l .

O n p o s t - e x e r c i s e  s t e r o i d  h o r m o n e  r e s p o n s e s  i n t r a i n e d  m a l e s .  E u r o p e a n  j o u r n a l  o f

2 9 .  D o a n B K ,  N e w t o n

D o n e l l y  et  a l .  E f f e c t s  o f  t r a i n i n g  o n  p l a s m a  a n a b o l i c  a n d  c a t a b o l i c  s t e r o i d  h o r m o n e s  a n d

a n d  t h e i r  r e s p o n s e  d u r i n g  p h y s i c a l  e x e r c i s e .  I n t e r n a t i o n a l  j o u r n a l  o f  s p o r t s

m e d i c i n e .  1 9 8 6 ; 7  S u p p l  1 : 2 7 .

H a y e s  L D ,  G r a c e  F M ,  B a k e r  J S ,  S c u l t h o r p e  N.  E x e r c i s e - i n d u c e d  r e s p o n s e s  i n s a l i v a r y  t e s t o s t e r o n e ,
c o r t i s o l ,  a n d  t h e i r  r a t i o s  i n m e n :  a  m e t a - a n a l y s i s .  S p o r t s  m e d i c i n e  ( A u k l a n d , N Z ) .  2 0 1 5 ; 4 5 (5 ) : 7 1 3.

C o e l h o  R W ,  K e l l e r  B ,  d a  S i l v a  A M.  E f f e c t  o f  p r e - e x e r c i s e  a n t i o x i d a n t  s u p p l e m e n t a t i o n  o n  p r o d u c t i v i t y a n d

m o t o r  s k i l l s .  2 0 1 0 ; 1 1 1 (1 ) : 1 5 8 .

K r a e m e r  W J ,  K w o n  Y H ,  S c h e e t  T P.  S a l i v a r y  c o r t i s o l ,  t e s t o s t e r o n e ,  a n d  T / C  r a t i o  r e s p o n s e s  d u r i n g  a 3 6-

3 0 .  T r e m b l a y  M S ,  C o p e l a n d  J L ,  V a n  H e l d e r  W.  I n f l u e n c e  o f  e x e r c i s e  d u r i n g  a 5 - k m  s h o o t i n g  c o m p e t i t i o n  o n

a p p l i e d  p h y s i o l o g y .  2 0 0 5 ; 9 4 (5 ) : 5 0 5 .

1 9 9 3 ; 6 7 (1 ) : 5 9 .

S n e g o v s k a y a  V ,  V i r u  A.  S t e r o i d  a n d  p i t u i t a r y  h o r m o n e  r e s p o n s e s  t o  r o w i n g :

p h y s i c a l  f i t n e s s .  2 0 0 0 ; 4 0 (2 ) : 1 3 9 .

3 3 .  K r a t s h a m a k e n s  e t  a l .  S t r e s s  ( A m s t e r d a m , N e r l e a n d s ) .  2 0 1 7 ; 2 0 (2 ) : 2 1 7 .

3 4 .  D r e s e n  M H ,  S l u i t e r  J K.  I n t e r n a t i o n a l  a r c h i v e s  o f  o c c u p a t i o n a l  a n d  e n v i r o n m e n t a l  h e a l t h .  2 0 1 2 ; 8 5 (8 ) : 8 4 9 .

1 2 6
Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Germany). VO2max was defined as the maximal oxygen uptake measured during a maximal exercise test on a bicycle ergometer (Ergoline GmbH, Bitz, Germany) to establish maximal aerobic capacity (VO2max). After an initial workload of 80W for 5 minutes, workload was subsequently increased by 20W/min until the participant could not maintain the required pedalling frequency of at least 60rpm.

Pilot testing in our lab showed that cortisol levels were not significantly variable between 12:00 and 17:00, assuring us that any changes in cortisol levels reflect changes in cortisol production rather than changes in cortisol availability. Swimming pool cortisol levels for swimmer A were 35.5 ± 8.2 ng/mL, and for swimmer B were 34.7 ± 7.6 ng/mL. Pre-exercise salivary cortisol levels were determined at 8:00, 10:00, and 12:00 on the morning of the early morning training session.

Saliva was collected on a weekly basis, just before the afternoon training in the swimming pool at 16:45h.

Data collection included a maximal exercise test on a bicycle ergometer (Ergoline GmbH, Bitz, Germany) to establish maximal aerobic capacity (VO2max). After an initial workload of 80W for 5 minutes, workload was subsequently increased by 20W/min until the participant could not maintain the required pedalling frequency of at least 60rpm.

Means ± SD are shown. BMI: Body mass index; Physical characteristics are determined during a VO2max test; bpm: beats per minute.
Chapter 7

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

Rieneke Terink
Renger Witkamp
Maria Hopman
Marco Mensink

Under review in: Sports, 2020
ABSTRACT

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 athlete's 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

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

which is often further classified into ‘functional’ (F-OR) and ‘non-functional’ (NF-OR) overreaching (1). Notwithstanding that the borders between these states differ from overreaching, which is less severe and of shorter duration, and OTS which refers to a state in which accumulation of training can be translated to OR

Sever al experimental study designs to simulate and study overreaching related states (ACSM) (1). However, this hol ds especially true when it comes to feasibility, external validity, and OTS in the real athletes’ world. For example, common disadvantages in these study designs relate to: not including performance tests, the lack of controls, and there is no consensus how study designs can be made for that design (13).

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 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.

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 physiological indicators to detect OTS in an early stage. While tracking for such physiological indicators to detect OTS differs from overreaching, which is less severe and of shorter duration, and performance capacity with or without related physiological and psychological signs change over as well. The psychological resilience of (potentially) overtrained persons. In this way, exercise stress markers collected with these single exercise studies can be used for a proper challenge mode to investigate the dynamic differences in physiological indicators to detect OTS 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.

How to perform testing and training protocols to study overreaching and overtraining - experimental protocols

and outcome measures revisited. A narrative Review
Definitions

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 decrements 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.” Overreaching is defined as “an accumulation of training and/or non-training stress resulting in short-term decrements in performance capacity”.

Study designs used in overtraining research

Acute stress model (single-exercise study design)

Exercise, which makes evaluation of individual changes easy. These single exercise simplicity and their controllability. Markers can be assessed directly pre- and post-exercise, primarily used to find exercise stress markers. An advantage of these studies is their training stress, muscle damage, or adaptations, and are therefore used as change after exercise. It is suggested that these changes indicate load or overload.
studies can be used in a ‘challenge’ mode to investigate the dynamic differences in

Retrospective/observational (field)/case studies

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

133
exercise, which makes evaluation of individual changes easy. These single exercise
simplicity and their controllability. Markers can be assessed directly pre- and post-
primarily used to find exercise stress markers. An advantage of these studies is their
exercise stress markers. These studies do not lead or intent to lead to OR, but are
change after exercise. It is suggested that these changes indicate load or overload (3),
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),

Another approach is monitoring athletes during long-term competition or training,
some factors, like competition anxiety, sleep deprivation and other external factors,
which included the definition of OTS and overreaching (OR). OTS is defined as “an
overtraining syndrome. Therefore, a joint consensus statement was formulated (1),
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
issues, like compliance, how to monitor athletes closely and intensively, and how to

Controlled intervention studies, employing increased training loads to induce a state
of overreaching and overtraining as stated elsewhere. More importantly, study
designs that have been described by various researchers will be discussed regarding
between functional and non-functional overreaching.

Study designs aiming to induce a state of overreaching

is that these designs don’t force athletes to follow specific
routines and programs, which makes it ethically less challenging and therefore, in practice, more
desired. Researchers should keep in mind that as soon as an
athlete is detected with underperformance, it should be diagnosed as OR, not OTS.

Another approach is monitoring athletes during training camp or long-term competition,
where it is ethically less challenging and therefore, in practice, more
possible. Researchers should keep in mind that as soon as an
athlete is detected with underperformance, it should be diagnosed as OR, not OTS.

Overreaching is defined as “an accumulation
of OR in athletes are also used (12, 19-25). With these experimental designs, athletes
may take

"primum non nocere" principle.
and directly after training camp to conclude if there is underperformance should be assessed and controlled, 3) performance tests should be included before checked for their general health and illnesses should be excluded, 2) dietary patterns In theory, monitoring athletes during a training camp, is a good method to study OR, status can be made for that design (13). Ishigaki et al (2005) did not include performance tests, so no conclusion about OR NF-OR cannot be concluded. The 8 day training camp, used as study design by include a recovery period, so whether those athletes were suffering from F-OR or promising, as both resulted in reduced performance (11, 12), however, both failed to executed in the study designs of Hedelin et al (2000) and Jürimäe et al (2004) seem known that those training camps can result in OR. The 6 days training camps lack baseline measurements, which makes before and after comparisons impossible. Results cannot or hardly be extrapolated. For example, most of the time, these reports 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, in this way, exercise psychological resilience of (potentially) overtrained persons. In this way, exercise studies can be used in a 'challenge' mode to investigate the dynamic differences in development of overtraining.

**Duration, volume and intensity**

there may also be a ‘mechanical’ explanation for this difference, as 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 recovery period points out that the athlete was functionally overreached.

**Inclusion of performance testing and a recovery period**

...
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. These single exercise simplicity and their controllability. Markers can be assessed directly pre- and post-exercise, which makes evaluation of individual changes easy. These single exercise stress markers. These studies do not lead or intend to lead to OR, but are primarily used to find exercise stress markers. An advantage of these studies is their control 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. Second, the load of these studies might be the most useful interventions for studying OR.

Nutrition

In the past, different terminologies were used to describe overreaching and overtraining. 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. In short, F-OR is an intentional part of an athlete's training schedule and leads, with several weeks-months of recovery (for NF-OR and OTS, respectively). These interventions can be prevented, because they lead to underperformance and require several days-weeks of recovery. The athlete consumes a diet that is adequate. However, such a diet is of course another burden for participants. 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 NF-OR and OTS. To be comprehensive, researchers should aim to assess dietary patterns before and during the training intervention. A 3-day food record could be an option. 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 NF-OR and OTS. To be comprehensive, researchers should aim to assess dietary patterns before and during the training intervention. A 3-day food record could be an option. 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 NF-OR and OTS. To be comprehensive, researchers should aim to assess dietary patterns before and during the training intervention. A 3-day food record could be an option. 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 NF-OR and OTS. To be comprehensive, researchers should aim to assess dietary patterns before and during the training intervention. A 3-day food record could be an option.
Experiences and learnings from our recent study

Based on the before mentioned considerations, we set out to test a ‘best practices’

We doubled training volume in four weeks’ time, without changes in intensity, and

Table 2

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>7.25 ± 1.11</td>
</tr>
<tr>
<td>VO2max (ml/kg/min)</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>6</td>
</tr>
<tr>
<td>BMI</td>
<td>25 ± 3</td>
</tr>
</tbody>
</table>

In theory, monitoring athletes during a training camp, is a good method to study OR,

Ishigaki et al (2005) did not include performance tests, so no conclusion about OR

NF-OR cannot be concluded. The 8 day training camp, used as study design by

include a recovery period, so whether those athletes were suffering from F-OR or

promising, as both resulted in reduced performance (11, 12), however, both failed to

known that those training camps can result in OR. The 6 days training camps

increased for training purposes is a popular and practical method (11-13), as it is

But preferably, other approaches should be used to induce overreaching in athletes.

controls may surge, and can be further evaluated by longitudinal studies.

From these comparisons, multiple

account. Some of these problems could be solved by using a large matched healthy

lack baseline measurements, which makes before and after comparisons impossible.

overtraining (8-10). These reports are useful for sport physicians to draw some

report about overtrained athletes and their symptoms after being diagnosed with

A large number of studies focussing on overtraining are case studies or studies that

Retrospective/observational (field)/case studies

development of overtraining.

overtrained persons are already at the point of overtraining and not in the

of markers for the early diagnosis of overtraining, because the (potentially)

stress markers collected with these single exercise studies can be used for a proper

psychological resilience of (potentially) overtrained persons. In this way, exercise

An advantage of the latter approach is that it provides insight into physical or even

studies can be used in a ‘challenge’ mode to investigate the dynamic differences in

the fourth and last week to 200%. Again participants kept track of their normal training with their

Par participants monitored their normal training program with a SPARK3 TomTom

participated and either triathletes or cyclists. Participants informed

in a larger overreaching study we planned to do. To this end, we included 7 healthy

We investigated whether that increase in volume would lead to non-functional

We doubled training volume in four weeks’ time, without changes in intensity, and

Of the 7 participants, 5 male, 2 female
Monitoring normal training

Adapted training (4 weeks)

Recovery (50%)

Figure 1. Study overview. Athletes monitored their usual training load in the first two weeks. Afterwards, they followed four weeks of adapted training with increased training volume, followed by two weeks of recovery. An overview of the study design can be seen in Figure 1.

Figure 2. Time trial protocol. Before the start of the warming up, athletes filled out a POMS Logbook. The time trial was without encouragement of any kind. Directly after four weeks of the increased load and one directly after two weeks of monitoring their usual training load, one ergometer: one after the two weeks of monitoring their usual training load, one after the four weeks of increased load and one directly after two weeks of recovery. An overview of the study design can be seen in Figure 1.

Definitions

In short, F-OR is an intentional part of an athlete’s training schedule and leads, with symptoms of overtraining in which restoration of performance capacity may take several days to several weeks. OTS may take two weeks of recovery training at 50% of their normal volume. They filled out a logbook and POMS questionnaires every second and fifth day of the week, during the total study. Therapy afterwards they followed four weeks of adapted training with increased training volume, followed by two weeks of recovery. An overview of the study design can be seen in Figure 1.

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Definitions

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and directly after training camp to conclude if there is underperformance should be assessed and controlled, 3) performance tests should be included before checked for their general health and illnesses should be excluded, 2) dietary patterns in theory, monitoring athletes during a training camp, is a good method to study OR, status can be made for that design (13).

NF-OR cannot be concluded. The 8 day training camp, used as study design by include a recovery period, so whether those athletes were suffering from F-OR or known that those training camps can result in OR. The 6 days training camps increased for training purposes is a popular and practical method (11-13), as it is executed in the study designs of Hedelin et al (2000) and Jürimäe et al (2004) seem

But preferably, other approaches should be used to induce overreaching in athletes. controls may surge, and can be further evaluated by longitudinal studies. markers that are significantly different between overtrained athletes and healthy account. Some of these problems could be solved by using a large matched healthy in addition, personal variation in levels of outcome measures cannot be taken into considerations. Some of these problems could be solved by using a large matched healthy retrospective/observational (field)/case studies

conclusions about symptoms and help them with diagnosing overtraining. However, overtraining (8-10). These reports are useful for sport physicians to draw some report about overtrained athletes and their symptoms after being diagnosed with overtraining. However, this approach does not result in a set results cannot or hardly be extrapolated. For example, most of the time, these reports

A large number of studies focussing on overtraining are case studies or studies that retrospective/observational (field)/case studies

development of overtraining.

studies can be used in a 'challenge' mode to investigate the dynamic differences in psychological resilience of (potentially) overtrained persons. In this way, exercise stress markers collected with these single exercise studies can be used for a proper

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

Table 3

<table>
<thead>
<tr>
<th>Participant</th>
<th>Basic: normal amount of training hours per week</th>
<th>Adapted: amount of training hours during adapted training</th>
<th>TT: time trial</th>
<th>HR: heart rate</th>
<th>2nd trial: increase or decrease in time between the first and second time trial</th>
<th>3rd trial: increase or decrease in time between the second and third time trial</th>
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Participants: 1-8; Basic: normal amount of training hours per week; Adapted: amount of training hours during adapted training; TT: time trial; HR: heart rate; 2nd trial: increase or decrease in time between the first and second time trial; 3rd trial: increase or decrease in time between the second and third time trial.
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. As research progresses, more and more outcome measures are taken into account in overtraining research. However, an advantage of such a design is the controllability of group size to end up with enough overreached athletes after 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 to achieve the required training load. Of course, this might result in more resilience to the increased training volume as compared to their training history at the time of this study. This might lead to more resilience to the increased training volume as compared to their training history at the time of this study. Interestingly, mood changes indicated more fatigue in our athletes, but performance decrement was not observed. At the same time, it is clear that these conditions are highly multi-factorial and individually classified as underperformance. We observed in only three participants such an overreached according to the definition. In addition, POMS mood scores indicated a significant increase in mental fatigue (p=0.030; one-tailed paired t-test). The test results did not show this. This points out that only questionnaires are not sufficient in diagnosing overreach, 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 stress markers indicate a possible state of NF-OR. In the second part of this review we will discuss the essential outcome measures to assess in overtraining research.
and directly after training camp to conclude if there is underperformance should be assessed and controlled, 3) performance tests should be included before status can be made for that design (13). Ishigaki et al (2005) did not include performance tests, so no conclusion about OR cannot be concluded. The 8 day training camp, used as study design by include a recovery period, so whether those athletes were suffering from F-OR or promising, as both resulted in reduced performance (11, 12), however, both failed to executed in the study designs of Hedelin et al (2000) and Jürimäe et al (2004) seem increased for training purposes is a popular and practical method (11-13), as it is controls may surge, and can be further evaluated by longitudinal studies.

In addition, personal variation in levels of outcome measures cannot be taken into markers that are significantly different between overtrained athletes and healthy lack baseline measurements, which makes before and after comparisons impossible. results cannot or hardly be extrapolated. For example, most of the time, these reports conclusions about symptoms and help them with diagnosing overtraining. However, overtraining (8-10). These reports are useful for sport physicians to draw some development of overtraining.

A large number of studies focussing on overtraining are case studies or studies that of markers for the early diagnosis of overtraining, because the (potentially) psychological resilience of (potentially) overtrained persons. In this way, exercise stress markers collected with these single exercise studies can be used for a proper 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 dimension of recovery or OTS. Performance depends on the type of exercise. With some sports, speed is

**Performance**

least the phrase ‘overtraining is an unwanted, long lasting period of underperformance’. Without (assessed) underperformance, one cannot diagnose OR

Which performance test is chosen in a study design depends on the type of athlete

relevant to overtrained persons are similar to clinical depression (49). These behavioural

**Mood/behaviour/cognition**

How to perform testing and training protocols to study overreaching and overtraining -experimental protocols

and outcome measures revisited. A narrative Review
Acute stress model (single-exercise study design)

Definitions

In short, F-OR is an intentional part of an athlete’s training schedule and leads, with several days to several weeks of recovery (for NF-OR and OTS, respectively). These conditions are highly multi-factorial and individually determined, and that there are no discrete boundaries between F-OR, NF-OR and OTS. At the same time it is clear that these conditions are highly multi-factorial and individually defined. 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.

Single-exercise studies, or acute exercise bouts, are used to identify markers that single training sessions don’t have detrimental effects on complex reaction time. More recently, immunological effects have been incorporated in overtraining research. 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 Colour Word Test compared to healthy athletes (50). And F-OR cycles had longer reaction times compared to a control group, although choise reaction times were found between fatigue and F-OR subjects who participated in this difference was just not significant (51). On the other hand, no differences in long-term performance, when assessed after physical exercise, and computerised assessment of fatigue were found for example. The differences between F-OR and control subjects were more substantial quantities of pro-inflammatory cytokines. These cytokines interact with white blood cells, and contribute to low-grade systemic inflammation. This leads to the release of adrenocorticotrophic hormone (ACTH), corticotropin-releasing hormone (CRH), adrenocortisol, prolactin (PRL), growth hormone (GH) and cortisol, all involved in this interaction. Proteins, like 5-hydroxytryptamine (5-HT), cytokines, like interleukin-1 (IL-1), and hormones like prolactin and cortisol are involved in this interaction. Both cytokines and hormones are linked to sickness behaviour and cortisol, prolactin, growth hormone and interleukin-1 are involved in this interaction. Proteins, like 5-hydroxytryptamine (5-HT), cytokines, like interleukin-1 (IL-1), and hormones like prolactin and cortisol are involved in this interaction. Both cytokines and hormones are linked to sickness behaviour and proteins like 5-hydroxytryptamine (5-HT) and hormones like prolactin and cortisol are linked to sickness behaviour.

Imune system

and competition. These “sickness” behaviours probably assist the athletes in

...
with the central nervous system and induce “sickness behaviour”.

Other outcome measures

Future research considerations and guidelines
Chapter 7

...
Table 1.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Ishigaki et al. (2005)</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Hedelin et al. (2000)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Jürimäe et al. (2004)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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</tbody>
</table>

An advantage of the latter approach is that it provides insight into physical or even psychological stress. Stress markers collected with these single exercise studies can be used for a proper comparison of markers that are significantly different between overtrained athletes and healthy controls. From these comparisons, multiple 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. However, this approach does not result in a set of markers for the early diagnosis of overtraining. How ever, this approach does not result in a set of markers for the early diagnosis of overtraining. How ever, this approach does not result in a set of markers for the early diagnosis of overtraining. How ever, this approach does not result in a set of markers for the early diagnosis of overtraining. How ever, this approach does not result in a set of markers for the early diagnosis of overtraining.

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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 athlete's 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 include:

- **Costill et al. 1988** (22)
  - 12 highly trained male swimmers
  - 10 days: 1.5 h/day, 5 days/week: 4,266 m per day, Intensity: 94% of their VO2max
  - 2 training sessions per day, 1.5 h per session.
  - Average training distance increased from 4,266 to 8,970 m/day.
  - 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 reduced muscle glycogen levels, this was a result of their abnormally low carbohydrate intake.
  - No overreaching in the group of swimmers.

- **Dressendorf 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.
  - No improvements observed; no overreaching.
and directly after training camp to conclude if there is underperformance and directly after training camp to conclude if there is underperformance should be assessed and controlled, 3) performance tests should be included before checked for their general health and illnesses should be excluded, 2) dietary patterns but there are 3 important issues that should be addressed: 1) athletes should be In theory, monitoring athletes during a training camp, is a good method to study OR, status can be made for that design (13). Ishigaki et al (2005) did not include performance tests, so no conclusion about OR NF-OR cannot be concluded. The 8 day training camp, used as study design by executed in the study designs of Hedelin et al (2000) and Jürimäe et al (2004) seem increased for training purposes is a popular and practical method (11-13), as it is Monitoring athletes during a training camp, where volume and/or intensity is But preferably, other approaches should be used to induce overreaching in athletes. controls may surge, and can be further evaluated by longitudinal studies. markers that are significantly different between overtrained athletes and healthy control group for between-group comparisons. From these comparisons, multiple account. Some of these problems could be solved by using a large matched healthy lack baseline measurements, which makes before and after comparisons impossible. results cannot or hardly be extrapolated. For example, most of the time, these reports conclusions about symptoms and help them with diagnosing overtraining. However, overtraining (8-10). These reports are useful for sport physicians to draw some report about overtrained athletes and their symptoms after being diagnosed with. In addition, personal variation in levels of outcome measures cannot be taken into study design. 1994 (46) considerations. Therefore, there is a need for a standardized diagnostic tool for overreaching and overtraining. However, there is not an established method to assess overreaching. Or, the diagnostic tool for overtraining and overreaching remains underdeveloped. Finally, we discuss the limitations and future perspectives in this review. at the point of overtraining and not in the diagnosis of overtraining in future. However, this approach does not result in a set psychological resilience of (potentially) overtrained persons. In this way, exercise studies can be used in a ‘challenge’ mode to investigate the dynamic differences in psychological resilience. At the same time, more longitudinal studies are required to better understand the development of psychological resilience. How to perform testing and training protocols to study overreaching and overtraining -experimental protocols
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<table>
<thead>
<tr>
<th>Study Authors</th>
<th>Year</th>
<th>Participants</th>
<th>Activity</th>
<th>Training Changes</th>
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</thead>
<tbody>
<tr>
<td>Halson et al.</td>
<td>2002</td>
<td>8 male endurance cyclists</td>
<td>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).</td>
<td>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 ± 2.6min. And a 29% increase in global mood disturbance. Overreaching criteria were: reduced performance on lab tests and negative mood. All 8 subjects completed the intensified training period and met the criteria for OR.</td>
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<tr>
<td>Hedelin et al.</td>
<td>2000</td>
<td>9 elite canoeists (6 men and 3 women)</td>
<td>6 days, training camp consisting of 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/anerobic training, 65% was endurance training, 10% was strength training.</td>
<td>Yes not</td>
</tr>
<tr>
<td>Hooper et al.</td>
<td>1993</td>
<td>5 male and 9 female elite swimmers</td>
<td>6 months</td>
<td>10 to 12 workouts/week and 6 days/week</td>
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<table>
<thead>
<tr>
<th>OT possible</th>
<th>Performance tests</th>
<th>Training load</th>
<th>Endurance</th>
<th>Intensity</th>
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<tr>
<td>Yes</td>
<td>No</td>
<td>Low</td>
<td>Endurance</td>
<td>Intensity</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Moderate</td>
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<td>Intensity</td>
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<tr>
<td>No</td>
<td>Yes</td>
<td>High</td>
<td>Endurance</td>
<td>Intensity</td>
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Retrospective/observational (field)/case studies

- Studies can be used in a 'challenge' mode to investigate the dynamic differences in response between athletes who are... study overreaching and overtraining -experimental protocols

and outcome measures revisited. A narrative Review

133

7
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.

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and directly after training camp to conclude if there is underperformance.

3 important issues that should be addressed:

1) Athletes should be checked for their general health and illnesses should be excluded.
2) Dietary patterns but there are
3) Performance tests should be included before

In theory, monitoring athletes during a training camp, is a good method to study OR, and controls may surge, and can be further evaluated by longitudinal studies.

Higher training volume resulted in reduced performance, however, both failed to include a recovery period, so whether those athletes were suffering from F-OR or

A large number of studies focussing on overtraining are case studies or studies that lack baseline measurements, which makes before and after comparisons impossible. Results cannot or hardly be extrapolated. For example, most of the time, these reports are useful for sport physicians to draw some conclusions about symptoms and help them with diagnosing overtraining. However, overtraining (8-10). These reports are useful for sport physicians to draw some conclusions about symptoms and help them with diagnosing overtraining. However, these reports are not enough to draw robust conclusions about the development of overtraining.

Overreaching in the 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

Psychological resilience of (potentially) overtrained persons. In this way, exercise stress markers collected with these single exercise studies can be used for a proper 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.

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To perform testing and training protocols to study overreaching and overtraining - experimental protocols

| Volume group: training volume was doubled from 85.9 km/week to 171.8 km/week in week 3. Intensity resulted in a decrease in running distance in week 3, and distance increased from 9 km/week in week 1 to 22.7 km/week in week 3. Higher intensity group.

| 4 days / week 31-33 |

| Submaximal and maximal endurance increment during training on a bike ergometer, and 2 4mmol lactate by 25% at medical or training + 3 recovery |

| Yes yes |

| Increase in volume approximately 12%. |

| No overreaching in the increased intensity training (103% increase in the increased intensity group) |

| Maximum performance by approximately 12%. |

| Lehmann et al. 1993 (28) |
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.

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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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- Smith et al. (2000) (21)
  - Male and female elite rowers
  - Increased training volume by 33% and training intensity per week
  - No OR or OT diagnosis possible

- Steinacker et al. (2000) (45)
  - Rowers in preparation for the World Championships in 1995
  - High training loads of 3.2 hours a day for 18 days
  - Incremental all-out rowing ergometer tests. The 2000m rowing speed was slower after training phase, and faster after taper and World Championships
  - Overreaching, and after taper recovery, so F-OR
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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
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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 performance capacity with or without related physiological and psychological signs and symptoms of overtraining in which restoration of performance capacity may take several days to several weeks or months."

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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

Rieneke Terink
Renger Witkamp
Maria Hopman
Els Siebelink
Huub Savelkoul
Marco Mensink

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ABSTRACT

We investigated the effects on exercise-induced stress and metabolic responses of acute (2 days) 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% Wmax.

Blood samples for cortisol, free fatty acids, glucose and ketones and saliva samples for immunoglobulin 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

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

used, including different ‘train high’ schedul
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/m² 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

Subjects were randomly assigned to start with either the low carbohydrate diet. Before intervention, participants’ characteristics were assessed, including a physical exercise test (VO_{2\text{max}} 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 washout period of at least two applied between both diets. An upper respiratory tract infection (URTI) questionnaire was filled out before.
Maximal aerobic capacity and body composition

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Maximal aerobic capacity and body composition were measured. VO2 peak was assessed by either 25W/minute or 40W/2 minute until the participant could not maintain the required pedal frequency of at least 60 rpm. Oxygen consumption was measured with indirect calorimetry (Oxycon Carefusion, Hoechberg, Germany) and VO2 peak was evaluated.

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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 100 grams), sunflower oil, margarine, nuts, muesli bars, fruit juices. For the LC diet these comprised: 48+ cheese (cheese with more fat per 100 grams), 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 all allowed) and herbs. Participants received electronically weighing scales (Impulse, Inter-East, B.V., Rosendaal) 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 weighed 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 while they already collected their morning urine at home to assess ketosis (ketosticks, 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 draw (LC breakfast: 588 kCal (average) ~34 En% fat, 17 En% protein, 6 En% carb; HC breakfast: 505 kCal (average) ~34 En% fat, 17 En% protein, 6 En% carb). At that point, body composition was assessed using a DEXA scan (GE Healthcare, Madison, WI). Scans were performed on the same time of the day during all sessions to minimize measurement errors. After that, a 90 minutes ergometer test (Lode Excalibur, Groningen, the Netherlands) at 60% of the athletes’ individual Wmax (~70% VO2max) 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...
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Blood sampling and analysis

Blood samples were collected in lithium-heparin, EDTA and serum tubes. Lithium-heparin tubes were used for chemiluminescence (sandwich) assay with Immulite XPi (Siemens, the manufacturer's protocol. Cortisol was measured with immunometric enzymatic assay (Sigma).

Concentrations are given as mean ± SD. Differences in concentrations between time points were analyzed by repeated measures analysis of variance (ANOVA) with factors time point, diet and their interaction. Post hoc analysis was performed with Bonferroni correction. Differences were considered significant at p < 0.05.

To the manufacturer’s protocol

Analysiss was performed according to the manufacturer’s protocol. Cortisol was measured with immunometric enzymatic assay (Sigma). β-Hydroxybutyrate (Sigma) was measured with a colorimetric assay. Glucose was measured with an enzymatic assay (Boehringer Mannheim, Germany). Plasma was frozen at -80 degrees Celsius until it was analyzed for free fatty acids (Sigma). Glucose was measured by means of an enzymatic assay (Boehringer Mannheim, Germany). Plasma was frozen at -80 degrees Celsius until it was analyzed for free fatty acids (Sigma). Glucose was measured by means of an enzymatic assay (Boehringer Mannheim, Germany). Glucose was measured by means of an enzymatic assay (Boehringer Mannheim, Germany).
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 drooling was performed (20). Participants were asked to bend their heads slightly downwards and first collect some saliva in their mouths before drooling into saliva collection aid (Salimetrix, 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 analyzed 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 analyze 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 analyzed 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 otherwise indicated.
RESULTS

Participant characteristics

<table>
<thead>
<tr>
<th>Participant characteristics</th>
<th>Value (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.9 ± 8.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>181.7 ± 4.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.4 ± 5.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.1 ± 1.4</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>61.9 ± 3.4</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>81.2 ± 4.4</td>
</tr>
<tr>
<td>BMC (kg)</td>
<td>3.2 ± 0.25</td>
</tr>
<tr>
<td>BMC (%)</td>
<td>4.2 ± 0.32</td>
</tr>
<tr>
<td>Body fat (kg)</td>
<td>11.2 ± 4.0</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>14.5 ± 4.6</td>
</tr>
<tr>
<td>Total training (hours/week)</td>
<td>5.6 ± 1.1</td>
</tr>
<tr>
<td>VO₂max (ml/kg/min)</td>
<td>57.3 ± 5.8</td>
</tr>
<tr>
<td>Max heart rate (bpm)</td>
<td>186.9 ± 8.8</td>
</tr>
<tr>
<td>Max Power (Watt)</td>
<td>346 ± 46</td>
</tr>
<tr>
<td>Max Power / kg body weight</td>
<td>4.5 ± 0.5</td>
</tr>
</tbody>
</table>

Means ± SD are shown. BMI: Body mass index; BMC: Bone mineral content.
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 (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 17 ± 0.21 vs 24 ± 0.93 En%, for LC and HC, respectively; p < 0.001), although this was not

Ener gy i nt ake bet ween t he LC ( 3104 ± 297 kCal )  and t he HC di et  ( 3075 ± 298 kCal )  
was not differ ent  ( p = 0. 221) .  As  i nt ended,  macr onut r i ent  i nt ake was  s i gni f i cant l y 
different between bot h di et s ,  w i t h  s i gni f i cant l y h ig her  f at  i nt ake i n t he LC di et  
compared t o 0. 001)  and i n l i ne wi t h t he exper i ment al  des i gn a l ower  car bohydr at e i nt ake i n t he 
LC di et  compar ed t o 0. 001)  and i n l i ne wi t h t he exper i ment al  des i gn a l ower  car bohydr at e i nt ake i n t he 

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 participants 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-hydroxybutyrate) 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).
Table 2.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Energy (kCal)</th>
<th>Carbohydrate (g)</th>
<th>Carbohydrate (En%)</th>
<th>Fat (g)</th>
<th>Fat (En%)</th>
<th>Protein (g)</th>
<th>Energy content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>3075 ± 298</td>
<td>64 ± 6</td>
<td>373 ± 38</td>
<td>6 ± 0</td>
<td>16 ± 1</td>
<td>8 ± 0</td>
<td>16.9 ± 0.9</td>
</tr>
<tr>
<td>HC</td>
<td>3104 ± 297</td>
<td>64 ± 6</td>
<td>373 ± 38</td>
<td>6 ± 0</td>
<td>16 ± 1</td>
<td>8 ± 0</td>
<td>16.9 ± 0.9</td>
</tr>
</tbody>
</table>

Body composition

Body mass was significantly lower after 2 weeks on each of the diets compared to baseline, for LC and HC diets respectively (p < 0.001). Body fat percentage was not different between diets (p = 0.101). Lean body mass percentage was significantly different between diets, with a decrease of 6% after LC and HC respectively; p < 0.001 and p = 0.005).
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.

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± 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 (170 ± 11 bpm vs 165 ± 11 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 the HC diet (17.00 ± 0.053). This difference in perceived exertion diminished after 2 weeks, but still tended to be higher on the LC diet (17.00 ± 0.053).
MATERIALS AND METHODS

Participants
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Table 3. Effects of a low carbohydrate diet on the exercise induced stress and metabolic response in well trained individuals

<table>
<thead>
<tr>
<th></th>
<th>LC</th>
<th>HC</th>
<th>Time effect</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Work (AUC kJ)</td>
<td>939 ± 163</td>
<td>1003 ± 129</td>
<td>&lt;0.001</td>
<td>0.016</td>
</tr>
<tr>
<td>RER (rest)</td>
<td>0.76 ± 0.03</td>
<td>0.77 ± 0.06</td>
<td>0.282</td>
<td>0.974</td>
</tr>
<tr>
<td>RER (t60)</td>
<td>0.82 ± 0.03</td>
<td>0.82 ± 0.03</td>
<td>0.681</td>
<td>0.974</td>
</tr>
<tr>
<td>HR (t60)</td>
<td>165 ± 13</td>
<td>170 ± 11</td>
<td>0.014</td>
<td>0.974</td>
</tr>
<tr>
<td>RPE score</td>
<td>18.0 ± 1.4</td>
<td>17.3 ± 1.7</td>
<td>0.151</td>
<td>0.974</td>
</tr>
</tbody>
</table>

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

Blood metabolites (Free fatty acids, glucose, cortisol and ketone bodies) 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 group (2.3 ± 0.6 and 2.2 ± 0.5 mmol/L after 2 days and 2 weeks, 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 generally 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 < 0.001) compared to baseline glucose levels. 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 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 (-0.018 ± 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).

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 (-0.018 ± 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 (622±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 cortisol response was highest after 2 days on the LC diet (622±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).
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) effect. *LC indicates that this difference was between 2 days and after 2 days. $ indicates significant differences between the LC and HC diet after 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 weeks. □
Salivary IgA

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 continuous line) and HC diet (grey continuous line). Significant p values represent a paired samples t-test for the HC vs LC diet.

URTI symptoms

“how i l l do you f eel t oday” 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 other participants with a 0. In addition, there were no significant correlations between URTI symptoms and IgA levels, p > 0.05.
DISCUSSION

responses, and compared this to a HC diet. Many previous studies didn’t take time

The 2 weeks of adaptation might be considered as short time frame to induce

as well as heart rate and perceived exertion showed a comparable or even higher

as reflected by an 81% increase of plasma cortisol levels, compared to ~20-30% at

hemoglobin concentration > 8.5 mmol/L, and they had not donated blood during six

inflammatory- and/or immunosuppressive medicines. All participants had a

in our study we investigated short-term (2 days) and prolonged (2 weeks) adaptation

lower RER, indicating more reliance on fat oxidation. Except for a further increase

2 days already higher circulating FFA and ketone levels, lower glucose levels, and a

as well as heart rate and perceived exertion showed a comparable or even higher

di et, in particular after two days, despite equal or even higher (perceived) exertion

di r ectly after initiation of the diet and after 2 weeks. In line with our expectations,

di r ectly after initiation of the diet and after 2 weeks. In line with our expectations,

not interfering with our results.

The other occasions. This effect had apparently disappeared after 2 weeks and was

exertion was not seen after 2 days on an LC diet, in contrast to the HC diet. However,

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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.

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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.

Further more, 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, with no 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

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

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MATERIALS AND METHODS

Stress response -
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. 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 –
Baseline glucose levels were lower after two weeks on the LC diet, this was not the result of a ketogenic diet as fat stores drop after 2 days on a LC diet is likely too short for fat stores to drop. The exercise intensity in our study was fairly high, reflected by heart rates above 160 bpm (187 bpm was their max HR at the VO2max 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 was initiated with already reduced values, and this may result in excess release of cortisol. Cortisol plays a role in the IGF1 pathway, which can affect the association with exercise-induced changes in IGF1. However, in the majority of studies no separate detection of IGF1 and IGF2 levels was performed and in contrast to studies showing a post-exercise decrease in IGF1, this protective role of a short-term LC diet on the exercise-induced decrease in IGF1 was debated (33), which could be interpreted as a favourable outcome of the LC diet. The reduced IGF1 (both in IGF1 and IGF2) is often linked to an increased risk for URTI (32), despite being also 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. The reduced IGF1 (both in IGF1 and IGF2) is often linked to an increased risk for URTI (32), despite being also 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. The reduced IGF1 (both in IGF1 and IGF2) is often linked to an increased risk for URTI (32), despite being also 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. The reduced IGF1 (both in IGF1 and IGF2) is often linked to an increased risk for URTI (32), despite being also 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. The reduced IGF1 (both in IGF1 and IGF2) is often linked to an increased risk for URTI (32), despite being also 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. The reduced IGF1 (both in IGF1 and IGF2) is often linked to an increased risk for URTI (32), despite being also 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.

Stress response -
Baseline glucose levels were lower after two weeks on the LC diet, this was not the result of a ketogenic diet as fat stores drop after 2 days on a LC diet is likely too short for fat stores to drop.
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.

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. Further more, 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 under reported energy intakes in the 3-day food records at intake, leading to a dietary advice with a lower energy intake than 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 under reporting 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
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/m² 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.

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

Perspectives

Metabolic and exercise-stress response...
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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

Rieneke Terink
Marco Mensink
Ben Meijer
Renger Witkamp
Huub Savelkoul
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, VO2max 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 washout period of >2 weeks in between. Two days and 2 weeks during both intervention periods, participants cycled for 90 min at 60% Wmax. Blood samples for cortisol, immune cell differential counts, proliferation, and homing marker 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 monocye 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 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, sport
INTRODUCTION

Exercise immunology suggests the occurrence of an ‘open window’ of

to as ‘homing’

This happens when recovery periods are short, or as part of a ‘train

high’ regime or as part of a so
viewpoints described above, the ‘open window’ and ‘redistribution’ theories, 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. 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 following strenuous exercise: a randomised cross-over trial (Figure 1). Subjects were randomly assigned to start with either the low- or the high-carbohydrate diet. Before the intervention, participants characteristics were 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 and a second test day to study the response following long-term intervention was performed after 2 weeks on the diet.
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.

Figure 1. Schematic overview of study design. 3DRF: 3-day food record; SQUASH: 7-day 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 (VO2max). 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 directly measured with a direct calorimetry (Oxycon Carefusion, Hoechberg, Germany), and VO2peak 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

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- 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/or the Medical Ethical Committee of the University of Groningen, the Netherlands.

Personalized diet plans were designed based on the participants’ habitual physical activity (SQUASH) (24), measured before the start of the intervention, using a standardized questionnaire for physical activity level (Short Questionnaire to Assess Health Promotion Activities). Habitual physical activity was assessed before the start of the intervention, using a standardized questionnaire for physical activity level (Short Questionnaire to Assess Health Promotion Activities). 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). 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). The diets were 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 (Figure 1). Subjects were randomly assigned to start with either the low- or the high-carbohydrate diet. Before the intervention, participants’ characteristics were measured in standardized household portions. Dietary intake was assessed by taking questionnaires. Furthermore, their dietary intake was measured in standardized household portions. Diets were provided foods which were weighed by distribution and return. Measurements and taking questionnaires. Furthermore, their dietary intake was recorded, which included a physical exercise test (VO2max test), body composition, and the following long-term intervention was performed after 2 weeks on the diet. This study had a cross-over design, with 2 times 2 weeks of dietary intervention following strenuous exercise: a randomised cross-over trial (24). Subjects were randomly assigned to start with either the low- or the high-carbohydrate diet. Before the intervention, participants’ characteristics were measured in standardized household portions. Dietary intake was assessed by taking questionnaires. Furthermore, their dietary intake was recorded, which included a physical exercise test (VO2max test), body composition, and the following long-term intervention was performed after 2 weeks on the diet. This study had a cross-over design, with 2 times 2 weeks of dietary intervention following strenuous exercise: a randomised cross-over trial (24).

Exercise test days

The first blood drawing took place at 08:30 AM. A standardized breakfast customized to their energy needs and current diet was provided after the first blood draw. Thereafter, a 90 min ergometer test (Lode Excalibur, Groningen, the Netherlands) at 60% of the athletes’ individual Wmax (~ 70% of their sustainable power output) was performed from 10:00 AM to 11:30 AM. Workload during the 90 min was measured during the 90 min ergometer test (Lode Excalibur, Groningen, the Netherlands) at 60% of the athletes’ individual Wmax (~ 70% of their sustainable power output) was performed from 10:00 AM to 11:30 AM. Workload during the 90 min ergometer test (Lode Excalibur, Groningen, the Netherlands) at 60% of the athletes’ individual Wmax (~ 70% of their sustainable power output) was performed from 10:00 AM to 11:30 AM. Workload during the 90 min ergometer test (Lode Excalibur, Groningen, the Netherlands) at 60% of the athletes’ individual Wmax (~ 70% of their sustainable power output) was performed from 10:00 AM to 11:30 AM.
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**Blood sampling and analysis for ketones and cortisol**

Blood sampling and analysis for ketones and cortisol were performed as follows. Blood samples for ketone analysis were collected in serum tubes (5 ml, Becton-Dickinson, New Jersey, America). Tubes were set aside for at least 30 min, whereafter 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; 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 plasma layer volume of 15 ml. Tubes were inverted 5 times and then centrifuged for 10 min at time. PBMC isolation was immediately aspirated and discarded. The PBMC layer was transferred to a 15 mL conical centrifuge tube and PBS was added until a final 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 (without brake, 20 °C). The medium (20% DMSO & 80% FCS) was added drop wise (final concentration 10% DMSO). The tube was placed in ‘Cryo 1 °C freezing containers’ (‘Mr. Frosty’s’). The freezing containers were placed in ‘Cryo 1 °C freezing containers’ (‘Mr. Frosty’s’). The freezing containers were kept in ‘Cryo 1 °C freezing containers’ (‘Mr. Frosty’s’). The freezing containers were kept in ‘Cryo 1 °C freezing containers’ (‘Mr. Frosty’s’). The freezing containers were kept in ‘Cryo 1 °C freezing containers’ (‘Mr. Frosty’s’). The freezing containers were kept in ‘Cryo 1 °C freezing containers’ (‘Mr. Frosty’s’).
Flow cytometric analysis for cell differentiation and homing

Table 1: Flow cytometric analysis for cell differentiation and homing

<table>
<thead>
<tr>
<th>Homing marker</th>
<th>Natural killer cells</th>
<th>Leucocytes</th>
<th>T helper cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α4β1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD16</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD14</td>
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<td></td>
<td></td>
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<tr>
<td>CD8</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CD3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For this panel, 1.5 × 10^6 isolated PBMCs were stained in a 96-well plate (NUNC). Antibodies used to analyse PBMCs for homing and cell differentiation were added to each well. After 2h, the samples were washed with PBS and washed cells were resuspended in 20 μl of PBS. The samples were analysed on a FacsCanto II flow cytometer (BD Biosciences) medium flow (30 μl/min) for 300s using a CytoFlex LX (Indianapolis, IN, USA).
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.
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Proliferation preparation and analysis (Ki-67)

PBMCs were plated in 96-well culture plates. Four types of stimuli were applied: medium control, LPS (1 μg/ml final concentration), ConA (1 μg/ml) and PWM (20 μg/ml). Cells were incubated at 37 °C at 5% CO2.

Extracellular staining

After 4 days, the 96-well 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-well 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 400g. 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 (Statistical analysis)

Data was analysed using a two-way repeated measures ANOVA (two factors, time and diet) using IBM SPSS version 25 Statistical Package for Social Sciences (IBM Corp., Armonk, NY, 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 otherwise indicated.

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RESULTS

Participant characteristics

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Body Mass Index (kg/m²)</th>
<th>VO₂max (ml/kg/min)</th>
<th>Max Power (Watt)</th>
<th>Max Heart Rate (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32.9 ± 8.2</td>
<td>181.7 ± 4.7</td>
<td>76.4 ± 5.4</td>
<td>23.1 ± 1.4</td>
<td>57.3 ± 5.8</td>
<td>346 ± 46</td>
<td>186.9 ± 8.8</td>
</tr>
</tbody>
</table>

Means ± SD are shown. BMI: Body mass index; Physical capacity: VO₂max

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carbohydrate (g/day)</th>
<th>Fat (g/day)</th>
<th>Protein (g/day)</th>
<th>Total Energy (kCal/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>180 ± 10</td>
<td>60 ± 5</td>
<td>100 ± 7</td>
<td>3104 ± 297</td>
</tr>
<tr>
<td>HC</td>
<td>100 ± 5</td>
<td>180 ± 10</td>
<td>60 ± 7</td>
<td>3075 ± 298</td>
</tr>
</tbody>
</table>

Dietary intake and blood and urine ketone levels

Compared to the HC diet (73 ± 1 vs 33 ± 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

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

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>LC diet</th>
<th>HC diet</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kCal)</td>
<td>2961 ± 528</td>
<td>3104 ± 297</td>
<td>3075 ± 298</td>
<td>0.221</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>116 ± 22</td>
<td>124 ± 12</td>
<td>112 ± 11</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Protein (En%)</td>
<td>16 ± 3</td>
<td>16 ± 1</td>
<td>15 ± 0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>318 ± 72</td>
<td>64 ± 6</td>
<td>373 ± 38</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Carbohydrate (En%)</td>
<td>43.4 ± 5.3</td>
<td>8 ± 0</td>
<td>49 ± 0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>122 ± 29</td>
<td>254 ± 25</td>
<td>116 ± 11</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total Fat (En%)</td>
<td>36 ± 6</td>
<td>73 ± 1</td>
<td>33 ± 0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Saturated Fat (g)</td>
<td>43 ± 13</td>
<td>68 ± 6</td>
<td>32 ± 3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Saturated Fat (En%)</td>
<td>13.1 ± 3.2</td>
<td>19.7 ± 0.3</td>
<td>9.3 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Monounsaturated Fat (g)</td>
<td>46 ± 13</td>
<td>127 ± 13</td>
<td>35 ± 3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Monounsaturated Fat (En%)</td>
<td>13.9 ± 3.3</td>
<td>36.8 ± 1.1</td>
<td>10.3 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Polyunsaturated Fat (g)</td>
<td>22 ± 7</td>
<td>39 ± 5</td>
<td>41 ± 5</td>
<td>0.002</td>
</tr>
<tr>
<td>Polyunsaturated Fat (En%)</td>
<td>6.6 ± 1.8</td>
<td>11.4 ± 0.4</td>
<td>12.1 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>354 ± 242</td>
<td>699 ± 57</td>
<td>165 ± 18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Dietary Fiber (g)</td>
<td>31 ± 6</td>
<td>28 ± 3</td>
<td>41 ± 4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Dietary Fiber (En%)</td>
<td>2 ± 0</td>
<td>5 ± 1</td>
<td>9 ± 2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Baseline serum βHB (mmol/L)</td>
<td>0.27 ± 0.13</td>
<td>0.07 ± 0.04</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Urine ketone levels (g/L)</td>
<td>0.26 ± 0.25</td>
<td>0.00 ± 0.00</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

*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 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 (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 (18.0 ± 1.4 vs 15.5 ± 2.7, for LC vs HC; *p* =...
A short-term low carbohydrate diet affects differential count, homing and proliferation rate of immune cells following strenuous exercise: a randomised cross-over trial

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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- 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).

Blood cortisol and ketone levels

Blood cortisol and ketone levels were significantly higher with the LC diet compared to those with the HC diet, but not after 2 weeks on the diet, at all time points (Figure 1). Subjects were randomly assigned to start with either the low- or the high carbohydrate diet. Before the intervention, participants characteristics were comparable between the two dietary interventions were followed for two weeks. A wash-out period of at least two days 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 and a second test day to study the response following long-term intervention was performed after 2 weeks on the diet.

Serum cortisol levels directly after exercise were highest after 2 days on the LC diet, and at both test days (p < 0.001). In summary, the exercise-induced increase in cortisol levels was estimated to gain insight in current eating habits and to estimate energy needs. Both measurements and taking questionnaires. Furthermore, their dietary intake was compared to baseline within 2 hours following exercise (also 23% decrease). During the low carbohydrate diet, the exercise-induced increase in CD45+ cell count was significant, both after 2 days and all participants gave written informed consent prior to participation.

Days: a first test day to investigate the short-term effects on the exercise-response, and all participants gave written informed consent prior to participation.

Immunological parameters: cell differential count, cell proliferation and homing

...(Continued)
Chapter 9

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). 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) did not differ significantly between the two diets at any time point after exercise.
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A short-term low carbohydrate diet affects differential count, homing and proliferation rate of immune cells following strenuous exercise: a randomised cross-over trial

![Graphs showing changes in immune cell counts and proliferation rates before, directly after, and 2 hours after exercise during low and high carbohydrate diets.](image-url)
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.
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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 continued line) and HC diet (grey continued 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...
± 0.50%; p = 0.024 and p = 0.015 respectively), whereas no significant decrease in
on the LC diet (p > 0.05). See Figure 4.

Figure 4. Means ± SE are shown. * indicates 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−/− 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.

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 exercise (p > 0.05), neither for the
exercised were compared to those 2 hours post-exercise. 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
A short-term low carbohydrate diet affects differential count, homing and proliferation rate of immune cells following strenuous exercise: a randomised cross-over trial

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Figure 5. Significant 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.
DISCUSSION

Many scientific studies on LC (ketogenic) diets have focused on body composition and/or sport performance, while their effects on the exercise-induced immune response appear to be largely understudied. Notwithstanding this, it has been speculated that a LC diet might induce immunosuppression via increased cortisol secretion. In order to elucidate this matter, we 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 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 hour 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 (acetacetate, acetone and $\beta$-hydroxybutyrat) 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).
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Immune response

Four-week 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 and/or immunosuppressive medication. Study enrolment took place between

Shortly after food intake, glucose remains the primary fuel source. As a consequence, strenuous exercise will

Glucagon (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 VO2max 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

As a role in the effects found in our study this cannot be concluded with our present findings. Interestingly, these effects were comparable after 2 weeks on the diets. Our findings are consistent with previous studies that 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

Interleukin-6 (31). The cellular response following strenuous exercise is induced by prior exercise and 2 days on a low carbohydrate diet (28). In a previous study from our

Count after 2 weeks on the diets was comparable. Our findings are consistent with previous studies that 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
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.

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-adrnergic 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 analyzed 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 Ki-67 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 Ki-67 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 analyzed 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 (URTI s) 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 has been reported before (40).
A short-term low carbohydrate diet affects differential count, homing and proliferation rate of immune cells following strenuous exercise: a randomised cross-over trial.

Studies are needed to more precisely study the kinetics of the homing process of T-immunocompetent and can be rapidly activated to fight these pathogens, an increased number of Th cells is required that are fully increasing the exposure to foreign pathogens in the respiratory tract. To be able to reflect of an exercise-induced phenotype of T-cell subsets homing to the upper airway. As these could be stimulated in vitro to proliferation. The was verified by the expression of homing markers on CD8+ and CD4+ Tc cells, while diet has less influence.

The blood is reflective of a redistribution induced by exercise in individuals on both carbohydrate diets. Before the intervention, participants characteristics were 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 measurements and taking questionnaires. Furthermore, their dietary intake was estimated to gain insight in current eating habits and to estimate energy needs. Both participants gave written informed consent prior to participation. The Declaration of Helsinki and approved by the Medical Ethical Committee of October 2018 and January 2019. The study was conducted in accordance with the and/or immunosuppressive medication. Study enrolment took place between 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- or immunosuppressive medication. Study enrolment took place between 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- or immunosuppressive medication. Study enrolment took place between 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- or immunosuppressive medication. 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‘LC diet and 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 signaling and the subsequent pro-inflammatory response (44). Furthermore, n-3 PUFAs can activate the anti-inflammatory transcription factor PPAR-γ and inhibit NF-κB and the subsequent pro-inflammatory cytokine production (45). Finally, previous studies from our own group have shown that endogenous lyophilized fatty acid 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 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 TLR4 activation, triggering its inflammatory response. Increased expression of TLRs in circulating macrophages will result in an increased production of pro-inflammatory cytokines (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 rates 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...
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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.

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23. h recall tool (Compleat) against interviewer
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Chapter 9


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Di f f er ent i al  modul at i on of  Tol l-l i ke r ecept or s  by f at t y aci ds :  pr ef er ent i al  i nhi bi t i on


45.  Cal der  PC.  Fat t y aci ds  and i nf l ammat i on:  t he cut t i ng edge bet ween f ood and

46.  de Bus  I ,  Wi t kamp R,  Zui l hof  H,  Al bada B,  Bal ver s  M.  The r ol e of  n-3


49.  Mor ei r a AP,  Texei r a TF,  Fer r ei r a AB,  Pel uzi o Mdo C,  Al f enas  Rde C.  Lipid mediators and inflammatory phenotype. Brain, behavior, and immunity. 2009;23(6):767-75

50.  Minalska A,  Thi l l i graph D,  M e t e l t s  A,  Uberti I,  W e l l s  C,  de Gouw  A,  et al .  PUFA-der i ved f at t y aci d der i vat i ves  and t hei r  oxygenat ed met abol i t es  i n t he

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53.  van der Kooy D,  S i c i s m i  A,  de Gouw  A,  Ho Knoppe  AL,  W e l l s  C.  PUFA-der i ved f at t y aci d der i vat i ves  and t hei r  oxygenat ed met abol i t es  i n t he

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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.


Blau C, Karlis AD, St-Pierre DH, Lamontagne L. Cross talk between intestinal microbiota, adipose tissue and skeletal muscle as an early event in low-grade inflammation and the development of obesity and diabetes. Diabetes/metabolism research and reviews. 2015;31(6):545

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- 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).

### MATERIALS AND METHODS

**Study design**

This study had a cross-over design, with 2 times 2 weeks of dietary intervention following long-term intervention was performed after 2 weeks on the diet.

**Participants**

Subjects were randomly assigned to start with either the low- or the high carbohydrate diet. This was followed by a wash-out period of at least two weeks. 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 following strenuous exercise: a randomised cross-over trial and a second test day to study the response two hours after exercise: a randomised cross-over trial.

**Measurements**

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 following strenuous exercise: a randomised cross-over trial and a second test day to study the response two hours after exercise: a randomised cross-over trial.

### Supplementary Table 1

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Directly after exercise</th>
<th>After 2 weeks on diet</th>
<th>LC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell differential counts are presented as percentage of total CD45+ cells (% of CD45+ cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T cell</td>
<td>9.3 ± 4.8</td>
<td>7.9 ± 4.9</td>
<td>7.9 ± 4.9</td>
<td>1.4 ± 1.1</td>
<td>1.5 ± 0.6</td>
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<tr>
<td>NK cell</td>
<td>1.6 ± 0.9</td>
<td>1.7 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>1.7 ± 0.5</td>
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<tr>
<td>Monocyte</td>
<td>14.1 ± 7.7</td>
<td>13.6 ± 6.7</td>
<td>13.6 ± 6.7</td>
<td>14.1 ± 7.7</td>
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<td><strong>p value</strong></td>
<td>0.195</td>
<td>0.106</td>
<td>0.106</td>
<td>0.288</td>
<td>0.288</td>
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</table>

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

Supplementary table 2

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After 2 days</th>
<th>After 2 weeks</th>
<th>After 2 days</th>
<th>After 2 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC</td>
<td>HC</td>
<td>LC</td>
<td>HC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.6 ± 3.0</td>
<td>7.0 ± 4.1</td>
<td>6.8 ± 3.6</td>
<td>7.0 ± 3.6</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.138</td>
<td>0.314</td>
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<tr>
<td></td>
<td>28.1 ± 16.1</td>
<td>26.8 ± 16.0</td>
<td>26.7 ± 16.0</td>
<td>27.5 ± 16.2</td>
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<td></td>
<td></td>
<td></td>
<td>0.319</td>
<td>0.292</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 hour post-exercise</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>LC</td>
<td>HC</td>
<td>4.7 ± 1.9</td>
<td>5.9 ± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5 ± 4.2</td>
<td>6.2 ± 2.5</td>
<td>6.1 ± 3.3</td>
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<td>0.432</td>
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<td>19.7 ± 15.1</td>
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<td>0.463</td>
<td>0.561</td>
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<td></td>
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<td></td>
<td>20.6 ± 13.2</td>
<td>20.6 ± 13.2</td>
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<td></td>
<td></td>
<td></td>
<td>0.561</td>
<td>0.561</td>
<td></td>
</tr>
</tbody>
</table>

Means ± SD are shown. P-values refer to a dependent sample t-test.
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 (VO2max 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.

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

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
Chapter 10

General discussion
back to baseline levels on the second day of walking. These observations were not
observed for total magnesium levels. Ionized magnesium levels restored
in this study was that plasma ionized magnesium level was more responsive to
deficiencies. In athletes participating in sports requiring weight control (e.g., wrestling, gymnastics)
deficiencies are not common when athletes consume a normal healthy diet (10). Still,
baseline differs per individual and that timing of blood withdrawal is crucial.
As our study did not address this issue the underlying mechanisms of our findings
plasma magnesium levels probably reflects a redistribution of the magnesium pool.
Factors like exercise type, intensity and duration will also affect the magnitude of
'return to homeostasis' remains of interest to further investigate.

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

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Chapter 2

Chapter 3

Chapter 5

Chapter 4

Chapter 6

Chapter 7

Chapter 8

Chapter 9

Chapter 10

Chapter 10
General discussion

Micronutrient status (during and directly after exercise)

...
back to baseline levels on the second day of walking. These observations were not observed for total magnesium levels. Ionized magnesium levels restored blood ionized magnesium levels after the first day of walking exercise, while this exercise compared to total magnesium. This was shown by a pronounced dip in this study was that plasma ionized magnesium level was more responsive to deficiencies. In athletes participating in sports requiring weight control (e.g., wrestling, gymnastics) deficiencies are not common when athletes consume a normal healthy diet (10). Still, frequently 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 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. 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.
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 were increased during the second exercise day. In addition, this research does not provide evidence on themselves in other ways to be fit enough to complete such a challenge.

General discussion

Interesting to notice was that these very old vital adults had good baseline magnesium levels, but 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 value to assess magnesium status, for example using nuclear magnetic resonance (NMR) or by administering and measuring of stable isotopes. Now magnesium distribution patterns, and to find out how our observations in this group of older people 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 analyzed a very vital group of older people. Not all men and women above 80 years of age are themselves in other ways to be fit enough to complete such a challenge.

Since total and ionized magnesium levels in blood are responsive to exercise, it is suggested that ionized magnesium should be the preferable parameter to determine magnesium status (15), but its reliability is subject of debate (16). Since 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 creates a demand for methodologies to assess magnesium status, for example using assessment of total magnesium in serum or plasma is most commonly used to reflect an acute response to exercise, and followed by a return to homeostasis. The equilibrium between bound and unbound magnesium, next to the effect of exercise, 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 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 assess magnesium status at all. The alternative, a balance study involving evaluation of stable isotopes, is questionable whether (ionized) magnesium in blood is a good reference.
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

**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...
General discussion

Exercise-induced immune response

of the ‘iron hormone’ hepcidin by the liver...
back to baseline levels on the second day of walking. These observations were not observed for total magnesium levels. Ionized magnesium levels restored to their pre-exercise levels after the first day of walking exercise, while this was shown by a pronounced dip in plasma magnesium levels probably reflects a redistribution of the magnesium pool. ATPase pumps in skeletal muscle (8). Furthermore, an increase in lipolysis probably increases the uptake of magnesium into adipocytes (9). Therefore, the decrease in Mg2+ uptake into muscle cells and regulates the magnesium dependent Na/K ATPase pumps in skeletal muscle (8).}

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Interestingly, 6 hours after exercise there were still 2 participants who were not back to their pre-exercise ionized magnesium levels. Which factors interfere with this 'return to homeostasis' remains of interest to further investigate.

Chapter 10

To further investigate this, we studied the effects of four consecutive days of walking exercise on exercise-induced cytokine responses.

Chapter 5

Chapter 3, we showed that cytokine levels significantly increased after exercise. Therefore, we examined magnesium levels during four consecutive days of walking exercise in order to further investigate this. We hypothesized that magnesium levels would decrease significantly during the first day of exercise and would gradually return to baseline levels within 3.5 hours. This is important to know, as monitoring magnesium levels in athletes is done very frequently, especially before plasma analysis, because this affects the status at least up to 3.5 hours.

Chapter 23

Chapter 20

Chapter 17

Chapter 14

Chapter 11

Chapter 8

Chapter 5

Chapter 3

Chapter 1

Chapter 8

Chapter 5
The effect of nutritional status on the exercise induced stress/immune response

...for investigated effects of clinical ‘true’ ketogenic diets, in chapter 8... in chapter 9...

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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.

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after exercise in the low carbohydrate condition compared to the high cytotoxic activity (44). After 2 days on the diets, T cell, T helper cell and B cell count between the diets, while cell differential count after 2 weeks on the diets was carbohydrate diet remains unknown.

showed increased carnitine acyl transferase (CAT) activity after 5 days on a high fat magnesium distribution patterns, and to find out how our observations in this group exercising during consecutive days. More research is needed to investigate these insight in the magnesium pattern that would occur in healthy athletes who are increased during the second exercise day. In addition, this research does not provide baseline levels at the start of the second walking day, or whether magnesium levels exercise, we don't know whether (ionized) magnesium levels were already back to As we didn't measure magnesium levels in the early morning before the start of themselves in other ways to be fit enough to complete such a challenge.

per day. Clearly these individuals walked on a regular basis or were training very vital group of older people. Not all men and women above 80 years of age are medicines, including proton pump inhibitors (14, 18). We realize that we analysed a suffering from bone loss, or, as mentioned above, for older adults who regularly use magnesium levels. Sub-optimal levels are reported for older adults, especially when exercise, we don't know whether (ionized) magnesium levels were already back to 17). However, only ~0.3% of our body magnesium is

to assess magnesium status, for example using measurement of 24-hour urine magnesium output is more laborious though. This creates a demand for methodologies to assess magnesium status, for example using that we showed that one bout of exercise affects both total and ionized magnesium, while consecutive bouts of exercise affects ionized magnesium, it becomes apparent that ionized magnesium should be the preferable parameter that we showed that one bout of exercise affects both total and ionized magnesium, while most of it is stored in muscles and bones, see Figure 2. Now to evaluate magnesium status (17). However, only ~0.3% of our body magnesium is free ionized magnesium is the active, directly available form involved in cellular processes, it is suggested that ionized magnesium should be the preferable parameter for assessment of total magnesium in serum or plasma is most commonly used to questionable if the assessment of the magnesium status should be done in blood. The

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reflection of an acute response to exercise, and followed by a return to homeostasis. The equilibrium between bound and unbound magnesium, next to the effect of exercise unbound magnesium. This could indicate that exercise has an acute effect on the caused by changes in plasma volume, but most likely by a shift between bound and

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 in involved in fat oxidation or transport of fatty acids. It has been shown that a 5 day

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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
of a network of surface ligands and receptors, often referred to as ‘homing’. The main finding regarding homing, was a decrease in T helper cell homing potential to the HC condition. Exercise itself affected cytotoxic T cell airway homing by an increased number of CD4+ T-cells that are fully immunocompetent and can be rapidly activated (29, 49). As a consequence, a delayed influx of CD8+ T-cells is required that are fully immunocompetent and can be rapidly activated. This would support the redistribution theory, stating that immune cells are in an elevated state of immune surveillance and improved immune regulation (46, 47). Time-dependent redistribution of immune cells to peripheral tissues, resulting in an increased number of CD3+ T-cells in blood might remain stable. Further studies are needed to investigate whether exercise-induced stress affects the stress and immune response, and it is therefore very important to take into account whether a training took place before plasma analysis, because this affects the status at least up to 3.5 hours.

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. Other suggestions that the reduction in cell counts after exercise reflects a transient and self-limited state of reduced immunocompetence (45). Our results suggest that decreased cell counts point towards immune-suppression and that a decrease in cell counts might indicate a period of time when the immune system is more susceptible to infections and illnesses during that period of time (45). Future study directions

Factors like exercise type, intensity and duration will also affect the magnitude of 'return to homeostasis' remains of interest to further investigate.
more research is needed to investigate these exercises, we don't know whether (ionized) magnesium levels were already back to normal in other ways to be fit enough to complete such a challenge.

Clearly these individuals walked on a regular basis or were training for more than 20 years to be able to exercise 4 consecutive days at which they walk 30-40 km (average ~ 8 hours) per day. According to these recommendations, they could help to answer the question whether there is a safe, healthy, and effective training plan or whether exercising is too little or too much.

We are biomarkers that are useful to discriminate between too much and too little fatigued, functional overreaching, non-functional overreaching and overtraining. So far, no studies have found (a set of) biomarkers that reliably and reproducibly detect and (or) predict overtraining from non-functional overreaching and overtraining. However, as we already pointed out in our review in chapter 2 and chapter 4, exercise affects micronutrient status. Moreover, the effect of exercise is dose-dependent, even though micronutrients are essential for health and performance. As daily training, even mental problems. On the other hand, exercise also puts homeostatic equilibrium between bound and unbound magnesium, next to the effect of exercise on magnesium distribution patterns, and to find out how our observations in this group can be assessed, the training background and circadian cycles should be taken into account.

Ideal strictly controlled training studies underline that exercise can decrease the risk for an early death, obesity and type 2 diabetes. Additionally, exercise can improve bone status, whereas in other, external factors.

On the other hand, exercise also puts homeostatic equilibrium between bound and unbound magnesium. This could indicate that exercise has an acute effect on the reflection of an acute response to exercise, and followed by a return to homeostasis. Still, it is very difficult to determine how much is too little and how much is too much exercise, and that this is individually determined and depending on other, external factors.

Interesting to notice was that these very old vital adults had good baseline isotopes. Nuclear magnetic resonance (NMR) or by administering and measuring of stable isotopes.
blood ionized magnesium levels after the first day of walking exercise, while this exercise compared to total magnesium. This was shown by a pronounced dip in this study was that plasma ionized magnesium level was more responsive to consecutive days of exercise in vital adults above 80 years of age. The main finding deficiencies. In athletes participating in sports requiring weight control (e.g., wrestling, gymnastics) deficiencies are not common when athletes consume a normal healthy diet (10). Still, It has been reported that magnesium status of most athletes is sufficient and that baseline differs per individual and that timing of blood withdrawal is crucial. Nevertheless, the results of this study indicated that return to sub-optimal levels in older people who are already at a higher risk for magnesium 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...
As we didn’t measure magnesium levels in the early morning before the start of the second walking day, or whether magnesium levels themselves in other ways to be fit enough to complete such a challenge. Clearly these individuals walked on a regular basis or were training able to exercise 4 consecutive days at which they walk 30-40 km (average ~ 8 hours) very vital group of older people. Not all men and women above 80 years of age are 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

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

While consecutive bouts of exercise affect ionized magnesium, it becomes that we showed that one bout of exercise affects both total and ionized magnesium, while most of it is stored in muscles and bones, see Figure 2. Now to evaluate magnesium status (17). However, only ~0.3% of our body magnesium is 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 determine magnesium status (15), but its reliability is subject of debate (16). 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 alternative, a balance study involving measurement of 24-hour 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.

Free ionized magnesium is the active, directly available form involved in cellular performance. This also applies to low carbohydrate, ‘ketogenic’, diets which have 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. This also applies to low carbohydrate, ‘ketogenic’, diets which have 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.

At present it is unknown to what extent these are interrelated with the ability to prevent development of cancer but can also be beneficial for improving treatment and lymphoma cells in vitro (50, 51). Another example is that more and more studies show that peak cell with a highly mature effect or phenotype were better redistributed after exercise, preventing development of cancer but can also be beneficial for improving treatment and lymphoma cells in vitro (50, 51). Another example is that more and more studies show that peak cell with a highly mature effect or phenotype were better redistributed after exercise,
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...
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Neubauer O, König D, Wagner KH. Recovery after an Ironman triathlon: Chapter 3, we therefore examined magnesium levels during four consecutive days of exercise in vital adults above 80 years of age. The main finding of 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 Mg2+ uptake into muscle cells and regulates the magnesium dependent Na/K receptor system and its role in physiological and pathological conditions. Clinical nutrition (Edinburgh, Scotland). 2019;38(6):2668–76.

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It has been reported that magnesium status of most athletes is sufficient and that Mg2+ uptake into adipocytes increases the uptake of magnesium into adipocytes. Therefore, the decrease in Mg2+ uptake into muscle cells and regulates the magnesium dependent Na/K receptor system and its role in physiological and pathological conditions. Clinical nutrition (Edinburgh, Scotland). 2019;38(6):2668–76.

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Factors like exercise type, intensity and duration will also affect the magnitude of 'return to homeostasis' remains of interest to further investigate. Nevertheless, the results of this study indicated that return to sub-optimal levels in older people who are already at a higher risk for magnesium deficiencies. In athletes participating in sports requiring weight control (e.g., wrestling, gymnastics) deficiencies are not common when athletes consume a normal healthy diet. Still, it has been reported that magnesium status of most athletes is sufficient and that use of specific medicines, in particular proton pump inhibitors, in this group (14).


insight in the magnesium pattern that would occur in healthy athletes who are exercising. We don't know whether (ionized) magnesium levels were already back to normal levels after a period of rest, as we didn't measure magnesium levels in the early morning before the start of an exercise session.

Per day. Clearly these individuals walked on a regular basis or were training intensively to be fit enough to complete such a challenge. These individuals are part of 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) 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 magnesium is such an important nutrient for many processes, it is suggested that ionized magnesium should be the preferable parameter to assess magnesium status at all. The alternative, a balance study involving the measurement of 24-hour urine magnesium output, is more laborious though. This 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 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 equilibrium between bound and unbound magnesium, next to the effect of exercise on magnesium levels, is 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 magnesium distribution patterns, and to find out how our observations in this group of very old vital adults are comparable with those of younger active individuals.

Interests. Isotopes. Nuclear magnetic resonance (NMR) or by administering and measuring of stable isotopes.

General discussion

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Martijn K, Jackson CF, Levy RG, Cooper PN. Ketogenic diet and other dietary treatments for epilepsy. The Cochrane database of systematic reviews. 2009;(9):CD005172.


Ionized magnesium levels restored back to baseline levels on the second day of walking. These observations were not observed for total magnesium levels. Ionized magnesium levels were shown to be more responsive to consecutive days of exercise in elderly adults above 80 years of age. Plasma ionized magnesium level was more responsive to exercise compared to total magnesium. This was demonstrated by a pronounced dip in ionized magnesium levels after the first day of walking exercise, while this dip was not observed for total magnesium levels. These findings are important as they provide evidence that exercise can cause a temporary decrease in magnesium levels, which may lead to suboptimal levels in older people who are already at a higher risk for magnesium deficiency. The gathered knowledge from our acute study, showing that exercise causes a temporary decrease in magnesium levels, is crucial for understanding the impact of exercise on magnesium homeostasis and the potential benefits of magnesium supplementation in elderly individuals. The decrease in magnesium status due to exercise may also be influenced by factors such as the type, intensity, and duration of exercise, as well as individual differences in magnesium status. Understanding these factors is crucial for the development of effective strategies for maintaining magnesium homeostasis in older individuals. 

References


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insight in the magnesium pattern that would occur in healthy athletes who are
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equilibrium between bound and unbound magnesium, next to the effect of exercise
unbound magnesium. This could indicate that exercise has an acute effect on the
caused by changes in plasma volume, but most likely by a shift between bound and


Part II: impact of latent cytomegalovirus infection and catecholamine sensitivity.

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 adaptations that occur directly following exercise, while the recovery after exercise concerns mechanisms to repair, refuel, replenish and return to homeostasis. Longer-term effects directly following exercise, while the recovery after exercise concerns adapta...
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 reviewed. Previous research showed that testosterone can be used to assess acute exercise stress but not prolonged training load over several weeks.

Samples were analysed for IL-6, IL-8, IL-10, TNF-α and IL-1β levels. In our study, ten male elite swimmers were monitored during 10 consecutive weeks of training, ending with a competition. We reviewed the existing literature regarding various research designs that were used to study overreaching and overtraining. We had noticed how the field of exercise science shifted its 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. In addition, this population was apparently healthy and vital, being 80+ years old and being able to walk 30-40 km every day for four consecutive days.

In chapter 7, we discussed the exercise-induced inflammatory response. 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 magnesium changes probably reflect re-distribution of magnesium through the body.

In chapter 4, we focused on the topic of acute stress responses to exercise. 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 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 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 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 increased directly after the first race and returned back to baseline levels within 2 hours after the last race.
After exercise, sub-optimal ionized and total magnesium levels did not drop after exercise. After exercise, sub-optimal ionized and total magnesium levels did not drop after exercise. After exercise, sub-optimal ionized and total magnesium levels did not drop after exercise. After exercise, sub-optimal ionized and total magnesium levels did not drop after exercise. After exercise, sub-optimal ionized and total magnesium levels did not drop after exercise.

Our results showed that ionized magnesium levels significantly dropped directly after the first day of walking, while total magnesium levels did not change. 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. We assessed the impact of exercise on the variation in blood magnesium levels before, during, and after exercise in a group of very old adults (>80 years) in a study conducted in 2019.

In chapter 8, we discussed the effect of exercise on magnesium levels in different diets. We found that the exercise response was different between diets, with clear exercise responses in the LC diet 2 hours after exercise. At baseline, immune cell counts and adherence were not different between diets, while differences between diets were homing on the LC diet 2 hours after exercise. At baseline, immune cell counts and adherence were not different between diets, while differences between diets were homing on the LC diet 2 hours after exercise. In chapter 9, we investigated the effects of exercise on magnesium levels in different diets. We showed that both ionized and total magnesium decreased after exercise direct after the first day of walking, while total magnesium did not change. The metabolic changes were comparable between short-term and prolonged adherence to the LC diet. The decrease in blood magnesium levels after exercise and subsequent increase a few hours after exercise was likely due to a diurnal cycle. The decrease in blood magnesium levels after exercise and subsequent increase a few hours after exercise was likely due to a diurnal cycle.

In conclusion, our results showed that exercise affects magnesium levels and timing of blood sampling to investigate whether nutritional status, i.e. high vs low carbohydrate intake, affects magnesium levels. Therefore, we examined changes in ionized and total magnesium levels before exercise and during recovery in well-trained cyclists and triathletes. We showed that both ionized and total magnesium decreased after exercise direct after the first day of walking, while total magnesium did not change. The decrease in blood magnesium levels after exercise and subsequent increase a few hours after exercise was likely due to a diurnal cycle. The decrease in blood magnesium levels after exercise and subsequent increase a few hours after exercise was likely due to a diurnal cycle.

The results of this study showed that short-term adherence to a LC diet already resulted in different immune cell counts and adherence. However, differences between diets were homing on the LC diet 2 hours after exercise. In chapter 8 and chapter 9, we discussed the effects of exercise on magnesium levels in different diets. We showed that both ionized and total magnesium decreased after exercise direct after the first day of walking, while total magnesium did not change. The metabolic changes were comparable between short-term and prolonged adherence to the LC diet. In chapter 9, we investigated the effects of exercise on magnesium levels in different diets. We showed that both ionized and total magnesium decreased after exercise direct after the first day of walking, while total magnesium did not change. The decrease in blood magnesium levels after exercise and subsequent increase a few hours after exercise was likely due to a diurnal cycle.

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Samples were analysed for IL-6, IL-8, IL-10, TNF-α and IL-1β. Again, blood samples were taken at baseline and every walking day after the first day of walking exercise. During competition, 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 the second week of training. During competition, both salivary cortisol and testosterone can be used to assess acute exercise stress but not prolonged training. We had noticed how the field 

In this study, 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.

Acute inflammatory response occurred after the first day of walking and that 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 exercise-induced inflammatory response was the topic of chapter 4 in this study. In this chapter, we reviewed the existing literature regarding various research designs that were used to study overreaching and overtraining. We had noticed how the field occurs within two weeks, but not after 2 days; and that exercise, more than diet, 

ew, can affect the immune system. 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.

In chapter 5, we concluded that adaptation to a LC diet in terms of metabolic and stress 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 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 was evident for immune cell differential count and cell proliferation rate.
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.
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.

### Dankwoord


### Marco Mensink

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.

### Renger

Previous research showed that the inflammatory response occurred after the first day of walking and that...
Chapter 11

Exercise induces a range of physiological responses involving different organs, tissues and systems, such as the immune, nervous, and endocrine systems. Micronutrients play a crucial role in these processes, and their assessment is essential to understand the impact of exercise on the body. We assessed the impact of exercise on the variation in blood magnesium status. This study aimed to investigate whether nutritional status, i.e., high vs. low carbohydrate intake, modulates the exercise-induced stress and immune response. We performed this investigation in a group of very old adults (≥80 years) in a group of young athletes, even though older persons might be at increased risks for micronutrient deficiencies. Therefore, we examined changes in ionized and total 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 levels directly after exercise and returned to pre-exercise levels within 3.5 hours after exercise. Longer-term effects directly following exercise, while the recovery after exercise concerns adaptations important for growth and supercompensation. The role of nutrition 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 training exercise was investigated. In addition, a dietary intervention was conducted.
The first day of walking exercise caused an increase in cytokine levels, thereafter, an acute inflammatory response occurred after the first day of walking and that individuals adapt rapidly to this type of repeated exercise.

In chapter 4 of the cytokine response during four consecutive days of walking exercise was examined. Again, blood samples were taken at baseline and every walking day after.

We reviewed the existing literature regarding various research designs that were used to study overreaching and overtraining. We had noticed how the field testosterone can be used to assess acute exercise stress but not prolonged training load in a group of elite swimmers. In our study, ten male elite swimmers were tested for and being able to walk 30-40km every day for four consecutive days.

In the second week of training, during competition, both salivary cortisol and testosterone levels remained unchanged. Hair testosterone levels were increased in periodization remained unclear. In our study, ten male elite swimmers were tested for their usefulness as indicators of long-term training load during a training periodization.

In this chapter, we discussed whether salivary and hair cortisol and testosterone could be used to assess training load in a group of elite swimmers in their usefulness as indicators of long-term training load during a training periodization.

For acute exercise stress, we assessed the acute inflammatory response, which can be used to assess acute exercise stress but not prolonged training load in a group of elite swimmers. We showed that plasma iron decreased across exercise in a group of healthy adults. We showed that plasma iron decreased across exercise in a group of healthy adults. We showed that plasma iron decreased across exercise in a group of healthy adults.

We analyzed the acute inflammatory response and acute exercise stress, which can be used to assess acute exercise stress but not prolonged training load in a group of elite swimmers. We showed that plasma iron decreased across exercise in a group of healthy adults. We showed that plasma iron decreased across exercise in a group of healthy adults.

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Summary

Exercise induces a range of physiological responses involving different organs, tissues, and systems, including the circulatory, endocrine, immune, muscular, and nervous systems. The acute exercise response refers to the metabolic and mechanical adaptations directly following exercise, while the recovery after exercise concerns mechanisms to repair, refuel, replenish, and return to homeostasis. Longer-term effects directly following exercise, while the recovery after exercise concerns adaptations important for growth and supercompensation. The role of nutrition mechanisms to repair, refuel, replenish, and return to homeostasis is substantial and demands further investigation.

In this thesis, we investigated the exercise response during acute and repeated exercise and the stress and immune response during repeated exercise and exercise training. Specifically, micronutrient status during training exercise was investigated. In addition, a dietary intervention was conducted to investigate whether nutritional status, i.e., high vs. low carbohydrate intake, would influence the exercise response.

We assessed the impact of exercise on the variation in blood magnesium levels. We observed a decrease in blood magnesium levels after exercise and a subsequent increase a few hours later, likely reflecting redistribution to muscles and blood respectively. We showed that both ionized and total magnesium levels 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 levels directly after exercise and returned to pre-exercise levels within 3.5 hours after exercise. 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 glycemic issues and protein turnover.

We showed that both ionized and total magnesium levels decreased directly after the first day of walking, while total magnesium levels significantly dropped directly after finishing. Our results showed that ionized magnesium levels collected at baseline (1 or 2 days before the first walking day) and every walking day did not drop after exercise. After exercise, sub-optimal ionized and total magnesium levels were observed, and these levels returned to pre-exercise levels within 3.5 hours after exercise. These observations suggest that magnesium status should be assessed before and after exercise to ensure adequate magnesium levels are maintained.

We concluded that exercise affects magnesium levels and timing of blood sampling to hours later likely reflects redistribution to muscles and to blood respectively. We showed that both ionized and total magnesium levels 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 levels directly after exercise and returned to pre-exercise levels within 3.5 hours after exercise.
While walking exercise caused an increase in cytokine levels on the first day, subsequent days showed a decrease in these levels. This suggests that individuals adapt rapidly to this type of repeated exercise. However, acute inflammatory response occurred after the first day of walking, indicating that even with constant daily workload, some stressors persist.

In this chapter, the focus was on prolonged training instead of acute exercise. We assessed whether salivary and hair cortisol and testosterone could be used to assess training load in a group of elite swimmers. We monitored plasma iron levels during 10 consecutive weeks of training, ending with a competition, and observed a decrease across the training period. Ferritin levels increased over subsequent days, and haptoglobin showed a decrease after the first day.

Salivary cortisol levels remained unchanged during competition, but testosterone levels were increased in the second week of training. The acute inflammatory response was evident after the first day of walking, even though daily workload remained constant. These results suggest that an iron loss occurred during the first day but increased during subsequent days. Ferritin and haptoglobin levels showed a decrease after the first day of walking. Nutritional changes, such as increased iron losses via foot strike haemolysis, sweat, and urine, were observed. Ferritin increased during subsequent days, while haptoglobin levels showed a decrease after the first day and increased over subsequent days. Haemoglobin did not change significantly after the first day of walking.

The iron losses and nutritional changes were monitored during 10 consecutive weeks of training, ending with a competition. They highlighted the importance of nutritional planning for elite swimmers to prevent iron deficiencies. In our study, ten male elite swimmers were monitored, and their usefulness as indicators of long-term training load during a training periodization remained unclear. Hair testosterone levels were increased in one-third of the participants.

Cortisol and testosterone were monitored, and their usefulness as indicators of long-term training load was assessed. The study showed that plasma iron decreased across the training period, and ferritin increased over subsequent days. These changes were mainly due to nutritional deficiencies and iron loss via sweat and urine. Haptoglobin levels showed a decrease after the first day of walking, indicating a decrease in acute exercise stress.

Shifting our focus to prolonged training instead of acute exercise, we first assessed the usefulness of cortisol and testosterone as indicators of exercise stress. These data were used to assess acute exercise stress in athletes, while iron losses occur during long-term training. The study suggested that cortisol and testosterone can be used to assess acute exercise stress in athletes, while iron losses via sweat and urine are due to nutritional deficiencies.

The study also showed that salivary cortisol levels remained unchanged during competition, but testosterone levels were increased in the second week of training. The acute inflammatory response was evident after the first day of walking, even though daily workload remained constant. These results suggest that an iron loss occurred during the first day but increased during subsequent days. Ferritin and haptoglobin levels showed a decrease after the first day of walking. Nutritional changes, such as increased iron losses via foot strike haemolysis, sweat, and urine, were observed. Ferritin increased during subsequent days, and haptoglobin levels showed a decrease after the first day of walking. Haemoglobin did not change significantly after the first day of walking.

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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.
Chapter 4

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 changes in cytokine levels were not associated with drop-out or health problems, suggesting that these were found in 88% and 16% of the participants, respectively. These sub-optimal iron levels were not associated with drop-out or health problems, suggesting that these were found in 88% and 16% of the participants, respectively.
Chapter 11

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. After having received her BSc in 2006, she enrolled in the Master’s programme ‘Cell Biology’ at Wageningen University and the Veterinary University of Utrecht. Her Internship was entitled: ‘Blood pressure in relation to sports background’, this was done at Sport Centre Pauweldal under supervision of a sports cardiologist. Her thesis was entitled: ‘Heart rate variability (HRV) and overtraining’ this was done at UMC Utrecht in the programme, namely ‘Nutrition’ and ‘Immunology’. Her Internship was entitled: ‘Nutrition at Wageningen University and Research’. The main topic of her PhD research was: to study the micronutrient state and exercise stress marker 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. In March 2015 Rieneke was appointed as a PhD candidate at the Division of Human and Sports’ for Edx. Rieneke completed her PhD in February 2020.

After finishing her master’s degree, she worked 2 years as a project leader at 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. Unfortunately this 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.

She helped with the development of the online course (MOOC) ‘Nutrition, Exercise and Sports’ for Edx. Rieneke completed her PhD in February 2020.

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 adaptations are important for growth and supercompensation. The role of nutrition directly following exercise and returned to pre-exercise levels within 3.5 hours after exercise. Furthermore, we observed an increase in both ionized and total magnesium levels directly after exercise and the stress and immune response during repeated and specific nutrients in the processes is substantial and demands further investigation. In this thesis, we investigated the exercise response during acute and repeated exercise and the stress and immune response during repeated and specific nutrients in the processes is substantial and demands further investigation. In this thesis, we investigated the exercise response during acute and repeated exercise and the stress and immune response during repeated and specific nutrients in the processes is substantial and demands further investigation.
Overview of completed training activities

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<tr>
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Previous research showed that the exercise-induced inflammatory response was the topic of a recent symposium on exercise and immunology. We also examined the impact of exercise-induced inflammation on hepcidin and iron status, especially increased iron losses via foot strike haemolysis, increased losses via sweat and urine, and being able to walk 30-40km every day for four consecutive days.

In addition, this population was apparently healthy and vital, being 80+ years old and able to walk long distances. Magnesium changes probably reflect redistribution of magnesium throughout the body.

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The research described in this thesis was financially supported by a grant from the Province of Gelderland, project EAT2MOVE.

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