# DISSERTATION

Titel der Dissertation

**Acute Stress and Recovery Responses to an Ironman Triathlon: Oxidative Stress, Antioxidant and Inflammatory Changes and their Relevance for DNA Stability**

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Doktor/in der Naturwissenschaften (Dr. rer.nat.)


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## **List of original publications** (see appendices)

The present thesis is a synthesis of the following original articles and reviews, which have been reproduced with kind permission of the respective publishers.

Original article I (basis for chapter 2):

**Neubauer O, König D, Kern N, Nics L, Wagner K-H** (2008). No Indications of Persistent Oxidative Stress in Response to an Ironman Triathlon. *Med Sci Sports Exerc*. 40 (12):2119-2128

Original article II (basis for chapter 3):

**Neubauer O, König D, Wagner K-H** (2008). Recovery after an Ironman Triathlon: Sustained Inflammatory Responses and Muscular Stress. *Eur J Appl Physiol*. 104 (3): 417-426

Review I (including original data; basis for chapter 4):

**Neubauer O, Reichhold S, Nersesyan A, König D, Wagner K-H** (2008). Exercise-induced DNA damage: Is there a relationship with inflammatory responses? *Exerc Immunol Rev*. 14:51-72

Original article III (relevant to the thesis, but not forming part of it):

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**König D, Neubauer O, Nics L, Kern N, Berg A, Bisse E, Wagner K-H** (2007). Biomarkers of exercise-induced myocardial stress in relation to inflammatory and oxidative stress. *Exerc Immunol Rev*. 13:15-36

Review III (relevant to the thesis, but not forming part of it):

**Reichhold S, Neubauer O, Bulmer AC, Knasmüller S, Wagner K-H** (2009). Endurance exercise and DNA stability: Is there a link to duration and intensity? *Mutat Res Rev*., in press

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## List of abbreviations used in the thesis

AOPP: advanced oxidation protein products

ANOVA: analysis of variance

BIA: bioelectric impedance analysis

BMI: body mass index

CAT: catalase

CBMN Cyt: cytokinesis block micronucleus cytome

CD: conjugated dienes

CD [..]-receptor: cluster of differentiation [..]-receptor

CK: creatine kinase

CRP: C-reactive protein

DNA: desoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

ENDO: endonuclease

FRAP: ferric reducing ability of plasma

FPG: formamidopyrimidine glycosylase

GSH: glutathione peroxidase

HDL: high-density lipoprotein

hs-CRP: high sensitive C-reactive protein

$H_2O_2$: hydrogen peroxide

HPLC: high-performance liquid chromatography

IAT: individual anaerobic threshold

IL: interleukin

LDL: low-density lipoprotein

MDA: malondialdehyde

MN: micronuclei

MPO: myeloperoxidase

NADP/$NADPH_2$: nicotinamide adenine dinucleotide / -phosphate

NF: nuclear factor

NPBs: nucleoplasmic bridges

Nbuds: nuclear buds

$O_2^{\bullet -}$: superoxide anion

ORAC: oxygen radical absorbance capacity

oxLDL: oxidized low-density lipoprotein

P: probability of error

PRE: pre-race

PMN: polymorphonuclear

PO: power output

POST: post-race

RDA: Recommended Dietary Allowance

RIA: radioimmunoassay

$ROO^{\bullet}$: peroxyl radical

ROS: reactive oxygen (and nitrogen) species

SCGE: single cell gel electrophoresis

SD: standard derivation

SOD: superoxide dismutase

TEAC: Trolox equivalent antioxidant capacity

TBARS: thiobarbituric acid reactive substances

TP: total protein

URTI: upper respiratory tract infection(s)

VLDL: very low-density lipoprotein

$VO_{2\,peak}$: peak oxygen consumption

WNET: weekly net endurance exercise time

8-OHdG: 8-hydroxy-2´-deoxyguanosine

# 1. A General Introduction: (Ultra-) Endurance Exercise and Health

The present doctoral thesis has been conducted within the framework of the research project *Risk Assessement of Participants of an Ironman Triathlon*: *Genome Stability, Oxidative, Muscular and Systemic Stress*, which was funded by the *Austrian Science Fund (FWF)*. Primum mobile of this project were the most recent health concerns about the continually growing number of athletes training for and competing in ultra-endurance races such as long-distance triathlons or ultra-marathon runs. According to the literature (21), ultra-endurance exercise is defined as exercise lasting more than four hours. Thereby, the Ironman triathlon (consisting of 3.8 km swimming, 180 km cycling and 42 km running) can be regarded as a – more and more popular – prototype of extremely demanding endurance exercise. Thus, Ironman triathletes are an exceptional group to investigate not only because of their training and performance level, their physiological characteristics (84) or the nutritional challenges (51), but urgently also to assess probable harmful effects as a result of ultra-endurance sports in general. This is of particular importance since some empirical as well as epidemiological data suggest that an extraordinary high volume of exercise is associated with oxidative DNA modulations (122) and an increased risk of developing cardiovascular disease (61, 68). At first sight, this seems paradoxically as, at the same time, there is compelling evidence that physical activity harvests numerous beneficial physiological effects and plays a key role in the prevention of various modern chronic diseases (138). A decreased overall morbidity has actually been shown in former top-level and particularly endurance (but not ultra-endurance) athletes in comparison with matched controls who were healthy at young age as well as with the general population (67, 146). However, Knez et al. (2006) recently reviewed epidemiological studies demonstrating that although mortality usually declines with increasing physical activity, some of these studies including the follow-up of the Harvard Alumni Health Study (68) implicate a reversal of this trend in individuals with the highest activity-derived energy expenditure (61). Cumulative oxidative stress, a subsequent increased oxidation of plasma lipoproteins and a consequent hypothesized contribution to atherosclerosis (153) are proposed to be potential mechanisms that might offset the positive outcome

imparted by regular physical training (61). Similarly to this hypothesized link on ultra-endurance exercise, oxidative stress and cardiovascular health, Poulsen et al. suggested a U-shaped dose-response curve relationship between exercise and health particularly in the context of oxidative damage to DNA (122). Interestingly, these proposed models resemble the originally postulated relationship concerning exercise immunology (96). Meanwhile, the latter is supported by good evidence suggesting an enhancement of parts of the innate immune system through regular physical training and an attenuation of many components of immunity after prolonged strenuous exercise (35, 45, 74).

Nevertheless, experimental data regarding certain stress phenomena following acute bouts of ultra-endurance exercise are limited. Up to date, there are only a few studies where oxidative stress was examined explicitly in competing long-distance triathletes (34, 61, 75, 100) or ultra-marathon runners (79, 99), whereas results among these investigations are inconsistent. On the emerging topic of genome stability, recent studies reported increases in DNA damage in peripheral blood cells mid-race of an ultra-marathon run (80), after a marathon run (165) or a short-distance triathlon (42). However, to the best of my knowledge, DNA effects hitherto have not been investigated following an Ironman triathlon or an exercise bout of a comparable duration. Crucially, it remains to be answered whether potential training-induced adaptations in protective mechanisms such as the endogenous antioxidant defence system (61, 124, 129) are sufficient to prevent oxidative damage to bio-macromolecules and patho-physiological consequences. Furthermore, information on immunological alterations after exhaustive endurance exercise is also scarce, since up to now these post-exercise responses rarely have been followed longer than one day (39, 75, 79).

Therefore, the overall scientific concept and objectives of the *FWF*-funded project were two-fold: First, this project was aimed to get a broader picture of certain biochemical, physiological or molecular-biological stress responses in an exceptional large cohort of Ironman competitors (42 triathletes completed the study). Second, it was intended to quantify the resolution of recovery up to 19 days after the Ironman race, and, thus longer than any other study that investigated divergent stress phenomena following

ultra-endurance exercise, to verify whether there are indications of persistent detrimental health consequences.

With these studies a thorough insight was gained into exercise-induced oxidative (92), cardiac (63), muscular, inflammatory and immuno-endocrine stress responses (93) as well as effects on genome stability (94, 135, 136). In parallel, specific issues have been addressed within the scope of the current doctoral thesis. Additional focuses have been drawn on related nutritional and training-physiological aspects (e.g. nutritional strategies to minimize exercise-induced stress responses, practical implications concerning the length of time needed for recovery after an Ironman triathlon). Accordingly to the published articles as first author (92-94), these topics have been structured into three main chapters as follows:

- Oxidative Stress and Antioxidant Responses to an Ironman Triathlon
- Acute and Recovery Responses in Inflammatory, Immune-endocrine and Muscle Damage Parameters after an Ironman Triathlon
- Consequences of Inflammation, Oxidative Stress and Antioxidant-related Factors on the DNA Stability in Response to an Ironman Triathlon

# 2. Oxidative Stress and Antioxidant Responses to an Ironman Triathlon

Main parts of the following chapter have been previously reported and published in an original article in *Medicine & Science in Sports & Exercise*, which is attached in the appendix. This article is cited as: **Neubauer O, König D, Kern N, Nics L, and Wagner KH**. No Indications of Persistent Oxidative Stress in Response to an Ironman Triathlon. *Med Sci Sports Exerc* 40: 2119-2128, 2008.

Furthermore, data on this issue have been presented at several scientific meetings by the author:

- *12th Annual Congress of the European College of Sport Science (ECSS)*, July 11th – 14th 2007, Jyväskyla, Finland: **Neubauer O, Kern N, Nics L, Wagner KH**: How Ironman Triathletes Balance Oxidative Stress (oral presentation, abstract: Book of Abstracts)
- *International conference "Oxidative Stress in Diseases"*, Apr. 24th 2008, Bratislava, Slovakia: **Neubauer O, Kern N, Nics L, Reichhold S, Wagner KH**: Oxidative stress and antioxidant responses after an Ironman triathlon (oral presentation, abstract: Book of Abstracts)
- *1st symposium of the Vienna Research Platform of Nutritional and Food Sciences*, Apr. 25th 2008, University of Vienna, Austria: **Neubauer O, Kern N, Nics L, Reichhold S, Wagner KH**: Enhanced Antioxidant Capacity after an Ironman Triathlon (poster presentation, abstract: *Annals of Nutrition and Metabolism* 52, 2008: 131)
- *Free Radical Summer School of the Society of Free Radical Research (SFRR)-Europe,* Aug. 30th – Sep. 5th 2008, Spetses, Greece: **Neubauer O, Kern N, Nics L, Reichhold S, Wagner KH**: Oxidative Balance in Ironman Triathletes (poster presentation, abstract: abstract booklet)

## 2.1. Introduction

After more than two decades of research, it is well documented that exercise of extreme duration or intensity can induce the generation of reactive oxygen (and nitrogen) species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{\bullet}$), hypochlorous acid (HOCl) and nitric oxide ($NO^{\bullet}$ ) (23, 32, 47, 144). Potential mechanisms for increased ROS production during and after particularly strenuous aerobic exercise include: inadequate electron transfer through the mitochondrial respiratory chain, inflammatory processes (e.g. via oxidative burst reactions of phagocytic cells), variations in perfusion (such as transient ischemic and hypoxic conditions in certain regions of the body) that trigger activity of xanthine oxidase (a pathway that generates superoxide), and autoxidation of haem proteins (32, 47, 64, 144, 173). If the rate of the production of ROS exceeds the body ´s capacity to detoxify them, a disruption of the cellular redox balance occurs that is collectively called oxidative stress (108, 123, 173). Under this condition, as stated by Ji et al. (2008), "ROS seriously threaten structural and functional integrity at the cellular, organic and systemic levels" (55). However, researcher in this area are still challenged by questions about the patho-physiological as well as physiological relevance of an exercise-induced ROS formation (54, 128, 137, 144, 173). As originally postulated by Davies et al. in 1982 (23), evidence has increased that ROS are not only damaging agents, but actually (also) act as signalling molecules to initiate exercise adaptations (39, 54, 128, 144) and to regulate muscle function (47, 137, 173). Nevertheless, it remains to be shown whether training-induced adaptations in the endogenous antioxidant mechanisms (124, 129) are capable to prevent harmful oxidative damage or whether these defences are surpassed by accumulative oxidative stress induced by both long-term training for and participating in (many) acute ultra-endurance events. If the latter is the case, as indicated by Knez et al. (2006), the population of athletes engaged in this type of sports, might be at higher risk of developing atherosclerotic lesions (61). The assumption that regular ultra-endurance activity may be associated with an enhanced susceptibility to cardiovascular disease is based on the oxidative modification hypothesis of atherosclerosis. This model predicts that atherosclerosis represents a state of heightened oxidative stress in the vascular wall in which oxidation of low-density lipoproteins (LDL) is an early event contributing to further artherogenesis (153). Research has

actually shown that intensive endurance exercise such as a marathon can increase the susceptibility of LDL to oxidation (71). Consequently, continuing (and nowadays often year-long (84)) engagement in ultra-endurance exercise may result in persisting oxidative stress and, in turn, an increased lipoprotein oxidation.

However, information on probable sustained adverse effects of oxidative stress after exhaustive endurance exercise is incomplete since, up to date, in only few studies related responses were followed for at least more than one day into recovery (44, 71, 75, 79, 165). Moreover, it is important to note, that research in the area of exercise-induced oxidative stress has quite frequently lead to controversial results (16, 32, 173). Inconsistencies in this context most likely originates from the diversity of study designs and methodologies that are used to induce and measure oxidative stress (48, 61, 64). Outcomes among the few studies that have specifically focussed on participants of ultra-endurance events are contradictory (34, 61, 62, 75, 99, 100), to a certain extent, probably due to differences in the durations, intensities and types (e.g. only running versus the triathlon-specific combination of three disciplines) of the exercise performed. In addition, differences in the study participants especially concerning their training status and, consequently, in potential adaptations of the endogenous antioxidant defence system might further contribute to the discrepancies in findings between these investigations (61).

The overall objective of this part of the current investigations was to comprehensively quantify oxidative stress and antioxidant responses to an Ironman triathlon race. Of particular importance, these responses were monitored up to 19 days into recovery to elucidate whether there are indications of a delayed onset of oxidative stress, persistent oxidative damage and possible detrimental consequences on health (e.g. LDL oxidation). The prolonged monitoring period and the large cohort of study participants were two of the main strengths of the study since oxidative stress markers as well as anti-oxidant related factors until now have not been followed in such a wide-ranging manner. Furthermore, a specific attention was drawn on factors that might have contributed to recent inconstant outcomes among the studies that have examined oxidative stress after ultra-endurance exercise. A broader spectrum of different markers

of lipid peroxidation and protein oxidation was applied to particularize the damage on blood cell components and blood lipids and to overcome potential limitations and inconsistencies caused by the use of diverse analytical approaches. Moreover, we particularly focussed on potential effects of regular endurance training on the oxidant/antioxidant balance. We expected that results should enable us to develop the hypothesis that even small differences in training levels within a larger group of well-trained male athletes can affect oxidative stress and antioxidant responses to extremely demanding endurance exercise.

## 2.2. Materials and Methods

### 2.2.1. Subjects

The study population comprised 48 non-professional well-trained healthy male triathletes, who participated in the 2006 Ironman Austria. Forty-two out of 48 completed the study and were included in the statistical analysis. The triathletes were recruited from all over Austria half a year before the event. They were informed about the purpose and risks of the study before they provided written informed consent. The Ethics Committee of the Medical University of Vienna approved the study. The physiological characteristics of the subjects, data on their training and their performance in the Ironman triathlon are shown in Table 1.

### 2.2.2. Study design

All participants of the study were physically fit, free of acute or chronic illnesses, within a normal range of body mass index and non-smokers. Furthermore, they were not taking prescribed medication and avoided taking more than 100% of Recommended Dietary Allowance (RDA) of antioxidants in form of supplements (in addition to their normal dietary intake) in the 6 weeks before the race and until the final blood sampling 19 days (d) post-race. Subjects were required to complete a medical and health-screening, a food frequency, a supplementation questionnaire, 24 h dietary recalls before each blood sampling and they had to document their training in the six months prior to the Ironman triathlon and thereafter until the end of the study (Table 1). Blood samples were taken 2 d pre-race, immediately (within 20 min), 1 d, 5 d and 19 d post-race. Additionally, at all these time-points body composition was measured via bioelectric impedance analysis

(BIA) (15). The athletes abstained from intense exercise 48 h before spiroergometry testing and before each blood sampling (except the Ironman itself). Additionally, the subjects had fasted overnight before the 2 d pre-race, 5 d and 19 d post-race blood samples (which were all taken between 08:00 and 09:00 a.m.). On race day and 1 d post-race, they were allowed to drink and eat *ad libitum*, and the quantities of intake were recorded within the framework of a diploma thesis by Anna Chalopek (15). After the Ironman race the study participants performed "recovery" training that was of moderate intensity and duration until the end of the study (Table 1). During this recovery period the participants were required to abstain from any training that was above the lactate threshold (respectively, the concomitant heart frequency) of each individual.

### 2.2.3. Race Conditions

The Ironman triathlon took place in Klagenfurt, Austria on July 16th 2006 and consisted of 3.8 km swimming, followed by 180 km cycling and 42.2 km running. When the race started at 07:00 a.m. the air temperature and relative humidity were 15°C and 77%, with the lake temperature at 25°C. Between 04:00 p.m. and 05:00 p.m. respectively by finishing time (median time for subjects approximately 05:43 p.m.), air temperature reached a maximum and was 27.2°C, and relative humidity had decreased to 36% (data provided by the Carinthian Centre of the Austrian Central Institute for Meteorology and Geodynamics).

### 2.2.4. $VO_2$ peak testing protocol

The triathletes were tested three weeks before the race on a cycle ergometer (Sensormedics, Ergometrics 900). The maximal test protocol started at an initial intensity of 50 W, followed by 50 W increments every 3 min until exhaustion. During the test oxygen and carbon dioxide fractions (via Sensormedics 2900 Metabolic measurement cart), power output (PO), heart rate, and ventilation were recorded continuously. In addition, earlobe blood samples for the measurement of the lactate concentration were taken at the beginning and at the end of each stage to determine further performance parameters including the individual anaerobic threshold (IAT) (141).

**TABLE 1:** Characteristics of the study participants and their performance in the Ironman triathlon

| | |
|---|---|
| Age (yr) | 35.3 ± 7.0 |
| Height (cm) | 180.6 ± 5.6 |
| Weight (kg) | 75.1 ± 6.4 |
| BMI ($kg/m^2$) | 23.0 ± 1.2 |
| Percentage of body fat (%) | 11.8 ± 4.1 |
| Cycling $VO_{2\ peak}$ ($ml\ kg^{-1}\ min^{-1}$) | 56.6 ± 6.2 |
| Peak power output (PO) ($W_{peak}$) | 357.9 ± 50 |
| Relative peak PO ($W_{peak}$ /kg) | 4.8 ± 0.5 |
| Individual anaerobic threshold (IAT) (W) | 219.4 ± 43.5 |
| Relative IAT (W/kg) | 2.9 ± 0.5 |
| PO at 2 mmol blood lactate * $L^{-1}$ (W) | 193.6 ± 46.5 |
| Relative PO at 2 mmol blood lactate * $L^{-1}$ (W/kg) | 2.6 ± 0.5 |
| PO at 3 mmol blood lactate * $L^{-1}$ (W) | 237.1 ± 46.0 |
| Relative PO at 3 mmol blood lactate * $L^{-1}$ (W/kg) | 3.1 ± 0.6 |
| **Training over a period of six months prior to the Ironman triathlon:** | |
| Weekly net endurance exercise time (WNET) (h * $wk^{-1}$) | 10.7 ± 2.6 |
| Swim training (km * $wk^{-1}$) | 4.8 ± 2.2 |
| Cycle training (km * $wk^{-1}$) | 144.0 ± 52.1 |
| Run training (km * $wk^{-1}$) | 36.4 ± 10.6 |
| **Performance in the Ironman triathlon:** | |
| Total race time (h:min:sec) | 10:51:52 ± 01:01:22 |
| 3.8 km swim time (h:min:sec) | 01:09:51 ± 00:10:28 |
| 180 km cycle time (h:min:sec) | 05:28:21 ± 00:29:08 |
| 42.2 km run time (h:min:sec) | 04:08:26 ± 00:31:36 |
| **Training after completion of the Ironman until 19 d post-race:** | |
| WNET (h * $wk^{-1}$) | 4.2 ± 2.4 |
| Swim training (km * $wk^{-1}$) | 2.1 ± 2.2 |
| Cycle training (km * $wk^{-1}$) | 66.3 ± 53.8 |
| Run training (km * $wk^{-1}$) | 12.3 ± 11.5 |

Data are mean ± SD, N=42

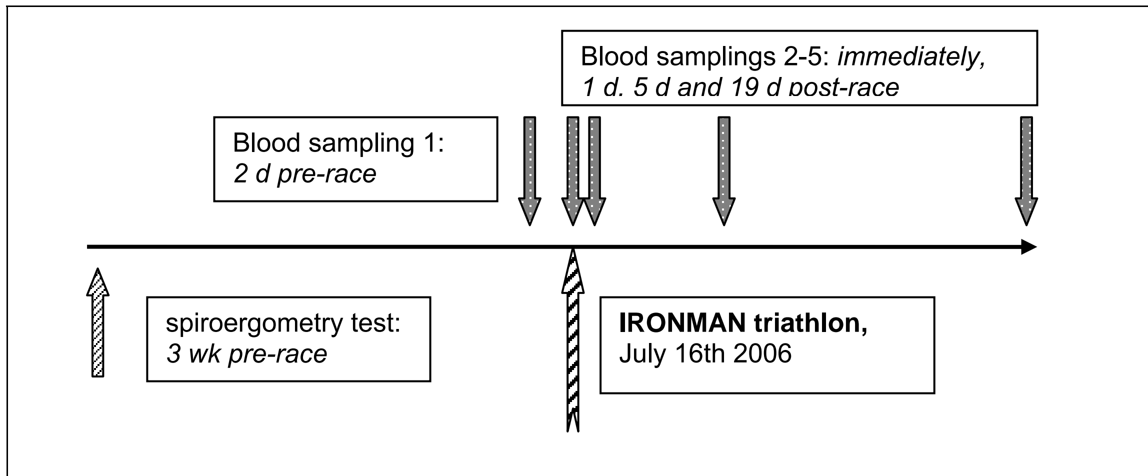



**FIGURE 1:** Study design

### 2.2.5. Blood sampling

At each blood sampling blood was collected using heparin, ethylenediamine-tetraacetic acid (EDTA) or serum vacutainers (Vacuette, Greiner, Austria). A field laboratory was installed at the race to ensure the appropriate collection of the first three blood samples. The blood was immediately cooled to 4°C and plasma or the serum separated at 1711 * g for 20 min at 4°C. Aliquots were immediately frozen at -80°C. Whole blood was taken for the haematological profile, and erythrocytes were also collected and frozen in aliquots at -80°C. All samples were analyzed within six months.

### 2.2.6. Haematological profile

The haematological profile was assessed with a MS4 Hematology 3-Part-Differential-Analyzer (Melet Schloesing Laboratories, Maria Enzersdorf, Austria). Exercise-induced percent changes in plasma volume were calculated (26) until 5 d post-race to assess expansion of plasma volume, which persists for 3 to 5 days following the cessation of demanding exercise (147). All results are reported adjusted for these changes, except for the plasma antioxidant capacity and ratios of oxidized LDL:LDL and Advanced Oxidation Protein Products (AOPP):total protein (TP). For these indices we used the data uncorrected for changes in plasma volume to consider their actual concentration to which the body responds. Correspondingly, “real” plasma concentrations of antioxidants (i.e. without adjusting for plasma volume changes) were used when potential associations with the plasma antioxidant capacity were examined. Mostly (and

otherwise reported), the adjustment for plasma volume changes had no significant effects on statistical calculations (e.g. when investigating differences between pre- and post-race values).

### 2.2.7. Plasma concentration of lipoproteins and biochemical variables

Plasma concentrations of total cholesterol, high-density lipoprotein (HDL), triglycerides, total protein (TP), total bilirubin and uric acid were measured using an automatic analyzer (Vitros DT 60 II module, Ortho-clinical Diagnostics, Germany) by Norbert Kern within the scope of a published diploma thesis (58) and by the author. Levels of very-low density lipoprotein (VLDL) and low-density lipoprotein (LDL) were calculated (VLDL= TG/2.2; LDL=TC – HDL – VLDL).

### 2.2.8. Plasma concentrations of markers of oxidative stress

Malondialdehyde (MDA) and conjugated dienes (CD) were both detected with high-performance liquid chromatography (HPLC) (pump, detector and integrator: Hitachi, column: Merck), as reported previously (132) within the framework of another published diploma thesis by Lukas Nics (95). Oxidized LDL (oxLDL) concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden). Advanced oxidation protein products (AOPP) were determined via a colorimetric assay kit (Immundiagnostik AG, Bensheim, Germany). For both, oxLDL and AOPP, absorbance of samples and standards were assessed with a Fluostar Optima microplate reader (BMG labtechnologies, Germany), and all measures were made in duplicate by the author.

### 2.2.9. Activities of antioxidant erythrocyte enzyme

Erythrocyte activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) were determined using methods reported previously (1, 10, 174) within the scope of a diploma thesis by Norbert Kern (58). Briefly, the principles of these methods were as follows: SOD activity was defined via its inhibition of the auto-oxidation of 1,2,3-trihydroxybenzol (pyrogallol) in the presence of superoxide anion ($O_2^{\cdot -}$), GSH-Px activity was defined in proportion to the oxidation of $NADPH_2$ to

$NADP^+$ and CAT activity was measured by the rate of breakdown of hydrogen peroxide ($H_2O_2$).

### 2.2.10. Total antioxidant capacity of plasma

Three approaches were applied to assess the total antioxidant capacity of plasma: the Trolox equivalent antioxidant capacity (TEAC), the ferric reducing ability of plasma (FRAP) and the oxygen radical absorbance capacity (ORAC) assay. Principally, the first two methods measure the ability of a substance to transfer one electron to reduce compounds like radicals or metals, while the ORAC assay is based on their ability to quench free radicals by hydrogen donation (60). Briefly, the TEAC assay is a spectrophotometric test, in which the 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical is oxidized by $ROO^•$ to a green-blue radical cation (83). The ability of antioxidants to delay the change of colour is expressed relative to Trolox (60). The test was performed as described previously (163). The FRAP assay was conducted within the framework of a published diploma thesis by Judit Valentini (169) according to Benzie and Strain (8) with modifications developed by the same authors (9). It depends upon the capacity of a substance to reduce the ferric tripyridyltriazine ($Fe^{3+}$-TPTZ) to the ferrous and intense blue coloured ($Fe^{2+}$-) TPTZ complex. Finally, the ORAC assay was measured within the scope of another diploma thesis by Barbara Stadlmayr (150) as described by Ou et al. (107) and Huang et al. (46). Thereby, 2,2´-azobis(2-amidinopropane)-dihydrochloride (AAPH) was used to generate peroxyl radicals. The principle of this test is based on the reaction of this peroxyl radical with a fluorescent probe. This reaction forms a non-fluorescent product, and it is delayed or inhibited by antioxidants in the biological sample (107, 126).

### 2.2.11. Plasma concentrations of nutritive antioxidants

Plasma concentrations of alpha- and gamma-tocopherol and carotenoids were detected with HPLC (Ultimate 3000, Dionex Cooperation) within the framework of the diploma thesis of Lukas Nics (95). Plasma vitamin C was analysed photometrically (95).

### 2.2.12. Data analysis

All statistical analyses were performed using SPSS 15.0 for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. The main effect of time was obtained by using the repeated measures analysis of variance (ANOVA). Then, all post-race values were compared with pre-race (= baseline) values by applying either paired t-tests (for normally distributed data) or Wilcoxon tests (for not normally distributed data) to assess time-point dependent differences in the test variables. To examine any significant relationships, Pearson ´s (for normally distributed data) or Spearman ´s correlations (for not normally distributed data) were used. Furthermore, subjects were divided into groups (percentiles) by exercise test variables including the relative IAT or the relative power output at $VO_{2\ peak}$. Subsequently, one-factorial ANOVA and *post hoc* analyses with Bonferroni ´s test were applied to assess whether differences in oxidative stress and antioxidant-associated variables were associated with this group distribution. In addition to the data analysis reported in the respective publication (92), further relationships between oxidative stress on the one hand and inflammatory responses, muscle damage (reported elsewhere (93); see also chapter 3) and plasma concentrations of nutritive antioxidants on the other were examined that are exclusively presented within the present doctoral thesis. Accordingly to the statistical procedure mentioned above, in the case of observed trends or significant correlations, subjects were divided into percentile groups by associated variables such as the the pre- to post-race change in the high-sensitive C-reactive protein. Again, one-factorial ANOVA analysis followed by Bonferroni ´s *post hoc* test was then used for multiple comparisons between these groups. Significance was set at a $P$ value of $<0.05$ and is reported as $P<0.05$, $P<0.01$ and $P<0.001$.

## 2.3. Results

### 2.3.1. Race results

The average completion time of the Ironman triathlon was 10 h 52 min ± 1 h 1 min (mean ± SD; Table 1). The estimated average antioxidant intake during the race was 393 ± 219 mg vitamin C and 113 ± 59 mg α-tocopherol. There were no differences in the amount of the consumed antioxidants between the groups divided by exercise test variables. Out of 48, three study participants failed to complete the race because of self-

reported fatigue. In addition, three subjects could not participate in one or more blood sample time points and thus were also excluded from the analysis.

### 2.3.2. Plasma concentrations of lipoproteins and biochemical variables

Plasma concentrations of lipoproteins, total bilirubin, TP and uric acid are shown in Table 2. LDL decreased significantly (P<0.001) immediately post-race (-15%) and 1 d post-race (-26%), and stabilized below pre-race concentrations until 5 d post-race (-8%; P<0.01). Total cholesterol significantly decreased to below pre-race values 1 d (-10%; P<0.001) and 5 d after the race (-4%; P<0.01), whereas HDL increased immediately (+9%; P<0.05) and 1 d post-race (+12%; P<0.01). VLDL and triglycerides significantly (P<0.001) increased post-race (+75 and +69%, respectively). Plasma levels of uric acid significantly (P<0.001) increased immediately after the triathlon (+49%). Thereafter, uric acid concentrations gradually declined, but remained significantly (P<0.001) elevated at all time-points (P=0.001 for 19 d post-race) versus pre-race values. Plasma total bilirubin was significantly (P<0.001) higher directly after the race (+39%) than pre-race, and increased further 1 d post-race (+65%). Subsequently, it significantly (P<0.001) declined below pre-race concentrations. Plasma TP increased immediately after the Ironman race (+4%; P<0.01), whereas there was a significant decrease to below pre-race thereafter (Table 2).

### 2.3.3. Plasma concentrations of markers of oxidative stress

Plasma concentrations of oxidative stress markers can be found in Table 3. The time-courses in CD, and the ratios of oxLDL:LDL and AOPP:TP are demonstrated in Figure 2. A considerable (+91%) and significant increase in CD occurred immediately after the Ironman triathlon (P<0.001). CD remained significantly elevated 1 d post-race (+13%; P<0.01) when compared with pre-race values. MDA trended to increase immediately after the race (+7%; P=0.06), then increased further and reached statistical significance 1 d post-race (+9%; P<0.01) (after correcting the values for the increase in plasma volume at this time-point). There was a significant decrease in oxidized low-density lipoprotein (oxLDL) below pre-race values immediately post-race (-13%; P<0.05) and 1 d post-race (-24%; P<0.01), whereas a tendency towards an increase in the oxLDL:LDL ratio 1 d post-race (+8%; P=0.07) occurred (Figure 2). Plasma AOPP concentrations

significantly (P<0.001) increased by 25% immediately post-race and remained significantly (P=0.01) elevated 1 d after the competition (+21%). Similarly, the AOPP:TP ratio peaked by 20% higher than pre-race immediately post-race (P<0.01), and remained significantly (P<0.05) elevated by 16% higher than pre-race values 1 d after the race (Figure 2). All markers of oxidative stress had returned to pre-race values 5 d after the Ironman triathlon and 19 d post-race all parameters were still similar to pre-race concentrations (Table 3).

### 2.3.4. Activities of antioxidant erythrocyte enzymes

Antioxidant enzyme activities can be found in Table 4. There was a significant decrease in the activities of erythrocyte SOD (-6%; P<0.001) and CAT (-4%; P<0.05) immediately post-race. There was a trend towards decreased GSH-Px activity 1 d post-race (-4%; P=0.08), but no significant changes during the monitoring period. SOD and CAT both followed a biphasic pattern during the recovery period and 19 d post-race, athletes had moderate, but significant decreases in the activities of SOD and CAT compared to pre-race values (-5%; P<0.001 and -6%; P<0.01, respectively) (Table 4) (58).

### 2.3.5. Antioxidant capacity of plasma

The time-course of the total plasma antioxidant capacity, as assessed by TEAC, FRAP (and ORAC, is shown in Figure 3. Similar time-courses were observed for all these assays. A sharp elevation of TEAC was observed in response to the Ironman triathlon (+48%; P<0.001), and values remained significantly (P<0.001) higher than pre-race until 1 d after the race (+25%). Five and 19 d post-race, TEAC values were similar to pre-race. FRAP significantly increased immediately post-race (+35%; P<0.001), remained significantly elevated 1 d post-race (+24%; P<0.001) and decreased to baseline 5 d post-race (169). ORAC peaked significantly immediately after the race (+18%; P<0.001), remained elevated 1 d post-race (+11%; P<0.05) and declined back to baseline levels 5 d after the Ironman race (150).

**TABLE 2:** Plasma values of biochemical variables.

| | PRE | POST | 1 d POST | 5 d POST | 19 d POST | Time effect (*P*) |
|---|---|---|---|---|---|---|
| Uric acid (µmol * L $^{-1}$) | 311 ± 54 | 465 ± 92 *** | 422 ± 63 *** | 338 ± 62 *** | 328 ± 53 *** | <0.001 |
| Total bilirubin (µmol * L $^{-1}$) | 9.73 ± 5.3 | 13.57 ± 5.4 *** | 16.04 ± 8.0 *** | 6.46 ± 3.8 *** | 9.25 ± 5.5 | <0.001 |
| Total protein (g * L $^{-1}$) | 7.6 ± 0.3 | 7.9 ± 0.7 ** | 7.0 ± 0.4 *** | 7.0 ± 0.6 *** | 7.1 ± 0.5 *** | <0.001 |
| Tot. cholesterol (mmol * L $^{-1}$) | 5.1 ± 0.9 | 5.1 ± 1.0 | 4.2 ± 0.8 *** | 4.7 ± 0.8 *** | 5.1 ± 0.9 | <0.001 |
| HDL (mmol * L $^{-1}$) | 1.9 ± 0.4 | 2.0 ± 0.4 * | 1.9 ± 0.4 | 1.8 ± 0.5 | 1.7 ± 0.4 * | <0.001 |
| LDL (mmol * L $^{-1}$) | 2.8 ± 0.8 | 2.4 ± 0.8 *** | 1.9 ± 0.7 *** | 2.5 ± 0.6 *** | 2.9 ± 0.8 | <0.001 |
| VLDL (mmol * L $^{-1}$) | 0.4 ± 0.2 | 0.7 ± 0.3 * | 0.4 ± 0.3 | 0.4 ± 0.2 | 0.4 ± 0.2 | <0.001 |
| Triglycerides (mmol * L $^{-1}$) | 0.9 ± 0.4 | 1.6 ± 0.6 *** | 0.9 ± 0.6 | 0.9 ± 0.4 | 0.9 ± 0.4 | <0.001 |

Values are mean ± SD; N=42; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; PRE, 2 d pre-race; POST, immediately post-race; 1 d POST, 1 d post-race; 5 d POST, 5 d post-race; 19 d POST, 19 d post-race; * significantly different from pre-race values, P<0.05; ** significantly different from pre-race values, P<0.01; *** significantly different from pre-race values, P<0.001

**TABLE 3:** Plasma values of oxidative stress markers.

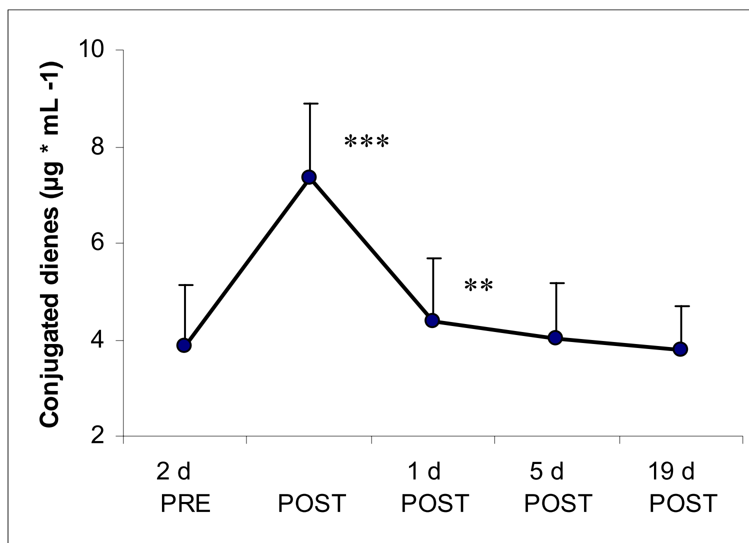
| | PRE | POST | 1 d POST | 5 d POST | 19 d POST | Time effect (*P*) |
|---|---|---|---|---|---|---|
| CD (µg * mL $^{-1}$) | 3.85 ± 1.27 | 7.35 ± 1.54 *** | 4.36 ± 1.30 ** | 4.03 ± 1.16 | 3.78 ± 0.91 | <0.001 |
| MDA (µmol * L $^{-1}$) | 2.39 ± 0.49 | 2.56 ± 0.67 | 2.60 ± 0.73 ** | 2.39 ± 0.68 | 2.42 ± 0.78 | 0.092 |
| OxLDL (U * L $^{-1}$) | 35.8 ±13.7 | 31.3 ± 12.4 * | 29.3 ± 13.6 ** | 32.3 ± 11.3 | 36.9 ± 13.6 | <0.001 |
| OxLDL:LDL (U * mmol * L$^{-1}$) | 13.3 ± 5.3 | 13.8 ± 6.2 | 14.4 ± 6.1 | 12.1 ± 5.2 | 13.1 ± 4.9 | 0.363 |
| AOPP (µmol * L $^{-1}$) | 39.8 ± 8.4 | 49.9 ± 14.8 *** | 48.1 ± 17.1 ** | 39.9 ± 6.7 | 41.7 ± 8.9 | <0.001 |
| AOPP:TP (µmol * g * dL$^{-1}$) | 5.28 ± 1.04 | 6.34 ± 1.88 ** | 6.12 ± 1.97 * | 5.47 ± 1.05 | 5.91 ± 1.45 | 0.005 |

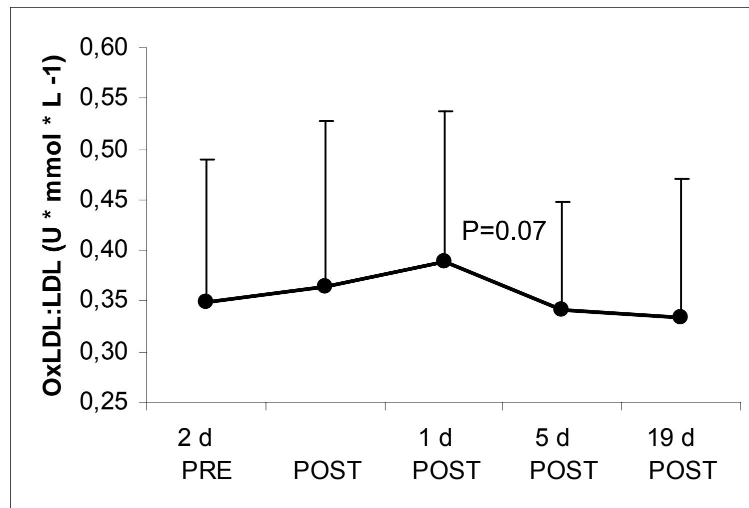
Values are mean ± SD; N=42; CD, conjugated dienes; MDA, malondialdehyde; oxLDL, oxidised LDL; AOPP, advanced oxidation protein products; TP, total protein; * significantly different from pre-race values, P<0.05; ** significantly different from pre-race values, P<0.01; *** significantly different from pre-race values, P<0.001.

**TABLE 4:** Plasma values of antioxidant erythrocyte enzymes.

| | PRE | POST | 1 d POST | 5 d POST | 19 d POST | Time effect (*P*) |
|---|---|---|---|---|---|---|
| SOD (IU * g $^{-1}$ Hb) | 1813 ± 257 | 1695 ± 156 *** | 1821 ± 194 | 1769 ± 177 *** | 1722 ± 159 *** | <0.001 |
| GSH-Px (IU * g $^{-1}$ Hb) | 22.5 ± 9.9 | 22.3 ± 9.7 | 21.7 ± 9.2 | 22.9 ± 9.4 | 22.0 ± 9.5 | 0.110 |
| CAT (IU * g $^{-1}$ Hb) | 282 ± 59 | 271 ± 56 * | 265 ± 55 *** | 273 ± 57 | 266 ± 47 ** | 0.036 |

Values are mean ± SD; N=42; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; PRE, 2 d pre-race; POST, immediately post-race; 1 d POST, 1 d post-race; 5 d POST, 5 d post-race; 19 d POST, 19 d post-race; * significantly different from pre-race values, P<0.05; ** significantly different from pre-race values, P<0.01; *** significantly different from pre-race values, P<0.001.



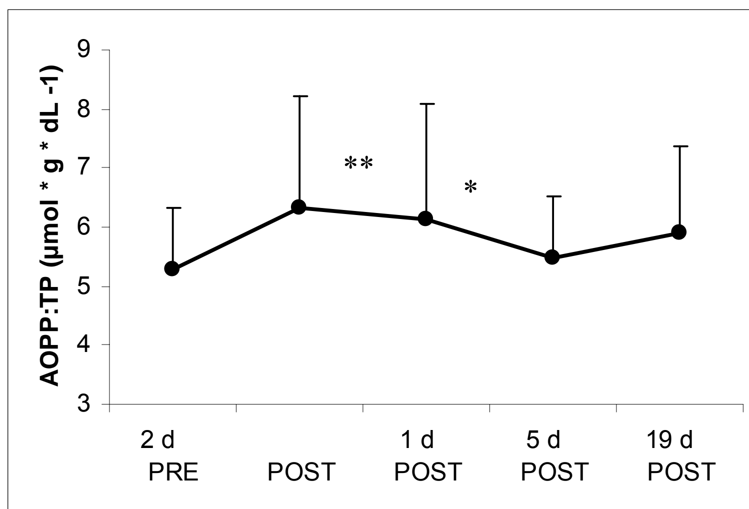




**FIGURE 2**: Changes in lipid peroxidation and protein oxidation as assessed by conjugated dienes (CD) (95), oxidised (ox) LDL:LDL ratio and Advanced Oxidation Protein Products (AOPP):total protein (TP) ratio 2 d pre-race (PRE), immediately, 1, 5 and 19 d post-race (POST). Data are mean ± SD; N = 42; *, **, ***, Change was significantly different from pre-race values, P<0.05, P<0.01, and P<0.001, respectively.

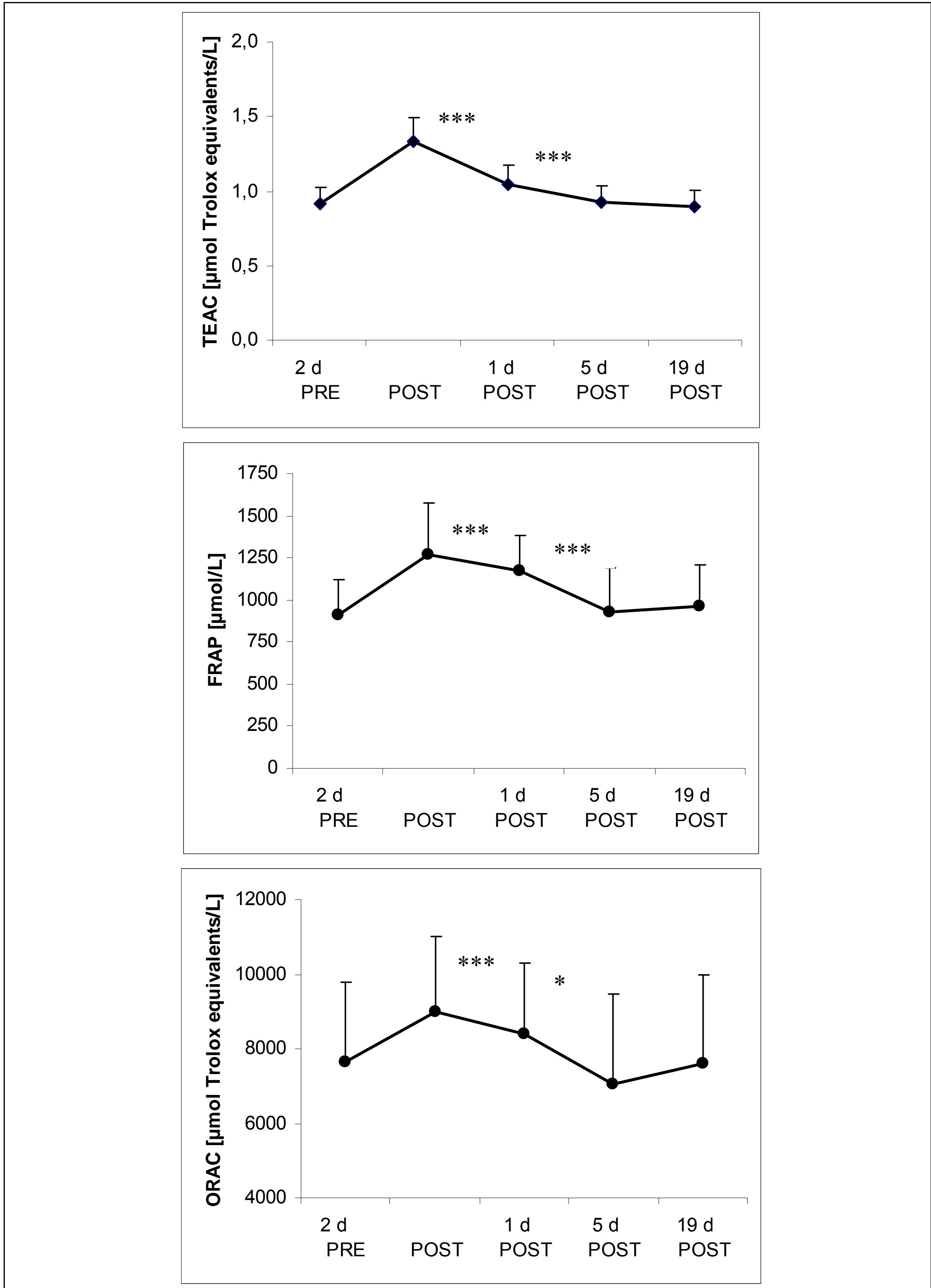



**FIGURE 3:** Time-course of the total plasma antioxidant capacity as assessed by the Trolox Equivalent Antioxidant (TEAC), the Ferric reducing ability of plasma (FRAP) (95) and the oxygen radical absorbance capacity (ORAC) assay (150) 2 d pre-race (PRE), immediately, 1, 5 and 19 d post-race (POST). Data are mean ± SD; N = 42; *, **, ***, Change was significantly different from pre-race values, $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

### 2.3.6. Plasma concentrations of nutritive antioxidants

The time-courses of the plasma concentrations of vitamin C, α-tocopherol, γ-tocopherol and carotenoids are summarised in Table 5. There was an immediate significant increase in response to the Ironman triathlon in both vitamin C and α-tocopherol (+54% and +18%, respectively; for both $P<0.01$). One day post-race vitamin C and α-tocopherol concentrations decreased to values similar to pre-race, whereas the following antioxidant nutrients significantly dropped to below pre-race concentrations: γ-tocopherol (-25%; $P<0.01$), lutein/zeaxanthin (-15%; $P<0.05$), cryptoxanthin (-8%; $P<0.05$) and β-carotene (-15%; $P<0.01$). Five days post-race, most nutritive antioxidant concentrations were similar to pre-race values, except for vitamin C, which was above pre-race (+21%; $P<0.01$).

### 2.3.7. Associations with training- and exercise test variables, performance and oxidative stress markers

Various significant negative correlations were obtained between parameters of lipid peroxidation and training- and exercise test variables. The most important of these associations are shown in Table 6. In addition, Figure 4 reflects the inverse correlation between the MDA concentration post-race and the relative peak power output. In contrast, the pre-race oxLDL:LDL ratio correlated positively with the weekly net endurance exercise time ($r=0.42$; $P<0.01$). Significant positive correlations were observed between post-race indices of protein oxidation and some exercise test variables, while changes in AOPP immediately after the Ironman race were inversely related with the total race time ($r= -0.30$; $P=0.053$) (Table 6). Additionally, triathlon-induced alterations in AOPP from pre- to post-race as well as from pre- to 1 d post-race correlated negatively with the cycle split time ($r= -0.35$, and $r= -0.38$, respectively; for both $P<0.05$).

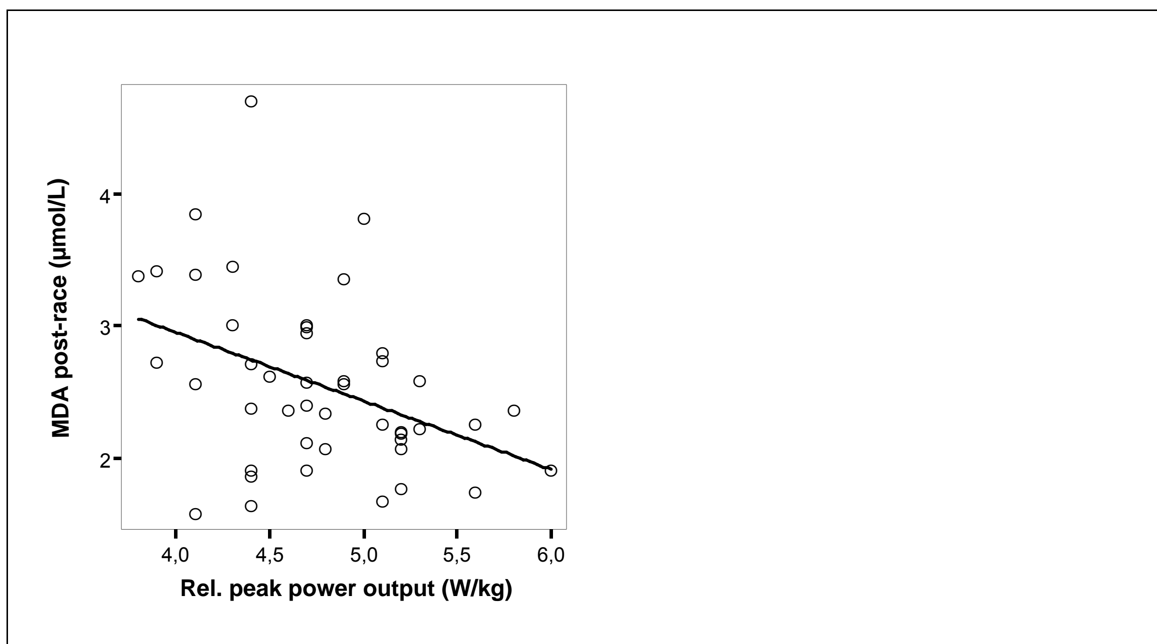



**FIGURE 4**: Inverse correlation between the malondialdehyde (MDA) plasma concentration immediately post-race and the relative peak power output assessed 3 weeks before the Ironman race (r= -0.40; P<0.01).

### 2.3.8. Associations with training- and exercise test variables, performance and antioxidant-related factors

There were multiple positive correlations with levels and changes in the total antioxidant capacity (assessed via the TEAC and FRAP assay) on the one side and pre-race training- and exercise test variables on the other. The most important of these correlations are summarized in Table 7. Exemplary, the change of TEAC from pre- to immediately post-race correlated with the percentage of maximum power output at 3 mmol * $L^{-1}$ blood lactate (r=0.56; P<0.001), which is shown Figure 5. In addition, exercise-induced changes in TEAC, uric acid and post-race FRAP levels correlated negatively with the total race time (r= -0.44, r= -0.48, and r= -0.42, respectively; all P<0.01). Significant positive correlations were observed between activities of erythrocyte GSH-Px with TEAC (Table 7). Furthermore, GSH-Px activities correlated positively with the percentage of maximum power output at 3 mmol * $L^{-1}$ blood lactate at pre-race (r=0.35; P<0.05), 1 d post-race (r=0.39; P<0.01) and 19 d post-race (r=0.36; P<0.05).

**TABLE 5:** Plasma concentrations of nutritive antioxidants.

| | PRE | POST | 1 d POST | 5 d POST | 19 d POST | Time effect (*P*) |
|---|---|---|---|---|---|---|
| Vitamin C (µmol * L $^{-1}$) | 66.6 ± 13.0 | 102.8 ± 26.6 ** | 68.2 ± 13.2 | 80.9 ± 15.2 ** | 82.8 ± 14.0 ** | <0.001 |
| α-tocopherol (µmol * L $^{-1}$) | 22.6 ± 7.3 | 26.6 ± 7.0 ** | 21.8 ± 6.1 | 22.5 ± 5.8 | 21.8 ± 5.8 | <0.001 |
| γ-tocopherol (µmol * L $^{-1}$) | 1.29 ± 0.67 | 1.22 ± 0.60 | 0.97 ± 0.38 ** | 1.29 ± 0.59 | 1.29 ± 0.48 | <0.01 |
| β-carotene (µmol * L $^{-1}$) | 0.88 ± 0.36 | 0.88 ± 0.36 | 0.75 ± 0.31 ** | 0.85 ± 0.36 | 0.84 ± 0.34 | 0.001 |
| Lutein/zeaxanthin (µmol * L $^{-1}$) | 0.45 ± 0.15 | 0.45 ± 0.15 | 0.40 ± 0.13 * | 0.42 ± 0.16 | 0.40 ± 01.5 | <0.05 |
| Cryptoxanthin (µmol * L $^{-1}$) | 0.12 ± 0.07 | 0.11 ± 0.06 * | 0.11 ± 0.06 * | 0.12 ± 0.06 | 0.13 ± 0.08 | <0.01 |
| Lycopene (µmol * L $^{-1}$) | 0.24 ± 0.13 | 0.26 ± 0.12 | 0.22 ± 0.10 | 0.25 ± 0.10 | 0.24 ± 0.10 | 0.001 |

Values are mean ± SD; N=42; PRE, 2 d pre-race; POST, immediately post-race; 1 d POST, 1 d post-race; 5 d POST, 5 d post-race; 19 d POST, 19 d post-race; * significantly different from pre-race values, P<0.05; ** significantly different from pre-race values, P<0.01; *** significantly different from pre-race values, P<0.001.

**TABLE 6:** Significant associations of oxidative stress markers with training- and exercise test variables.

| | $VO_{2\,peak}$ | rel. $W_{peak}$ | IAT | rel. IAT | PO La 2 | rel. PO La 2 | PO La 3 | rel. PO La 3 | rel. PO La 3 $\%W_{peak}$ | WNET | Cycle training |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **CD** *1 d POST* | | | | | −0.33 * | −0.37 * | −0.36 * | −0.46 ** | | | |
| *Δ PRE- to 1 d POST* | | −0.31 * | | | | | | | | | |
| **MDA** *PRE* | | | | | | | | | | −0.38 * | −0.37 * |
| *POST* | −0.30 * | −0.40 ** | | | | | | | | | −0.35 * |
| *Δ PRE- to POST* | | −0.34 * | | | | | | | | | |
| *1 d POST* | | −0.32 * | | | | | | | | −0.34 * | −0.39 * |
| *Δ PRE- to 1 d POST* | | −0.32 * | | | | | | | | | |
| **oxLDL** *PRE* | | −0.30 * | | −0.29 * | −0.35 * | −0.37 * | −0.34 * | −0.36 * | | | |
| *POST* | | −0.37 * | | −0.30 * | | | | | | | |
| *1 d POST* | | | | | −0.33 * | −0.37 * | | | | | |
| **oxLDL:LDL** *PRE* | | | | | | | | | | 0.42 ** | 0.33 * |
| **AOPP** *POST* | | | 0.31 * | 0.33 * | | | 0.31 * | | 0.38 ** | | |
| *Δ PRE- to POST* | | | | 0.34 * | 0.34 * | 0.39 * | 0.34 * | 0.37 * | 0.35 * | | |
| *1 d POST* | | | | | | | | | | | |
| *Δ PRE- to 1 d POST* | | | | | | | | | | | |
| **AOPP:total protein** *POST* | | | 0.36 * | 0.31 * | 0.32 * | | 0.38 * | | 0.44 ** | | |
| *Δ PRE- to POST* | | | | 0.33 * | 0.33 * | 0.39 ** | 0.33 * | 0.36 * | 0.32 * | | |

CD, conjugated dienes; MDA, malondialdehyde; oxLDL, oxidised LDL; AOPP, advanced oxidation protein products; $W_{peak}$, peak power output; rel., relative; IAS, individual anaerobic threshold; PO La 2 (3), power output at 2 (3) mmol blood lactate * $L^{-1}$; WNET, weekly net endurance exercise time; PRE, 2 d pre-race; POST, immediately post-race; 1 d POST, 1 d post-race; Δ PRE to (1 d) POST, change from pre- to immediately (1 d) post-race; * P<0.05; ** P<0.01.

**TABLE 7:** Significant associations with total plasma antioxidant capacity.

| | $VO_{2\,peak}$ | $W_{peak}$ | rel. $W_{peak}$ | IAT | rel. IAT | rel. PO La 3 $\%W_{peak}$ | Total race time | 180 km cycle time | 42.2 km run time | Uric acid | Total bilirubin | Total protein | Vitamin C | GSH-Px |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***TEAC*** *PRE* | | | | | | | | | | | | | | 0.41 ** |
| *POST* | 0.33 * | 0.30 * | 0.39 ** | 0.34 * | 0.36 * | 0.42 ** | −0.31 * | | | | | | | 0.37 ** |
| *Δ PRE to POST* | 0.38 * | 0.41 ** | 0.47*** | 0.45 ** | 0.49*** | 0.56*** | −0.44** | −0.40** | −0.35** | 0.54 *** | | | 0.33 * | |
| *1 d POST* | | | | | | | | | | | | | | 0.40 ** |
| *Δ PRE to 1 d POST* | | | | 0.34 * | 0.31 * | 0.33 * | | | | | | | | |
| *5 d POST* | | | | | | | | | | | | | | 0.37 * |
| *19 d POST* | | | | | | | | | | | | | | 0.47 ** |
| ***FRAP*** *POST* | | 0.35 * | | 0.37 * | | | −0.42 ** | | | | | | | |
| *Δ PRE to POST* | | | | | | | | | | 0.73 *** | 0.42 ** | | | |
| *5 d POST* | | 0.36 * | | 0.41 ** | 0.35 * | | | | | | | | | |
| ***ORAC*** *PRE* | | | | | | | | | | | | 0.44 ** | | |
| *POST* | | | | | | | | | | | | 0.46 ** | | |
| *Δ PRE to POST* | | | | | | | | | | 0.36 * | | | | |
| *5 d POST* | | | | | | | | | | | | 0.39 * | | |

TEAC, Trolox equivalent antioxidant capacity; FRAP, Ferric Reducing Ability of Plasma; ORAC, Oxygen radical absorbance capacity; $W_{peak}$, peak power output; rel., relative; IAT, individual anaerobic threshold; rel. PO La 3 % $W_{peak}$, relative power output at 3 mmol blood lactate * $L^{-1}$ as percentage of peak power output; PRE, 2 d pre-race; POST, immediately post-race; 1 (5) [19] d POST, 1 d (5) [19] post-race; Δ PRE to POST, change from pre- to immediately post-race; Δ PRE to 1 d POST, change from pre- to 1 d post-race; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

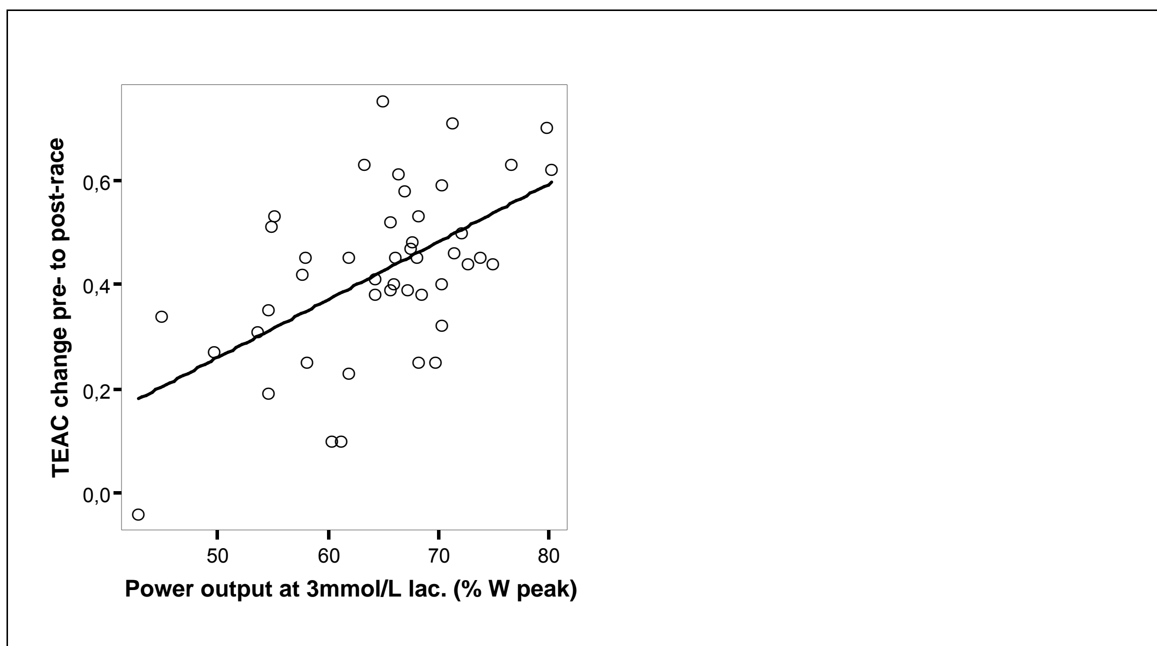



**FIGURE 5:** Correlation between the pre- to post-race change in the plasma antioxidant capacity detected via the Trolox Equivalent Antioxidant Capacity (TEAC) assay and the power output at a blood lactate concentration of 3 mmol/L (described as percentage of the peak power output) assessed 3 weeks before the Ironman race (r=0.56; P<0.001).

### 2.3.9. Groups divided by the relative power output at $VO_{2\,peak}$ and the relative IAT: effects on LDL oxidation and plasma antioxidant capacity

Based on the group distribution into percentiles by the relative power output at $VO_{2\,peak}$, a trend was observed insofar as lower oxLDL concentrations immediately post-race were associated with higher levels in relative power output at $VO_{2\,peak}$ (differences between all groups: P=0.056). Furthermore, athletes in the group with the highest relative power output at $VO_{2\,peak}$ (top percentile) had significantly (P<0.05) lower oxLDL concentrations immediately post-race than those athletes in the group with the lowest relative power output at $VO_{2\,peak}$ (lowest percentile) and an according trend was noted with pre-race oxLDL concentrations (P=0.059). TEAC increased with the relative IAT across the percentiles and the differences between all groups were P=0.018. Moreover, the TEAC response was significantly (P<0.05) higher in the subject group with the highest relative IAT (top percentile) compared with the group with the lowest IAT (lowest percentile).

### 2.3.10. Associations with nutritive and endogenous antioxidants and plasma antioxidant capacity

The associations with the total plasma antioxidant capacity, as detected by different assays, are summarised in Table 7. Positive correlations were observed between the pre- to post-race changes in uric acid on the one side and TEAC ($r=0.54$; $P<0.001$), FRAP ($r=0.73$; and $P<0.001$) and ORAC ($r=0.36$; $P<0.05$) on the other. Furthermore, correlations were found between pre- to post-race changes in TEAC and in vitamin C ($r=0.33$; $P<0.05$), pre- to post-race changes in FRAP and in total bilirubin ($r= 0.42$; $P<0.01$), and pre- and post-race ORAC levels with total protein (Table 7). No links were found between antioxidant and oxidative stress responses.

### 2.3.11. Associations with inflammatory, muscular and oxidative stress responses

Significant correlations were observed between post-race concentrations of AOPP and myoglobin ($r=0.47$; $P=0.001$), and between 1 d post-race levels of the AOPP:TP ratio and interleukin (IL)-6 ($r=0.47$; $P<0.01$) (Figure 6). Furthermore, both high-sensitive C-reactive protein (hs-CRP) immediately and 1 d post-race ($r=0.39$; $P=0.01$, and $r=0.46$; $P<0.01$, respectively) correlated with concentrations of CD 1 d post-race. In addition, based on the group distribution into percentiles by pre- to post-race responses in hs-CRP, we observed that athletes in the group with the highest hs-CRP responses (top percentile) had significantly higher increases in CD from pre- to 1 d post-race than the subjects in the group with the lowest hs-CRP alterations (lowest percentile) ($P<0.05$).

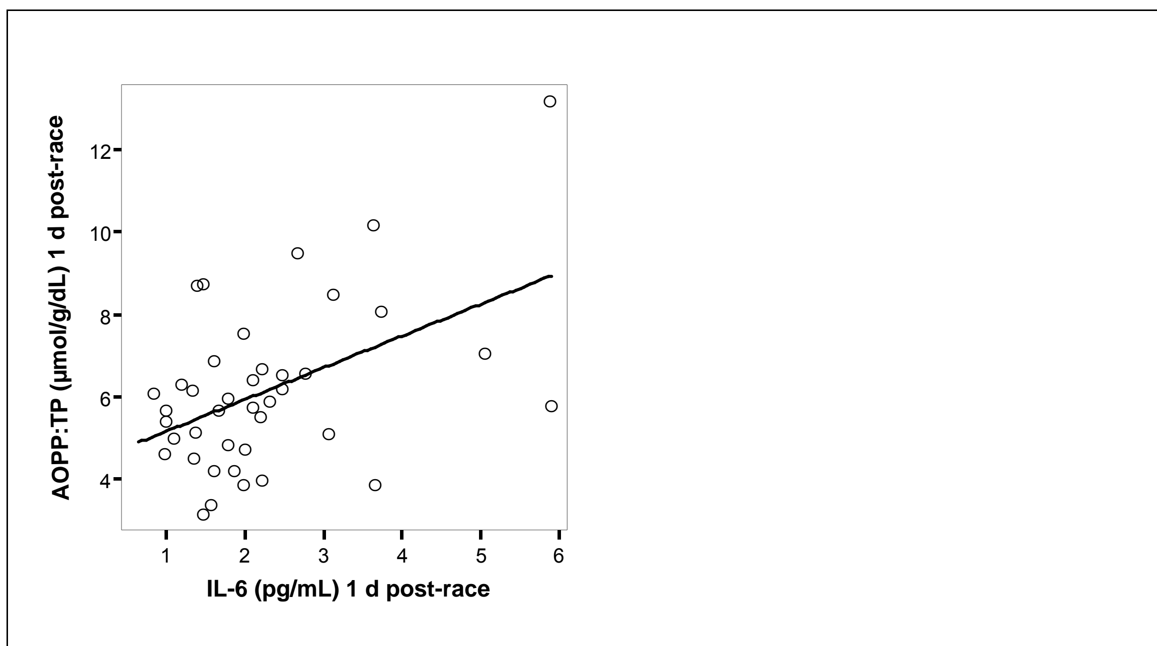



**FIGURE 6**: Correlation between the Advanced Oxidation Protein Products:total plasma protein (AOPP:TP) ratio and plasma concentration of interleukin-6 (IL-6) 1 d post-race ($r=0.47$; $P<0.01$).

## 2.4. Discussion

A key finding of the present study was that there are no indications of persistent oxidative damage after a single bout of ultra-endurance exercise, despite a transient increase in most, but not all oxidative stress markers. The current results suggest that training- and acute exercise-induced antioxidant responses in well-trained athletes were capable to counteract severe or persistent oxidative damage to cell compounds and blood lipids following exhaustive endurance exercise. Thereby, acute exercise-induced alterations in the plasma antioxidant capacity of the athletes appear to play a major role, and these changes were related with a variety of physiological training-related determinants. Considering the current concerns about health consequences for ultra-endurance athletes, these findings provide important and novel information since oxidative stress and recovery responses after ultra-endurance exercise, up to date, have not been followed for such a long time-course.

### 2.4.1. Differences in the responses of divergent oxidative stress markers: a potential explanation for inconsistencies among previous studies and an indication for various ROS-generating mechanisms

In most, but not all previous studies on this topic increased levels of various indices of lipid peroxidation such as MDA (62), lipid hydroperoxides (99), or $F_2$-isoprostanes (79, 99, 100) were found after long-distance triathlons (62, 100), an 80-km race (99), and another 50-km ultra-marathon (79). On the contrary, no changes in methemoglobin, disulfide glutathione, and thiobarbituric acid reactive substances (TBARS) (75) or even a decline in the susceptibility of plasma lipids to peroxidation, as measured by CD (34), were reported following other long-distance triathlon races. As mentioned previously, inconsistencies among these studies may at least partly be explained by the use of different methods. Therefore, a brief consideration of the limitations of determination of biomarkers for oxidative stress is warranted since some of the quite frequently used methods are carried out under un-physiological conditions and are prone to artefact formation (60). Among these, above all the TBARS assay has been criticized for insufficient accuracy, specificity and validity (48, 61, 64). Although one of the most common methods to measure MDA as a stable by-product of the oxidation of polyunsaturated fatty acids (PUFAs), there are a number of drawbacks with this assay including the sample preparation as well as the reactivity of thiobarbituric acid with substances other than MDA (60). However, both sensitivity and reproducibility of the measurement of MDA can be arguably increased by detection using HPLC (60-62), which has been conducted in the present investigation. In addition, CD were measured as this method is considered as a specific marker for the initial phase of lipid peroxidation (32, 60, 168, 170). Noteworthy, the study is the first in which attention is drawn on the effects of ultra-endurance exercise on oxLDL and AOPP. Both parameters are referred as novel and reliable biomarkers that indicate long-term effects of oxidative stress (25, 120) such as following a high volume training period. AOPPs comprise predominantly albumin and its oxidized aggregates, which are mainly formed by chlorinated oxidants generated by the enzyme myeloperoxidase (MPO). Therefore, AOPPs are also seen as markers of neutrophil activation and, moreover, as an independent risk factor for cardiovascular disease (56, 60). Using the same method as in the present work, oxLDL levels were analysed in a recent study with the objective to

characterise cardiovascular risk factors in former athletes more sensitive than with traditional biochemical parameters such as LDL cholesterol (120).

The assumption that disparity in results reported in the literature also originates from the application of different methods is supported by the finding that there were different amplitudes and kinetics of diverse oxidative stress markers in the current investigation. There was an immediate and marked rise in CD (followed by a rapid decline) whereas MDA increased only slightly immediately after race completion, but rose to significant levels 1 d after the Ironman triathlon. The differences in the changes of these indices might at least partly be explained insofar CD are primary oxidation products formed during initial reactions of lipid peroxidation (conjugation of double bounds of PUFAs), while MDA is produced at a latter stage of the lipid peroxidation chain reactions (32, 168). Our data also suggest that enhanced post-race antioxidant defences (described later in detail) might have played a role in preventing a more pronounced rise in MDA. Importantly, oxLDL even significantly decreased below pre-race values after the race. This change is most likely a consequence of an enhanced lipoprotein metabolism and the decline of LDL cholesterol itself as demonstrated after another Ironman race (34). However, the oxLDL:LDL ratio showed a modest and only temporary trend to increase 1 d after the competition. Concerning protein oxidation, both AOPP concentrations and AOPP:TP ratio peaked immediately post-race.

Interestingly, opposite to the inverse links among triathlon-induced effects with markers of lipid peroxidation (discussed in section 2.4.2. in more detail), AOPP and AOPP:TP ratio were positively related to some training-associated variables. In addition with the finding that AOPP rose with the performance in the cycle split time in the Ironman race, these data might imply that there is an intensity-related response in protein oxidation since better trained athletes were capable of competing the Ironman at higher intensities and therefore had more pronounced changes in protein oxidation. Based on previous observations in exercised animals (127), these results possibly suggest that proteins are more prone to free-radical induced oxidation during strenuous endurance exercise than lipids.

A further important finding was that, despite a decrease (from immediately post-race to 1 d post-race) in CD, AOPP and AOPP:TP ratio, all these markers in addition to MDA remained significantly above pre-race values 1 d post-race. This apparently indicates that peroxidation of membranes or/and blood lipids as well as oxidative modification of plasma proteins are sustained for at least 1 d after prolonged strenuous exercise. A delayed removal of oxidized products cannot be excluded as an explanation. However, our findings that concentrations of most nutritive antioxidants dropped below pre-race values 1 d post-race (95) (probably reflecting increased antioxidant consumption associated with the counteracting of increased ROS formation) further support the concept of continued oxidative stress responses. While augmented susceptibility of LDL particles to oxidation (71) and increased lipid peroxides levels (44) persisted over 4 to 8 days after a marathon run, Mastaloudis et al. (79) reported that $F_2$-isoprostanes (together with IL-6) had returned to pre-race values 1 d post-race in ultra-marathon runners. In the present study, correlations were observed between protein oxidation markers and markers of muscle damage and inflammation (such as IL-6 as shown in Figure 5) that might point to muscular inflammatory processes as a source of this low-grade oxidative stress response 1 d after the Ironman race (32, 64, 173). Mechanisms such as the hypothesised mitochondrial electron "leakage" or transient ischemic conditions appear to be the predominant contributors to the enhanced ROS generation *during* intense endurance exercise (173). In contrast, the delayed oxidative stress responses *after* muscle-damaging exercise probably result from oxidative burst reactions of phagocytic cells (64, 105, 111, 154). Alternatively, IL-6, vice versa, also could have been induced by oxidatively damaged plasma proteins since AOPPs are postulated to mediate pro-inflammatory activities (60, 176). Further associations were found between CD and hs-CRP that probably support evidence that exercise-induced inflammatory responses are related to oxidative stress (64, 103). Beside the increased ROS production derived from neutrophils and macrophages, an enhanced cytokine formation as well as an increased MPO activity are discussed as biochemical key mechanisms that link inflammation with oxidative stress in response to exercise (64, 111, 173). Beyond, recent research has proposed that redox-sensitive signal transduction pathways such as those involving nuclear factor (NF) κB regulate cytokine production during exercise (54, 111). Related aspects and findings concerning the hypothesised interaction between exercise-induced

inflammatory, muscular and oxidative stress responses are also discussed in chapters 3 and 4.

### 2.4.2. Relevance of training status on oxidative stress responses and implications for health

Crucially, since all oxidative stress markers had returned to pre-race values 5 d post-race and remained at pre-race levels 19 d post-race, there are no indications of persistent oxidative stress in the course of an Ironman triathlon. In line with observations of Ginsburg et al. (34), who reported a reduced susceptibility of plasma lipids to peroxidation in male Ironman competitors, the finding of a decrease in LDL oxidation suggests that a single bout of ultra-endurance exercise might not contribute to the development or progression of atherosclerosis lesions based upon the oxidative modification of LDL hypothesis (153). Of further interest, a wide range of correlations with training volume, training status and oxidative stress markers were present (Table 6). Consistent with a previous study of Knez et al. (62), who reported a dose-response relationship of resting MDA concentrations with time spent training, we observed a positive correlation between the pre-race oxLDL:LDL ratio and weekly net endurance exercise. In contrast to the demonstrated oxLDL responses to acute ultra-endurance exercise, this might reflect cumulative oxidative stress that had been attributed to high training volumes or overload training (32, 76). On the other hand, our data revealed that those athletes with the highest power output at $VO_{2\ peak}$ had significantly lower plasma oxLDL (but not LDL) concentrations after the triathlon compared with the subjects with the lowest power output at $VO_{2\ peak}$ (percentile distribution). Additionally, MDA pre-, post- and 1 d post-race concentrations were also lower with higher training status and with increasing weekly training loads (Figure 4). Furthermore, a number of negative associations with markers of lipid peroxidation and variables associated with training status, both pre-race as well as in response to the race (Table 6) indicate that better training levels might confer enhanced protection against oxidative stress and consequent damage of lipids. Thus, the results from the present study support the idea that endurance training reduces post-exercise oxidative stress (32, 64). The exact biochemical and molecular mechanisms behind this maintenance of oxidative/antioxidative homeostasis remain to be elucidated, but the prevention of

persistent oxidative stress responses may result from *both* increased antioxidant defences (discussed in section 2.4.4. in more detail) and a decrease in ROS production (32). The latter could be explained by an adaptive increase in mitochondrial volume (resulting in a relatively lower oxidative load), an improved efficiency of the electron transfer through the mitochondrial respiratory chain or less pronounced muscle damage and inflammatory responses in well-trained individuals (61, 159, 173).

Based on the findings of the present investigation one might conclude that the population of ultra-endurance athletes might not be at significantly higher risk of developing cardiovascular disease. The investigated individuals in the previously mentioned epidemiological study (68) who had the highest physical activity energy expenditure (ca. > 3.500 kcal per week) and a non-significant increase in the age-standardised mortality rate (see chapter 1), were most likely *not* ultra-endurance athletes. Therefore, a comparison of the cohort group in this epidemiological study and ultra-endurance athletes appear to be inadequate. Given that typically triathlon training requires 500 – 900 kcal per hour (depending on the intensity and the individual fitness level) (49), participants of the current study with a weekly net endurance exercise time of 10.7 ± 2.6 hour per week) expended about 5.350 – 9.630 kcal per week just on their training. However, the high training status of the athletes engaged in ultra-endurance exercise is probably associated with significant adaptations in protective systems (55, 61, 128). This assumption is in line with results of a recent cross-sectional study that was conducted to investigate whether cardiovascular health depends on the previous athletic activity (120). This study showed that physically active former top-level athletes (who previously participated in endurance sports events and sports games) are characterized by a significantly lower cardiovascular risk profile including a substantially lower oxidative stress status compared both with sedentary ex-athletes and non-athletic controls (120). Nevertheless, further research is required to clarify the effects of long-term (year-long) participating in explicitly ultra-endurance exercise.

### 2.4.3. Implications for further investigations: necessity to use multiple biomarkers and time-course considerations

In summary, results from the current research work reveal a complex picture of oxidative stress in the course of exhaustive endurance exercise. With regards to future study designs and methodology, the present findings emphasize the importance to use multiple markers and to monitor them in a longer time-course for several reasons. First, recent research has shown that there are optimal time-points for the detection of maximum concentrations of oxidative stress markers (82, 173). Furthermore, delayed oxidative stress responses resulting from muscle inflammatory processes might be sustained for or appear days after muscle-damaging exercise, and, thus, may be missed in investigations with shorter monitoring periods (105). Second, our results support findings (127, 129) that lipids and proteins might be affected differently by exercise-induced oxidative stress. Since, up to now, there is limited data regarding the effects of exercise on protein oxidation (173), which has striking consequences for cell function (129), it seems crucial not to focus exclusively on indices of lipid peroxidation. Finally, little information is available on the complete resumption of recovery in these indices especially after ultra-endurance exercise (75, 79), which might be important for assessing possible deleterious health effects.

### 2.4.4. Effects of training and acute ultra-endurance exercise on the antioxidant system

As suggested in the literature (60, 126), a "battery" of different measurements was applied to adequately assess the total antioxidant capacity in plasma. Importantly, although based on different methodological principles (60), time-courses of the values obtained by the TEAC, FRAP and ORAC assay were similar for the whole monitoring period (Figure 3). In agreement with previous studies in marathon runners (71, 170), but probably investigated for the first time in ultra-endurance athletes, plasma antioxidant capacity rose markedly following the Ironman race. As detected by all three assays, this immediate alteration in the total plasma antioxidant capacity can be seen as an early adaptive response to oxidative stress (71, 126), that might have prevented initiation of lipid peroxidation to a certain degree. One day after the race, plasma antioxidant capacity declined, but still remained elevated above pre-race values. While TEAC was

still found to be increased 4 days after a marathon (71), it had returned to pre-race levels 5 d post-race along with oxidative stress markers in the present study. Our data suggest several mechanisms for the observed post-race increase in the antioxidant capacity of plasma (Table 7). First, the increase in TEAC might be a result of the elevation of vitamin C (which change correlated with that of TEAC) and probably also α-tocopherol. Correspondingly to a study in ultra-marathon runners (79), the immediate increase in vitamin C and α-tocopherol probably can be attributed to both, the intake of these antioxidants during the race as well as mobilization of ascorbic acid from the adrenal glands (38) and/or leukocytes (171). The rise in the plasma concentration of α-tocopherol might be associated with exercise-induced changes in the lipoprotein metabolism (78, 79). Second, further correlations were observed between changes in FRAP levels and the total bilirubin concentration (which increase possibly resulted from an enhanced haemolysis (155)) and between ORAC levels and the total plasma protein. Both links point to the contribution of protein-associated thiol (sulfhydryl) groups to plasma antioxidant capacity (32, 71). It also has to be noted in this context that actually no single test fully reflects the overall antioxidant capacity of plasma due to the different principles of these assays and an overestimation of hydrophilic substances (60, 177). Thus, despite active interactions among antioxidants located in the hydrophilic and lipophilic plasma compartments (177), it is different to obtain correlations between these assays (27). This was also demonstrated within the scope of the diploma theses of Judit Valentini (169) and Barbara Stadlmayr (150). Third, concomitant with previous findings (71, 79), the current results imply that uric acid is responsible for the rise in the total plasma antioxidant capacity to a considerable extent (Table 7). Noteworthy, this is reflected by associations of changes in uric acid with those in the TEAC, FRAP as well as the ORAC assay. Plasma concentrations of the potent hydrophilic antioxidant uric acid are known to rise during intense exercise being produced from increased purine metabolism (32, 64, 173) and, possibly also because of impaired renal clearance (79). We found that TEAC, FRAP and uric acid increased with performance in the Ironman triathlon. Consequently, these results suggest that those athletes with a higher training- and performance status could push themselves harder, which in turn resulted in higher concentrations of uric acid after the race. This phenomenon can not be considered as a specific training adaptation, but it obviously contributes to the performance-linked

increase in the plasma antioxidant capacity. Of further interest, we observed that the Ironman-induced change of TEAC was associated with the relative IAT (percentile distribution), i.e. TEAC increased in athletes with greater performance ability. Crucially, a wide range of correlations were found between other training-related variables on the one side and FRAP and TEAC on the other, which are summarized in Table 7. Thus, collectively considered, several training physiological determinants (associated with training and performance capacity at different exercise intensities) seem to play important roles in promoting such a protective response in antioxidant defences of plasma. Interestingly, TEAC was positively related to GSH-Px activities throughout the monitoring period (Table 7), which may imply a synergistic interaction between erythrocytes and plasma antioxidant capacity. However, although we noted that GSH-Px was linked with training status, it is unclear whether or to which extent training-induced adaptations of endogenous antioxidant defences (in particular antioxidant enzymes) might have contributed to the rise in the plasma antioxidant capacity following the Ironman race.

Several studies showed increased antioxidant erythrocyte enzyme activities after relatively short bouts of aerobic exercise (32), whereas different patterns or opposite effects (decreases) were seen after a 171 km cycling mountain stage (2) or a marathon (44). Only a small number of studies have examined adaptations to or acute effects of ultra-endurance exercise on the antioxidant system (62, 75). Recently, Knez et al. (2007) reported that activities of all key antioxidant enzymes in erythrocytes declined immediately after an Ironman race (62). Except for GSH-Px (which only trended to decrease), our data further confirm these somewhat unexpected results as we also observed a significant decrease in the activities of SOD and CAT following the competition. In general, modifications of antioxidant enzyme activities after exercise characterize either adaptation (an increase in the activity at first) or utilization (a decrease if oxidative stress is overwhelming) (32). These decreases had, hypothetically, been attributed to a modification of the catalytic centres and subsequent inactivation of enzymes due to a disturbed red-ox balance induced by augmented oxidative stress (2, 62, 173). Contrary to the acute effects of the Ironman triathlon, the attenuation of SOD and CAT activities 19 d after the competition to below pre-race values likely can be

explained by a down-regulation during the recovery period (32). Moreover, we noted that activities of GSH-Px were positively associated with the percentage power output at a blood lactate concentration of 3 mmol * $L^{-1}$. This result supports data that this enzyme may be highly responsive to endurance training in general (62, 75) and to training at higher-intensities in particular. This specific finding is in accordance with previous investigations in the skeletal muscle response to exercise (124). Taken together, data from the current study provide further evidence that (endurance) training results in protective adaptations in the organism to cope with future "stress loads" (173) particularly with regards to endogenous antioxidant defences (39, 54, 128, 129, 144).

### 2.4.5. Consequences for the antioxidant intake in ultra-endurance exercise

An additional finding of the present study was that with the exception of the reported relationship between the changes in vitamin C and TEAC, there was no further association between plasma levels of nutritive antioxidants and alterations in the plasma antioxidant capacity or oxidative stress responses. Despite individual differences in the plasma concentrations of nutritive antioxidants this observation may also be due to the fact that antioxidant status of all subjects was in a normal physiological range. It is important to note in this context, that the current study was not designed to investigate effects of antioxidant supplementation, but to examine oxidative stress and antioxidant responses under "real" race conditions, i.e. without any intervention. Subjects were precisely instructed to avoid antioxidant supplementation at larger doses (i.e. more than 100% of RDA), and antioxidant intake during the race was *ad libitum* and carefully documented. However, it has to be emphasised, that the athletes´ plasma concentrations of vitamin C and β-carotene throughout the study period (Table 4) were above those levels which are considered as beneficial in preventing against cancer and cardiovascular disease (22). Therefore, the results of the present study support the assumption that the requirements for vitamin C and β-carotene can be achieved by a "normal" diet rich in fruits and vegetables (143). Endurance athletes seem to meet the general recommendations for these antioxidants probably also due to their increased daily energy expenditure and food intake, provided that the nutritional density is adequate (143). On the other side, the current findings also indicate that the vitamin E intake of ultra-endurance athletes must be considered carefully. Although in a normal

physiological range (22), plasma α-tocopherol concentrations of the subjects were relatively low (Table 3). The theoretically increased risk for a deficit in this fat-soluble vitamin could partly be explained by the fact that these athletes are usually very concerned about a high carbohydrate proportion in their nutrition, whereas an increased ingestion of tocopherols requires a substantial increase in the consumption of foods that are also rich in (poly-unsaturated) fatty acids, such as nuts, margarine and certain oils. Nevertheless, it has to be mentioned that a relatively moderate and energy-expenditure-associated increase in the intake of lipids – and in turn also vitamin E – does not necessarily cause an imbalance in the macronutrient intake (49, 91).

Concerning the antioxidant demands *during* ultra-endurance exercise, one cannot rule out that the (recorded) intake of vitamin C and α-tocopherol along with the ingestion of specified and vitamin-fortified beverages and food (e.g. carbohydrate bars) during the race, at least in part, might have preserved antioxidant responses. This is also supported by the mentioned finding that the exercise-induced change in TEAC was related with the elevation in the plasma vitamin C concentration, which may partly be associated with vitamin C intake. On the contrary, the observation that plasma concentrations of all investigated nutritive antioxidants decreased 1 d post-race, partly significantly to below pre-race values (Table 5), may indicate an increased requirement for antioxidant nutrients in the early recovery period after an acute bout of ultra-endurance exercise. Similarly, Palazzetti et al. (2004) reported a drop in serum concentrations of nutritive antioxidants after four weeks of overloaded training, which was compensated by an antioxidant mixture at doses that can be provided by a diversified and well-balanced diet. Interestingly, the protective effects of this antioxidant mixture were shown mostly by the alleviated muscle damage (110). This finding could also be attributed to delayed oxidative stress responses as a result of local inflammatory processes in injured skeletal muscle. Whatever the mechanisms involved, Palazzetti and co-authors suggested that the physiologically-dosed antioxidant supplementation helped to preserve the antioxidant system during heavy training in subjects with initially low antioxidant intakes (110). However, endurance athletes should be encouraged to abstain from supra-physiological doses of antioxidants for the following reasons: First, evidence of benefits of antioxidant supplementation in athletes in general is still scarce and inconsistent (32,

64, 123, 168). In previous studies, high-dosed antioxidant supplementation in competitors of ultra-endurance races had either beneficial (79), adverse (i.e. pro-oxidant) (62, 100) or no effects (99) on oxidative stress changes. Second, the optimal bioavailability and combined action of multiple phytochemical and antioxidant compounds derived from fruits and vegetables cannot be replaced by supplementation (22, 125, 143). Third, in the light of the postulation that ROS play important physiological roles as signalling molecules (e.g. by stimulating training adaptations in the endogenous antioxidant defences) (39, 54, 128, 144), exceptional high levels of antioxidant supplementation may even blunt certain exercise-induced cellular adaptations by complete elimination of ROS (123, 173).

## 2.5. Conclusion

The present data indicate that a single bout of ultra-endurance exercise is associated with a systemic acute and elevated oxidative stress response. Although the disturbance in the oxidant/antioxidant balance was sustained for at least one day after the Ironman triathlon, there are no indications of persistent detrimental health effects due to oxidative stress. Moreover, the current results provide further evidence that there are chronic training-induced biochemical adaptations (resulting either in a decrease in ROS production and/or in an enhancement of the antioxidant defences) and that manifold training-associated determinants might be responsible for these protective responses to a certain extent. Weekly training loads as well as training at different intensities seem to be factors in the improvement of antioxidant defence mechanisms after prolonged intense exercise. The present investigation illustrates that even minor differences in the training status among well-trained athletes can result in significantly different outcomes in the training- and exercise-induced responses of oxidative stress and antioxidant-related parameters. Furthermore, the current results point to the importance of an adequate intake of nutritive antioxidants to preserve antioxidant responses to ultra-endurance exercise (especially during the early recovery period). Thereby, athletes should be encouraged to increase their intake of antioxidant nutrients through a diversified diet (rich in vegetables, fruits, nuts, etc.) rather than using high-dosed antioxidant supplements to avoid probable adverse consequences such as pro-oxidative effects or an attenuation of training-induced adaptations in the endogenous antioxidant

system. Generally, these data imply that acute ultra-endurance exercise does not cause longer lasting alterations in systemic oxidative stress markers, probably due to improved antioxidant responses to strenuous exercise in well-trained athletes.

# 3. Acute and Recovery Responses in Inflammatory, Immune-endocrine and Muscle Damage Parameters after an Ironman Triathlon

This chapter is based on another original article that has been published in *European Journal of Applied Physiology*. The paper is attached subsequently and is cited as: **Neubauer O, König D, Wagner KH**. Recovery after an Ironman triathlon: sustained inflammatory responses and muscular stress. *Eur J Appl Physiol* 104: 417-426, 2008.

Furthermore, results on this topic have been presented at scientific meetings by the author:

- *1$^{st}$ symposium of the Vienna Research Platform of Nutritional and Food Sciences*, Apr. 25$^{th}$ 2008, University of Vienna, Austria: **Neubauer O, König D, Reichhold S, Wagner K-H**: Recovery Responses in Ironman Triathletes (poster presentation)
- *13$^{th}$ Annual Congress of the European College of Sport Science*, July 9$^{th}$ – 12$^{th}$ 2008, Estoril, Portugal: **Neubauer O, König D, Wagner K-H**: Recovery Responses in Ironman Triathletes (oral presentation, abstract: Book of Abstracts)

## 3.1. Introduction

Regular physical training appears to enhance parts of the innate immune system while prolonged strenuous physical exercise attenuates many components of immunity (35, 74, 116). When the integrity of the organism is challenged by vigorous endurance exercise, a systemic inflammatory response is induced. This stereotypical and evolutionary conserved reaction to major physical stressors protects the organism by eliminating antigens, cellular debris, tissue fragments, by preventing further damage and by promoting tissue repair. However, it also elicits a temporary dysfunction of various aspects of immunity, which may increase the risk of subclinical and clinical infection (30, 35, 116). The regulation of recruitment and distribution of leukocytes is complex and includes a plethora of signals and mediators such as hormones and cytokines (74, 86, 116). Beyond the ultra-structural damage of muscle tissue as a potential stimuli for the production and release of cytokines, exhaustive endurance exercise induces several factors such as metabolic, hormonal, thermal and oxidative stress, all of which can give rise to the release of cytokines and other acute phase proteins in addition to the activation of several cell sub populations within the immune system. Nevertheless, crucially, up to now only a small number of studies have investigated the relationships between muscle damage and the release of cytokines or acute phase reactants explicitly after ultra-endurance exercise (40, 53, 98, 100, 155). Moreover, exercise-induced inflammatory or immune responses rarely have been followed longer than one day post-race (40, 75, 79). Further questions arise on this topic as it is hypothesised that repetitive skeletal muscle tissue trauma caused by heavy training and competition could result in a persistent systemic cytokine response, which may be associated with a chronic inflammatory state, immune dysfunction and a poorly understood condition of overreaching/overtraining (41) or underperformance syndrome (139, 140, 148). Thus, detailed knowledge concerning the circumstances that alter the time-course of the restoration in muscular, inflammatory and immune parameters after ultra-endurance exercise is essential for designing training schedules that avoid eventual negative consequences of high volumes and intensities of physical activity.

The clinical relevance of research work within this area is further emphasised because there are important similarities between the systemic inflammatory response to heavy

endurance exercise and acute conditions such as sepsis and trauma (30, 86, 116, 119). A range of common factors are involved in both, trauma- and exercise-induced systemic inflammatory responses, including ROS and cytokines, albeit there are significant quantitative differences (e.g. in the concentrations of diverse cytokines) (30). Exercise-induced stress usually results in a positive adaptation to the stimuli within days whereas infection or physical trauma leads to more severe and sustained complications (30). Identifying the circumstances for these divergent outcomes (e.g. factors that trigger and counter-regulate the exercise-induced inflammatory and immuno-modulatory effects) might be of high importance for a better understanding of the aetiology of patho-physiological inflammatory states (119) as well as age-related processes (115).

The primary aim of this part of the study was to investigate a range of muscular, inflammatory and immune-endocrine parameters in response to an Ironman triathlon not only to quantify the magnitude directly after an acute bout of ultra-endurance exercise, but importantly also to get a thorough picture of the resolution of the recovery. Since alterations in these responses *after* extremely demanding endurance exercise are poorly documented, their time-course was monitored until 19 days after the race. Furthermore, attention was drawn on potential links between these parameters to verify potential interactions between muscle damage, cytokines/acute phase reactants, steroid hormones and leukocyte dynamics. In addition to the previously reported data (93), probable effects of antioxidants and carbohydrates/glucose were exclusively examined within the scope of the present dissertation. Importantly, results from this study were expected to provide practical implications concerning the length of time needed to recover from muscle damage and inflammatory responses induced by ultra-endurance exercise.

## 3.2. Materials and Methods

Characteristics of the study participants, the study design, race conditions, the spiroergometry testing protocol, the blood sampling procedure as well as the assessment of the haematological profile are reported in the respective part in chapter 2.

### 3.2.1. Adjustment for exercise-induced changes in the plasma volume

As described in section 2.2.6, all results were reported adjusted for exercise-induced changes in plasma volume (until 5 days post-race due to the sustained expansion of the plasma volume (147)), except for glucose, cortisol and testosterone. For factors that are metabolically and hormonally controlled or released in an endocrine manner it is important to consider their actual circulating concentration.

### 3.2.2. Data analysis

Statistical analysis was identical as described in section 2.2.12. In addition to correlation analysis, subjects were divided into percentile groups by concentrations of nutritive antioxidants and the intake of vitamin C, α-tocopherol and carbohydrates during the race. One-factorial ANOVA and *post hoc* analysis (Bonferroni) were applied subsequently to determine probable associations of immune-endocrine and inflammatory parameters with these nutritional factors.

### 3.2.3. Plasma concentrations of cortisol, testosterone and glucose

Both parameters were determined with radioimmunoassay based on the competition between radioactive and non-radioactive antigen by the working group of Karl-Heinz Wagner. Testosterone was assessed with Active ® Testosterone RIA DSL-4000 (Diagnostic Systems Laboratories, Inc., Webster, TX, USA), Cortisol with Corti-Cote ® Cortisol Antibody Coated Tube - 125I RIA Kit (MP Biomedicals, Illkirch, France). Plasma glucose was detected using an automatic analyzer by the author (Vitros DT 60 II module; Ortho-clinical Diagnostics, Germany).

### 3.2.4. Plasma concentrations of myeloperoxidase and polymorphonuclear elastase

Myeloperoxidase (MPO) concentrations were measured using the immundiagnostik MPO ELISA kit (Immundiagnostik AG, Bensheim, Germany) by two-site sandwich technique by the author. The absorbance of samples and standards were read with a Fluostar Optima microplate reader (BMG labtechnologies, Germany) at 450nm. Polymorphonuclear (PMN) elastase was determined using a quantitative enzyme immunoassay (Milena Biotec GmbH, Bad Nauheim, Germany) in the laboratories of the

Freiburg University Hospital in Germany (working group of Daniel König (93). All measures were made in duplicate.

### 3.2.5. Plasma markers of muscle damage and of inflammation

Plasma creatine kinase (CK) activity was detected using an automatic analyzer (Vitros DT 60 II module; Ortho-clinical Diagnostics, Germany) by the author. Concentrations of myoglobin and high sensitive C-reactive protein (hs-CRP) were analyzed nephelometrically (Dade Behring, Marburg, Germany). Plasma interleukin (IL)-6 and IL-10 were determined by the Quantikine HS Immunoassay kit (R&D Systems GmbH, Wiesbaden, Germany) in the laboratories of the Freiburg University Hospital in Germany (working group of Daniel König (93). All measures were made in duplicate.

## 3.3. Results

### 3.3.1. Race results

Race results are reported in section 2.3.1 and shown in Table 1. Furthermore, the average carbohydrate intake of the study participants during the race was 753 ± 297 g (according to 69 ± 29 g per hour).

### 3.3.2. Total leukocyte counts, leukocyte subsets and markers of neutrophil activation

Total leukocyte count increased significantly (+237%; P<0.001) immediately after the Ironman triathlon versus pre-race and remained significantly (+56%; P<0.001) elevated until 1 d post-race. Changes in leukocyte subpopulations are shown in Figure 7. Plasma MPO and plasma PMN elastase concentrations significantly (both P<0.001) increased by 342% and 424%, respectively immediately post-race. Both markers remained elevated above the pre-race values 1 d after the race (+70% and +108%, respectively; both P<0.001). MPO and PMN elastase values had returned to pre-race 5 d post-race and 19 d post-race there was a significant (both P<0.001) reduction (-28% and -21%, respectively) below pre-race values (Table 8).

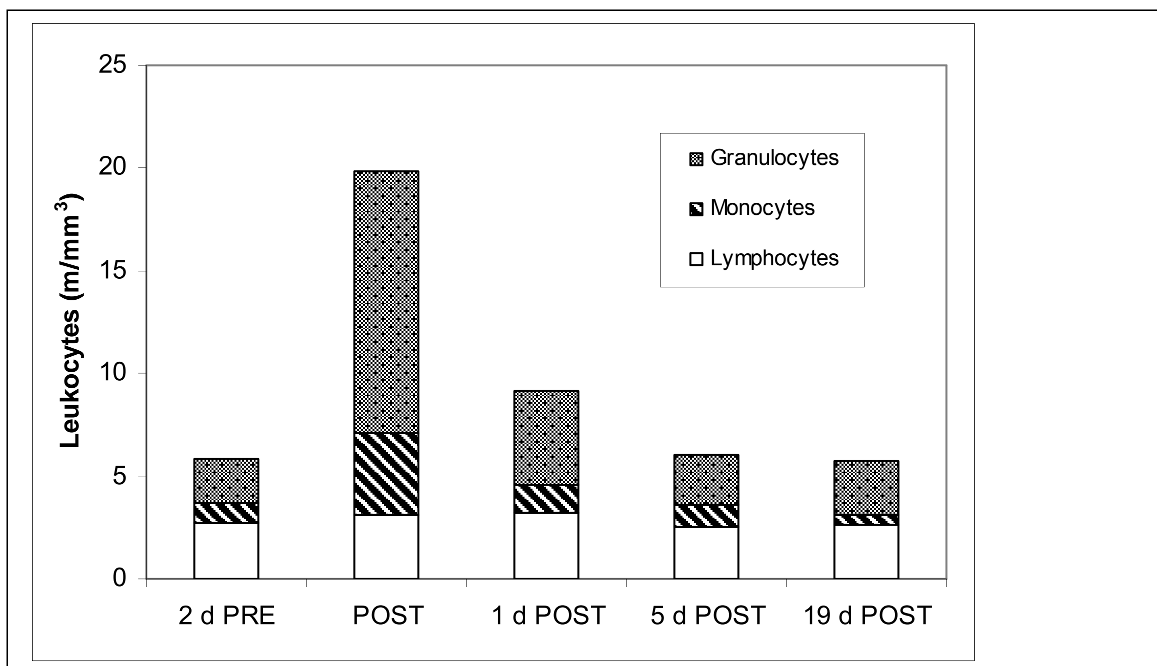



**FIGURE 7:** Mean changes in blood concentrations of leukocytes and subpopulations 2 d pre-race (PRE), immediately post-race (POST), 1 d post-race, 5 d post-race and 19 d post-race for 42 subjects.

### 3.3.3. Plasma cortisol, testosterone, testosterone:cortisol ratio and glucose

There was a significant increase immediately post-race for cortisol (+241%; P<0.001), whereas testosterone dropped below pre-race (-53%; P<0.001) at the same time point. 1 d post-race cortisol decreased sharply by 47% to below pre-race values (P<0.001). 5 and 19 d post-race cortisol concentrations remained moderately but not-significantly lower. The testosterone concentration levelled off below pre-race values 1 d after the competition (-50%; P<0.001) returning to pre-race values 5 d post-race. The testosterone to cortisol ratio was significantly (P<0.001) decreased by 86% directly after the race compared to pre-race. Glucose was significantly increased immediately post-race (+35%; P<0.001) (Table 8).

### 3.3.4. Markers of muscle damage

Plasma CK activity increased significantly (P<0.001) by 1195% immediately post-race, with a maximum concentration observed 1 d post-race, when it was 4316% higher than pre-race values (P<0.001). CK values remained significantly (P<0.001) higher than pre-race until 5 d post-race (+281%). Plasma myoglobin peaked by 3842% higher than pre-race instantly post-race (P<0.001), and remained significantly (P<0.001) elevated by

964% higher than pre-race values 1 d after the race. Thereafter myoglobin concentrations declined, but remained elevated by 5 d (+45%) and 19 d (+30%) after the competition (both P<0.001) (Figure 7).

### 3.3.5. Plasma cytokines and high sensitive C-reactive protein concentrations

Plasma IL-6 increased dramatically in response to the race (+10408%; P<0.001), and despite a sharp decline, values remained significantly elevated 1 d (+345%; P<0.001) and 5 d (+79%; P<0.001) after the race. The plasma IL-10 concentration was also elevated immediately after the race (+287%; P<0.001), and remained above pre-race concentrations 1 d post-race (+37%; P<0.01). 5 d post-race, IL-10 had declined by 4% below pre-race concentrations (P<0.05), and 19 d post-race levels were similar to pre-race. The plasma hs-CRP concentration rose significantly (P<0.001) immediately after the race by 543% and had increased by 7702% 1 d post-race (P<0.001). Hs-CRP subsequently decreased, but values remained significantly higher than pre-race concentrations 5 d (+881%; P<0.001) and 19 after the competition (+38%; P<0.01) (Figure 8).

### 3.3.6. Associations with neutrophil dynamics, changes in cytokines and high sensitive C-reactive protein

Significant positive correlations were obtained between the change in total leukocyte count and changes in plasma myoglobin, CK activity, cortisol and IL-6, which are summarised in Table 9. Moreover, a positive correlation was observed between the pre- to immediately post-race change of IL-6 and the pre- to 1 d post-race change of MPO (r=0.45, P<0.01). Moderate positive correlations were observed between the pre- to post-race changes of IL-6 and markers of muscle damage. In addition, the pre- to immediately post-race responses in IL-10 correlated positively with cortisol (r=0.46, P<0.01) and were inversely related with performance-associated variables (Table 9). There were also several stronger positive correlations between hs-CRP and markers of muscle damage that are also shown in Table 9.

### 3.3.7. Associations with immune-endocrine parameters, antioxidants and carbohydrate intake

No significant associations were found between immune-endocrine or muscle damage parameters on the one side and nutritive antioxidant concentrations or intake, carbohydrate ingestion during the race or plasma glucose concentrations on the other.

**TABLE 8:** Plasma values of myeloperoxidase (MPO), polymorphonuclear (PMN) elastase, cortisol, testosterone, testosterone:cortisol ratio and glucose

| | PRE | POST | 1 d POST | 5 d POST | 19 d POST | Time effect (*P*) |
|---|---|---|---|---|---|---|
| MPO (g * L $^{-1}$) | 57 ± 31 | 253 (122) *** | 97 (82) *** | 61 (58) | 41 (25) *** | <0.001 |
| PMN elastase (µg * L $^{-1}$) | 46 (23) | 239 (137) *** | 95 (104) *** | 44 (31) | 36 (16) *** | <0.001 |
| Cortisol (nmol * L $^{-1}$) | 282 (112) | 957 (696) *** | 149 (66) *** | 249 (107) | 273 (110) | <0.001 |
| Testosterone (nmol * L $^{-1}$) | 11.4 (5.6) | 5.3 (3.6) *** | 5.5 (2.9) *** | 12.7 (6.9) | 12.3 (6.3) | <0.001 |
| Testosterone:cortisol ratio | 0.040 (0.037) | 0.006 (0.009) *** | 0.037 (0.027) | 0.051 (0.036) | 0.045 (0.032) | <0.001 |
| Glucose (mmol * L $^{-1}$) | 4.0 ± 0.7 | 5.4 ± 1.4 *** | 4.6 ± 0.9 ** | 4.6 ± 0.7 *** | 4.6 ± 0.7 *** | <0.001 |

Values are mean (SD); n=42; PRE, 2 d pre-race; POST, immediately post-race; 1 d POST, 1 d post-race; 5 d POST, 5 d post-race; 19 d POST, 19 d post-race; * significantly different from pre-race values, P<0.05; ** significantly different from pre-race values, P<0.01; *** significantly different from pre-race values, P<0.001.

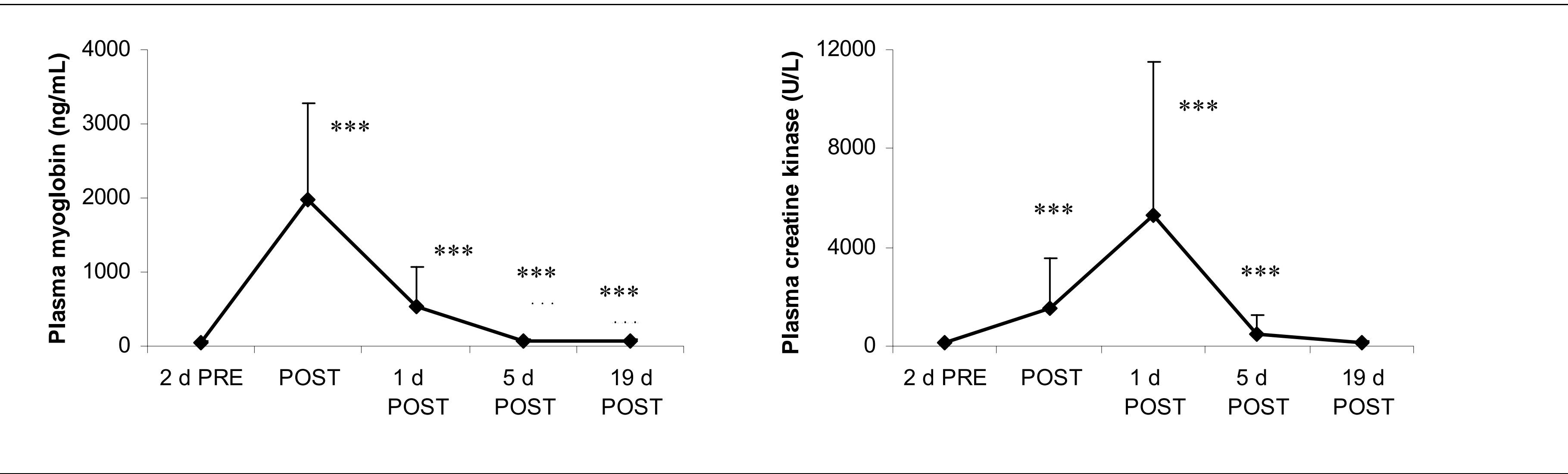



**FIGURE 8:** Changes in plasma myoglobin concentrations (left) and plasma creatine kinase activity (right) 2 d pre-race, immediately post-race, 1 d post-race, 5 d post-race and 19 d post-race. Data are mean ± SD; N=42; ** significantly different from pre-race values, P<0.001.

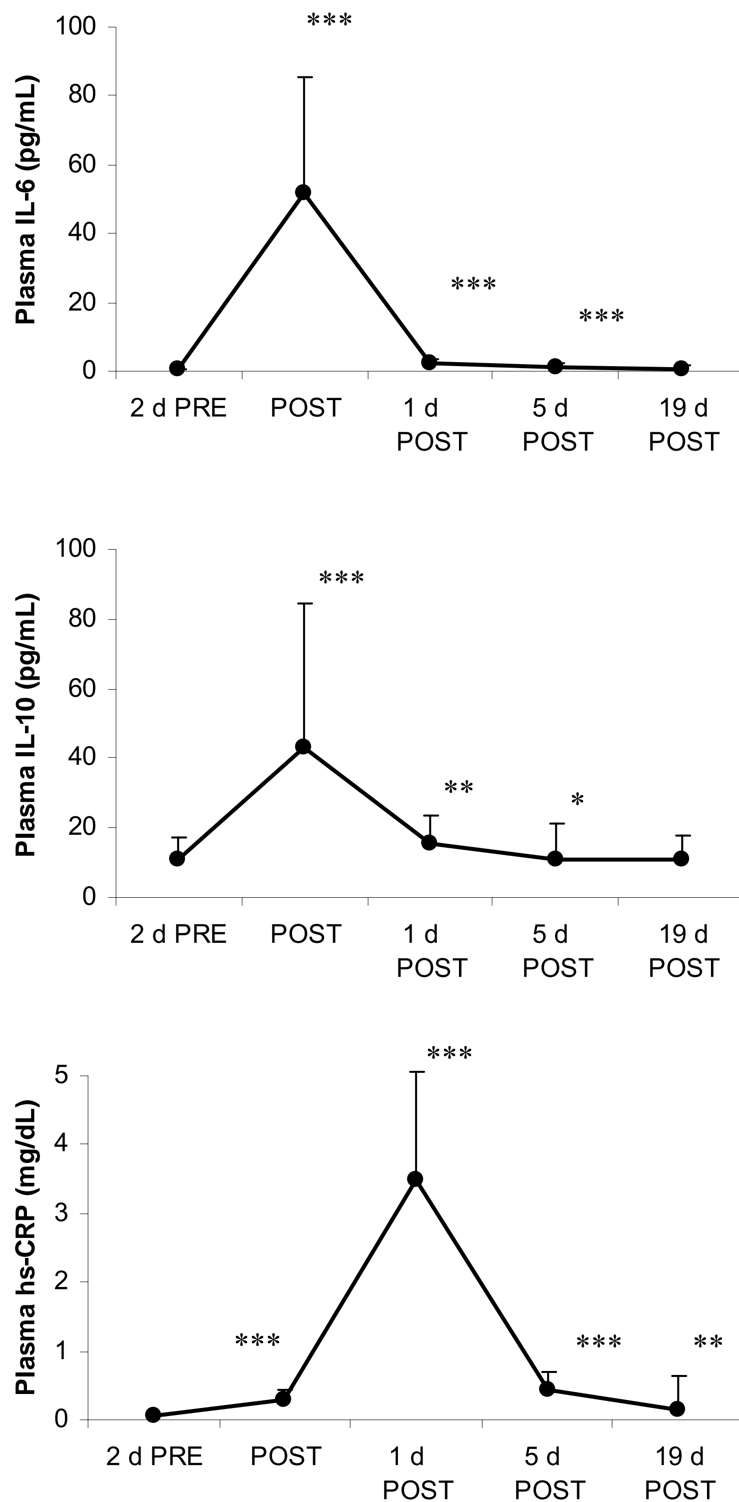



**FIGURE 9:** Changes of the plasma concentrations of interleukin (IL)-6), IL-10 and high sensitive C-reactive protein (hs-CRP) 2 d pre-race, immediately post-race, 1 d post-race, 5 d post-race and 19 d post-race. Data are mean ± SD; n=42; *** significantly different from pre-race values, P<0.001; ** significantly different from pre-race values, P<0.01.

**TABLE 9:** Significant associations with exercise-induced responses of total leukocyte counts, interleukin (IL)-6, IL-10 and high sensitive C-reactive protein (hs-CRP)

| | MPO | cortisol | CK | myoglobin | IL-6 | total race time | run split |
|---|---|---|---|---|---|---|---|
| **Leukocytes** *Δ PRE to 1 d POST* | | *Δ PRE to POST:* 0.42 ** | *Δ PRE to 1 d POST:* 0.47 ** | *Δ PRE to POST:* 0.44 **; *Δ PRE to 1 d POST:* 0.44 ** | *Δ PRE to 1 d POST:* 0.48 ** | | |
| **IL-6** *Δ PRE to POST* | *Δ PRE to 1 d POST:* 0.45 ** | | | | | | |
| *…1 d POST* | | | *1 d POST:* 0.38 ** | *1 d POST:* 0.35 * | | | |
| **IL-10** *POST* | | | | | | - 0.43 ** | - 0.47 ** |
| *Δ PRE to POST* | | *Δ PRE to POST:* 0.44 ** | | | | | - 0.42 ** |
| **Hs-CRP** *POST* | | | *POST:* 0.44 ** | | | | |
| *1 d POST* | | | *1 d POST:* 0.52 *** | *1 d POST:* 0.58 *** | | | |
| *Δ PRE to 1 d POST* | | | *Δ PRE to POST:* 0.60 ***; *Δ PRE to 1 d POST:* 0.54 *** | *Δ PRE to POST:* 0.61 *** | | | |
| *5 d POST* | | | *5 d POST:* 0.53 *** | | | | |

MPO, myeloperoxidase; CK, creatinkinase; POST, immediately post-race; 1 (5) [19] d POST, 1 d (5) [19] post-race; Δ PRE to POST, change from pre- to immediately post-race; Δ PRE to 1 d POST, change from pre- to 1 d post-race; * P<0.05; ** P<0.01; *** P<0.001

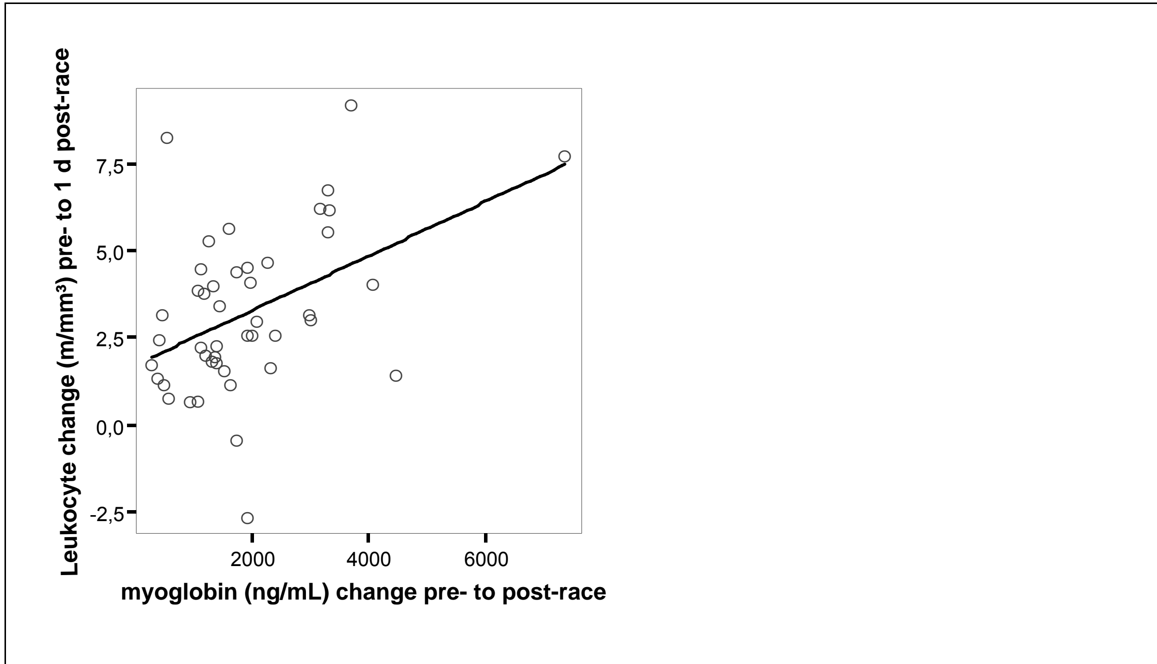



**FIGURE 10:** Correlation between the change in the plasma myoglobin concentration from pre- to immediately after the Ironman race and the pre- to 1 d post-race change of the total leukocyte counts (r=0.44; P<0.01).

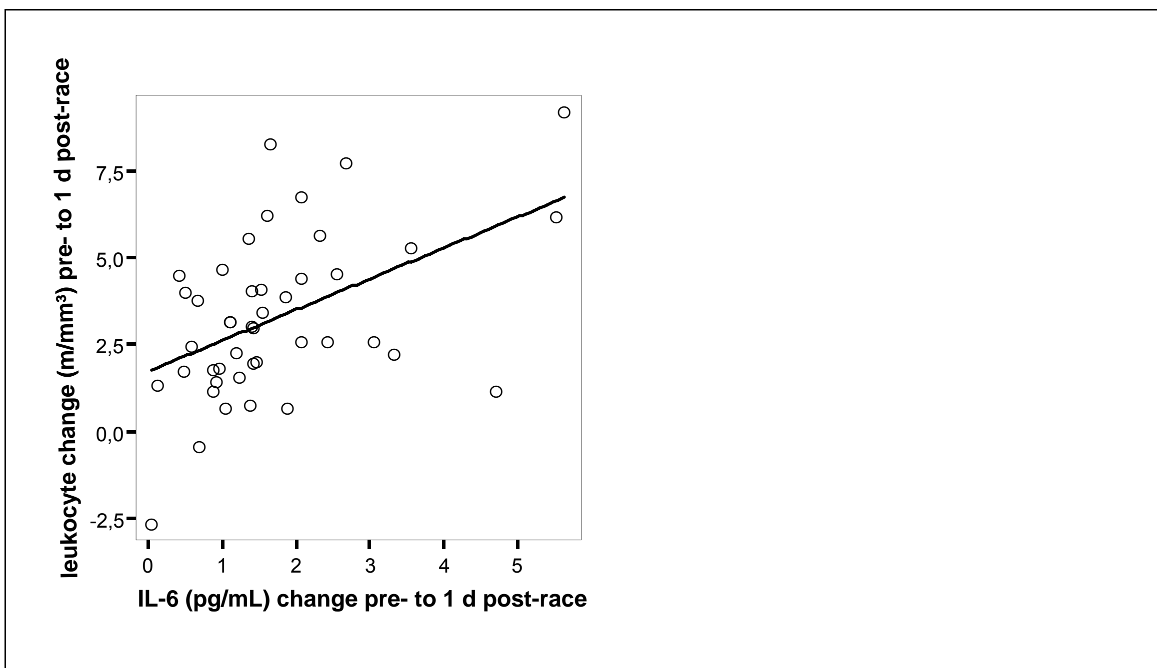



**FIGURE 11:** Correlation between the pre- to 1 d post-race change in the plasma concentration of interleukin (IL)-6 and the change of the total leukocyte counts from pre- to 1 d after the Ironman race (r=0.48; P<0.01).

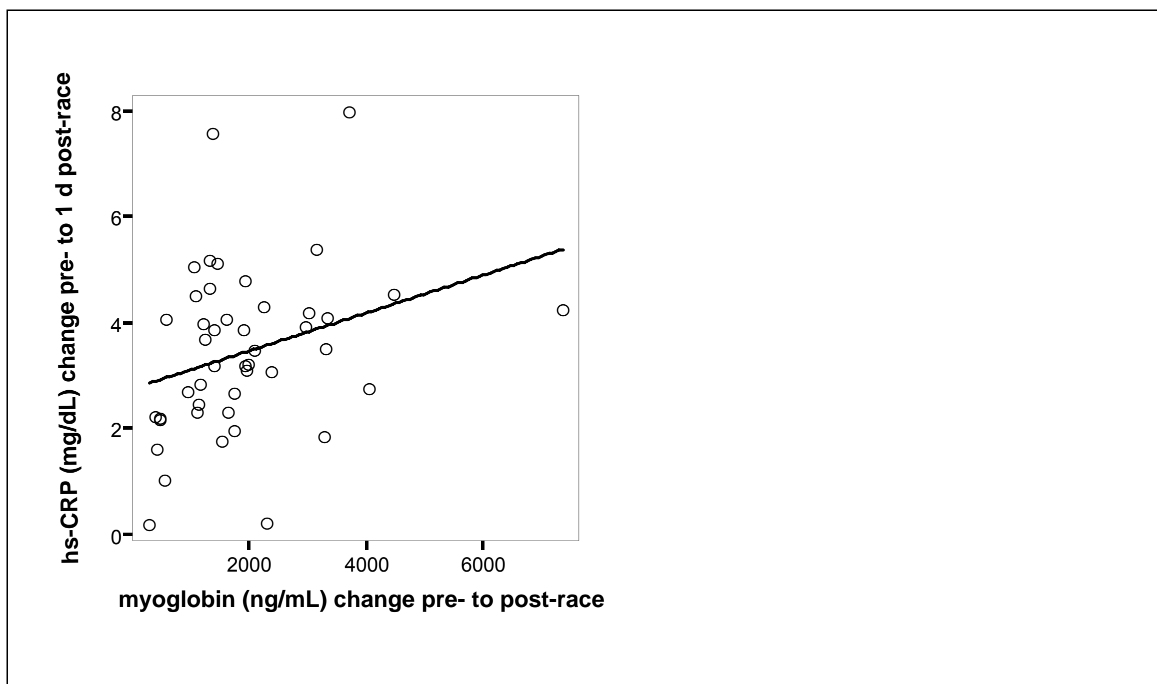



**FIGURE 12:** Correlation between the pre- to post-race change in the plasma concentration of myoglobin and the change in the plasma concentration of high sensitive C-reactive protein (hs-CRP) from pre- to 1 d after the Ironman race (r=0.61; P<0.001).

## 3.4. Discussion

The time-course of recovery after acute ultra-endurance exercise is a critical question that has hardly ever been answered. By quantifying the recovery responses until 19 days after the race in a large cohort of Ironman competitors we addressed this issue in a comprehensive manner. The main finding of the present study is that markers of muscle damage, IL-6 and hs-CRP were still altered 5 d after the long-distance triathlon. Moreover, the large number of study participants enabled us to substantiate scarce findings regarding associations between muscular stress and the inflammatory response with a high statistical power.

### 3.4.1. Response of cortisol and testosterone and the testosterone:cortisol ratio as an indicator of the balance between anabolic and catabolic effects of exercise

The response of the steroid hormones following the Ironman triathlon revealed the major systemic stress caused by the competition. Corresponding to previous studies (12, 24, 158), plasma cortisol rose significantly, whereas testosterone decreased in response to prolonged exercise. While plasma testosterone remained lower than pre-race 1 d post-

race, cortisol levels recovered and fell below pre-race values. The ratio of testosterone to cortisol that hypothetically indicates the balance between anabolic and catabolic effects of exercise (41) declined as an immediate response to the triathlon, but normalised within the first day of recovery. Exercise-induced changes of both hormones are known to affect the immune system (116), and in the present study cortisol was associated with the exercise-induced leukocytosis as well as with the increase of IL-10.

### 3.4.2. Indications for an initial ultra-structural damage of skeletal muscle and incomplete muscle regeneration 5 d after the Ironman triathlon

In agreement with other studies examining muscle damage following a long distance triathlon (40, 75, 155), the initiate injury of skeletal muscle was indicated by the leakage of myofibre proteins into the blood plasma. Mechanical stress (associated with exercise involving frequent eccentric/lengthening contractions such as in the marathon split of the Ironman triathlon) in addition to metabolic stress are believed to be the most important initial factors leading to exercise-induced muscle damage (159). In the present investigation, the time-course of muscle damage markers is similar to that characteristically found after protocols, in which metabolic stress is suggested to be heavily involved in muscle injury (75, 159, 175). The highest circulating myoglobin concentrations were found immediately after the race and, presumed that there was no further increase according to previous data (175), CK activity also peaked within 1 d after the triathlon. Since the training intensity of the subjects in the first days after the Ironman race was only modest compared to the tapering period before the competition, the prolonged appearance of myofibre proteins in the plasma until at least 5 d post-race is most likely related to subsequent muscle repair processes and probable local inflammatory responses. Although data is not consistent (149), there is good evidence to suggest that neutrophils and macrophages infiltrate damaged muscle (149, 162). Even though this is a desirable response in terms of muscle repair and probably also muscle adaptation (74, 162) it may trigger further muscular inflammatory processes and damage, partly through the increased formation of ROS (64). As discussed in previous and following sections (2.4.1. and 3.4.4., respectively) the assumption that muscular, inflammatory and oxidative stress responses are related with each other is supported by several associations that were observed within the current investigation. The slightly,

but significantly elevated plasma myoglobin concentration 19 d post-race might rather be attributed to the gradual resumption of running training (despite its moderate intensity and volume) than to direct race responses. Otherwise, this could also indicate that muscle regeneration was still not complete (159).

### 3.4.3. Leukocyte trafficking in blood in response to the Ironman triathlon and related factors

With regards to the cellular immune system the systemic inflammatory response was characterised by a pronounced leukocytosis immediately after the Ironman triathlon (Figure 7). Subsequently, the total leukocyte count declined, but was still significantly increased until 1 d post-race. Despite a significant decrease in lymphocyte percentages, there was an increase in total lymphocyte counts post- and actually also 1 d post-race, whereas lymphocyte concentrations trended to decrease 5 d after the competition. Although the mechanisms that are responsible for lymphocyte trafficking and (blood) redistribution, hitherto, remain unclear, prolonged strenuous exercise is known to induce an initial lymphocytosis followed by a lymphocytopenia in the recovery phase (66, 74, 116). Potential explanations are discussed within the context of chapter 4.

In support of previous studies (113, 157), a number of correlations between CK and myoglobin changes and alterations in total leukocyte count within the present study indicate that polymorphonuclear leukocytes (i.e. primarily neutrophils) are mobilized in response to exercise-induced muscle damage. The increase in circulating neutrophils following muscle damage is possibly due to an activation of the alternative complement pathway, which is stimulated by the appearance of damaged muscle tissue fragments in the blood (113, 157). Inflammatory mediators such as IL-6 have also been associated with the release of leukocytes (i.e. particularly neutrophils (154)), which is supported by the present data. Furthermore, the observed associations between the exercise-induced increase in cortisol and the changed leukocyte numbers 1 d after the competition provides further evidence to the proposed time-lapsed role of cortisol in leukocyte trafficking (64, 74, 116). In addition, the amplified response of plasma concentrations of MPO and PMN elastase in the course of the Ironman triathlon reflects a rapid neutrophil activation and degranulation. Despite a marked decrease towards pre-race, both granular

enzymes remained elevated after 1 d of recovery. In the case of MPO, the observed relationship between its change in plasma from pre- to 1 d post-race and the the IL-6 response immediately after the race may point to a delayed IL-6 stimulated neutrophil degranulation, as shown in a study of marathon running (154). Only few studies have investigated MPO levels following a marathon race (81, 154) and the present study appears to be the first to investigate the effects of an Ironman triathlon or exercise of a similar duration on MPO. Myeloperoxidase is also used as a novel marker for myocardial injury (81). However, data within the current study indicate that elevations in MPO as well as in PMN elastase most likely were part of the exercise-induced systemic inflammatory response, as the post-race increase in cardiac troponin T and brain natriuretic peptide were not associated with inflammatory stress (63). Both enzymes possess lytic capacities and thus they assist in the destruction of damaged tissue or in the destroying of infectious agents (12, 113, 162).

### 3.4.4. Initial and sustained responses of cytokines and high-sensitive C-reactive protein, and influencing factors

IL-6 is one of the most potent mediators of the early exercise-induced systemic inflammatory response and, consistent with the findings of previous ultra-endurance studies (53, 79, 99, 100, 155), plasma IL-6 concentration rose considerably (more than 100-fold) following the race. The relationship between the leukocytosis and the rise in IL-6 1 d after the competition probably indicates that IL-6 has promoted leukocyte mobilisation. A similar relationship is also reported in a previous marathon study (154). In addition, we found correlations between 1 d post-race concentrations of IL-6 and myoglobin along with CK (Table 9), but these associations were weaker than directly after a 160-km run race (98). It is plausible, that, lined up with recent research on that topic (33, 114, 152), contracting muscles accounted for the release of IL-6. In the year 2000, Steensberg et al. identified (working) skeletal muscle as an IL-6 producing organ (152), whereupon this cytokine has been classified as a “myokine” by Pedersen et al. (2007) (114). Moreover, there is evidence to suggest that the initial increase of plasma IL-6 is not solely responsible (113) for or actually independent (114) from the simultaneous exercise-induced muscle damage. Originally, Gleeson (2000) pointed to the possibility that other signals such as low glycogen stores may play a more important

role (36). The synthesis and subsequent release of IL-6 from exercising muscles, in turn, might not only modulate immunological, but also metabolic responses to acute exercise (e.g. by increasing fat oxidation or endogenous glucose output) (33). Previous studies have reported that plasma IL-6 concentrations had returned to pre-race values 1 d after an ultra-marathon (79) and after an Ironman triathlon (53), whereas after another Ironman race IL-6 levels were significantly elevated 1 d post-race (155), but IL-6 concentrations were not documented more than 1 d post-race. Importantly, we found that IL-6 levels fell down 1 d after the long-distance triathlon, but were sustained significantly above pre-race values until 5 d post-race. Similarly, Robson-Ansley et al. (2007) reported a low-grade, but prolonged elevation of systemic IL-6 following an acute period of intense running training (discussed in section 3.4.6. in more detail) (139). The migration of cytokine-releasing macrophages involved in muscle repair and persistent glycogen depletion are suggested to be the main reasons for the longer-lasting post-exercise elevation in IL-6 (139). Even despite an adequate carbohydrate intake, exercise-induced muscle damage can impair appropriate glycogen re-synthesis (51) and, as mentioned above, it is known that carbohydrate availability in skeletal muscles modulates IL-6 production (114). Thus, the low-level, but longer-standing IL-6 response into recovery in the present study may be associated with a delayed glycogen restoration as well as with regenerative processes modulated by recruited inflammatory cells (as another potential source of IL-6 production). Thereby, IL-6 might exert influence on the recovery, repair and adaptation of muscle after exercise (36). Moreover, although IL-6 is often classified as a pro-inflammatory cytokine, data also suggest that this cytokine has anti-inflammatory properties and may negatively regulate the acute phase response (33, 114, 119).

Consistent with previous studies (99, 154, 155), increased IL-10 concentrations were found immediately after the competition, whereupon IL-10 values remained significantly elevated until 1 d after the race. Based on the concept that the appearance of counter-regulatory anti-inflammatory cytokines such as IL-10 after strenuous exercise attenuates the inflammatory and immune response to prevent overshooting inflammation, it is suggested that well-trained athletes are able to balance the acute exercise-induced inflammation (30). Results of a recent study in Ironman triathletes

imply that there is a strong compensatory anti-inflammatory cytokine response (155). Our data support this idea because the pronounced *initial* inflammatory response was rapidly diminished in the course of the recovery from the competition. Regarding the potential stimuli of the anti-inflammatory cytokine response, the present data provide further indirect evidence that exercise intensity is a major factor in the elevation of IL-10. In a study investigating well-trained male runners, Peake et al. (2005) reported that factors related to the intensity of exercise such as stress hormones had a stronger influence on the production of anti-inflammatory cytokines than muscle damage (112). In line with these results, we observed that the exercise-induced change in IL-10 was negatively related to the run split time as well as the change in cortisol immediately post-race. Furthermore, the IL-10 increase was negatively correlated with the total race time and the run split time (i.e. IL-10 concentration rose with performance).

The delayed increase of CRP following intense endurance exercise is a sign that the inflammatory response had reached a systemic level (116). CRP is released from the liver induced by IL-6 (33) and is responsible for the recognition and clearance of damaged cells (121). CRP has also been established as a novel marker of inflammation and is suggested to provide additional information concerning atherosclerotic lesions (121). Whereas evidence increases, that regular physical activity reduces CRP concentrations (121), information concerning the response of CRP to an acute bout of ultra-endurance exercise is limited (59, 79, 155). In the present study, plasma CRP concentrations were significantly increased even immediately post-race, which is in contrast to another study in Ironman finishers (155). This rapid response in plasma CRP was followed by a marked further elevation 1 d post-race. Thereafter levels declined, but were still augmented 5 d post-race corresponding to the results of another ultra-endurance study, in which CRP levels after an ultra-marathon were determined over a longer time-course (79). Crucially, we found a number of associations between CRP and markers of muscle damage within 20 min, 1 d and 5 d after the Ironman. Therefore, our data support very recent findings (59) that muscle damage and the subsequent repair processes are important inducers of CRP. The slightly elevated CRP levels 19 d post-race (in parallel with moderately increased myoglobin concentrations) might be related to incomplete muscle recovery, but, alternatively, this could also be a sign of low pre-

race values. Interestingly, as reported in chapter 2, the observed association between pre- to post-race changes of CRP and the increase in lipid peroxidation (CD) from pre- to 1 d post-race, might indicate that the systemic inflammatory response propagates delayed oxidative stress responses (64).

### 3.4.5. Nutritional aspects in exercise immunology: importance of carbohydrates and antioxidants

There is huge interest in potential nutritional strategies to improve or maintain performance and to prevent fatigue, oxidative and/or immune-endocrine stress, immune dysfunction and probable detrimental health consequences following competitive endurance exercise (3, 11, 51, 97, 111). A main rationale for the ingestion of carbohydrates before and during competition especially concerning immune function is based on the (inverse) link between plasma concentrations of glucose and stress hormones such as cortisol (3). Cortisol is known for its role in maintaining neutrophilia and lymphocytopenia (116) and for its suppressive effects on several leukocyte functions (11). A decrease in blood glucose is an important stimulus for the activation of the hypothalamic-pituitary-adrenal axis which in turn leads to stimulation of the adrenal production and release of cortisol (3, 11). This hypothesis has been verified in several recent studies (12, 90, 116). The ingestion of a 6% carbohydrate beverage before and at regular intervals (0.25 litres every 15 minutes) during 2.5 hours of high-intensity treadmill running significantly raised plasma glucose levels, whereas plasma concentrations of cortisol as well as IL-6 were attenuated (90). In the present study, no link was found between carbohydrate intake during the race, plasma glucose concentrations and immune-endocrine or inflammatory parameters. A potential explanation for this finding is that inter-individual differences in the carbohydrate intake and blood glucose might have been too small to detect significant effects on immune-endocrine parameters including cortisol. On an average, study participants ingested sufficient amounts of carbohydrates during the race (69 ± 29 g per hour) (15), which apparently contributed to the maintenance of plasma glucose concentrations (post-race levels were 5.4 ± 1.4 mmol * L $^{-1}$). Importantly, the subjects´ carbohydrate supply during the Ironman race met the current guidelines concerning carbohydrate ingestion strategies in triathlon (50, 51). A carbohydrate intake of 60–70 g per hour is known to

enhance endurance capacity by maintaining blood glucose levels and high rates of carbohydrate oxidation (50, 51). During Ironman races or equivalent events an intake of up to 90 g of carbohydrate per hour is required to minimize negative energy balance, but according rates of absorption and oxidation of exogenous carbohydrate increase the risk of gastro-intestinal distress and can only be achieved by combinations of multiple transportable carbohydrates (49, 50, 52). However, as demonstrated in two recent randomized and placebo-controlled studies, ingestion of similar or even lower carbohydrate amounts (i.e. ca. 60 g per hour during intense treadmill running for 2.5 hours (90) or ca. 30 g per hour immediately before, during and after ergometer cycling for 2 hours (12)) were effective in alleviating the rise plasma cortisol.

Contrary to the results on carbohydrate ingestion as a countermeasure to the immune suppressive outcomes of intense prolonged exercise, data concerning beneficial effects of antioxidant supplementation on inflammatory and immune responses are not convincing (97, 111). Especially vitamin C has received much attention in the context of exercise immunology since this vitamin might, in theory, counter immuno-suppression, e.g. by reducing the auto-oxidative effect of neutrophil phagocytosis (11, 97, 116). Furthermore, in some (117), but not all recent studies (99) vitamin C has been reported to attenuate exercise-related increases in cortisol. However, in sum, there is only limited evidence that supplementation with vitamin C is an effective nutritional countermeasure to exercise-induced immune dysfunction and a probable increased risk of infection (3, 11, 97). Moreover, given the link between inflammation and oxidative stress (as discussed in section 2.4.1), antioxidants may hypothetically influence pathways that regulate cytokine production and, in turn, restrict the extent of the inflammatory cascade (64, 111). Nevertheless, findings in recent studies on the efficacy of antioxidant supplementation on inflammatory responses following ultra-endurance exercise are highly variable (111). Whereas supplementation with vitamin C attenuated plasma concentrations of IL-10, IL-1β and cortisol after a 90 km ultra-marathon (101, 117), both, supplementation with vitamin C alone (99) as well as a combination of vitamin C and α-tocopherol (79) did not attenuate immune changes after other ultra-marathon races with a distance of a mean of 69 km (99) and 50 km (79), respectively. In a recent vitamin E supplementation study, Nieman et al. reported even a higher cytokine

response after an Ironman triathlon in the supplement versus the placebo group, which was related to concomitant pro-oxidant effects of high doses of α-tocopherol (100). Possible explanations for these variations among study results might be attributed to differences in the supplementation regimen (with regards to dosage, timing, the supplementation period, and the biological activity of nutritive antioxidants), the mode of exercise and the extent of exercise-induced muscle damage (111). In the present investigation, no effects of both antioxidant intake during the race as well as plasma concentrations of nutritive antioxidants on endocrine, immune, inflammatory and muscle damage parameters were observed. As suggested previously in the context with oxidative stress (see section 2.4.4.), the lack of significant effects of antioxidants on exercise-induced alterations in these markers might be explained by the fact that the subjects´ plasma concentrations of nutritive antioxidants (both pre- and post-race) on average were in a normal physiological range. Exemplarily, the mean pre-race plasma concentration of vitamin C (66.6 ± 13.0 μmol * $L^{-1}$) was beyond the level that has been observed to be sufficient to saturate immuno-competent cells (> 50 μmol * $L^{-1}$) (22, 69). Furthermore, since antioxidant supplementation in the study participants was only modest (as a requirement to participate in the study) the differences in the plasma levels of nutritive antioxidants between the study participants, compared to supplementation studies (79), apparently were too small to obtain significant findings. In addition, the pronounced inflammatory response to ultra-endurance exercise likely might exceed the capacity of antioxidants to attenuate cytokine production (79, 111), probably also because there are several other factors than ROS that modulate the cytokine response. Nevertheless, it is important to emphasize that maintaining a normal nutritional status concerning the antioxidant intake undoubtedly plays a key role in preserving an adequate immune function in training and competing athletes (11, 37).

### 3.4.6. Practical implications: the length of time needed for recovery after an Ironman triathlon

In order to avoid eventual negative consequences of training for and competing in ultra-endurance races, it is crucial to determine the length of time necessary to recover from the inflammatory and muscular responses induced by competitions such as an Ironman triathlon. To the best of my knowledge, these stress and recovery responses, up to now,

have not been followed for such a long time course and in such a comprehensive manner as in the framework of the present research work. Hence, these results provide valuable novel knowledge since they are highly relevant for designing training schedules and achieving an appropriate balance between training and competition stresses and recovery especially after completing such a race. This is of particular importance for athletes who wish to maintain a high level of performance to compete in another event, e.g. those athletes who compete in an Ironman event to qualify for the Ironman World Champion Ship at Hawaii, which usually takes place two to four months after the qualifying race.

Prolonged periods of heavy training and competition can result in longer lasting immune dysfunctions that, in turn, may compromise the resistance to common minor illnesses including upper respiratory tract infections (URTI) (3, 35, 37). Beyond, a disproportionate balance between training, competition and recovery is suggested to lead to overreaching or overtraining (41). According to a definition by Halson and Jeukendrup (2004), states of both overreaching and overtraining are characterised by fatigue and a reduction in the performance capacity with or without related physiological and psychological symptoms. Importantly, the major difference in these states is the duration needed for performance restoration. Overreaching may occur as a consequence of the normal training process that, if adequate rest and recovery is provided, may stimulate a super-compensation effect and a higher performance level within a relatively short amount of time (days to approximately two weeks). On the contrary, continual periods of intensified training and competition without sufficient recovery are thought to progressively cause the more severe state of overtraining in which restoration of performance might take several weeks or months (41). Although it seems plausible that overreaching and overtraining is associated with an impairment of the immune system and an increased susceptibility to infections, there is only little scientific data available to confirm this assumption (41, 74). Moreover, up to now, knowledge on the mechanisms which can cause a state of overtraining is incomplete. In a recent study, Robson-Ansley and co-authors (2007) observed chronically elevated IL-6 levels in triathletes following a highly intensive training period. This finding support the hypothesis that the overtraining or underperformance syndrome could be related to

abnormal cytokine responses (139). Initially, Smith (2000) proposed a model through which the state of declining performance, fatigue and depression might be caused by repeated micro trauma and subsequent local inflammatory reactions within muscle and other tissues that result in a chronic systemic inflammatory condition (148). In light of this hypothesis, Robson (2003) suggested that a dysregulated overproduction of and/or intolerance to IL-6 induced by excessive training and competition, insufficient recovery and psychological stressors could be implicated in the aetiology of the underperformance syndrome (140). Beside the potentially long-term debilitating outcomes in the form of overtraining, lack of appropriate recovery may impair an athletes ´s ability to attain training requirements (e.g. to train at the required intensities), and it may also predispose to injury (7).

With regards to the duration needed for full recovery, it is important to note that the study participants performed "recovery" training in the period between the Ironman race and 19 d post-race. This is described in section 2.2.2., and shown in table 1. As mentioned above, the moderate, but significant elevations in IL-6, hs-CRP and markers of muscle damage (linked with hs-CRP) until at least 5 d after the Ironman race can probably be attributed to sustained muscle repair and associated inflammatory processes. Nineteen days after the triathlon, most immune-associated and muscular stress parameters had returned to (or even below) pre-race values, except for hs-CRP and myoglobin, which remained slightly, but significantly above pre-race concentrations. The latter could also be related to incomplete muscle recovery, but, as discussed, there might be alternative explanations. Together with the other findings of our research (no indications of persistent oxidative stress and DNA damage; see chapters 2 and 4), the recovery processes may at least be advanced and in a final stage 19 d after the Ironman triathlon. I ´m aware that the circumstances concerning the duration of recovery responses after ultra-endurance exercise should be discussed cautiously since more data is required. However, from the perspective of the observed muscle repair and related inflammatory processes, abstention from training at higher intensities and from muscle-damaging exercise (i.e. exercise involving frequent eccentric/lengthening muscle contractions including running) for at least two to three weeks after such a race is apparently advisable. Recovery modalities such as active

recovery most likely help athletes e.g. to reduce the severity and duration of muscle injury and delayed onset of muscle soreness (7) before they might gradually return to a more intensive training. Of note, appropriate nutritional practices can help to optimise the recovery processes. Thereby, the quantities of carbohydrates and proteins consumed after exercise are regarded as one of the most important nutritional factors determining muscle glycogen (re-) synthesis, muscle protein balance and repair. For more detailed information on the nutritional demands of triathlon the reader is referred to a recent review by Jeukendrup et al. (51).

Finally, in the context of the hypothesised role of a chronic inflammatory state in the development of the overtraining or underperformance syndrome, the three participants of the current study, although limited to three subjects, another observation of this study is noteworthy. Interestingly, the three subjects who dropped out from the Ironman race due to self-reported fatigue symptoms (but without any apparent systemic symptoms of an infection) had much higher pre-race CRP concentrations than any other study participant with levels ranging from 316 to 1442% above the mean pre-race concentration. In two of them, these values exceeded the normal clinical range, and in parallel noticeable higher MPO levels (in both) and IL-6 concentration that was nearly 300% higher than the mean pre-race IL-6 level (in one subject) were found. The anomalies in these inflammatory markers may represent a prolonged inflammatory condition following the prolonged training period and thus might provide some additional evidence to the novel cytokine hypothesis of the unexplained underperformance syndrome (139, 140, 148).

## 3.5. Conclusion

The most important finding of the current study was that although the marked initial inflammatory response induced by an Ironman triathlon subsided rapidly, a low-grade systemic inflammatory response was sustained for at least five days of recovery. The prolonged moderate, but significant elevation of IL-6 and CRP might be associated with inflammatory processes and/or impaired glycogen replenishment within damaged muscle. Athletes may be more susceptible to infections due to this attenuated immune competence within this first period of recovery after demanding endurance exercise.

Furthermore, inadequate rest following prolonged, intensive exercise might cause a chronic systemic inflammatory state that in turn leads to a syndrome of impaired performance and progressive fatigue. However, due to the continuous demands of Ironman competition on training schedules, competitive athletes might not have sufficient recovery between the races. Thus, finding an appropriate balance between training, competition and recovery is an essential challenge to maintain a high level of performance and to minimise potential health consequences. Based on the current results, one might recommend athletes to abstain from training at higher intensities and from muscle-damaging exercise for at least two to three weeks following an event such as an Ironman-distance triathlon.

# 4. Consequences of Inflammation, Oxidative Stress and Antioxidant-related Factors on DNA Stability in Response to an Ironman Triathlon

This chapter is based on a review that includes original data from the current study. This review article has been published in *Exercise Immunology Review*. The paper is also attached subsequently to this chapter and is cited as: **Neubauer O, Reichhold S, Nersesyan A, König D, Wagner K-H** (2008): Exercise-induced DNA damage: Is there a relationship with inflammatory responses? *Exerc Immunol Rev.* 14:51-72.

Results on this issue have also been presented at the following scientific meetings by the author:

- *2nd Copenhagen Workshop on DNA Oxidation*, Jan. 29th – 30th 2009, University of Copenhagen, Denmark: **Neubauer O, Reichhold S, Knasmüller S, König D, Wagner K-H**: Exercise and DNA damage: Significance of Inflammatory and Antioxidant Responses (poster presentation, abstract: abstract booklet)
- *24th Meeting of the Gesellschaft für Umwelt-Mutationsforschung (GUM; German section of the European Enviromental Mutagen Society),* Feb. 17th – 20th 2009, University of Vienna, Austria: **Neubauer O, Reichhold S, König D, Knasmüller S, Wagner K-H**: Consequences of Inflammatory and Antioxidant Responses on DNA Damage after Ultra-endurance Exercise (oral presentation, abstract: abstract booklet)

The effects of the Ironman triathlon on DNA stability were investigated within the framework of another doctoral thesis by Stefanie Reichhold (133). For more detailed information on this part of the research project the reader is referred to original articles by Reichhold et al. (135, 136). Furthermore, our working group has most recently reviewed investigations that have studied the impact of exercise on DNA stability with a focal point on the effects of various exercise intensities and duration (134). These papers are attached subsequently to the present dissertation.

## 4.1. Introduction

After extensive research in the past decades, the effects of exercise on the immune system are well documented (35, 74, 116). However, researchers in this area are still puzzled by questions about the underlying molecular mechanisms of the observed immunological alterations (73, 74). Extremely demanding endurance exercise has been shown to induce both a systemic inflammatory response (30, 93, 116, 154) as well as DNA damage (42, 80, 122, 135, 165). Exercise-induced DNA damage in peripheral blood cells appear to be mainly a consequence of an increased production of ROS during and after vigorous aerobic exercise (122). Beside oxidative stress, other factors such as metabolic, hormonal and thermal stress in addition to the ultra-structural damage of muscle tissue are characteristic responses to prolonged strenuous exercise, that can lead to the release of cytokines, acute phase proteins and to the activation or inhibition of certain lines of the cellular immune system (30, 64). In addition to these effectors, exercise-induced modifications in DNA of immuno-competent cells have been hypothesized to be related with immune and inflammatory responses to prolonged intensive physical activity, either by resulting from exercise-induced inflammatory processes and/or by playing a causative role (42, 88, 102, 116). Based on the pioneer findings on exercise-induced DNA damage, researchers in this field questioned whether damage to cellular DNA after vigorous exercise could also induce apoptosis and whether programmed cell death, in turn, may be related to the exercise-induced regulation of leukocyte counts and, particularly, lymphocyte trafficking and redistribution in the blood (116). Nevertheless, only few studies investigated both, certain endpoints of DNA damage and immuno-endocrine and inflammatory parameters in the context of exercise (13, 79, 80, 102, 118). Hence, experimental data as well as a more mechanistic understanding regarding this relationship are still incomplete.

To the best of my knowledge, the current study was the first that investigated DNA stability in the course of an acute bout of exercise of such duration. Endpoints of DNA and chromosomal damage in lymphocytes were assessed by the single cell gel electrophoresis (SCGE or COMET) (136) and the cytokinesis block micronucleus cytome (CBMN Cyt) assays (135) (briefly described in section 4.2.2.). It was another major aim of our research to test the hypothesis whether there is a relationship between

DNA damage in lymphocytes on the one hand and immunological alterations, inflammatory stress and markers of muscle damage (as probable indirect indices for local inflammatory processes post-race) on the other. Furthermore, by concomitantly exploring oxidative stress markers and antioxidant-related factors, we aimed to particularize a potential interaction of oxidative stress between inflammation and DNA effects over a period of 19 days into recovery after the Ironman race. Whereas the Ironman-induced changes in oxidative stress, antioxidant-related and immunological factors as well as endpoints of DNA stability have been reported and discussed in the previous chapters by the author and by Stefanie Reichhold within her doctoral thesis (133), the following chapter focuses on the associations between these effects.

## 4.2. Materials and Methods

For detailed information on the study participants, the study design, race conditions and methodological aspects with regards to the biochemical analysis of oxidative stress markers, antioxidant-related factors, immunological/inflammatory parameters and markers of muscle damage the reader is refered to the respective sections in chapters 2 and 3.

### 4.2.1. Subjects

Of the entire study group 20 and 28 subjects were randomly selected for the CBMN Cyt and the SCGE assays, respectively.

### 4.2.2. DNA and chromosomal damage in lymphocytes

For the analysis of DNA and chromosomal damage in lymphocytes, blood samples were processed instantly as described by Reichhold (2009) (133). As reported previously (135, 136), the SCGE and CBMN Cyt- assays were carried out according the methods described by Tice et al. (161) and Fenech (31), respectively. Within the SCGE-assay, oxidative DNA base damage was assessed on the basis of the protocols of Collins et al. (18), Collins and Dusinska (17) and Angelis et al. (4). Analysed endpoints within the SCGE assay included: determination of DNA migration 1.) under standard conditions to measured single and double strand breaks (determined as percentage of DNA in the tail), and 2.) using lesions specific enzymes endonuclease (ENDO) III and

formamidopyrimidine glycosylase (FPG) to detect oxidized pyrimidines and purines, respectively. Biomarkers within the CBMN Cyt block included the number of 1.) micronuclei (MN) (resulting from chromosomal breakage or loss), 2.) nucleoplasmic bridges (NPBs) (indicating chromosome rearrangements), 3.) nuclear buds (Nbuds) (that are formed as a consequence of gene amplification), and 4.) necrotic and apoptotic cells.

### 4.2.3. Data analysis

Details of the data analysis concerning the endpoints of the SCGE and CBMN Cyt-assays have been described previously (135, 136). The additional correlation analysis that is reported in this chapter was identical as described in section 2.2.12. Briefly, Pearson ´s correlation was used to examine significant relationships. In case of observed trends or significant correlations, subjects were divided into percentile groups by the associated variables (e.g. IL-6). One-factorial ANOVA and *post hoc* analyses with Scheffé ´s test were then applied to assess whether differences in endpoints of DNA or chromosomal damage were associated with the percentile distribution. Significance was set at a P-value <0.05 and is reported P<0.05, P<0.01 and P<0.001.

## 4.3. Results

### 4.3.1. Race results

Race results are described in the sections 2.3.1., 3.3.1., and summarized in Table 1. There were neither significant differences in the performance nor in the consumed antioxidants between the whole study group and the subgroups that were tested for genome stability.

### 4.3.2. Endpoints of DNA and chromosomal damage, apoptosis and necrosis

As reported by Reichhold (2009) (133) and Reichhold et al. (2008, 2009) (135, 136), the results concerning DNA and chromosomal damage were as follows: Within the SCGE assay, a decrease was observed in the level of strand breaks immediately after the race. One day post-race the levels of strand breaks increased (P<0.01), then returned to pre-race 5 d post-race, and decreased further to below the initial levels 19 d post-race (P<0.01). Immediately post-race there was a trend in ENDO III and FPG-sensitive sites

to decrease. Compared to pre-race, a tendency towards an increase in the ENDO III-sensitive sites (P=0.09) occurred, but levels decreased until 19 d (P<0.05). No significant changes were observed in the levels of FPG-sensitive sites throughout the monitoring period (136).

Within the CBMN Cyt assay, the number of MN significantly (P<0.05) decreased immediately post-race, and declined further to below pre-race levels 19 d after the Ironman competition (P<0.01). There were no changes in the frequency of NPBs and Nbuds as an immediate response to the triathlon, but 5 d thereafter the frequency of Nbuds was significantly (P<0.01) higher than levels immediately post-race. However, 19 d post-race the frequency of Nbuds returned to pre-race levels, while the number of NPBs was significantly (P<0.05) lower than pre-race (135). The overall number of apoptotic cells decreased significantly (P<0.01) immediately post-race, and declined further until 19 d after the race (P<0.01). Similarly, the overall number of necrotic cells significantly (P<0.01) declined immediately post-race, and remained at a low level 19 d after the Ironman (133).

### 4.3.3. Associations between endpoints of genome stability and immuno-endocrine, Inflammatory and Muscle Damage Parameters

No significant correlations were found between all these markers at all time-points with the exception of a link between IL-6 and necrosis. Immediately post-race, the plasma concentration of IL-6 correlated positively with the number of necrotic cells (r=0.528; P<0.05). In addition, significant associations were observed on the basis of a group distribution into percentiles by the IL-6 concentrations immediately post-race. First, the numbers of necrotic cells increased with IL-6 across the percentiles, and the differences between all groups were P=0.012. Second, necrosis in lymphocytes was significantly (P=0.017) higher in the subject group with the highest IL-6 concentrations (top percentile) compared with the lowest IL-6 values (lowest percentile).

### 4.3.4. Associations between oxidative DNA damage and antioxidant capacity of plasma

Significant positive correlations were observed between ENDO III-sensitive sites and ORAC immediately after (r= -0.54; P<0.01) and also 1 d (r= -0.65; P<0.05) (Figure 3).

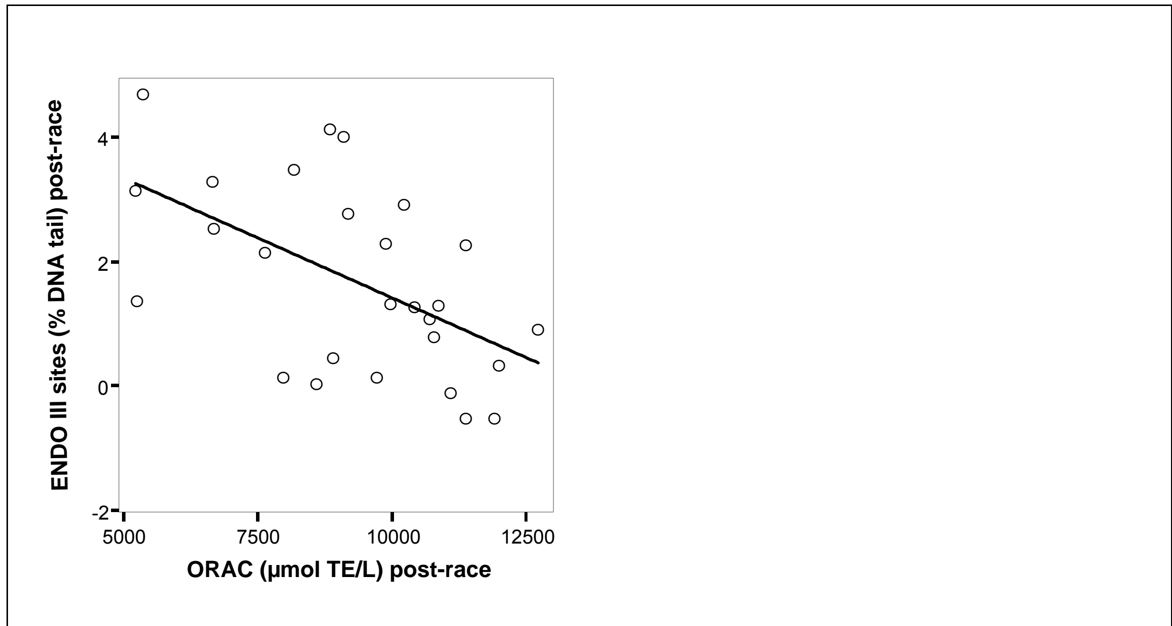



**FIGURE 13:** Inverse correlation between the total plasma antioxidant capacity as assessed by the Oxygen Radical Absorbance Capacity (ORAC) assay and oxidative DNA damage as detected by the use of the lesion specific enzyme endonuclease (ENDO) III immediately after the Ironman triathlon (r= -0.54, P<0.01).

### 4.3.5. Associations between endpoints of genome stability and training-related determinants

As previously reported together with Stefanie Reichhold and co-authors of our working group (2008, 2009), DNA stability was related with the training status of the study participants (135, 136). First, the level of DNA strand breaks immediately post-race was inversely correlated with the weekly net endurance exercise time (r= -0.40, P<0.05) (136). A second training-associated effect was observed when dividing the total group of study participants randomized for the CBMN Cyt assay into two sub-groups according their $VO_{2\ peak}$ (cut-off point: 60 ml kg $^{-1}$ min $^{-1}$). The decrease in the number of MN was most pronounced in the group with a $VO_{2\ peak}$ of more than 60 ml kg $^{-1}$ min $^{-1}$ (135).

## 4.4. Discussion

A major finding of the present investigation is that there were no associations between different markers of DNA and chromosomal damage and parameters of muscle damage and inflammation in participants of an Ironman triathlon as a prototype of ultra-endurance exercise with the exception of a link between IL-6 and necrosis. The current results suggest that there might be no *direct* relationship between exercise-induced DNA effects and immune and inflammatory responses, while oxidative DNA damage may have been counteracted by training- and exercise-induced antioxidant responses. Beyond the framework of, but relevant to the current thesis, the findings of Reichhold (133) indicate that an acute bout of ultra-endurance exercise does not cause sustained DNA or chromosomal damage in well-trained triathletes.

### 4.4.1. A brief overview: (ultra-)endurance exercise and DNA stability – no indications for sustained DNA or chromosomal damage in well-trained athletes

As most recently reported in reviews (94, 134), there is growing evidence that strenuous exercise can lead to DNA damage. With regards to competitive (ultra-)endurance exercise, with few exceptions (80), signs for DNA damage have not been observed before 24 h after a short-distance triathlon (42), a half-marathon (102) or a marathon run (165). Thus, changes in DNA stability might have been missed in studies with shorter monitoring periods (13, 145, 167). However, results are not consistent. Exemplarily, no DNA damage was detected after 2.5 hours of intense treadmill running in well-trained endurance athletes (118). On the one side, the diversity of methods, used sample matrices and endpoints are likely to contribute to inconsistencies among the studies on this issue. The most commonly applied approaches to examine DNA stability in exercise studies are the SCGE assays, the MN assay, and its further developed version, the CBMN Cyt assay as well as measurement of 8-oxo-7,8-dihydro-2-deoxyguanosine (8-OHdG). Naturally, these methods have their own principles and also limitations, which are described in the literature (19, 31, 60, 122) including our reviews (94, 134). On the other side, the heterogeneity in study cohorts (varying in gender, age and training status) and different study designs (i.e. divergent exercise protocols and sampling time-points) make if difficult to determine the exact circumstances under which DNA damage occurs (94, 134). Moreover, a clear-dose response relationship

regarding the level of exercise that could be detrimental cannot yet be established. Results of the few studies that have investigated genome stability just after ultra-endurance exercise, indicate that adaptations of endogenous protective antioxidant and/or repair mechanisms may prevent severe and persistent DNA damage in well-trained endurance athletes (80, 85, 130). The current findings in the Ironman triathletes further support this assumption. Compared to pre-race values only temporary increased levels of DNA strand breaks (1 d post-race) (136) and nuclear buds (5 d post-race) (135) were observed in response to the Ironman race. Other endpoints of DNA or chromosomal damage remained unchanged or, such as the number of binucleated cells with MN, even decreased during the recovery period following the Ironman (135). Hence, whereas epidemiological and empirical data indicate protective effects of physical activity on site-specific cancer risk (122, 142, 160), currently, there are no indications that extremely demanding exercise increases the risk for cancer and other diseases via DNA damage. However, it remains to be clarified whether perturbances in the DNA stability of immuno-competent cells are involved in the temporary dysfunction of certain aspects of immunity after intense prolonged exercise.

#### 4.4.2. No indications for a direct relationship of DNA damage in lymphocytes with inflammation and immunological alterations

The data from this study indicate that DNA damage is neither causally involved in the initial systemic inflammatory response nor in the low-grade inflammation that was sustained at least until 5 d after the Ironman race (discussed in chapter 3). Instead, based on several assessed relationships between leukocyte dynamics, cortisol, muscle damage markers and cytokines, the pronounced but temporary systemic inflammatory response was most likely induced by stressors other than DNA modulations. In fact, consistent with previous studies in this context, factors such as the initial ultra-structural injury of skeletal muscle (113, 157), changes in concentrations of cortisol (116) and IL-6 (154) apparently mediated leukocyte mobilization and activation. Vice versa, missing links between all these markers in the present study indicate that exercise-induced inflammatory responses do not promote DNA damage in lymphocytes (discussed later in more detail). These results support those of Mastaloudis et al. (2004), who demonstrated that inflammatory and muscle damage responses, indeed, do not *directly*

interact with the mechanisms of oxidative DNA damage (79, 80, 164). Mastaloudis et al. (2004) reported that DNA damage in leukocytes increased temporarily mid-race of an ultra-marathon, but returned to baseline 2 h after the competition and, similarly with the findings in the previous study, even decreased to below baseline values by 6 d post-race (80). As probable causes for this decrease in the proportion of cells with DNA damage, the authors suggested enhanced repair mechanisms, increased clearance and/or a redistribution of damaged cells. Of note, plasma concentrations of inflammatory parameters, $F_2$-isoprostanes and antioxidant vitamins were investigated in the same subjects (79). Although acute oxidative and inflammatory responses were observed, no correlations between either of these markers with DNA damage were reported (80). Furthermore, supplementation with vitamins C and E prevented increases in lipid peroxidation, but had no noticeable effects on DNA damage, on inflammation and on muscle damage. The authors concluded that the mechanism of oxidative damage is operating independently of the inflammatory and muscle damage processes (79, 80, 164).

### 4.4.3. ROS as a link between inflammation and DNA damage: relevance of antioxidant responses

Nevertheless, the lack of links in these studies do not rule out the possibility that inflammatory processes can trigger oxidative stress via oxidative burst reactions of circulating neutrophils and an increased cytokine formation (30, 57, 64, 111, 156) (see section 2.4.1.). In turn, the latter might lead to secondary (oxidative) DNA damage in immuno-competent cells (165). Considering the very few studies in which markers of DNA damage were found to correlate with signs of inflammation (102) or muscle damage (165), DNA damage in peripheral immuno-competent cells, indeed, most likely resulted from an increased generation of ROS due to initial systemic inflammatory responses or the delayed muscle inflammatory processes. As one of the earlier works in the context on the effects of particularly competitive endurance exercise on DNA damage, Niess et al. (1998) found that neutrophil counts 1 h after a half-marathon run correlated with levels of DNA strand breaks in leukocytes 24 h after the race (102). Although no markers of oxidative stress were examined in this study, the authors supposed that ROS released by neutrophils might have been responsible for the

observed delayed DNA damage, which, in turn, may be the key mechanism for the modifications in the immune cell counts (102). Similarly, based on their findings in short-distance triathletes (no signs for oxidatively damaged DNA immediately, but highest values within the standard SCGE assay 3 d after the competition), Hartmann et al. (1998) hypothesised that DNA damage may occur due to inflammatory reactions in the course of the muscle damage rather than acute oxidative stress (42). However, whereas no immune or inflammatory parameters were measured in the investigation of Hartmann el al. (1998) (42), likewise no correlations were reported in other exercise studies that actually examined both endpoints of DNA damage on the one hand and immune/inflammatory (13, 79, 80, 104, 118) or muscle damage markers (43, 85, 106, 109, 130, 145, 167) on the other. Tsai et al. (2001) was, to my knowledge, the only working group that observed links between DNA damage and exercise-induced muscle damage in humans (165). The prolonged monitoring period of up to 14 d after a marathon race might account for the observed significant correlations between peak levels of ENDO III-sensitive sites and urinary 8-OHdG on the one side and plasma parameters of muscle damage and lipid peroxidation on the other. In support of the conclusions of Hartmann et al. (1998) (42), the authors suggested that inflammatory cells infiltrating into injured skeletal muscle tissue and activated phagocytes were responsible for the increased production of ROS and consequently the delayed oxidative DNA damage during the reparative processes after the marathon (165). This idea is supported by a study in rats, in which DNA damage in circulating white blood cells was closely related to muscle damage due to exercise (166). Nevertheless, based on these findings it is not possible to draw a clear conclusion as to whether oxidative DNA modifications in peripheral immuno-competent cells are casually related with immune disturbances or whether DNA damage in leukocytes, in fact, results from oxidative stress that occurs through inflammatory processes after strenuous exercise.

Collectively considered, the results from the present investigations reveal a complex picture how inflammatory, oxidative and DNA stress responses to ultra-endurance might interact with each other. As reported in chapter 2, there were temporary increases in both, protein and lipid peroxidation markers as well as in the plasma antioxidant capacity following the Ironman triathlon. There were moderate oxidative stress

responses 1 d after the Ironman race, which, based on observed correlations between markers of oxidative stress, muscle damage and inflammation (discussed in more detail in section 2.4.1.), were probably related with inflammatory processes during ongoing muscle repair and regeneration. Nevertheless, training- and acute exercise-induced responses in the antioxidant defence system were apparently able to prevent severe or longer-lasting oxidative damage post-race. While these strong antioxidant responses most likely played a significant role in counteracting sustained oxidative stress post-race in the current study, it seems that antioxidant defences in the study group of Tsai et al. (165) were not sufficient to confer protection against delayed oxidative damage to lipids and DNA due to reparative processes of muscular tissue. Whatever the reasons for these discrepancies in the oxidant/antioxidant balance are (differences in training-induced biochemical adaptations, antioxidant status and/or antioxidant intake during the race, etc.), this might be a major explanation for the inconsistencies between the findings of Tsai et al. (165) and the current results. In fact, the observed negative correlations between the ORAC and ENDO III-sensitive sites immediately (Figure 15) and 1 d after the Ironman race suggest that an enhanced plasma antioxidant capacity might have prevented oxidative DNA damage (136). These findings are in line with a recent animal study (6), which demonstrated the protective role of an enhanced serum antioxidant capacity in lymphocyte apoptosis. Interestingly, the temporary trend to increase in ENDO III lesions 5 d after the Ironman competition was found simultaneously with the reported moderate prolongation of inflammatory processes (see section 3.4.2.) and at the same time as levels in the plasma antioxidant capacity had returned to baseline values (Figure 3). Since no additional links were observed among these responses, it remains uncertain whether ROS derived from inflammatory cells accounted for this tendency towards an increase in oxidized pyrimidines. However, in this context it is also important to note the general difficulty in determining correlations between markers of oxidative DNA damage and other biomarkers of oxidative stress, partly due to differences in the biological sites where oxidative damage occurred (27).

Taken together, whenever correlations between DNA damage in immuno-competent cells and inflammation (102) or muscle damage (165) were observed, ROS derived from inflammatory cells, appear to be the key effectors that link inflammation with

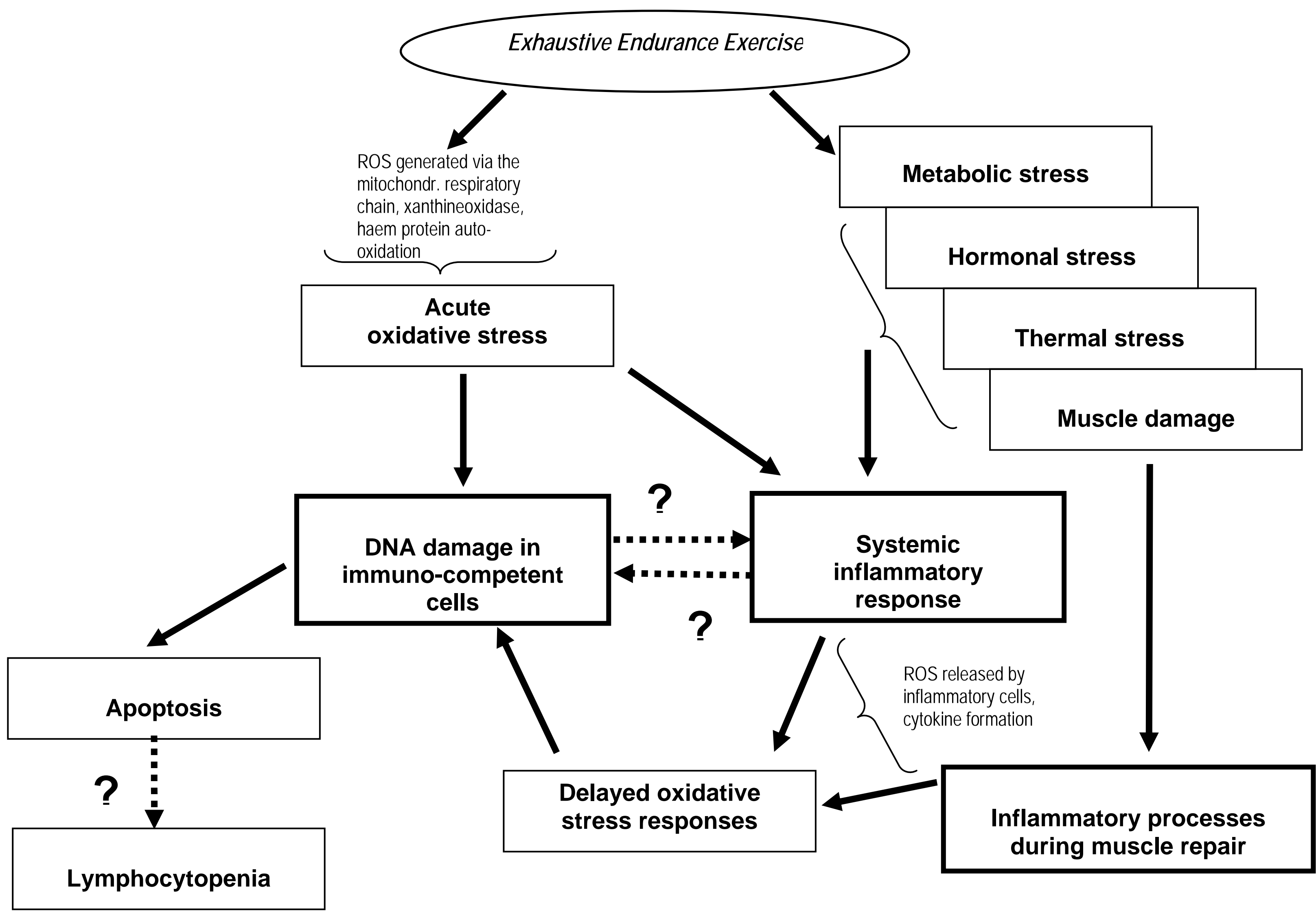



**FIGURE 14:** Proposed model of exercise-induced DNA damage and inflammatory responses; adapted from (94)

DNA damage after vigorous exercise. Figure 14 is a schematic illustration of the relationships between these stress responses to exhaustive endurance exercise. It may be argued that results from our study fit well into this picture insofar that antioxidant mechanisms neutralized an enhanced generation of ROS potentially resulting from inflammatory processes due to the injury of skeletal muscle tissue, and consequently were able to prevent lymphocyte DNA damage. Beyond, it should also be noted that, similar to DNA effects, muscle inflammatory processes and related oxidative stress responses might be sustained for or appear days after muscle-damaging exercise (105). Hence, potential links between these outcomes might have been missed in investigations with shorter monitoring periods (13, 88, 118, 145, 151).

**4.4.4. Implications of apoptosis and necrosis on immunological alterations**

In response to cellular stressors that lead to DNA damage, apoptosis is vital in preventing the propagation of severely damaged DNA and in maintaining genomic stability (70). Exercise-induced apoptosis in particular is also regarded as an important auto-reactive immune cells (65, 87). However, since apoptotic processes are suggested to influence the characteristic decline of lymphocyte blood concentration after strenuous prolonged exercise, these alterations may have consequences for the post-exercise immune dysfunction (65, 74, 116). Mars et al. (1998) were the first to describe apoptosis in lymphocytes after exhaustive exercise (treadmill running) that was paralleled by DNA damage (77). By applying flow cytometry and annexin-V to label apoptotic cells, Mooren et al. (2002, 2004) confirmed that either short maximal exercise (in untrained subjects) (87) as well as competitive endurance exercise (a marathon run) (88) has the potential to induce lymphocyte apoptosis. Nevertheless, the authors concluded that the changes in the proportions of apoptotic cells after exhaustive exercise were small and, if at all, might only partially account for the concomitantly observed significant decline of lymphocytes to below baseline levels.

In the present investigations levels of apoptosis decreased immediately after the Ironman race and remained at these low levels throughout the whole monitoring period (136). In addition, our data revealed no link between apoptosis and post-race changes in lymphocyte counts or any other leukocyte subpopulation. In agreement with the results

of the current study, previous research in this context suggests that intensive endurance exercise does neither automatically induce apoptosis in lymphocytes nor cause DNA damage (assessed immediately and 3 h post-exercise), provided that subjects are well-trained (118). Since there was no correlation between the (non-significant) decrease in circulating lymphocytes and the percentage lymphocyte apoptosis after a 2.5 h treadmill run at 75% $VO_{2\ max.}$, Peters et al. (118) concluded that the typical post-exercise lymphocytopenia is not due to apoptotic regulation by the immune system. The latter results are consistent with another study which was conducted with a similar exercise protocol, but in untrained subjects (151). Steensberg et al. (151) noted that the lymphocytes which left the circulation during the first 2 h post-exercise were characterised by not being apoptotic. Thus, mechanisms other than apoptosis seem to play a more important role in inducing lymphocytopenia after exercise, including a redistribution of lymphocytes and/or a lack of mature cells that can be recruited (116). Noteworthy, in most recent animal studies Krüger et al. (2007, 2009) demonstrated that exercise-induced lymphocyte trafficking as well as lymphocyte apoptosis are systemic phenomena occurring in various lymphoid and non-lymphoid organs, while only a small fraction of lymphocytes can be found transiently in the circulation (65, 66). The mechanisms responsible for both lymphocyte recirculation and apoptosis in response to exercise are still not fully understood. To a certain extend, these processes might be explained by exercise-induced changes in the expression of certain adhesion molecules and surface receptors concerning lymphocyte homing (66), and cell death receptors and ligands with regards to apoptosis (88). In addition, it has been shown that exhaustive endurance exercise results in both a shift to a lymphocyte population with a higher density of these cell surface death (Fas or CD95) receptors (87). Also, Atamaniuk et al. (2008) demonstrated that a shift to a pro-apoptotic state in the balance between pro- and anti-apoptotic genes occurred after an ultra-marathon (5). Moreover, exercise-induced alterations in corticosteroids and catecholamines are known to play a major role in post-exercise leukocytosis and lymphocytopenia (see also section 3.4.3.) (35, 89, 116). However, contrary to previous findings (45), recent results imply that cortisol affects the cellular immune system more by other pathways than via apoptotic regulation (118). Furthermore, the occurrence of DNA damage in the course of exercise does not

necessarily implicate induction of apoptosis (88). Alternative cellular outcomes to prevent the propagation of DNA damage include cell cycle arrest or DNA repair (70).

Otherwise, the decrease of apoptotic cells in the present study and 1 d after a marathon run (88) might also be explained by an overshooting removal of apoptotic leukocytes by phagocytic cells in order to protect tissue from overexposure to inflammatory and immunogenic contents of dying cells (72, 88). Based on the concept that the phagocytic clearance of apoptotic immuno-competent cells plays a critical role in the resolution of inflammation (72, 172), this could be a further explanation for the lack of a link between inflammatory responses on the one hand, and DNA damage and/or apoptotic cell death on the other hand.

Similarly, as to the decrease in apoptosis, levels of necrosis also decreased immediately after the Ironman race. The observed association between IL-6 concentration and the number of necrotic cells immediately post-race in the present study may indicate that lymphocytes partly undergo an unregulated cell death in athletes experiencing an overshooting inflammatory response. Based on recent research on the role of IL-6 in exercise (30, 33, 114), it is questionable whether IL-6, probably released by contracting muscles (33, 114), directly modulates necrosis in lymphocytes. In this case, plasma IL-6 concentrations may just serve as a marker for the pronounced initial systemic inflammatory response. However, the (patho-) physiological relevance of this association cannot be generalised based upon the present results, since the overall number of necrotic cells declined significantly to below pre-race values after the acute bout of ultra-endurance exercise, and remained at these levels at all time-points investigated.

#### 4.4.5. Enhanced DNA stability despite inflammatory responses after exhaustive endurance exercise: the role of training status

The results of our research provide further evidence to suggest that enhanced DNA stability is associated with protective adaptations due to (endurance) training. As mentioned above and reported by Reichhold et al. (2008, 2009) (135, 136), data from the present investigation in the Ironman triathlon participants indicate that even an acute

bout of ultra-endurance exercise does neither lead to prolonged levels of DNA strand breaks or oxidized bases nor to chromosomal alterations. These findings are in accordance with two studies of Radak et al. (2000) and Miyata et al. (2008). Both research groups determined urinary 8-OHdG levels and markers of muscle damage in competitors of ultra-marathon events with a duration of 2 (130) and 5 days (85), respectively. No propagation of oxidative DNA damage was observed after the first race day in both studies (85, 130). Reflecting the average rate of oxidative DNA damages in all cells of the body (122), and in case of muscle-damaging exercise probably largely DNA damage of skeletal muscles (130), 8-OHdG significantly decreased to levels below their peak values during the race on the second day (85), and on the fourth race day (130), respectively. Both research groups suggested that a rapid induction of antioxidant and repair systems occurred (85, 128). In contrast, parameters for muscle damage continuously increased during the 2-day-race period (85) and until the third day of the 4-day-race (130), and no correlations were reported with 8-OHdG. Taken together, these data may show that, even if myofibrillar injury occurs, an adaptive up-regulation of repair and nucleotide sanitization mechanisms is capable of preventing further damage of DNA. Interestingly, Mooren et al. (2004) demonstrated that apoptotic sensitivity apparently is also inversely related to the training status, since analysis of two subgroups of marathon runners revealed that programmed cell death occurred only in less trained, but not in highly-trained athletes (88). A similar effect was observed in the present study concerning the chromosomal stability (135). The number of MN declined in the total as well in the both sub-groups that were formed according to their $VO_{2\ peak}$ (as one of the major determinants for endurance performance (84)), but this decrease was most pronounced in the sub-group with the higher $VO_{2\ peak}$ In addition, the formation of DNA strand breaks immediately after the Ironman competition decreased with increasing weekly training loads (136). Both associations indicate that DNA stability depends, at least to a certain extend, on training status. Hence, a reason that may also account for the lack of correlations within most of the relatively few studies that have addressed this issue is that the majority of these investigations have been conducted in trained individuals (42, 80, 85, 106, 109, 118, 130, 135). Accumulating evidence points to adaptations in protective mechanisms due to (endurance) training – including improved endogenous antioxidant defences and

enhanced repair mechanisms (128) – that appear to be responsible for maintaining genome integrity in immuno-competent cells in response to extremely demanding endurance exercise. While these protective mechanisms were suggested to prevent DNA damage and/or apoptosis in a number of studies (85, 88, 104, 109, 118, 130, 135), several other exercise-associated factors induce, mediate and counter-regulate a systemic inflammatory response (30, 116). This indirectly further implies that DNA damage in immuno-competent cells, if it occurs at all, might *not* be a major determinant of exercise-induced inflammation. As mentioned in the introduction of chapter 3, the systemic inflammatory response to exhaustive endurance exercise (or major physical stressors in general) is a stereotypical and regulated reaction, which, in general, protects the organism and contributes to repair and adaptation (30, 64). Or in other words, while prolonged strenuous exercise leads to a – physiologically desirable – systemic inflammatory response also in highly trained individuals, protective systems might be up-regulated in a selective manner according to their physiological importance, i.e. in first line to prevent and/or repair DNA damage due to exercise stress (128, 129).

## 4.5. Conclusion

The current results indicate that DNA effects in lymphocytes are not responsible for exercise-induced inflammatory and immunological responses. Furthermore, this investigation shows that inflammatory processes, vice versa, do not promote DNA damage, neither directly or via an increased formation of ROS derived from inflammatory cells. In general, there is only little evidence concerning a *direct* relationship between DNA damage and inflammatory responses after strenuous prolonged exercise. The most conclusive picture that emerges from the available data is that oxidative stress seems to be the main link between exercise-induced inflammation and DNA damage. Considering the very few studies in which markers of DNA damage were found to correlate with signs of inflammation or muscle damage, DNA damage in peripheral immuno-competent cells, indeed, most likely resulted from an increased generation of ROS due to initial systemic inflammatory responses or the delayed inflammatory processes in response to muscle damage (Figure 14). Data from the present study support this hypothesis insofar as oxidative DNA damage through ROS-generating inflammatory processes such as oxidative burst reactions of circulating

neutrophils might have been counteracted by training- and exercise-induced antioxidant responses. Furthermore, the lack of correlations between these exercise-induced responses in most of the studies might also be explained by the fact that the monitoring period was too short. Hence, particular attention should be paid to the characteristic time-course of inflammatory and oxidative stress events on the one hand and DNA effects on the other hand. Though obvious differences exist in the manifestation and outcomes a comparable relationship is reported in patho-physiological conditions including carcinogenesis, where (chronic) inflammation induces DNA damage and mutations via oxidative stress (28). However, there might be further mechanisms that link exercise-induced DNA modulations, inflammatory responses and ROS. It has been shown, that redox-sensitive signal transduction pathways including nuclear factor (NF) κB or p53 cascades are involved in inflammation as well as "cell stress management" in response to DNA damage (54, 70). Recent explorations of the gene expression responses to exercise have already shed a light on hitherto unknown molecular mechanisms in exercise immunology (14, 20, 29, 131, 178, 179). In the future, the combination of these powerful modern techniques (*transcriptomics, proteomics*) with state-of-the-art biochemical biomarkers should therefore enable researchers in this field to provide novel insights into potential further interactions between genome stability and inflammation.

# 5. General Conclusions

The current doctoral thesis has been conducted within the framework of the research project *Risk Assessment of Participants of an Ironman Triathlon: Genome Stability, Oxidative, Muscular and Systemic Stress*, which was funded by the *Austrian Science Fund (FWF).* Based on the most recent health concerns about the continually growing number of athletes engaged in ultra-endurance sports (61), the overall objective of this project was to get a broader picture of biochemical, physiological and molecular-biological stress responses to an Ironman triathlon as a prototype of ultra-endurance exercise (i.e. exercise of more than four hours (21)). Given the scarceness of data concerning certain stress phenomena following extremely demanding endurance exercise results from these studies contribute to an increased knowledge on several important issues that have emerged in the field of sport sciences. Thereby, it was a main strength of our research to monitor these responses up to 19 days into recovery in 42 Ironman competitors to examine whether there are indications of persistent detrimental health consequences. Hence, these results provide novel key information insofar stress and recovery responses to extremely demanding endurance exercise, to my knowledge, until now have not been followed for such a long time-course and in such a comprehensive manner. Unique information is also provided on the effects of an exercise bout of such duration on DNA stability, which was investigated within the framework of another doctoral thesis of Stefanie Reichhold (133). Comparing the international literature, it was a major finding of the current study that there are no indications of persistent DNA or chromosomal damage following the Ironman race (135, 136). Furthermore, König et al. (2007) reported that the transient increase in biomarkers for myocardial stress after the long-distance triathlon was not associated with exercise-induced inflammation or oxidative stress (63).

The following specific topics have been addressed within the scope of the present doctoral thesis: 1.) oxidative stress and antioxidant responses, 2.) inflammatory, immuno-endocrine and muscular responses, and 3.) the interactions of these responses with exercise-induced effects on DNA stability. These issues have been reported and published in original articles (92, 93) and a review that includes original data (94), and

they have been used for structuring the current dissertation. Beyond, additional considerations concerning related nutritional and training-physiological aspects have been included in the present thesis.

Of utter importance, the current results disprove the recent hypothesis about cumulative oxidative stress and subsequent LDL oxidation after ultra-endurance exercise (61). This study indicates that despite temporary increases in most lipid peroxidation and protein oxidation markers immediately and 1 d post-race (except for oxidised LDL), there is no persistent oxidative damage on blood cell components and blood lipids. As a general picture that emerges from the data of this *FWF* funded project, enhanced antioxidant defences and/or repair mechanisms, apparently induced by training and acute exercise, are able to counteract severe and sustained oxidative damage. Interestingly, weekly training loads as well as training at different intensities were associated with these biochemical adaptations that may lead to both a decrease in ROS production and enhanced antioxidant mechanisms. Taken together, these findings provide further evidence to the idea that (endurance) training results in protective adaptive responses in the organism to cope with future "stress loads" particularly with regards to the oxidant/antioxidant balance and DNA stability (55, 128, 173). Furthermore, attention was drawn on factors which might have contributed to recent inconstant outcomes of the few studies that have examined oxidative stress explicitly in participants of long-distance triathlons (34, 62, 75, 100) or ultra-marathon races (79, 99). On the one hand, the current results suggest that divergent effects on different markers of lipid peroxidation and protein oxidation (e.g. differences in time-kinetics, amplitues and intensity-related responses) apparently have contributed to inconsistencies among previous investigations. One the other hand, this study illustrates that even minor differences in the training status and potentially associated adaptations in the endogenous antioxidant system may be responsible for significant different outcomes. Moreover, the present findings underline the importance of an adequate intake of nutritive antioxidants to preserve antioxidant responses especially in the early recovery period *after* and probably also *during* ultra-endurance exercise. Noteworthy, the plasma concentrations of antioxidant nutrients of the study participants (who avoided taking more than 100% of RDA of antioxidants in form of supplements) were in a normal

physiological range. Plasma concentrations of vitamin C and β-carotene were actually above those levels which are considered as beneficial in preventing against cancer and cardiovascular disease (22), while plasma α-tocopherol was below this level. Thus, the intake of α-tocopherol requires specific attention. However, in general, data from this study support previous investigations in competitive athletes (143) insofar as the requirements for nutritive antioxidants apparently can be achieved by a diversified, balanced and energy-sufficient diet. In contrast, high-dosed antioxidant supplementation may be associated with adverse consequences such as pro-oxidant effects (62, 100) or a blunting in cellular training adaptations (123, 173).

Consistent with the few previous studies in which muscle damage and inflammatory parameters were explicitly investigated after ultra-endurance (53, 98, 100, 155), there was a pronounced initial systemic inflammatory response after the Ironman triathlon that subsided rapidly. Significant relationships between leukocyte dynamics, cortisol, markers of muscle damage, cytokines and hs-CRP after the Ironman triathlon were noted, substantiating previous findings regarding the complex regulation of inflammatory and immune responses to exhaustive endurance exercise (98, 113, 116, 154, 157). Furthermore, observed associations between lipid and protein oxidation markers on the one hand, and muscle damage and inflammatory parameters on the other suggest that delayed (low-grade) oxidative stress responses might occur as a consequence of inflammation (e.g. via oxidative burst reactions of phagocytic cells). The present results also support the idea that well-trained individuals are able to balance this systemic inflammation (30, 155), to a certain extent due to an intensity-related counter-regulation through anti-inflammatory cytokines such as IL-10 (112). A key finding beyond previous investigations was that a low-grade systemic inflammation was sustained for at least 5 d after the Ironman race. Similarly to a recent study in triathletes who had chronically increased plasma IL-6 concentrations after a period of intense running training (139), both the prolonged moderate, but significant elevation of IL-6, hs-CRP and muscle damage markers as well as links among these parameters point to ongoing inflammatory processes within damaged muscle. These findings provide valuable information concerning the length of time needed for recovery after an Ironman triathlon. Although more data is needed, from the perspective of the observed

incomplete muscle repair, at least two to three weeks of active recovery seem to be advisable after such a race to avoid potential detrimental consequences of a disproportionate balance between training, competition and recovery (e.g. prolonged immune dysfunctions or overtraining). Interestingly, both the observation of such a sustained inflammatory state post-race and the finding of anomalies in inflammatory markers in the three subjects who dropped out from the Ironman race due to self-reported fatigue symptoms may provide some additional evidence for the cytokine hypothesis of the hitherto unexplained overtraining or underperformance syndrome (140, 148).

Finally, collectively considered, the results from the present study reveal a complex picture how inflammatory, oxidative stress, antioxidant and DNA responses to ultra-endurance exercise might be related with each other. The data from this study indicate that DNA modulations in lymphocytes are neither responsible for the systemic inflammatory response immediately after the Ironman race, nor for the sustained low-grade inflammation 5 d post-race nor for any other immunological alteration. As pointed out in chapter 3, other exercise-induced factors than DNA damage (if it occurs at all) play more significant roles in inducing and mediating exercise-induced inflammatory responses. Vice versa, no indications were found that inflammatory processes had promoted DNA damage, neither directly nor through an increased formation of ROS derived from inflammatory cells. Considering the very few studies in which correlations between endpoints of DNA damage and signs of inflammation (102) or muscle damage (165) were found, DNA damage in peripheral immuno-competent cells, most likely, resulted from an increased ROS generation due to initial systemic inflammatory responses or the delayed inflammatory processes in response to muscle damage. Importantly, the current results suggest that an enhanced plasma antioxidant capacity might have prevented oxidative DNA damage in lymphocytes immediately and 1 d after the Ironman race. This finding supports the postulation (42, 102, 165) that ROS derived from inflammatory processes appear to be the biochemical key effectors that link inflammation with DNA damage. Furthermore, the finding that training and acute exercise-induced adaptations in protective mechanisms such as endogenous

antioxidant defences might have played a major role in maintaining DNA stability fits well into the overall conclusion of this investigation.

In sum, data from this research project provide important and in many respects up to now unique information on acute stress and recovery responses to an Ironman triathlon concerning oxidative and antioxidant (92), cardiac (63), (skeletal) muscular, inflammatory and immune-endocrine responses (93) and endpoints of DNA stability (135, 136). Crucially, there are no indications of harmful health consequences such as sustained oxidative stress or DNA damage. Based on these findings, the population of ultra-endurance athletes might not be at a higher risk of developing cardiovascular disease or diseases through DNA damage. This assumption is in line with both epidemiological data demonstrating a decreased overall morbidity especially in former endurance athletes (67, 146) and results of a recent cross-sectional study indicating a lower cardiovascular risk profile in physically still active, former top-level endurance and sports games athletes compared with sedentary control groups (120). However, the effects of long-term (year-long) training for and competing in ultra-endurance races requires further investigation. Additional work is also recommended to elucidate the underlying molecular basis of training adaptations, acute as well as recovery responses to exercise. Combining well-established "classical" biochemical biomarkers with powerful new tools such as transcriptome and proteome analysis has the great potential to generate new hypothesis about the molecular and cellular events that are involved in the physiological responses to exercise stimuli researchers in this area. It should also be emphasised here, that investigating the physiological, biochemical and molecular-biological regulatory mechanisms in response to extremely demanding endurance exercise provides a unique model to study various stress responses *in vivo*. Therefore, research in this area exerts important implications for biomedical research in general.

# 6. Summary

## 6.1. Background and aims of the study

There is compelling evidence that physical activity harvests numerous beneficial physiological effects and plays a key role in the prevention of various modern chronic diseases. However, some data imply that there are potentially harmful effects as a result of exceptional high volumes of exercise such as cumulative oxidative stress. In this context the *Austrian Science Fund*-funded research project *Risk Assessment of Participants of an Ironman Triathlon* was aimed to get a broader picture of biochemical, physiological and molecular-biological stress responses to an Ironman triathlon (3.8 km swimming, 180 km cycling, 42 km running) as a prototype of ultra-endurance exercise. As a part of this project the following specific issues have been addressed and conducted within the scope of the present doctoral thesis: 1.) oxidative stress and antioxidant responses, 2.) inflammatory, immuno-endocrine and (skeletal) muscular responses, and 3.) interactions of these responses with exercise-induced effects on DNA stability.

## 6.2. Subjects and methods

Blood samples were taken from 42 well-trained, male, so called "age-group" (i.e. non-professional) triathletes (cycling $VO_{2\ peak}$: 56.6 ± 6.2 ml * kg $^{-1}$ * min $^{-1}$, mean ± SD) 2 days (d) before an Ironman triathlon, then immediately post-race, 1, 5 and 19 d later. Beside for the haematological profile, blood plasma was analyzed above all for the following parameters: conjugated dienes (CD), malondialdehyde (MDA), oxidized low-density lipoprotein (oxLDL), oxLDL:LDL ratio, advanced oxidation protein products (AOPP), AOPP:total protein (TP) ratio, Trolox equivalent antioxidant capacity (TEAC), ferric reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC), vitamin C, α-tocopherol, γ-tocopherol and carotenoids, uric acid, total bilirubin, myeloperoxidase (MPO), polymorphonuclear (PMN) elastase, cortisol, testosterone, creatine kinase (CK) activity, myoglobin, interleukin (IL)-6, IL-10 and high-sensitive C-reactive protein (hs-CRP). Furthermore, activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) were investigated in erythrocytes.

Correlation analyses and one-factorial ANOVA plus *post hoc* tests were applied to assess significant associations among antioxidant, oxidative stress, inflammatory, immune-endocrine responses, muscle damage and exercise-induced effects on endpoints of DNA stability. Within the framework of another doctoral thesis by Stefanie Reichhold (133), 20 and 28 subjects were randomly selected for evaluation of DNA damage in lymphocytes measured via the cytokinesis block micronucleus cytome (CBMN Cyt) and the single cell gel electrophoresis (SCGE) assays including lesion specific enzymes such as endonuclease (ENDO) III, respectively.

## 6.3. Results

### 6.3.1. Oxidative stress and antioxidant responses

Immediately post-race there were significant increases in CD, AOPP, TEAC, FRAP, ORAC, total bilirubin, uric acid (for all $P<0.001$), AOPP:TP, vitamin C and α-tocopherol (for all $P<0.01$). MDA rose significantly ($P<0.01$) 1 d post-race, while TEAC, FRAP ($P<0.001$), CD, AOPP (for both $P\leq0.01$), AOPP:TP and ORAC (for both $P<0.05$) remained elevated. OxLDL:LDL trended to increase, whereas oxLDL significantly ($P<0.01$) decreased 1 d post-race. One day post-race, vitamin C and α-tocopherol returned to pre-race concentrations, while most nutritive antioxidants dropped significantly below. Except for GSH-Px ($P=0.08$), activities of SOD ($P<0.001$) and CAT ($P<0.05$) significantly decreased post-race. All oxidative stress markers had returned to pre-race values 5 d post-race. Furthermore, several relationships between training status and oxidative stress markers, total antioxidant capacity and GSH-Px activities were noted.

### 6.3.2. Inflammatory, immuno-endocrine and muscular stress responses

Immediately post-race there were significant ($P<0.001$) increases in total leukocyte counts, MPO, PMN elastase, cortisol, CK activity, myoglobin, IL-6, IL-10 and hs-CRP, while testosterone significantly ($P<0.001$) decreased compared to pre-race. With the exception of cortisol, which decreased below pre-race values ($P<0.001$), these alterations persisted 1 d post-race ($P<0.001$; $P<0.01$ for IL-10). Five days post-race CK activity, myoglobin, IL-6 and hs-CRP had decreased, but were still significantly ($P<0.001$) elevated. Nineteen days post-race most parameters had returned to pre-race

values, except for MPO and PMN-elastase, which had both significantly ($P<0.001$) decreased below pre-race concentrations, and myoglobin and hs-CRP, which were slightly, but significantly higher than pre-race. Furthermore, significant relationships between leukocyte dynamics, cortisol, markers of muscle damage, cytokines and hs-CRP after the Ironman triathlon were observed.

#### 6.3.3. Consequences of exercise-induced inflammatory, oxidative stress and antioxidant responses on DNA stability

Increased DNA instability was detected by the SCGE technique (increased level of strand breaks 1 d post-race compared to pre-race) and the CBMN Cyt assay (increased nuclear buds 5 d post-race), but these changes were only temporarily. All endpoints within the SCGE and the CBMN Cyt assay decreased to or even below pre-race values 19 d post-race. No correlations were found between endpoints of DNA stability on the one hand and immuno-endocrine, inflammatory and muscle damage parameters on the other. ENDO III-sensitive sites correlated negatively with oxygen radical absorbance capacity (ORAC) immediately ($r= -0.54$; $P<0.01$) and 1 d post-race ($r= -0.65$; $P<0.05$).

### 6.4. Conclusions

This study indicates that despite a temporary increase in most oxidative stress markers, there is no persistent oxidative damage to blood cell compounds and blood lipids in response to an Ironman triathlon, probably due to training- and exercise-induced protective alterations in the antioxidant defense system. Furthermore, findings from the current investigation illustrates that requirements by ultra-endurance athletes for nutritive antioxidants, to a great extend, can be achieved by a diversified, balanced and energy-sufficient diet, while the antioxidant intake during and in the early recovery phase after ultra-endurance exercise requires specific attention. The pronounced initial systemic inflammatory response induced by the acute bout of ultra-endurance exercise declines rapidly, but a low-grade systemic inflammation persisted until at least 5 d post-race, possibly reflecting incomplete muscle recovery. Moreover, the current results indicate that DNA effects in lymphocytes are not responsible for exercise-induced inflammatory responses, and that, vice versa, inflammatory processes do not promote DNA damage. Oxidative DNA damage via an increased formation of ROS derived from

inflammatory cells might have been prevented by enhanced antioxidant responses. The general picture that emerges from the data of this research project is that training- and acute exercise-induced adaptations in protective mechanisms including improved endogenous antioxidant defences and repair systems counteract severe oxidative stress and persistent DNA damage. Based on these findings, training for and participating in an ultra-endurance event such as an Ironman triathlon, might not be associated with a higher risk of developing cardiovascular disease or diseases through DNA damage. However, the enormous capability to cope with and recover from certain forms of physiological stress requires an adequate training status.

# 7. Zusammenfassung

## 7.1. Hintergrund und Ziele der Studie

Körperliche Aktivität bewirkt eine Reihe von günstigen physiologischen Effekten und beugt damit erwiesenermaßen chronischen Erkrankungen vor. Paradoxerweise deuten indes einige Daten auf mögliche Gesundheitsrisiken (wie z.B. kumulativen oxidativen Stress) durch extreme sportliche Ausdauerbelastungen hin. Das Hauptziel des vom *Österreichischen Wissenschaftsfonds* geförderten Forschungsprojekts: *Risikobeurteilung von Teilnehmern eines Ironman Triathlons* war daher, einen umfassenden Einblick in biochemische, physiologische und molekular-biologische Stressreaktionen nach einem Ironman Triathlon (3,8 km Schwimmen, 180 km Radfahren, 42 km Laufen) als einem Prototyp einer Ultra-Ausdauerbelastung zu gewinnen. Als Teil dieses Projekts wurden folgende Aspekte im Rahmen der vorliegenden Dissertation untersucht: 1.) Oxidativer Stress und antioxidative Reaktionen, 2.) Entzündungs-, immuno-endokrine und (skelett-) muskuläre Reaktionen sowie 3.) Wechselwirkungen dieser Reaktionen mit belastungsbedingten Effekten auf die DNA-Stabilität.

## 7.2. Studienteilnehmer und Methodik

Das Studienkollektiv bestand aus 42 gut trainierten, männlichen, sogenannten "Altersklasse" Triathleten ($VO_{2\ peak}$ am Fahrradergometer: 56,6 ml ± 6.2 ml * kg $^{-1}$ * min $^{-1}$, Mittelwert ± Standardabweichung). Blutproben wurden zu folgenden Zeitpunkten entnommen: zwei Tage vor dem Ironman Triathlon, unmittelbar nach Beendigung des Wettkampfes sowie einen Tag, fünf und 19 Tage nach dem Ironman. Neben dem hämatologischen Profil wurden im Blutplasma insbesondere folgende Parameter analysiert: konjugierte Diene (CD), Malondialdehyde (MDA), oxidiertes LDL (oxLDL), oxLDL:LDL Ratio, Advanced Oxidation Protein Products (AOPP), AOPP:Gesamtprotein (TP) Ratio, Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Ability of Plasma (FRAP), Oxygen Radical Absorbance Capacity (ORAC), Vitamin C, α-Tocopherol, γ-Tocopherol und Carotenoide, Harnsäure, Gesamtbilirubin, Myeloperoxidase (MPO), Polymorphonukleäre (PMN) Elastase, Cortisol, Testosteron, Creatine Kinase (CK) Aktivität, Myoglobin, Interleukin (IL)-6,

IL-10 und hoch-sensitives C-reaktives Protein (hs-CRP). Weiters wurden die Aktivitäten der antioxidativen erythrozytären Enzyme Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px) und Catalase (CAT) untersucht. Korrelationsanalysen, einfaktorielle ANOVA und daran gekoppelte *post hoc* Tests wurden zur Eruierung potentieller Zusammenhänge zwischen antioxidativen Reaktionen, oxidativem Stress, immun-endokrinen und Entzündungsreaktionen, skelett-muskulären Schäden und belastungsinduzierten Effekten auf Endpunkte zur Beschreibung der DNA-Stabilität herangezogen. Zur Untersuchung von DNA Schäden in Lymphozyten wurde im Rahmen einer weiteren Dissertation von Stefanie Reichhold (133) 20 bzw. 28 Studienteilnehmer für den Mikrokern-Test bzw. den Einzelzellgelelektrophorese-Test (einschließlich der Analyse mit schadensspezifischen Enzymen wie der Endonuklease (ENDO) III) randomisiert.

## 7.3. Resultate

### 7.3.1. Oxidativer Stress und antioxidative Reaktionen

Unmittelbar nach dem Ironman Triathlon wurden bei folgenden Parametern signifikante Anstiege verzeichnet: CD, AOPP, TEAC, FRAP, ORAC, TBIL, Harnsäure (jeweils $P<0.001$), AOPP:TP, Vitamin C und α-Tocopherol (jeweils $P<0.01$). MDA stieg einen Tag nach dem Triathlon signifikant ($P<0.01$) an; gleichzeitig blieben TEAC, FRAP (jeweils $P<0.001$), CD, AOPP (jeweils $P\leq0.01$), AOPP:TP und ORAC (jeweils $P<0.01$) erhöht. OxLDL:LDL stieg tendenziell an, während oxLDL einen Tag nach dem Rennen signifikant ($P<0.01$) abfiel. Einen Tag nach dem Ironman fielen die Plasmakonzentrationen von Vitamin C und α-Tocopherol wieder auf das Ausgangsniveau zurück; jene der meisten anderen nutritiven Antioxidantien jedoch signifikant darunter. Bei den Aktivitäten der erythrozytären Enzyme wurde nach dem Triathlon ein – mit Ausnahme der GSH-Px ($P=0.08$) – signifikanter Abfall festgestellt ($P<0.001$ für SOD, $P<0.05$ für CAT). Sämtliche Marker für oxidativen Stress waren fünf Tage nach dem Ironman wieder auf das Ausgangsniveau zurückgekehrt. Die Ergebnisse zeigten darüber hinaus signifikante Zusammenhänge zwischen Trainingsstatus, Markern für oxidativen Stress, der antioxidativen Plasmakapazität und GSH-Px an.

### 7.3.2. Inflammatorische-, immuno-endokrine und muskuläre Stressreaktionen

Unmittelbar nach dem Ironman Triathlon stiegen die Leukozyten-Gesamtzahl, MPO, PMN Elastase, Cortisol, die CK Aktivität, Myoglobin, IL-6, IL-10 und hs-CRP signifikant ($P<0.001$) an, während Testosteron signifikant ($P<0.001$) unter das Ausgangsniveau abfiel. Mit der Ausnahme von Cortisol (Abfall unter den Ausgangswert; $P<0.001$), blieben diese Veränderungen auch einen Tag nach dem Triathlon bestehen ($P<0.001$; $P<0.01$ für IL-10). Trotz eines Absinkens waren die CK-Aktivität, Myoglobin, IL-6 und hs-CRP auch fünf Tage nach dem Ironman noch signifikant ($P<0.001$) erhöht. Neunzehn Tage nach der extremen Ausdauerbelastung waren die meisten Parameter auf die Werte von vor dem Ironman zurückgekehrt. Dagegen fielen MPO und PMN-Elastase signifikant ($P<0.001$) unter das Ausgangsniveau, während Myoglobin ($P<0.01$) und hs-CRP ($P<0.001$) moderat, aber weiterhin signifikant darüber lagen. Zahlreiche Korrelationen weisen auf Zusammenhänge zwischen dem Leukozytenanstieg, Cortisol, Muskelschädigungsmarkern, Zytokinen und hs-CRP nach dem Ironman Triathlon hin.

### 7.3.3. Auswirkungen von belastungsinduzierten inflammatorischen Reaktionen, oxidativem Stress und antioxidativen Reaktionen auf die DNA Stabilität

Sowohl im Rahmen des SCGE- (Zunahme der Höhe an DNA-Strangbrüchen nach dem Ironman Triathlon) als auch des CBMN Cyt-Assays (erhöhtes Level an Kernkörperchen fünf Tage nach dem Triathlon) wurden temporäre DNA Instabilitäten detektiert. Spätestens 19 Tage nach der sportlichen Extrembelastung lagen sämtliche Marker für DNA und Chromosomen-Schäden jedoch auf oder unter dem Ausgangsniveau. Es wurden keine Korrelationen zwischen Parametern für die DNA Stabilität auf der einen und immuno-endokrinen, inflammatorischen und Muskelschädigungsgrößen auf der anderen Seite festgestellt. Hingegen zeigten die ENDO III sensitiven Schadstellen einen signifikant negativen Zusammenhang mit den Ergebnissen des ORAC-Assays, sowohl unmittelbar ($r= -0.54$; $P<0.01$) als auch einen Tag nach dem Ironman ($r= -0.65$; $P<0.05$).

## 7.4. Schlussfolgerungen

Diese Studie dokumentiert, dass die Belastung eines Ironman Triathlons, trotz vorübergehender Anstiege bei den meisten Markern für oxidativen Stress, zu keiner

nachhaltigen oxidativen Schädigung an zellulären Bestandteilen und Lipiden im Blut führt. Diese Studienresultate weisen auf protektive Veränderungen im antioxidativen Schutzsystem hin, welche sowohl durch Training als auch durch die akute Belastung induziert werden. Darüber hinaus veranschaulichen die vorliegenden Ergebnisse, dass der Bedarf von Ultra-Ausdauerathleten an nutritiven Antioxidantien weitgehend über eine vielfältige, ausgewogene und in der Energiebilanz ausgeglichene Ernährung gedeckt werden kann. Allerdings erfordert die Antioxidantienaufnahme sowohl während als auch in der frühen Regenerationsphase nach einem Ironman Triathlon (oder einer damit vergleichbaren körperlichen Belastung) spezielles Augenmerk. Die durch den Ironman Triathlon initiierte markante systemische Entzündungsreaktion nahm rasch wieder ab. Dahingegen geben die, bis mindestens fünf Tage nach dem Triathlon moderat, aber signifikant erhöhten Entzündungsparameter sowie Korrelationen mit Muskelschädigungsmarkern, Hinweise auf Entzündungsvorgänge infolge noch nicht abgeschlossener Reparaturprozesse in der Skelettmuskulatur. Die Ergebnisse dieser Studie lassen auf keine ursächliche Zusammenhänge zwischen DNA Effekten in Lymphozyten und belastungsbedingten Entzündungsreaktionen schließen. Eine oxidative DNA-Schädigung durch eine erhöhte Bildung von reaktiven Sauerstoffspezies von an Entzündungsvorgängen beteiligten Leukozyten wurde vermutlich durch verstärkte antioxidative Reaktionen verhindert. In Summe weisen die Daten dieses Forschungsprojekts auf eine sowohl durch Training als auch die akute Belastung induzierten Adaptationen von z.B. endogenen antioxidativen Schutzsystem und/oder Reparatursystemen hin. Diese Anpassungen protektiver Mechanismen dürften schwerwiegenden und nachhaltigen Schäden durch oxidativen Stress und DNA Schädigungen entgegenwirken. Diese Resultate sind ein Indiz dafür, dass die Vorbereitungen auf und Teilnahme an einem Ultra-Ausdauerwettkampf wie einem Ironman Triathlon mit keinen erhöhten Gesundheitsrisiken in Form von Herz-Kreislauferkrankungen oder Erkrankungen im Zusammenhang mit DNA Schädigungen verbunden sein dürften. Diese enorme physiologische Belastungsverträglichkeit und Regenerationsfähigkeit setzt allerdings einen entsprechenden Trainingszustand der Athleten voraus.

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# **8. Appendices** (original publications)

# Original article I

(Basis for chapter 2)

# No Indications of Persistent Oxidative Stress in Response to an Ironman Triathlon

OLIVER NEUBAUER[1], DANIEL KÖNIG[2], NORBERT KERN[1], LUKAS NICS[1], and KARL-HEINZ WAGNER[1]

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**ABSTRACT**

NEUBAUER, O., D. KÖNIG, N. KERN, L. NICS, and K.-H. WAGNER. No Indications of Persistent Oxidative Stress in Response to an Ironman Triathlon. *Med. Sci. Sports Exerc.*, Vol. 40, No. 12, pp. 2119–2128, 2008. **Introduction**: Training for and competing in ultraendurance exercise events is associated with an improvement in endogenous antioxidant defenses as well as increased oxidative stress. However, consequences on health are currently unclear. **Purpose**: We aimed to examine the impact of training- and acute exercise-induced changes in the antioxidant capacity on the oxidant/antioxidant balance after an ironman triathlon and whether there are indications for sustained oxidative damage. **Methods**: Blood samples were taken from 42 well-trained male triathletes 2 d before an ironman triathlon, then immediately postrace, 1, 5, and 19 d later. Blood was analyzed for conjugated dienes (CD), malondialdehyde (MDA), oxidized low-density lipoprotein (oxLDL), oxLDL:LDL ratio, advanced oxidation protein products (AOPP), AOPP:total protein (TP) ratio, Trolox equivalent antioxidant capacity (TEAC), uric acid (UA) in plasma, and activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) in erythrocytes. **Results**: Immediately postrace, there were significant increases in CD, AOPP, TEAC, UA (for all $P < 0.001$), and AOPP:TP ($P < 0.01$). MDA rose significantly ($P < 0.01$) 1 d postrace, whereas CD ($P < 0.01$), AOPP ($P = 0.01$), AOPP:TP ($P < 0.05$), and TEAC ($P < 0.001$) remained elevated. OxLDL:LDL trended to increase, whereas oxLDL significantly ($P < 0.01$) decreased 1 d postrace. Except for GSH-Px ($P = 0.08$), activities of SOD ($P < 0.001$) and CAT ($P < 0.05$) significantly decreased postrace. All oxidative stress markers had returned to prerace values 5 d postrace. Furthermore, several relationships between training status and oxidative stress markers, TEAC, and antioxidant enzyme activities were noted. **Conclusions**: This study indicates that despite a temporary increase in most (but not all) oxidative stress markers, there is no persistent oxidative stress in response to an ironman triathlon, probably due to training- and exercise-induced protective alterations in the antioxidant defense system. **Key Words:** ULTRAENDURANCE EXERCISE, LIPID PEROXIDATION, PROTEIN OXIDATION, PLASMA ANTIOXIDANT CAPACITY, ANTIOXIDANT ENYZMES



There is overwhelming evidence that physical activity harvests many beneficial physiological effects that improve physical fitness and play a major role in the prevention of various chronic disease states (30). Research has even shown an increased life expectancy in former top-level athletes including long distance runners and cross-country skiers (but not ultraendurance athletes) (14,32). However, some empirical as well as epidemiologic data, recently reviewed by Knez et al. (10), paradoxically suggest that an exceptionally high volume of exercise is associated with an increased risk of developing cardiovascular disease (15). Oxidative stress is proposed to be one of the main potential mechanisms that, at least partly, might offset the positive outcome imparted by regular physical training (6,10), probably due to the increased oxidation of plasma lipoproteins and the consequent hypothesized contribution to atherosclerosis (10,34). Thus, concerns have arisen about the growing number of athletes engaged in ultraendurance sports because extremely demanding exercise such as an ironman triathlon is associated with an increased formation of reactive oxygen species (ROS). Probable mechanisms for increased ROS production during strenuous aerobic exercise include inadequate electron transfer through the mitochondrial respiratory chain during oxygen metabolism, inflammatory responses, increased xanthine oxidase activity triggered by transient hypoxic conditions (that even may occur during predominantly aerobic exercise caused by blood-redistribution), and autoxidation of haem proteins (6,13,39).

Nevertheless, research in the area of exercise-induced oxidative stress has lead to controversial results, and to date there is little conclusive information. For example, it remains unclear whether the exercise-induced production of free radicals results in persisting oxidative stress responses and adverse effects on health such as LDL oxidation (6,10,36,39). Training appears to lead to adaptations of the endogenous antioxidant defense system (11,25,28); however, it is unknown whether these up-regulated protective mechanisms are sufficient to prevent cumulative oxidative stress and oxidative damage. Moreover, the lack of consensus most likely

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TABLE 1. Characteristics of the study participants and their performance in the Ironman triathlon.

| | |
|---|---|
| Age (yr) | 35.3 ± 7.0 |
| Height (cm) | 180.6 ± 5.6 |
| Weight (kg) | 75.1 ± 6.4 |
| BMI ($kg \cdot m^{-2}$) | 23.0 ± 1.2 |
| Percentage of body fat (%) | 11.8 ± 4.1 |
| Cycling $VO_{2peak}$ ($mL \cdot kg^{-1} min^{-1}$) | 56.6 ± 6.2 |
| Peak PO ($W_{peak}$) | 357.9 ± 50 |
| Relative peak PO ($W_{peak} \cdot kg^{-1}$) | 4.8 ± 0.5 |
| IAT (W) | 219.4 ± 43.5 |
| Relative IAT ($W \cdot kg^{-1}$) | 2.9 ± 0.5 |
| Power output at 2 mmol blood lactate$\cdot L^{-1}$ ($W \cdot kg^{-1}$) | 193.6 ± 46.5 |
| Relative PO at 2 mmol blood lactate$\cdot L^{-1}$ ($W \cdot kg^{-1}$) | 2.6 ± 0.5 |
| Power output at 3 mmol blood lactate$\cdot L^{-1}$ ($W \cdot kg^{-1}$) | 237.1 ± 46.0 |
| Relative PO at 3 mmol blood lactate$\cdot L^{-1}$ ($W \cdot kg^{-1}$) | 3.1 ± 0.6 |
| Training over a period of 6 months before the Ironman triathlon | |
| Weekly net endurance exercise time (WNET) ($h \cdot wk^{-1}$) | 10.7 ± 2.6 |
| Swim training ($km \cdot wk^{-1}$) | 4.8 ± 2.2 |
| Cycle training ($km \cdot wk^{-1}$) | 144.0 ± 52.1 |
| Run training ($km \cdot wk^{-1}$) | 36.4 ± 10.6 |
| Performance in the Ironman triathlon | |
| Total race time (h:min:s) | 10:51:52 ± 01:01:22 |
| 3.8-km swim time (h:min:s) | 01:09:51 ± 00:10:28 |
| 180-km cycle time (h:min:s) | 05:28:21 ± 00:29:08 |
| 42.2-km run time (h:min:s) | 04:08:26 ± 00:31:36 |
| Training after completion of the Ironman triathlon until 19 d postrace | |
| WNET ($h \cdot wk^{-1}$) | 4.2 ± 2.4 |
| Swim training ($km \cdot wk^{-1}$) | 2.1 ± 2.2 |
| Cycle training ($km \cdot wk^{-1}$) | 66.3 ± 53.8 |
| Run training ($km \cdot wk^{-1}$) | 12.3 ± 11.5 |

Data are presented as mean ± SD, $N = 42$.

also originates from the diversity of study designs and methodological approaches that are used to induce and measure oxidative stress (9,11,13). In particular, different durations, intensities, and types of exercise probably contribute to inconsistencies even among the few studies that have examined oxidative stress specifically in competitors of ultraendurance races such as long-distance triathlons (7,11,17,23) or ultramarathon running (19,22).

The data presented here are part of a larger study that aimed to get a broader picture of certain stress responses to vigorous aerobic exercise in a large cohort of athletes and to explore hypothesized associations between oxidative, muscular, cardiac (12), inflammatory, immunoendocrine stress, and genome stability. The primary aim of the present study was to comprehensively quantify antioxidant and oxidative stress responses to an ironman triathlon. Of utter importance, we monitored these responses 19 d into recovery to verify whether there are indications of delayed onset of oxidative stress, sustained oxidative damage, and health consequences. Furthermore, the relevance of training status on the magnitude of oxidative stress markers and the antioxidant capacity was examined. We hypothesized that even small differences in training levels within a large group of well-trained athletes would affect the changes of oxidative stress and endogenous antioxidant variables after an acute bout of ultraendurance exercise. Finally, due to recent inconsistent outcomes that were probably related with diverse analytical approaches, we aimed to particularize the damage on blood cell components and blood lipids by using various markers to detect different phases of lipid peroxidation as well as protein oxidation.

## MATERIALS AND METHODS

**Subjects.** The study population comprised 48 nonprofessional well-trained healthy male triathletes who participated in the 2006 Ironman Austria; 42 of them completed the study and were included in the statistical analysis. The subjects were recruited from all over Austria half a year before the event. They were informed about the purpose and the risks of the study before they provided written informed consent. The Ethics Committee of the Medical University of Vienna approved the study. The characteristics of the subjects and their performance in the ironman triathlon are shown in Table 1.

**Study design.** All participants of the study were physically fit, free of acute or chronic illnesses, within a normal range of body mass index and nonsmokers. Furthermore, they were not taking prescribed medication and avoided taking more than 100% of RDA of antioxidants (as sup plements in addition to their normal dietary intake) in the 6 wk before the race and until the final blood sampling 19 d postrace. Subjects were required to complete a medical and health screening, a food frequency, a supplementation questionnaire, and 24-h dietary recalls before each blood sampling, and they had to document their training in the 6 months before the ironman triathlon and thereafter until the end of the study (Table 1). Blood samples were taken 2 d prerace, immediately (within 20 min), 1, 5, and 19 d postrace. The athletes abstained from intense exercise 48 h before spiroergometry testing and before each blood sampling

TABLE 2. Plasma values of biochemical variables.

| | PRE | POST | 1 d POST | 5 d POST | 19 d POST | Time Effect (*P*) |
|---|---|---|---|---|---|---|
| UA ($\mu mol \cdot L^{-1}$) | 311 ± 54 | 465 ± 92*** | 422 ± 63*** | 338 ± 62*** | 328 ± 53*** | <0.001 |
| TC ($mmol \cdot L^{-1}$) | 5.1 ± 0.9 | 5.1 ± 1.0 | 4.2 ± 0.8*** | 4.7 ± 0.8*** | 5.1 ± 0.9 | <0.001 |
| HDL ($mmol \cdot L^{-1}$) | 1.9 ± 0.4 | 2.0 ± 0.4* | 1.9 ± 0.4 | 1.8 ± 0.5 | 1.7 ± 0.4* | <0.001 |
| LDL ($mmol \cdot L^{-1}$) | 2.8 ± 0.8 | 2.4 ± 0.8*** | 1.9 ± 0.7*** | 2.5 ± 0.6*** | 2.9 ± 0.8 | <0.001 |
| VLDL ($mmol \cdot L^{-1}$) | 0.4 ± 0.2 | 0.7 ± 0.3* | 0.4 ± 0.3 | 0.4 ± 0.2 | 0.4 ± 0.2 | <0.001 |
| TG ($mmol \cdot L^{-1}$) | 0.9 ± 0.4 | 1.6 ± 0.6*** | 0.9 ± 0.6 | 0.9 ± 0.4 | 0.9 ± 0.4 | <0.001 |

Values are presented as mean ± SD; $N = 42$.
* Significantly different from prerace values, $P < 0.05$.
** Significantly different from prerace values, $P < 0.01$.
*** Significantly different from prerace values, $P < 0.001$.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein; PRE, 2 d prerace; POST, immediately postrace; UA, uric acid; TC, total cholesterol; 1 d POST, 1 d postrace; 5 d POST, 5 d postrace; 19 d POST, 19 d postrace.





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TABLE 3. Plasma values of oxidative stress markers.

| | PRE | POST | 1 d POST | 5 d POST | 19 d POST | Time Effect ($P$) |
|---|---|---|---|---|---|---|
| CD (μg·mL$^{-1}$) | 3.85 ± 1.27 | 7.35 ± 1.54*** | 4.36 ± 1.30** | 4.03 ± 1.16 | 3.78 ± 0.91 | <0.001 |
| MDA (μmol·L$^{-1}$) | 2.39 ± 0.49 | 2.56 ± 0.67 | 2.60 ± 0.73** | 2.39 ± 0.68 | 2.42 ± 0.78 | 0.092 |
| OxLDL (U·L$^{-1}$) | 35.8 ±13.7 | 31.3 ± 12.4* | 29.3 ± 13.6** | 32.3 ± 11.3 | 36.9 ± 13.6 | <0.001 |
| OxLDL:LDL rat. (U·mmol·L$^{-1}$) | 13.3 ± 5.3 | 13.8 ± 6.2 | 14.4 ± 6.1 | 12.1 ± 5.2 | 13.1 ± 4.9 | 0.363 |
| AOPP (μmmol·L$^{-1}$) | 39.8 ± 8.4 | 49.9 ± 14.8*** | 48.1 ± 17.1** | 39.9 ± 6.7 | 41.7 ± 8.9 | <0.001 |
| AOPP:TP (μmmol·g·dL$^{-1}$) | 5.28 ± 1.04 | 6.34 ± 1.88** | 6.12 ± 1.97* | 5.47 ± 1.05 | 5.91 ± 1.45 | 0.005 |

Values are presented as mean ± SD; $N$ = 42.
* Significantly different from prerace values, $P < 0.05$.
** Significantly different from prerace values, $P < 0.01$.
*** Significantly different from prerace values, $P < 0.001$.
CD, conjugated dienes; MDA, malondialdehyde; oxLDL, oxidized LDL; AOPP, advanced oxidation protein products; TP, total protein.

(except the ironman itself). The subjects had fasted overnight before the 2-d prerace and the 5- and 19-d postrace blood samples, but on race day and 1 d postrace, they were allowed to drink and eat *ad libitum*, and the quantities of intake were recorded. After the triathlon, the athletes performed "recovery" training that was of moderate intensity and duration until the end of the study (Table 1).

**Race conditions.** The ironman triathlon was held in Klagenfurt, Austria, on July 16, 2006, and consisted of a 3.8-km swim, followed by 180-km cycling and 42.2-km running. When the race started at 7:00 a.m., the air temperature and relative humidity were 15°C and 77%, with the lake temperature at 25°C. Between 4:00 and 5:00 p.m., respectively, by finishing time (median time for subjects approximately 5:43 p.m.), air temperature reached a maximum and was 27.2°C, and relative humidity had decreased to 36% (data provided by the Carinthian Centre of the Austrian Central Institute for Meteorology and Geodynamics).

**$\dot{V}O_{2peak}$ testing protocol.** The triathletes were tested 3 wk before the race on a cycle ergometer (Ergometrics 900, Sensormedics GmbH, Höchberg, Germany). The maximal test protocol started at an initial intensity of 50 W, followed by 50-W increments every 3 min until exhaustion. During the test oxygen and carbon dioxide fractions (via Sensormedics 2900 Metabolic measurement cart), power output (PO), heart rate, and ventilation were recorded continuously. Earlobe blood samples for the measurement of the lactate concentration were taken at the beginning and at the end of each stage to determine performance parameters including the individual anaerobic threshold (IAT) (31).

**Blood sampling.** Each blood sample was collected into heparin, ethylenediaminetetraacetic acid, or serum vacutainers (Vacuette, Greiner, Austria). A field laboratory was installed at the race to ensure the appropriate collection of the first three blood samples. The blood was immediately cooled to 4°C and plasma or the serum separated at 1711*g* for 20 min at 4°C. Aliquots were immediately frozen at −80°C. Whole blood was taken for the hematological profile, and erythrocytes were also collected and frozen in aliquots at −80°C. All samples were analyzed within 6 months.

**Hematological profile.** The hematological profile was assessed with an MS4 Hematology 3-Part-Differential-Analyzer (Melet Schloesing Laboratories, Maria Enzersdorf, Austria). Exercise-induced changes in plasma volume were calculated (5) until 5 d postrace to assess expansion of plasma volume, which persists for 3 to 5 d after the cessation of demanding exercise (33). All results are reported adjusted for these changes, except for Trolox equivalent antioxidant capacity (TEAC) and ratios of oxLDL:LDL and AOPP:TP. For these indices, we used the data uncorrected for changes in plasma volume to consider their actual concentration to which the body responds.

**Plasma concentration of lipoproteins and biochemical variables.** Concentrations of total cholesterol (TC), HDL, triglycerides (TG), total protein (TP), and uric acid (UA) were measured using an automatic analyzer (Vitros DT 60 II module, Ortho-clinical Diagnostics, Germany). Levels of VLDL and LDL were calculated (VLDL = TG/2.2; LDL = TC − HDL − VLDL).

**Plasma concentrations of markers of oxidative stress.** Malondialdehyde (MDA) and conjugated dienes (CD) were both detected with high-performance liquid chromatography (HPLC) as reported previously (29). Oxidized LDL (oxLDL) concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden). Advanced oxidation protein products (AOPP) were determined via a colorimetric assay kit (Immundiagnostik AG, Bensheim, Germany). For both

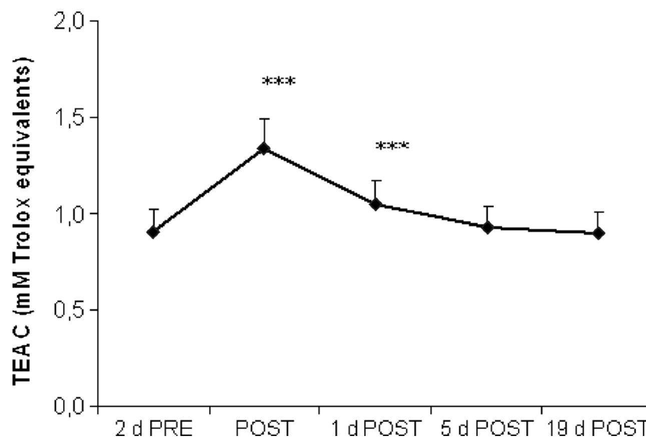


**FIGURE 1—Changes in Trolox equivalent antioxidant capacity (TEAC) 2 d prerace (PRE) and immediately, 1, 5, and 19 d postrace (POST). Data are mean ± SD; $N$ = 42. ***Change was significantly different from prerace values ($P$ < 0.001).**

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TABLE 4. Plasma values of antioxidant erythrocyte enzymes.

| | PRE | POST | 1 d POST | 5 d POST | 19 d POST | Time Effect (*P*) |
|---|---|---|---|---|---|---|
| SOD (IU·g$^{-1}$ Hb) | 1813 ± 257 | 1695 ± 156*** | 1821 ± 194 | 1769 ± 177*** | 1722 ± 159*** | <0.001 |
| GSH-Px (IU·g$^{-1}$ Hb) | 22.5 ± 9.9 | 22.3 ± 9.7 | 21.7 ± 9.2 | 22.9 ± 9.4 | 22.0 ± 9.5 | 0.110 |
| CAT (IU·g$^{-1}$ Hb) | 282 ± 59 | 271 ± 56* | 265 ± 55*** | 273 ± 57 | 266 ± 47** | 0.036 |

Values are presented as mean ± SD; $N = 42$.
* Significantly different from prerace values, $P < 0.05$.
** Significantly different from prerace values, $P < 0.01$.
*** Significantly different from prerace values, $P < 0.001$.
SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; PRE, 2 d prerace; POST, immediately postrace; 1 d POST, 1 d postrace; 5 d POST, 5 d postrace; 19 d POST, 19 d postrace.

oxLDL and AOPP, absorbance of samples and standards were read with a Fluostar Optima microplate reader (BMG labtechnologies, Germany), and all measures were made in duplicate.

**Activities of antioxidant erythrocyte enzyme and antioxidant capacity of plasma.** Erythrocyte activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) were determined using methods reported previously (1,3,40). Briefly, the principles of these methods were as follows: SOD activity was defined via its inhibition of the auto-oxidation of 1,2,3-trihydroxybenzol (pyrogallol) in the presence of superoxide anion ($O_2^{-}$), GSH-Px activity was defined in proportion to the oxidation of $NADPH_2$ to $NADP^+$, and CAT activity was measured by the rate of breakdown of hydrogen peroxide ($H_2O_2$). Trolox equivalent antioxidant capacity (TEAC) of plasma was analyzed photometrically as described previously (36).

**Data analysis.** Data were tested for normal distribution using the Kolmogorov–Smirnov test. The main effect of time was obtained by using the repeated-measures ANOVA. Dependent on normal distribution of data, either paired *t*-tests (for normally distributed data) or Wilcoxon tests (for not normally distributed data) were then used to assess differences in the test variables, and all postrace values were compared with prerace (baseline) values. Pearson's or Spearman's correlations were used to examine any significant relationships. Subjects were divided into groups (percentiles) by exercise test variables including the relative IAT or the relative PO at $\dot{V}O_{2peak}$. One-factorial ANOVA and *post hoc* analyses with Bonferroni's test were then applied to assess whether differences in oxidative stress- and antioxidant-associated variables were associated with the percentile distribution. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Significance

TABLE 5. Significant associations with training and exercise test variables, performance, and oxidative stress markers.

| | $\dot{V}O_{2peak}$ | Rel. $W_{peak}$ | IAT | Rel. IAT | PO La 2 | Rel. PO La 2 | PO La 3 | Rel. PO La 3 | Rel. PO La 3 %$W_{peak}$ | WNET | Cycle Training | 180-km Cycle Time |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CD | | | | | | | | | | | | |
| 1 d POST | | | | | −0.33* | −0.37* | −0.36* | −0.46** | | | | |
| Δ PRE to 1 d POST | | −0.31* | | | | | | | | | | |
| MDA | | | | | | | | | | | | |
| PRE | | | | | | | | | | −0.38* | −0.37* | |
| POST | −0.30* | −0.40** | | | | | | | | | −0.35* | |
| Δ PRE to POST | | −0.34* | | | | | | | | | | |
| 1 d POST | | −0.32* | | | | | | | | −0.34* | −0.39* | |
| Δ PRE to 1 d POST | | −0.32* | | | | | | | | | | |
| oxLDL | | | | | | | | | | | | |
| PRE | | −0.30* | | −0.29* | −0.35* | −0.37* | −0.34* | −0.36* | | | | |
| POST | | −0.37* | | −0.30* | | | | | | | | |
| 1 d POST | | | | | −0.33* | −0.37* | | | | | | |
| oxLDL:LDL | | | | | | | | | | | | |
| PRE | | | | | | | | | | 0.42** | 0.33* | |
| AOPP | | | | | | | | | | | | |
| POST | | | 0.31* | 0.33* | | | 0.31* | | 0.38** | | | −0.31* |
| Δ PRE to POST | | | | 0.34* | 0.34* | 0.39* | 0.34* | 0.37* | 0.35* | | | −0.35* |
| 1 d POST | | | | | | | | | | | | −0.34* |
| Δ PRE to 1 d POST | | | | | | | | | | | | −0.38* |
| AOPP:total protein | | | | | | | | | | | | |
| POST | | 0.36* | 0.31* | 0.32* | | 0.38* | | 0.44** | | | | |
| Δ PRE to POST | | | | 0.33* | 0.33* | 0.39** | 0.33* | 0.36* | 0.32* | | | |

CD, conjugated dienes; MDA, malondialdehyde; oxLDL, oxidized LDL; AOPP, advanced oxidation protein products; $W_{peak}$, peak PO; rel., relative; IAT, individual anaerobic threshold; PO La 2 (3), power output at 2 (3) mmol blood lactate·L$^{-1}$; WNET, weekly net endurance exercise time; PRE, 2 d prerace; POST, immediately postrace; 1 d POST, 1 d postrace; Δ PRE to (1 d) POST, change from prerace to immediately (1 d) postrace; *$P < 0.05$; **$P < 0.01$.





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was set at a $P$ value of <0.05 and is reported as $P < 0.05$, $P < 0.01$, and $P < 0.001$.

## RESULTS

**Race results.** The average completion time was 10 h 52 min ± 61 min (mean ± SD; Table 1). The estimated average antioxidant intake during the race was 393 ± 219 mg vitamin C and 113 ± 59 mg alpha-tocopherol. There were no differences in the amount of the consumed antioxidants between the groups divided by exercise test variables. Out of 48, three study participants failed to complete the race due to self-reported fatigue. In addition, three subjects could not participate in one or more blood sample time points and thus were excluded from the analysis.

**Plasma concentrations of lipoproteins and biochemical variables.** Plasma concentrations of lipoproteins and UA can be found in Table 2. LDL decreased significantly ($P < 0.001$) immediately postrace (−15%) and 1 d postrace (−26%) and stabilized below prerace concentrations until 5 d postrace (−8%; $P < 0.01$). TC significantly decreased to below prerace values 1 d (−10%; $P < 0.001$) and 5 d after the race (−4%; $P < 0.01$), whereas HDL increased immediately (+9%; $P < 0.05$) and 1 d postrace (+12%; $P < 0.01$). VLDL and TG significantly ($P < 0.001$) increased postrace (+75 and +69%, respectively). Plasma levels of UA significantly ($P < 0.001$) increased immediately after the triathlon (+49%). Thereafter, UA concentrations gradually declined but remained significantly ($P < 0.001$) elevated at all time points ($P = 0.001$ for 19 d postrace) versus prerace values (Table 2).

**Plasma concentrations of markers of oxidative stress.** Plasma concentrations of oxidative stress markers are shown in Table 3. A considerable (+91%) and significant increase in CD occurred immediately after the ironman triathlon ($P < 0.001$) and remained significantly elevated 1 d postrace (+13%; $P < 0.01$) when compared with prerace values. MDA trended to increase immediately after the race (+7%; $P = 0.06$), then increased further and reached statistical significance 1 d postrace (+9%; $P < 0.01$). There was a significant decrease in oxidized low-density lipoprotein (oxLDL) below prerace values immediately postrace (−13%; $P < 0.05$) and 1 d postrace (−24%; $P < 0.01$), whereas a tendency toward an increase in the oxLDL:LDL ratio 1 d postrace (+8%; $P = 0.07$) occurred. Plasma AOPP concentrations significantly ($P < 0.001$) increased by 25% immediately postrace and remained significantly ($P = 0.01$) elevated 1 d after the competition (+21%). Similarly, the AOPP:TP ratio peaked by 20% higher than prerace immediately postrace ($P < 0.01$) and remained significantly ($P < 0.05$) elevated by 16% higher than prerace values 1 d after the race. All markers of oxidative stress had returned to prerace values 5 d after the ironman triathlon, and 19 d postrace, all parameters were still similar to prerace concentrations (Table 3).

TABLE 6. Significant associations with plasma antioxidant capacity.

| | $\dot{V}O_{2peak}$ | $W_{peak}$ | Rel. $W_{peak}$ | IAT | Rel. IAT | PO La 2 | Rel. PO La 2 | PO La 3 | Rel. PO La 3 | Rel. PO La 3 %$W_{peak}$ | Total Race Time | 180-km Cycle Time | 42.2-km Run Time | UA | GSH-Px |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TEAC | | | | | | | | | | | | | | | |
| PRE | | | | | | | | | | | | | | | 0.41** |
| POST | 0.33* | 0.30* | 0.39** | 0.34* | 0.36* | 0.33* | 0.36* | 0.36* | | 0.42** | −0.31* | | | | 0.37** |
| Δ PRE to POST | 0.38* | 0.41** | 0.47*** | 0.45** | 0.49*** | 0.46*** | 0.49*** | 0.48*** | 0.44** | 0.56*** | −0.44** | −0.40** | −0.35** | 0.54*** | |
| 1 d POST | | | | | | | | | | | | | | | 0.40** |
| Δ PRE to 1 d POST | | | | 0.34* | 0.31* | | | 0.31* | | 0.33* | | | | | |
| 5 d POST | | | | | | | | | | | | | | | 0.37* |
| 19 d POST | | | | | | | | | | | | | | | 0.47** |

TEAC, Trolox equivalent antioxidant capacity; $W_{peak}$, peak PO; rel., relative; IAT, individual anaerobic threshold; PO La 2 (3), power output at 2 (3) mmol blood lactate·$L^{-1}$; PRE, 2 d prerace; POST, immediately postrace; 1 (5) [19] d POST, 1 d (5) [19] postrace; Δ PRE to POST, change from pre- to immediately postrace; Δ PRE to 1 d POST, change from prerace to 1 d postrace; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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**Antioxidant capacity of plasma and activities of antioxidant erythrocyte enzymes.** The time course of TEAC is shown in Figure 1, and antioxidant enzyme activities can be found in Table 4. A sharp elevation of TEAC was observed in response to the ironman triathlon (+48%; $P < 0.001$), and values remained significantly ($P < 0.001$) higher than prerace until 1 d after the race (+25%). Five and 19 d postrace, TEAC values were similar to prerace. There was a significant decrease in the activities of erythrocyte SOD (−6%; $P < 0.001$) and CAT (−4%; $P < 0.05$) immediately postrace. There was a trend toward decreased GSH-Px activity 1 d postrace (−4%; $P = 0.08$) but no significant changes during the monitoring period. SOD and CAT both followed a biphasic pattern during the recovery period and 19 d postrace, and athletes had moderate but significant decreases in the activities of SOD and CAT compared with prerace values (−5%; $P < 0.001$ and −6%; $P < 0.01$, respectively; Table 4).

**Associations with training and exercise test variables, performance, and oxidative stress markers.** Various significant negative correlations were obtained between parameters of lipid peroxidation and training and exercise test variables, which are shown in Table 5. In contrast, the prerace oxLDL:LDL ratio correlated positively with the weekly net endurance exercise time ($r = 0.42$; $P < 0.01$). Significant positive correlations were observed between postrace indices of protein oxidation and some exercise test variables, whereas triathlon-induced changes in AOPP were inversely related with the cycle split time ($P < 0.05$; Table 5) and the total race time ($P = 0.053$).

**Associations with training and exercise test variables, performance, and plasma antioxidant capacity and antioxidant enzyme activities.** There were multiple positive correlations with changes in TEAC and prerace training and exercise test variables that are summarized in Table 6. Exemplary, the change of TEAC from pre- to immediately postrace correlated with the percentage of maximum PO at 3 mmol·L$^{-1}$ blood lactate ($r = 0.56$; $P < 0.001$). In addition, positive correlations were noted between the exercise-induced changes in TEAC and UA ($r = 0.54$; $P < 0.001$; Table 6), and both changes in TEAC and UA correlated negatively with the total race time ($r = -0.44$ and $r = -0.48$, respectively; both $P < 0.01$). Significant positive correlations were observed between activities of erythrocyte GSH-Px with TEAC (Table 6). Furthermore, GSH-Px activities correlated positively with the percentage of maximum PO at 3 mmol·L$^{-1}$ blood lactate at prerace ($r = 0.35$; $P < 0.05$), 1 d postrace ($r = 0.39$; $P < 0.01$), and 19 d postrace ($r = 0.36$; $P < 0.05$).

**Groups divided by the relative PO at $\dot{V}O_{2peak}$ and the relative IAT: effects on LDL oxidation and plasma antioxidant capacity.** On the basis of the group distribution into percentiles by the relative PO at $\dot{V}O_{2peak}$, a trend was observed insofar as lower oxLDL concentrations immediately postrace were associated with higher levels in relative PO at $\dot{V}O_{2peak}$ (differences between all groups: $P = 0.056$). Furthermore, athletes in the group with the highest relative PO at $\dot{V}O_{2peak}$ (top percentile) had significantly ($P < 0.05$) lower oxLDL concentrations immediately postrace than those athletes in the group with the lowest relative PO at $\dot{V}O_{2peak}$ (lowest percentile), and an according trend was noted with prerace oxLDL concentrations ($P = 0.059$). The association of pre- to postrace changes in TEAC with the percentile distribution by the relative IAT is shown in Figure 2. TEAC increased with the relative IAT across the percentiles, and the differences between all groups were $P = 0.18$. Moreover, the TEAC response was significantly ($P < 0.05$) higher in the subject group with the highest relative IAT (top percentile) compared with the group with the lowest IAT (lowest percentile; Fig. 2).

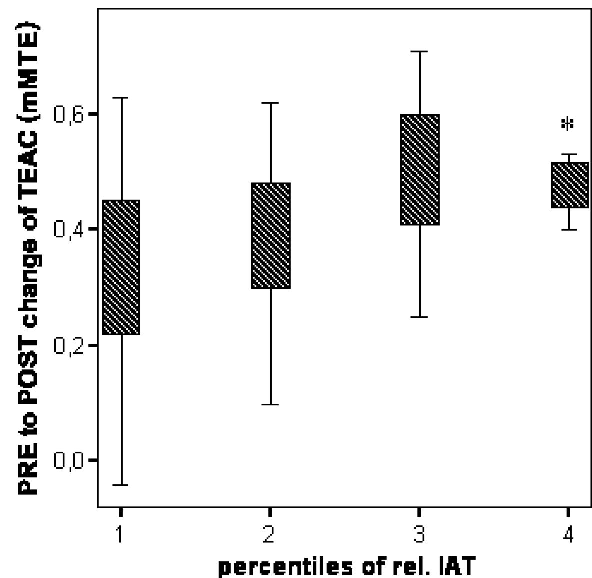


**FIGURE 2—Association of the prerace (PRE) to postrace (POST) change of Trolox equivalent antioxidant capacity (TEAC) with the percentiles of the relative IAT (rel. IAT). TE indicates Trolox equivalents; percentile 1, ≤2.64 W·kg$^{-1}$; percentile 2, 2.65–2.89 W·kg$^{-1}$; percentile 3, 2.90–3.24 W·kg$^{-1}$; percentile 4, ≥3.25 W·kg$^{-1}$. *Significant different from percentile 1 ($P < 0.05$); differences between all percentiles: $P = 0.18$.**

## DISCUSSION

The major finding of the present study was that there are no indications of persistent oxidative damage during a single bout of ultraendurance exercise. Although most (but not all) oxidative stress markers temporarily increased after the ironman triathlon, the current results indicate the importance of the acute exercise-induced alterations in antioxidant capacity of the athletes, which were associated with a variety of physiological training-related determinants. Our data suggest that these training- and/or exercise-induced biochemical and physiological responses in the antioxidant defense system are able to counteract severe or persistent oxidative damage to cell compounds and blood lipids after extremely demanding exercise. Considering the current concerns about health consequences for ultraendurance





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athletes, these findings provide important and novel information because oxidative stress and recovery responses after ultraendurance exercise, up to date, have not been followed for such a long time course.

Most but not all previous studies have shown increased oxidative stress as immediate responses to acute bouts of ultraendurance exercise. Various indices of lipid peroxidation such as MDA (11), lipid hydroperoxides (22), or $F_2$-isoprostanes (19,22,23) were found to be elevated after long-distance triathlons (11,23), an 80-km race (22), and another 50-km ultramarathon (19). Contrary, after other long-distance triathlon races, authors reported either no evidence of oxidative stress (17) or even a decrease in the susceptibility of plasma lipids to peroxidation (7). Some of these methods such as the TBARS assay have been criticized for insufficient accuracy, specificity, and validity (9,10,13). Therefore, we measured CD, as this method is considered as a specific marker for the initial phase of lipid peroxidation (6,36,37), and detected MDA by high-performance liquid chromatography (HPLC). Moreover, the study is the first in which attention is drawn on the effects of ultraendurance exercise on oxLDL and AOPP. Both parameters are seen as novel and reliable biomarkers that indicate long-term effects of oxidative stress (4,24) such as after a high volume training period.

In the current investigation, different amplitudes and kinetics were observed in the examined oxidative stress markers. Although there was an immediate and marked rise in CD, followed by a rapid decline, MDA increased only slightly immediately after race completion but rose to significant levels 1 d after the ironman triathlon. One explanation for the differences in the changes of these indices is that CD are primary oxidation products formed during initial reactions of lipid peroxidation, whereas MDA is produced at a latter stage of the lipid peroxidation chain reactions (6,36). Furthermore, our data suggest that enhanced postrace antioxidant defenses (described later in detail) might have played a role in preventing a more pronounced rise in MDA. AOPP concentrations and AOPP:TP ratio peaked immediately postrace. Importantly, despite a decrease (from immediately postrace to 1 d postrace) in CD, AOPP, and AOPP:TP ratio, all these markers in addition to MDA remained significantly above prerace values 1 d postrace. This apparently indicates that peroxidation of membranes or/and blood lipids as well as oxidative modification of plasma proteins are sustained for at least 1 d after prolonged strenuous exercise. Although a delayed removal of oxidized products cannot be excluded, our findings that concentrations of nutritive antioxidants dropped below prerace values 1 d postrace (21) (probably reflecting increased antioxidant consumption associated with the counteracting of increased ROS formation) further support the concept of continued oxidative stress responses. Although augmented susceptibility of LDL particles to oxidation (16) and increased lipid peroxides levels (8) persisted over 4 to 8 d after a marathon run, Mastaloudis et al. (19) reported that $F_2$-isoprostanes (together with IL-6) had returned to prerace values 1 d postrace in ultramarathon runners. Observed correlations between protein oxidation markers and markers of muscle damage and inflammation (unpublished results) might point to muscular inflammatory processes as a source of this low-grade oxidative stress response 1 d postrace (6,13,38). Crucially, oxLDL even significantly decreased below prerace values after the race. This change is most likely a consequence of an enhanced lipoprotein metabolism and the decline of LDL cholesterol itself as demonstrated after another ironman race (7). However, the oxLDL:LDL ratio showed a modest and only temporary trend to increase 1 d after the competition. In line with observations of Ginsburg et al. (7), who reported a reduced susceptibility of plasma lipids to peroxidation in male ironman competitors, this finding suggests that a single bout of ultraendurance exercise might not contribute to the development or progression of atherosclerosis lesions based upon the oxidative modification of LDL hypothesis (34). Moreover, as all oxidative stress markers had returned to prerace values 5 d postrace and remained at prerace levels 19 d postrace, there are no indications of persistent oxidative stress during an ironman triathlon.

A wide range of correlations with training volume, training status, and oxidative stress markers were present (Table 5). Consistent with a previous study of Knez et al. (11), who reported a dose–response relationship of resting MDA concentrations with time spent training, we observed a positive correlation between the prerace oxLDL:LDL ratio and the weekly net endurance exercise. In contrast to the demonstrated oxLDL responses to acute ultraendurance exercise, this might reflect cumulative oxidative stress that had been attributed to high training volumes or overload training (6,18). On the other hand, our data revealed that those athletes with the highest PO at $\dot{V}O_{2peak}$ had significantly lower plasma oxLDL (but not LDL) concentrations after the triathlon compared with the subjects with the lowest PO at $\dot{V}O_{2peak}$ (percentile distribution). Additionally, MDA before, after, and 1 d postrace concentrations were also lower with higher training status and with increasing weekly training loads. Furthermore, we found many negative associations with markers of lipid peroxidation and variables associated with training status, both prerace as well as in response to the race (Table 5), indicating that better training levels might confer enhanced protection against oxidative stress and consequent damage of lipids and/or result in a decrease in free radical formation. Thus, the results from the present study support the idea that endurance training reduces postexercise oxidative stress (6,13).

Interestingly, opposite to the triathlon-induced effects on markers of lipid peroxidation, AOPP and AOPP:TP ratio were positively related to some training-associated variables. In addition with the finding that AOPP rose with the performance in the cycle split time in the ironman race, these data might imply that there is an intensity-related response in protein oxidation because better trained athletes were capable of competing the ironman at higher

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intensities and therefore had more pronounced changes in protein oxidation. On the basis of previous observations in exercised animals (27), these results possibly suggest that proteins are more prone to free-radical–induced oxidation during strenuous endurance exercise than lipids. Taken together, our results reveal a complex picture of oxidative stress during exhaustive endurance exercise that emphasizes the importance to use multiple markers and to monitor them in a longer time course for several reasons. First, recent research has shown that there are optimal time points for the detection of maximum concentrations of oxidative stress markers (20,38). Second, our results support findings (27,28) that lipids and proteins might be affected differently by exercise-induced oxidative stress. Since, up to now, there is limited data regarding the effects of exercise on protein oxidation (38), which has striking consequences for cell function (28), it seems crucial not to focus exclusively on indices of lipid peroxidation. Finally, little information is available on the complete resumption of recovery in these indices especially after ultraendurance exercise (17,19), which might be important for assessing possible deleterious health effects.

In agreement with previous studies in marathon runners (16,37), but probably investigated for the first time in ultraendurance athletes, plasma antioxidant capacity rose markedly after the ironman race. The alteration in the total antioxidant capacity of plasma can be seen as an early adaptive response to oxidative stress (26), which might have prevented initiation of lipid peroxidation to a certain degree. One day after the race, TEAC declined but still remained elevated above prerace values. Although TEAC was still found to be increased 4 d after a marathon (16), it had returned to prerace levels 5 d postrace along with oxidative stress markers in the present study. Our data suggest several mechanisms for the observed postrace increase in the antioxidant capacity of plasma. On the one hand, the increase in TEAC might be a result of the elevation of vitamin C (which change correlated with that of TEAC) and alpha-tocopherol (21), attributed to the intake of these antioxidants during the race as well as tissue mobilization (19). On the other hand, concomitant with previous findings (16,19), the current results imply that UA is responsible for the rise in TEAC to a considerable extent (Table 4). Plasma concentrations of the potent hydrophilic antioxidant UA are known to rise during intense exercise being produced from increased purine metabolism (6,13,38) and possibly also due to impaired renal clearance (19). We found that both TEAC and UA increased with performance in the ironman triathlon. Consequently, these results suggest that those athletes with a higher training and performance status could push themselves harder, which in turn resulted in higher concentrations of UA after the race. This phenomenon cannot be considered as a specific training adaptation, but it contributes to the performance-linked increase of TEAC. Of further interest, we observed that the ironman-induced change of TEAC was associated with the relative IAT (percentile distribution), that is, TEAC increased in athletes with greater performance ability (Fig. 2). Moreover, several other training physiological determinants (associated with training and performance capacity at different exercise intensities) seem to play important roles in promoting such a protective response in antioxidant defenses of plasma (Table 6). Interestingly, TEAC was positively related to GSH-Px activities throughout the monitoring period (Table 6), which may imply a synergistic interaction between erythrocytes and plasma antioxidant capacity. However, although we noted that GSH-Px was linked with training status, it is unclear whether or to which extent training-induced adaptations of endogenous antioxidant defenses (in particular antioxidant enzymes) might have contributed to the rise of TEAC after the ironman race. In previous studies, high-dosed antioxidant supplementation in competitors of ultraendurance races had either beneficial (19), adverse (i.e., pro-oxidant) (23,11), or no effects (22) on oxidative stress changes. With the exception of the reported relationship between the changes in vitamin C and TEAC, there was no association found between plasma levels of nutritive antioxidant and oxidative stress or antioxidant responses in the present study. Despite individual differences in the plasma concentrations of nutritive antioxidants, this observation may be because antioxidant status of all subjects was in a normal physiological range (21).

Several studies showed increased antioxidant erythrocyte enzyme activities after relatively short bouts of aerobic exercise (6), whereas different patterns or opposite effects (decreases) were seen after a 171-km cycling mountain stage (2) or a marathon (8). Only a small number of studies have examined adaptations to or acute effects of ultraendurance exercise on the antioxidant system (11,17). Recently, Knez et al. (11) reported that activities of all key antioxidant enzymes in erythrocytes declined immediately after an ironman race. Except for GSH-Px (which only trended to decrease), our data further confirm these somewhat unexpected results as we also observed a significant decrease in the activities of SOD and CAT after the competition. In general, modifications of antioxidant enzyme activities after exercise characterize either adaptation (an increase in the activity at first) or utilization (a decrease if oxidative stress is overwhelming) (6). These decreases had, hypothetically, been attributed to a modification of the catalytic centers and subsequent inactivation of enzymes due to a disturbed redox balance induced by augmented oxidative stress (2,11,38). Contrary to the acute effects of the ironman triathlon, the attenuation of SOD and CAT activities 19 d after the competition to below prerace values likely can be explained by a down-regulation during the recovery period (6). Moreover, we noted that activities of GSH-Px were positively associated with the percentage PO at a blood lactate concentration of 3 $mmol \cdot L^{-1}$. This finding supports data that this enzyme may be highly responsive to endurance training in general (11,17) and to training at higher-intensities in particular. This conclusion is



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supported by evidence in the skeletal muscle response to exercise (25).

## CONCLUSION

The present data indicate that a single bout of ultraendurance exercise is associated with a systemic acute and elevated oxidative stress response. Although the disturbance in the oxidant/antioxidant balance was sustained for at least 1 d after the ironman triathlon, there are no indications of persistent detrimental health effects due to oxidative stress. Moreover, our results provide further evidence that there are chronic training-induced biochemical adaptations (resulting either in a decrease in free radical production and/or in an enhancement of the antioxidant defenses) and that manifold training-associated determinants might be responsible for these protective responses to a certain extent. Weekly training loads as well as training at *different* intensities seem to be factors in the improvement of antioxidant defense mechanisms after prolonged intense exercise. Finally, the present investigation illustrates that even minor differences in the training status among well-trained athletes can result in significantly different outcomes in the training- and exercise-induced responses of oxidative stress and antioxidant-related parameters. Generally, these data imply that acute ultraendurance exercise does not cause longer lasting alterations in systemic oxidative stress markers, probably due to improved antioxidant responses to strenuous exercise in well-trained athletes.

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# Original article II

(Basis for chapter 3)

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ORIGINAL ARTICLE

# Recovery after an Ironman triathlon: sustained inflammatory responses and muscular stress

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**Abstract** Ultra-endurance exercise, such as an Ironman triathlon, induces muscle damage and a systemic inflammatory response. As the resolution of recovery in these parameters is poorly documented, we investigated indices of muscle damage and systemic inflammation in response to an Ironman triathlon and monitored these parameters 19 days into recovery. Blood was sampled from 42 well-trained male triathletes 2 days before, immediately after, and 1, 5 and 19 days after an Ironman triathlon. Blood samples were analyzed for hematological profile, and plasma values of myeloperoxidase (MPO), polymorphonuclear (PMN) elastase, cortisol, testosterone, creatine kinase (CK) activity, myoglobin, interleukin (IL)-6, IL-10 and high-sensitive C-reactive protein (hs-CRP). Immediately post-race there were significant ($P < 0.001$) increases in total leukocyte counts, MPO, PMN elastase, cortisol, CK activity, myoglobin, IL-6, IL-10 and hs-CRP, while testosterone significantly ($P < 0.001$) decreased compared to prerace. With the exception of cortisol, which decreased below prerace values ($P < 0.001$), these alterations persisted 1 day post-race ($P < 0.001$; $P < 0.01$ for IL-10). Five days post-race CK activity, myoglobin, IL-6 and hs-CRP had decreased, but were still significantly ($P < 0.001$) elevated. Nineteen days post-race most parameters had returned to prerace values, except for MPO and PMN elastase, which had both significantly ($P < 0.001$) decreased below prerace concentrations, and myoglobin and hs-CRP, which were slightly, but significantly higher than prerace. Furthermore, significant relationships between leukocyte dynamics, cortisol, markers of muscle damage, cytokines and hs-CRP after the Ironman triathlon were noted. This study indicates that the pronounced initial systemic inflammatory response induced by an Ironman triathlon declines rapidly. However, a low-grade systemic inflammation persisted until at least 5 days post-race, possibly reflecting incomplete muscle recovery.

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**Keywords** Ultra-endurance exercise · Muscle damage · Systemic inflammatory response · Immunoendocrine responses · Recovery phase

## Introduction

Regular physical training appears to enhance parts of the innate immune system while prolonged strenuous physical exercise attenuates many components of immunity (Gleeson 2007; Malm 2004; Pedersen and Hoffman-Goetz 2000). When the integrity of the organism is challenged by vigorous endurance exercise, a systemic inflammatory response is induced. This stereotypical and evolutionary conserved reaction to major physical stressors protects the organism by eliminating antigens, cellular debris, tissue fragments, by preventing further damage and by promoting tissue repair. However, it also elicits a temporary dysfunction of various aspects of immunity, which may increase the risk of subclinical and clinical infection (Fehrenbach and Schneider 2006; Gleeson 2007; Pedersen and Hoffman-Goetz 2000). The regulation of recruitment and distribution of leukocytes is complex and includes a plethora of signals and mediators such as hormones and cytokines (Malm

2004; Moldoveanu et al. 2001; Pedersen and Hoffman-Goetz 2000). Beyond the ultra-structural damage of muscle tissue as a potential stimuli for the production and release of cytokines, exhaustive endurance exercise induces several factors such as metabolic, hormonal, thermal and oxidative stress, all of which can give rise to the release of cytokines and other acute phase proteins in addition to the activation of several cell sub populations within the immune system (Fehrenbach and Schneider 2006; König et al. 2001). Nevertheless, crucially, up to now only very few studies have investigated the relationships between muscle damage and the release of cytokines or acute phase reactants explicitly after ultra-endurance exercise (Jeukendrup et al. 2000; Gomez-Merino et al. 2006; Nieman et al. 2004, 2005; Suzuki et al. 2006). Moreover, exercise-induced inflammatory or immune responses rarely have been followed longer than 1 day post-race (Gomez-Merino et al. 2006; Margaritis et al. 1997; Mastaloudis et al. 2004). Further questions arise on this topic as it is hypothesized that repetitive skeletal muscle tissue trauma caused by heavy training and competition could result in a persistent systemic cytokine response, which may be associated with a chronic inflammatory state, immune dysfunction and a poorly understood condition of overreaching/overtraining (Halson and Jeukendrup 2004) or underperformance syndrome (Robson-Ansley et al. 2007; Robson 2003; Smith 2000). Thus, detailed knowledge concerning the circumstances that alter the time-course of the restoration in muscular, inflammatory and immune parameters after ultra-endurance exercise is essential for designing training schedules that avoid eventual negative consequences of high volumes and intensities of physical activity.

Ironman triathletes are extraordinary in their level of training and in the endurance and intensity of exercise performed. Thus, they are an exceptional group to investigate not only because of their physiological characteristics and demands (Millet et al. 2003), the nutritional challenges (Jeukendrup et al. 2005), but urgently also to assess potentially harmful effects as a result of ultra-endurance sports in general (Knez et al. 2006). This is of particular importance since the number of non-professional athletes training for and competing in ultra-endurance events also continually increases. Furthermore, the clinical relevance of research work within this area is further emphasized because there are important similarities between the systemic inflammatory response to heavy endurance exercise and acute conditions such as sepsis and trauma (Fehrenbach and Schneider 2006; Pedersen and Hoffman-Goetz 2000).

The present study aimed to further elucidate the physiological, biochemical and molecular-biological stress responses to an Ironman triathlon in a large cohort of athletes. Specifically, we examined a range of muscular, inflammatory and hormonal parameters not only to quantify their magnitude directly after an acute bout of ultra-endurance exercise, but importantly also to verify the resolution of recovery. Information on the alterations in these responses *after* extremely demanding endurance exercise is limited. Therefore, it was a primary aim of the current study to get a thorough picture of these recovery responses including potential interactions between the immune system and skeletal muscle that are still not fully understood.

## Materials and methods

### Subjects

The study population comprised 48 non-professional well-trained healthy male triathletes, who participated in the 2006 Ironman Austria; 42 of them completed the study and were included in the statistical analysis. The characteristics of the subjects and their performance in the Ironman triathlon are shown in Table 1. The subjects were recruited from all over Austria half a year before the event. They were informed about the purpose and risks of the study before they provided written informed consent. The Ethics Committee of the Medical University of Vienna approved the study.

**Table 1** Characteristics of the study participants and their performance in the Ironman triathlon

| | |
|---|---|
| Age (y) | 35.3 (7.0) |
| Height (cm) | 180.6 (5.6) |
| Weight (kg) | 75.1 (6.4) |
| BMI (kg m$^{-2}$) | 23.0 (1.2) |
| Body fat (%) | 11.8 (4.1) |
| Cycling $VO_{2\,peak}$ (ml kg$^{-1}$ min$^{-1}$) | 56.6 (6.2) |
| Power output at $VO_{2\,peak}$ (W) | 358.3 (49.9) |
| Training over a period of 6 months prior to the Ironman triathlon | |
| Weekly net endurance exercise time (WNET) (h week$^{-1}$) | 10.7 (2.6) |
| Swim training (km week$^{-1}$) | 4.8 (2.2) |
| Cycle training (km week$^{-1}$) | 144 (52.1) |
| Run training (km week$^{-1}$) | 36.4 (10.6) |
| Performance in the Ironman triathlon | |
| Total race time (h:min:s) | 10:51:52 (01:01:22) |
| 3.8 km swim time (h:min:s) | 01:09:51 (00:10:28) |
| 180 km cycle time (h:min:s) | 05:28:21 (00:29:08) |
| 42.2 km run time (h:min:s) | 04:08:26 (00:31:36) |
| Training after completion of the Ironman triathlon until 19 days post-race | |
| WNET (h week$^{-1}$) | 4.2 (2.4) |
| Swim training (km week$^{-1}$) | 2.1 (2.2) |
| Cycle training (km week$^{-1}$) | 66.3 (53.8) |
| Run training (km week$^{-1}$) | 12.3 (11.5) |

Data are mean (SD), $n = 42$

### Study design

All participants of the study were required to complete a medical and health-screening, a food frequency and a supplementation questionnaire and to document their training in the 6 months prior to the Ironman triathlon and thereafter until the end of the study (Table 1). All participants were not taking prescribed medication and avoided taking more than 100% of RDA in form of antioxidant supplements in addition to their normal dietary antioxidant consumption in the 6 weeks before the race and until the final blood sampling. Subjects were physically fit, free of acute or chronic illnesses, within a normal range of body mass index and non-smokers. In order to investigate exercise-induced effects in addition to recovery responses, blood samples were taken 2 days prerace, immediately (within 20 min), 1, 5 and 19 days post-race. Furthermore, subjects completed a 24 h dietary recall at each time point. The athletes abstained from intense exercise 48 h before the spiroergometry testing as well as before each blood sampling (except the Ironman itself). In addition, the athletes had fasted overnight before the 2-days prerace, 5- and 19-days post-race blood samples (which were all taken between 8:00 and 9:00 a.m.). On race day and 1 day post-race, they were allowed to drink and eat ad libitum, and the quantities of intake were recorded. After the triathlon the subjects performed “recovery” training that was of moderate intensity and duration until the end of the study (Table 1). During this recovery period the athletes were required to abstain from any training that was above the lactate threshold (respectively, the concomitant heart frequency) of each individual.

### Race conditions

The Ironman triathlon was held in Klagenfurt, Austria on 16 July 2006 and consisted of 3.8 km swimming, 180 km cycling and 42.2 km running on a flat course. When the race started at 7:00 a.m. the air temperature and relative humidity were 15°C and 77%, with the lake temperature at 25°C. Between 4:00 p.m. and 5:00 p.m. respectively by finishing time (median time for subjects approximately 5:43 p.m.), air temperature reached a maximum and was 27.2°C, and relative humidity had decreased to 36% (data provided by the Carinthian Centre of the Austrian Central Institute for Meteorology and Geodynamics).

### $VO_{2\,peak}$ testing protocol

The triathletes were tested 3 weeks before the race on a cycle ergometer (Sensormedics, Ergometrics 900). The maximal test protocol started at an initial intensity of 50 W, followed by 50 W increments every 3 min until exhaustion. During the test oxygen and carbon dioxide fractions (via Sensormedics 2900 Metabolic measurement cart), power output, heart rate, and ventilation were recorded continuously and earlobe blood samples for the measurement of the lactate concentration were taken at the beginning and at the end of each stage.

### Blood sampling

At each blood sampling blood was collected into heparin, EDTA or serum vacutainers (Vacuette, Greiner, Austria). A field laboratory was installed at the race to ensure the appropriate collection of the first three blood samples. The blood was immediately cooled to 4°C and plasma separated at $1{,}711 \times g$ for 20 min at 4°C. Aliquots were immediately frozen at −80°C. Whole blood was taken for the hematological profile.

### Hematological profile

The hematological profile was assessed with a MS4 Hematology 3-Part-Differential-Analyzer (Melet Schloesing Laboratories, Maria Enzersdorf**,** Austria). Exercise-induced changes in plasma volume were calculated (Dill and Costill 1974) until 5 days post-race to assess expansion of plasma volume, which persists for 3–5 days following the cessation of demanding exercise (Shaskey and Green 2000). All results are reported adjusted for these changes, except for cortisol and testosterone as for factors released in an endocrine manner it is important to consider their actual circulating concentration.

### Plasma cortisol and testosterone concentrations

Both parameters were determined with radioimmunoassay based on the competition between radioactive and non-radioactive antigen. Testosterone was assessed with Active® Testosterone RIA DSL-4000 (Diagnostic Systems Laboratories, Inc., Webster, TX, USA), Cortisol with Corti-Cote® Cortisol Antibody Coated Tube - 125I RIA Kit (MP Biomedicals, Illkirch, France). The coefficients of variation were 5.3 and 6.4% for cortisol and testosterone, respectively.

### Plasma myeloperoxidase (MPO) concentration and polymorphonuclear elastase (PMN) concentration

Myeloperoxidase concentrations were measured using the immundiagnostik MPO ELISA kit (Immundiagnostik AG, Bensheim, Germany) by two-site sandwich technique. The absorbance of samples and standards were read with a Fluostar Optima microplate reader (BMG labtechnologies, Germany) at 450 nm. Polymorphonuclear elastase was determined using a quantitative enzyme immunoassay (Milena Biotec GmbH, Bad Nauheim, Germany). All mea-

sures were made in duplicate. The coefficients of variation were 5.6 and 7.3% for PMN elastase and MPO, respectively.

### Plasma markers of muscle damage and of inflammation

Plasma creatine kinase (CK) activity was detected using an automatic analyzer (Vitros DT 60 II module; Ortho-clinical Diagnostics, Germany). Concentrations of myoglobin and high-sensitive C-reactive protein (hs-CRP) were analyzed nephelometrically (Dade Behring, Marburg, Germany). Plasma interleukin (IL)-6 and IL-10 were determined by the Quantikine HS Immunoassay kit (R&D Systems GmbH, Wiesbaden, Germany). The coefficients of variation were 3.0, 4.5, 5.0, 7.8 and 8.6% for CK activity, myoglobin, hs-CRP, IL-6 and IL-10, respectively.

### Data analysis

Data were tested for normal distribution using the Kolmogorov–Smirnov test. The main effect of time was obtained by using the repeated measures analysis of variance (ANOVA, general linear model). Dependent on normal distribution of data, either paired *t*-tests (for normally distributed data) or Wilcoxon tests (for not normally distributed data) were then used to assess differences in the test variables, whereas all post-race values were compared with prerace (= baseline) values. Either Pearson's (for normally distributed data) or Spearman's correlation (for not normally distributed data) was used to examine significant relationships. All statistical analyzes were performed using SPSS 15.0 for Windows. $P$ values were considered as follows: $P < 0.05$ significant, $P < 0.01$ highly significant and $P < 0.001$ extremely significant.

## Results

### Race results

The completion time was 10 h 52 min ± 1 h 1 min (mean ± SD; Table 1). Three study participants failed to complete the race because of self-reported fatigue. In addition, three subjects could not participate in one or more blood sample time points and thus were excluded from the analysis.

### Total leukocyte counts, leukocyte subpopulations and markers of neutrophil activation

Total leukocyte count increased significantly (+237%; $P < 0.001$) immediately after the Ironman triathlon versus prerace and remained significantly (+56%; $P < 0.001$) elevated until 1 day post-race. Changes in leukocyte subpopulations are shown in Fig. 1. Plasma MPO and plasma PMN elastase concentrations significantly (both $P < 0.001$) increased by 342% and 424%, respectively immediately post-race. Both the variables remained elevated above the prerace values 1 day after the race (+70 and +108%, respectively; both $P < 0.001$). Myeloperoxidase, polymorphonuclear elastase values had returned to prerace 5 day post-race and 19 days post-race there was a significant (both $P < 0.001$) reduction (−28 and −21%, respectively) below prerace values (Table 2).

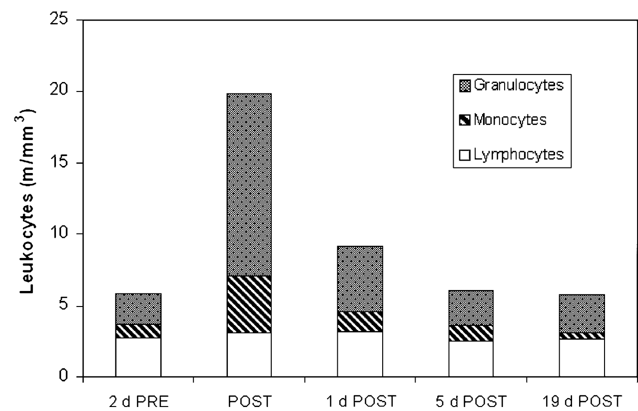


**Fig. 1** Mean changes in blood concentrations of leukocytes and subpopulations 2 days prerace (*PRE*), immediately post-race (*POST*), 1 day post-race, 5 days post-race and 19 days post-race for 42 subjects

### Plasma concentrations of cortisol and testosterone, and testosterone:cortisol ratio

There was a significant increase immediately post-race for cortisol (+241%; $P < 0.001$), whereas testosterone dropped below prerace (−53%; $P < 0.001$) at the same time point. One day post-race cortisol decreased sharply by 47% to below prerace values ($P < 0.001$). Five and nineteen days post-race cortisol concentrations remained moderately but not-significantly lower. The testosterone concentration levelled off below prerace values 1 day after the competition (−50%; $P < 0.001$) returning to prerace values 5 day post-race. The testosterone to cortisol ratio was significantly ($P < 0.001$) decreased by 86% directly after the race compared to prerace (Table 2).

### Markers of muscle damage

Plasma CK activity increased significantly ($P < 0.001$) by 1,195% immediately post-race, with a maximum concentration observed 1 day post-race, when it was 4,316% higher than prerace values ($P < 0.001$). Creatine kinase values remained significantly ($P < 0.001$) higher than prerace until 5 days post-race (+281%). Plasma myoglobin peaked by 3,842% higher than prerace instantly post-race ($P < 0.001$), and remained significantly ($P < 0.001$) elevated by 964% higher than prerace values 1 day after

**Table 2** Plasma values of myeloperoxidase (MPO), polymorphonuclear (PMN) elastase, cortisol, testosterone and testosterone:cortisol ratio

| | Pre | Post | 1 day post | 5 days post | 19 days post | Time effect (*P*) |
|---|---|---|---|---|---|---|
| MPO (μg $L^{-1}$) | 57 (31) | 253 (122)*** | 97 (82)*** | 61 (58) | 41 (25)*** | <0.001 |
| PMN elastase (μg $L^{-1}$) | 46 (23) | 239 (137)*** | 95 (104)*** | 44 (31) | 36 (16)*** | <0.001 |
| Cortisol (nmol $L^{-1}$) | 282 (112) | 957 (696)*** | 149 (66)*** | 249 (107) | 273 (110) | <0.001 |
| Testosterone (nmol $L^{-1}$) | 11.4 (5.6) | 5.3 (3.6)*** | 5.5 (2.9)*** | 12.7 (6.9) | 12.3 (6.3) | <0.001 |
| Testosterone:cortisol ratio | 0.040 (0.037) | 0.006 (0.009)*** | 0.037 (0.027) | 0.051 (0.036) | 0.045 (0.032) | <0.001 |

Values are mean (SD); $n = 42$

*Pre* 2 days prerace; *Post* immediately post-race; *1 day post* 1 day post-race; *5 days post* 5 days post-race; *19 days post* 19 days post-race

*** Significantly different from prerace values, $P < 0.001$

the race. Thereafter myoglobin concentrations declined, but remained elevated by 5 days (+45%) and 19 days (+30%) after the competition (both $P < 0.001$) (Fig. 2).

### Plasma cytokines and high-sensitive C-reactive protein concentrations

Plasma IL-6 increased dramatically in response to the race (+10,408%; $P < 0.001$), and despite a sharp decline, values remained significantly elevated 1 day (+345%; $P < 0.001$) and 5 days (+79%; $P < 0.001$) after the race. The plasma IL-10 concentration was also elevated immediately after the race (+287%; $P < 0.001$), and remained above prerace concentrations 1 day post-race (+37%; $P < 0.01$). Five days post-race, IL-10 had declined by 4% below prerace concentrations ($P < 0.05$), and 19 days post-race levels were similar to prerace. The plasma hs-CRP concentration rose significantly ($P < 0.001$) immediately after the race by 543% and had increased by 7,702% 1 day post-race ($P < 0.001$). High-sensitive C-reactive protein subsequently decreased, but values remained significantly higher than prerace concentrations 5 days (+881%; $P < 0.001$) and 19 days after the competition (+38%; $P < 0.01$) (Fig. 3).

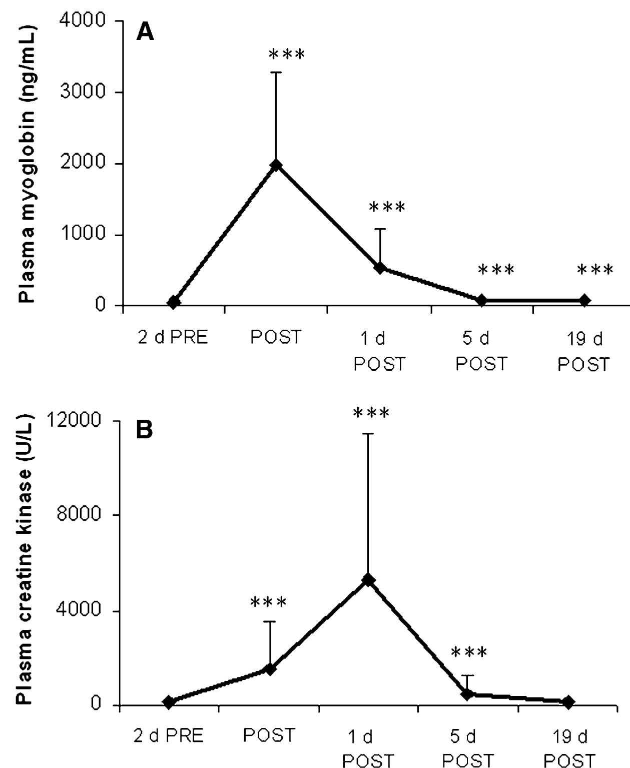


**Fig. 2** Changes in plasma myoglobin concentrations (**a**) and plasma creatine kinase activity (**b**) 2 days prerace, immediately post-race, 1 day post-race, 5 days post-race and 19 days post-race. Data are mean ± SD; $n = 42$; ** significantly different from prerace values, $P < 0.001$

### Associations with neutrophil dynamics, changes in cytokines and hs-CRP

Significant positive correlations were obtained between the change in total leukocyte count and changes in plasma myoglobin, CK activity, cortisol and IL-6, which are summarized in Table 3. Moreover, a positive correlation was observed between the pre- to immediately post-race change of IL-6 and the pre- to 1 day post-race change of MPO ($r = 0.45$; $P < 0.01$). Moderate positive correlations were observed between the pre- and post-race changes of IL-6 and markers of muscle damage. In addition, the pre- to immediately post-race responses in IL-10 correlated positively with cortisol ($r = 0.46$; $P < 0.01$) and were inversely related with performance-associated variables (Table 3). There were also several stronger positive correlations between hs-CRP and markers of muscle damage that are also shown in Table 3.

## Discussion

The time-course of recovery after acute ultra-endurance exercise is a critical question that has rarely ever been answered. By quantifying the recovery responses until 19 days after the race in a large cohort of Ironman competitors we addressed this issue in a comprehensive manner.

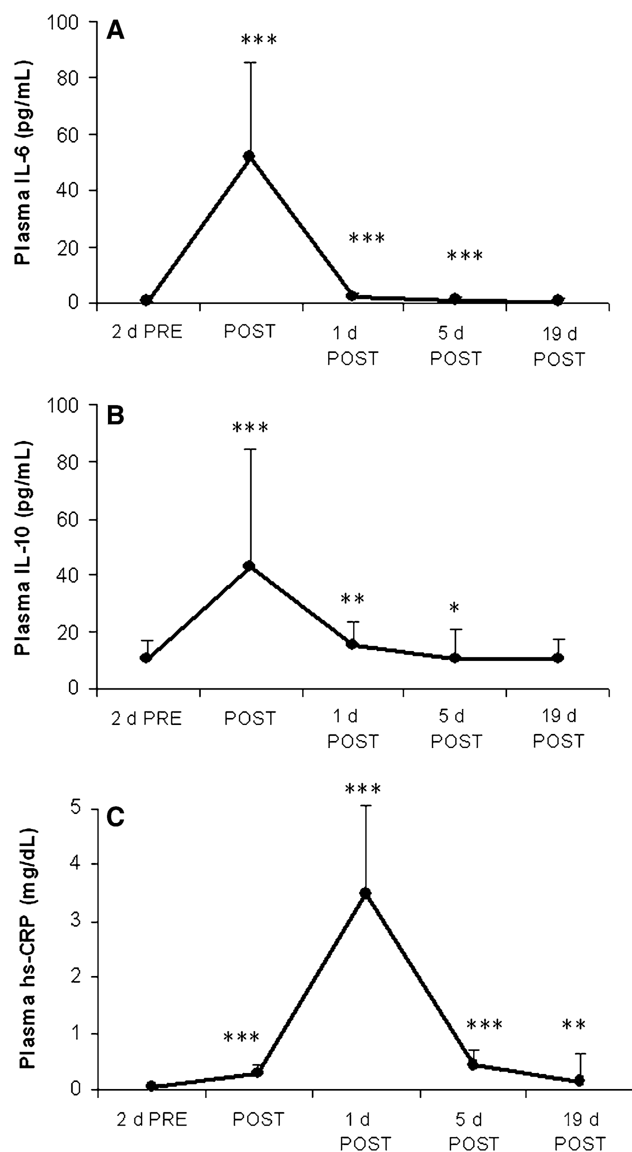


**Fig. 3** Changes in the plasma concentrations of interleukin (IL)-6 (**a**), IL-10 (**b**) and high-sensitive C-reactive protein (hs-CRP) (**c**) 2 days prerace, immediately post-race, 1 day post-race, 5 days post-race and 19 days post-race. Data are mean ± SD; $n = 42$; *** significantly different from prerace values, $P < 0.001$; ** significantly different from prerace values, $P < 0.01$

The main finding of the present study is that markers of muscle damage, IL-6 and hs-CRP were still altered 5 days after the long-distance triathlon. Moreover, the large number of study participants enabled us to substantiate scarce findings regarding associations between muscular stress and the inflammatory response with a high statistical power.

The response of the steroid hormones following the Ironman triathlon revealed the major systemic stress caused by the competition. Corresponding to previous studies (Bishop et al. 2003; Davison and Gleeson 2006; Suzuki et al. 2000), plasma cortisol rose significantly, whereas testosterone decreased in response to prolonged exercise. While plasma testosterone remained lower than prerace 1 day post-race, cortisol levels recovered and, probably also reflecting the circidian rhythm of steroidhormones, fell below prerace values. The ratio of testosterone to cortisol that hypothetically indicates the balance between anabolic and catabolic effects of exercise (Halson and Jeukendrup 2004) declined as an immediate response to the triathlon, but normalized within the first day of recovery. Exercise-induced changes of both hormones are known to affect the immune system (Pedersen and Hoffman-Goetz 2000), and in the present study cortisol was associated with the exercise-induced leukocytosis as well as with the increase of IL-10.

In agreement with other studies examining muscle damage following a long distance triathlon (Gomez-Merino et al. 2006; Margaritis et al. 1997; Suzuki et al. 2006), the initiate injury of skeletal muscle injury was indicated by the leakage of myofibre proteins into the blood plasma. Mechanical stress (associated with exercise involving frequent eccentric or lengthening contractions such as in the marathon split of the Ironman triathlon) in addition to metabolic stress are believed to be the most important initial factors leading to exercise-induced muscle damage (Tee et al. 2007). In the present investigation, the time-course of muscle damage markers is similar to that characteristically found after protocols, in which metabolic stress is suggested to be heavily involved in muscle injury (Margaritis et al. 1997; Tee et al. 2007; Whyte et al. 2000). The highest circulating myoglobin concentrations were found immediately after the race and, presumed that there was no further increase according to previous data (Whyte et al. 2000), CK activity also peaked within 1 day after the triathlon. Since the training intensity of the subjects in the first day after the Ironman race was only modest compared to the tapering period before the competition, the prolonged appearance of myofibre proteins in the plasma until at least 5 days post-race is most likely related to subsequent muscle repair processes and probable local inflammatory responses. Although data is not consistent (St Pierre Schneider and Tiidus 2007), there is good evidence to suggest that neutrophils and macrophages infiltrate damaged muscle (St Pierre Schneider and Tiidus 2007; Tidball 2005). Even though this is a desirable response in terms of muscle repair and probably also muscle adaptation (Malm 2004; Tidball 2005) it may trigger further muscular inflammatory processes and damage, partly through the increased formation of reactive oxygen species (König et al. 2001). The slightly, but significantly elevated plasma myoglobin concentration 19 days post-race might rather be attributed to the gradual resumption of run training (despite its moderate intensity and volume) than to direct race responses. Otherwise, this could also indicate that muscle regeneration was not complete (Tee et al. 2007).

**Table 3** Significant associations with exercise-induced responses of total leukocyte counts, interleukin (IL)-6, IL-10 and high-sensitive C-reactive protein (hs-CRP)

| | MPO | Cortisol | CK | Myoglobin | IL-6 | Total race time | Run split |
|---|---|---|---|---|---|---|---|
| Leukocytes | | | | | | | |
| Δ pre to 1 day post | | Δ pre to post: 0.42** | Δ pre to 1 day post: 0.47** | Δ pre to post: 0.44**; Δ pre to 1 day post: 0.44** | Δ pre to 1 day post: 0.48** | | |
| IL-6 | | | | | | | |
| Δ pre to post | Δ pre to 1 day post: 0.45** | | Δ pre to 1 day post: 0.35* | Δ pre to 1 day post: 0.38** | | | |
| IL-10 | | | | | | | |
| Post | | | | | | −0.43** | −0.47** |
| Δ pre to post | | Δ pre to post: 0.44** | | | | | −0.42** |
| Hs-CRP | | | | | | | |
| Post | | | post: 0.44** | | | | |
| 1 day post | | | 1 day post: 0.52*** | 1 day post: 0.58*** | | | |
| Δ pre to 1 day post | | | Δ pre to post: 0.60***; Δ pre to 1 day post: 0.54*** | Δ pre to post: 0.61*** | | | |
| 5 days post | | | 5 days post: 0.53*** | | | | |

*MPO* myeloperoxidase; *CK* creatinkinase; *Post* immediately post-race; *1, 5, 19 post* 1, 5, 19 days post-race; *Δ pre to post* change from pre- to immediately post-race; *Δ pre to 1 day post* change from pre- to 1 day post-race

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

With regards to the cellular immune system the systemic inflammatory response was characterized by a pronounced leukocytosis immediately after the Ironman triathlon (Fig. 1). Subsequently, the total leukocyte count declined, but still significantly increased until 1 day post-race. Despite a significant decrease in lymphocyte percentages, there was an increase in total lymphocyte counts post- and 1 day post-race, whereas lymphocyte concentrations tend to decrease 5 days after the competition. In support of previous studies (Peake et al. 2005b; Suzuki et al. 1999), a number of correlations between CK and myoglobin changes and alterations in total leukocyte count within the present study indicate that polymorphonuclear leukocytes (i.e., primarily neutrophils) are mobilized in response to exercise-induced muscle damage. The increase in circulating neutrophils following muscle damage is possibly due to activation of the alternative complement pathway and stimulated by the appearance of damaged muscle tissue fragments in the blood (Peake et al. 2005b; Suzuki et al. 1999). Inflammatory mediators such as IL-6 have also been associated with the release of leukocytes [i.e., particularly neutrophils (Suzuki et al. 2003)], which is supported by the present data. Furthermore, the observed associations between the exercise-induced increase in cortisol and the changed leukocyte numbers 1 day after the competition provides further evidence to the proposed time-lapsed role of cortisol in leukocyte trafficking (König et al. 2001; Malm 2004; Pedersen and Hoffman-Goetz 2000). In addition, the amplified response of plasma concentrations of MPO and PMN elastase in the course of the Ironman triathlon reflects a rapid neutrophil activation and degranulation. Despite a marked decrease towards prerace, both granular enzymes remained elevated after 1 day of recovery. In the case of MPO, the observed relationship between its change in plasma from pre- to 1 day post-race and the the IL-6 response immediately after the race may point to a delayed IL-6 stimulated neutrophil degranulation, as shown in a study of marathon running (Suzuki et al. 2003). Only few studies have investigated MPO levels following a marathon race (Melanson et al. 2006; Suzuki et al. 2003) and the present study appears to be the first to investigate the effects of an Ironman triathlon or exercise of a similar duration on MPO. Myeloperoxidase is also used as a novel marker for myocardial injury (Melanson et al. 2006). However, data within the current study indicate that elevations in MPO as well as in PMN elastase most likely were part of the exercise-induced systemic inflammatory response, as the post-race increase in cardiac troponin T and brain natriuretic peptide were not associated with inflammatory stress (König et al. 2007). Both enzymes possess lytic capacities and thus they assist in the destruction of damaged tissue or in the destruction of infectious agents (Bishop et al. 2003; Peake et al. 2005b; Tidball 2005).

IL-6 is one of the most potent mediators of the early exercise-induced systemic inflammatory response and, consistent with the findings of previous ultra-endurance studies (Jeukendrup et al. 2000; Mastaloudis et al. 2004; Nieman et al. 2002, 2004; Suzuki et al. 2006), plasma IL-6 concentration rose considerably (greater than 100-fold) following the race. The relationship between the leukocytosis and the rise in IL-6 1 day after the competition probably indicates that IL-6 has promoted leukocyte mobilization. A similar relationship is also reported in a previous marathon study (Suzuki et al. 2003). In addition, we found correlations between 1 day post-race changes of IL-6 and myoglobin along with CK, but these associations were weaker than directly after a 160-km run race (Nieman et al. 2005). It is plausible, that, lined up with recent research on that topic (Fischer 2006; Pedersen et al. 2007), contracting muscles account for the release of IL-6. Nevertheless, there is evidence to suggest that exercise-induced muscle damage is not solely responsible for the simultaneous initial increase of plasma IL-6 (Peake et al. 2005b) and that other signals such as low glycogen stores may play a more important role (Pedersen et al. 2007). Previous studies have reported that plasma IL-6 concentrations had returned to prerace values 1 day post-race after an ultramarathon (Mastaloudis et al. 2004) and after an Ironman triathlon (Jeukendrup et al. 2000), whereas after another Ironman race IL-6 levels were significantly elevated 1 day post-race (Suzuki et al. 2006), but IL-6 concentrations were not documented more than 1 day post-race. Importantly, we found that IL-6 levels fell 1 day after the long-distance triathlon, but were sustained significantly above prerace values until 5 days post-race. Similarly, Robson-Ansley et al. (2007) reported a low-grade, but prolonged elevation of systemic IL-6 following an acute period of intense running training. The migration of cytokine-releasing macrophages involved in muscle repair and/or in persistent glycogen depletion is suggested to be the main reasons for the long-lasting post-exercise elevation in IL-6 (Robson-Ansley et al. 2007). Even despite an adequate carbohydrate intake, exercise-induced muscle damage can impair appropriate glycogen re-synthesis (Jeukendrup et al. 2005) and it is known that carbohydrate availability in skeletal muscles modulates IL-6 production (Pedersen et al. 2007). Thus, the low-level, but long-standing IL-6 response into recovery in the present study may be associated with a delayed glycogen restoration as well as with regenerative processes modulated by recruited inflammatory cells (as another potential source of IL-6 production).

Consistent with previous studies (Nieman et al. 2002; Suzuki et al. 2003, 2006), we found increased IL-10 concentrations immediately after the competition and IL-10 values remained significantly elevated until 1 day after the race. Based on the concept that the appearance of counter-regulatory anti-inflammatory cytokines such as IL-10 after strenuous exercise attenuates the inflammatory and immune response to prevent overshooting inflammation, it is suggested that well-trained athletes are able to balance the acute exercise-induced inflammation (Fehrenbach and Schneider 2006). Results of a recent study in Ironman triathletes imply that there is a strong compensatory anti-inflammatory cytokine response (Suzuki et al. 2006). Our data support this idea because the pronounced *initial* inflammatory response was rapidly diminished in the course of the recovery from the competition. Regarding the potential stimuli of the anti-inflammatory cytokine response, the present data provide further indirect evidence that exercise intensity is a major factor in the elevation of IL-10. In a study investigating well-trained male runners, Peake et al. (2005a) reported that factors related to the intensity of exercise such as stress hormones had a stronger influence on the production of anti-inflammatory cytokines than muscle damage. In line with these results, we observed that the exercise-induced change in IL-10 was negatively related to the run split time as well as the change in cortisol immediately post-race. Furthermore, the IL-10 increase was negatively correlated with the total race time and the run split time (i.e., IL-10 concentration rose with performance).

The delayed increase of CRP following intense endurance exercise is a sign that the inflammatory response had reached a systemic level (Pedersen and Hoffman-Goetz 2000). C-reactive protein is released from the liver induced by IL-6 (Fischer 2006) and is responsible for the recognition and clearance of damaged cells (Plaisance and Grandjean 2006). C-reactive protein has also been established as a novel marker of inflammation and is suggested to provide additional information concerning atherosclerotic lesions (Plaisance and Grandjean 2006). Whereas evidence increases, that regular physical activity reduces CRP concentrations (Plaisance and Grandjean 2006), information concerning the response of CRP to an acute bout of ultra-endurance exercise is limited (Kim et al. 2007; Mastaloudis et al. 2004; Suzuki et al. 2006). In the present study, plasma CRP concentrations were significantly increased even immediately post-race, which is in contrast to another study on Ironman finishers (Suzuki et al. 2006). This rapid response in plasma CRP was followed by a marked further elevation 1 day post-race. Thereafter levels declined, but were still augmented 5 days post-race corresponding to the results of another ultra-endurance study, in which CRP levels after an ultra-marathon were determined over a longer time-course (Mastaloudis et al. 2004). Crucially, we found a number of associations between CRP and markers of muscle damage within 20 min, 1 day and 5 days after the Ironman. Therefore, our data support very recent findings (Kim et al.

2007) that muscle damage and the subsequent repair processes are important inducers in CRP. The slightly elevated CRP levels 19 days post-race (in parallel with moderately increased myoglobin concentrations) might be related to incomplete muscle recovery, but alternatively, this could also be a sign of low prerace values.

Interestingly, the three subjects, who dropped out from the race due to self-reported fatigue symptoms (but without any apparent systemic symptoms of an infection) had much higher prerace CRP concentrations than any other study participant with levels ranging from 316 to 1,442% above the mean prerace concentration. In two of them, these values exceeded the normal clinical range, and in parallel noticeable higher MPO levels (in both) and IL-6 concentration that was nearly 300% higher than the mean prerace IL-6 level (in one subject) were observed. The anomalies in these inflammatory markers in these subjects may represent a prolonged inflammatory condition following the prolonged training period and the subsequent tapering and thus might provide some additional evidence to the cytokine hypothesis of the unexplained underperformance syndrome (Robson-Ansley et al. 2007; Robson 2003; Smith 2000).

The most important finding of the current study was that although the marked initial inflammatory response induced by Ironman triathlon subsided rapidly, a low-grade systemic inflammatory response was sustained for at least 5 days of recovery. The prolonged moderate, but significant elevation of IL-6 and CRP might be associated with inflammatory processes and/or impaired glycogen replenishment within damaged muscle. Athletes may be more susceptible to infections due to this attenuated immune competence within this first period of recovery after demanding endurance exercise. Furthermore, inadequate rest following prolonged, intensive exercise might cause a chronic systemic inflammatory state that in turn leads to a syndrome of impaired performance and progressive fatigue. However, due to the continuous demands of Ironman competition on training schedules, competitive athletes might not have sufficient recovery between the races. Thus, finding an appropriate balance between training, competition and recovery is an essential challenge to maintain a high level of performance and to minimize potential health consequences. From the perspective of the observed muscle repair and inflammatory processes at least 2–3 weeks of active recovery is advisable before gradually returning to more intensive training.

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# Review I

(Including original data; basis for chapter 4)

# *Exercise-induced DNA damage: Is there a relationship with inflammatory responses?*

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## ABSTRACT

*Both a systemic inflammatory response as well as DNA damage has been observed following exhaustive endurance exercise. Hypothetically, exercise-induced DNA damage might either be a consequence of inflammatory processes or causally involved in inflammation and immunological alterations after strenuous prolonged exercise (e.g. by inducing lymphocyte apoptosis and lymphocytopenia). Nevertheless, up to now only few studies have addressed this issue and there is hardly any evidence regarding a direct relationship between DNA or chromosomal damage and inflammatory responses in the context of exercise. The most conclusive picture that emerges from available data is that reactive oxygen and nitrogen species (RONS) appear to be the key effectors which link inflammation with DNA damage. Considering the time-courses of inflammatory and oxidative stress responses on the one hand and DNA effects on the other, the lack of correlations between these responses might also be explained by too short observation periods. This review summarizes and discusses the recent findings on this topic. Furthermore, data from our own study are presented that aimed to verify potential associations between several endpoints of genome stability and inflammatory, immune-endocrine and muscle damage parameters in competitors of an Ironman triathlon until 19 days into recovery. The current results indicate that DNA effects in lymphocytes are not responsible for exercise-induced inflammatory responses. Furthermore, this investigation shows that inflammatory processes, vice versa, do not promote DNA damage, neither directly nor via an increased formation of*

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*RONS derived from inflammatory cells. Oxidative DNA damage might have been counteracted by training- and exercise-induced antioxidant responses. However, further studies are needed that combine advanced –omics based techniques (transcriptomics, proteomics) with state-of-the-art biochemical biomarkers to gain more insights into the underlying mechanisms.*

**Key words:** DNA damage, systemic inflammatory response, lymphocytopenia, muscle inflammatory responses, endurance exercise

## INTRODUCTION

Due to extensive research in the past decades, the effects of exercise on the immune system are well documented [20, 33, 53]. However, researchers in this area are still puzzled by questions about the underlying molecular mechanisms of the observed immunological alterations [32, 33]. Extremely demanding endurance exercise has been shown to induce both a systemic inflammatory response [15, 42, 53, 71] as well as DNA damage [21, 36, 58, 62, 80]. Exercise-induced DNA damage in peripheral blood cells appear to be mainly a consequence of an increased production of reactive oxygen and nitrogen species (RONS) during and after vigorous aerobic exercise [58]. Besides oxidative stress, other factors such as metabolic, hormonal and thermal stress in addition to the ultra-structural damage of muscle tissue are characteristic responses to prolonged strenuous exercise, that can lead to the release of cytokines, acute phase proteins and to the activation or inhibition of certain lines of the cellular immune system [15, 29]. In addition to these effectors, exercise-induced modifications in DNA of immuno-competent cells have been hypothesised to be related with immune and inflammatory responses to prolonged intensive physical activity, either by playing a causative role and/or by resulting from exercise-induced inflammatory processes [21, 40, 44, 53]. Nevertheless, both experimental data as well as a more mechanistic understanding regarding this relationship are still incomplete.

The aim of this review is to outline the findings and current state of knowledge on potential associations between DNA modulations and inflammatory responses after exercise. In the first part of this article, a short description of the most commonly applied techniques to evaluate genome stability is provided. This is followed by a brief summary of studies that have investigated the effects of exercise on DNA in general. The latter issue has been presented elsewhere in detail with a focal point on methodology in an article by Poulsen et al. [58]. In the second part of this review the focus is on studies that have investigated *both*, certain endpoints of DNA damage *and* immuno-endocrine and inflammatory parameters in the context of exercise. Since apoptosis (programmed cell death) has been suggested to influence the regulation of leukocyte counts after exercise [53], we also addressed studies on this topic in the present review. Furthermore, we included the few investigations that examined exercise-induced DNA modulations and markers of muscle damage, since this issue might give some indirect evidence for inflammatory processes following exercise. Finally, data from our own study is presented, which aimed to get a broader and more thorough insight into oxidative [43], myocardial [28], skeletal muscular, inflammatory and immuno-endocrine

stress responses [42] as well as genome stability [62, 63] in a large cohort of Ironman competitors. By investigating a range of divergent parameters and by quantifying the resolution of recovery up to 19 days (d) after the Ironman race, the results specifically enabled us to verify potential interactions between several endpoints of DNA and chromosomal damage on the one hand and inflammation and muscle damage on the other hand.

**Commonly Applied Techniques to Monitor DNA and Chromosomal Stability in Exercise**

A number of different approaches have been used to evaluate DNA stability in exercise studies. The aim of this part of the present article is to give a brief overview on the principles of the most frequently applied methods, since this topic has been comprehensively reviewed in the scientific literature [8, 17, 26, 58].

Many studies in this context applied the single cell gel electrophoresis (SCGE or COMET) assay due to its sensitivity and simplicity [8]. This technique is based on the determination of the migration of damaged DNA out of the nucleus in an electric field, whereas the migrated DNA resembles the shape of a comet [21, 26]. The standard version (under alkaline conditions) enables the detection of DNA single and double strand breaks, and apurinic sites [77], while the use of the lesion specific enzymes endonuclease III (ENDO III) and formamidopyrimidine glycosylase (FPG) allows the detection of oxidized purines and pyrimidines, respectively [7, 8]. Regarding the interpretation of the results that are obtained by the SCGE assay it is important to bear in mind that endpoints are differently reported as tail lengths of the comets, percentage DNA in tail and tail moment [8].

Contrary to the SCGE assay, the cytokinesis block micronucleus cytome (CBMN Cyt) assay allows to assess persistent chromosomal damage [16, 21]. Endpoints of this precise method includes the formation of micronuclei (MN) resulting from chromosomal breakage or loss, nucleoplasmic bridges (NPBs) indicating chromosome rearrangements, and nuclear buds (Nbuds) that are formed as a consequence of gene amplification [16, 18]. The reliability of this MN in pathophysiological conditions has been substantiated by a recent study which has shown an association between MN frequency and cancer incidence [3].

In several exercise studies 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG), was investigated, which is formed through oxidative modification of guanine, and mainly detected in urine or in leukocytes [26]. Measurement of urinary 8-oxodG is thought to be the result of the repair of these lesions in DNA, excretion into the plasma and subsequently into urine [58]. Hence, it does not necessarily reflect the steady-state of un-repaired DNA damage [80]. Moreover, urinary 8-oxodG represents a general oxidative damage marker for the whole body, and consequently, is not specific to DNA damage in white blood cells [60, 80]. Attention should also be given in the interpretation of this biomarker due to methodological drawbacks and discrepancies among divergent approaches which are currently used to analyse 8-oxodG [26, 58].

**Effects of Different Kinds of Exercise on DNA**

Epidemiological as well as empirical data indicate protective effects of physical activity on site-specific cancer risk [58, 64, 76]. However, similarly to the con-

cerns about ultra-endurance exercise and cardiovascular health [27], Poulsen et al. hypothesised a U-shaped curve relationship between exercise and health particularly in the context of oxidative DNA modifications [58]. Data are available now on the effects of acute bouts of very prolonged (ultra-endurance) exercise on genome stability, which will also be presented in the following overview. According to the literature [10], ultra-endurance is defined as exercise lasting more than 4 hours (h).

*Ultra-endurance Exercise (> 4 h)*

Increased DNA instability as detected by the SCGE technique [36, 63] or with the CBMN Cyt assay [62] or by analysis of urinary 8-OHdG concentrations [37, 60] were found after an Ironman triathlon [62, 63] and ultra-marathon races [36, 37, 60]. Importantly, changes regarding the SCGE assays as well as urinary 8-OHdG were only temporary [36, 37, 60, 62, 63] and endpoints of DNA damage measured with the CBMN Cyt assay even decreased in response to an Ironman race and declined further 19 d post-race [62]. These responses are discussed later in detail within the scope of our own observations.

*Competitive Endurance Exercise (< 4 h)*

Data regarding competitors of endurance races with a duration of less than four hours are partly inconclusive, albeit in most studies increased DNA migration was detected in SCGE assays after a half-marathon [44], a marathon [80] or a short-distance triathlon race [21]. On the contrary, neither changes in the levels of strand breaks nor in the FPG-sensitive sites, but increased ENDO III sites were observed after a half-marathon- and a marathon [4]. However, the subjects of the latter study were monitored only immediately post-race, while other investigations demonstrated that major DNA modulations were sustained until 5 d post-race in six short-distance triathletes [21] and for even 14 d following a marathon [80]. Nevertheless, based on the finding of an unaltered frequency in MN, Hartmann et al. [21] concluded that intense exercise with a mean duration of 2.5 h does not lead to chromosome damage.

*Submaximal and Maximal Exercise Under Laboratory Conditions*

Several studies conducted submaximal aerobic exercise protocols under laboratory conditions to investigate DNA effects. DNA damage was neither seen after intense treadmill running in male subjects of different training status [82] nor in well-trained endurance athletes [54]. In addition, Sato et al. showed that acute mild exercise as well as chronic moderate training does not result in DNA damage, but rather leads to an elevation in the sanitization system of DNA damage [66]. Interestingly, in an experiment that aimed to examine the influence of a downhill run before and after supplementation with vitamin E, no effect was found on the levels of leukocyte 8-OHdG in both 16 young and 16 older physically active men [65]. However, it has to be mentioned that DNA responses were not followed until at least 1 d post-exercise in most of these studies [54, 65, 82].

Conflicting findings were reported when maximal exercise protocols, i.e. tests until exhaustion, were conducted under laboratory conditions. Increased levels of DNA strand breaks were observed after exhaustive treadmill running in subjects of different training status [22, 45]. Moller et al. [38] demonstrated DNA strand breaks

and oxidative DNA damage after an maximal cycle ergometer test under high-altitude hypoxia, but not normal (normoxic) conditions. In another study, elevated levels of MN were reported after exhaustive sprints; however, the six subjects were of divergent training levels and gender and included one smoker [67]. On the contrary, Pittaluga et al. [56] detected no effects of a maximal exercise test on MN in 18 young subjects with different training status, but the authors noted chronic cellular stress including higher MN levels at rest in the athlete group. Furthermore, there were no differences in urinary 8-OHdG concentrations before and after supplementation with β-carotene within the 3 d following a cycle ergometer test to exhaustion [70].

*Periods of intensified training*

A few studies have examined whether periods of intensified training affect genome stability. Increased urinary 8-OHdG levels were observed in 23 healthy males in response to a vigorous physical training programme (about 10 h of exercise for 30 d) [57] and in male long-distance runners throughout a training period for 8 d compared to a sedentary period [47]. However, in a longitudinal study no differences in urinary excretion of 8-OHdG between a group of long-distance runners and a sedentary control group were observed [55]. In two separate studies that comprised a similar group of male triathletes, Palazzetti et al., reported either no [48] or increased DNA damage [49] after 4 weeks (wk) of overload training as detected by the SCGE assay, probably due to inter-individual differences.

In conclusion, there is growing evidence that strenuous exercise can lead to DNA damage that with few exceptions [36] is predominantly observed not before 24 h after the resolution of exercise [21, 44, 45, 80]. However, the diversity of methods and endpoints used to assess DNA modifications and different study designs (i.e. divergent exercise protocols and sampling time-points) make it difficult to determine the exact circumstances under which DNA damage occurs. Crucially, in addition to the aforementioned factors, the heterogeneity of study cohorts (varying in gender, age and training status) most likely contributes to inconsistencies among the studies on this topic. Nevertheless, results of the few studies that have examined the effects of ultra-endurance exercise on genome stability indicate that adaptations of endogenous protective antioxidant and/or repair mechanisms prevent severe and persistent DNA damage in well-trained athletes [36, 37, 45, 60, 62]. Thus, a clear dose-response relationship regarding the level of exercise that could be detrimental cannot yet be established. Currently, there are no indications that exhaustive endurance exercise increases the risk for cancer and other diseases via DNA damage. However, it remains to be clarified whether perturbances of the genomic stability of immuno-competent cells are involved in the post-exercise temporary dysfunction of certain aspects of immunity, which may increase the risk of subclinical and clinical infection [15, 20, 53].

**Findings on Exercise-induced DNA Damage and/or Apoptosis and Inflammatory Responses**

Table 1 summarizes the small number of studies that have examined the effects of exercise on DNA and/or apoptosis on the one side *and* inflammatory responses on the other. As one of the earlier works in the context of the effects of particularly competitive endurance exercise on DNA damage, Niess et al. [44] found that neu-

trophil counts 1 h after a half-marathon run correlated with DNA damage in leukocytes, assessed 24 h post-race. Without examining markers of oxidative stress, the authors could only speculate that RONS released by neutrophils might have been responsible for the formation of DNA strand breaks. However, their results led them to suppose that the observed DNA damage might be the key mechanism for the modifications in the immune cell counts [44]. On the contrary,

**Table 1.** Studies investigating exercise-induced DNA damage and/or apoptosis and inflammatory/immune parameters

| Reference | Experimental protocol and sampling time-points | Subjects | Endpoints of DNA damage and/or apoptosis | Inflammatory and immune parameters (plasma) | Correlations between DNA damage/apoptosis and inflammation | Main other results |
|---|---|---|---|---|---|---|
| ***Ultra-endurance exercise (> 4 h)*** | | | | | | |
| Mastaloudis et al. 2004 [35, 36] | Ultra-marathon (50km) Pre-, mid-, 2 h post-, daily for 6 d post-race (supplementation with vitamins C and E versus placebo) | 11 ♀and 11 ♂ runners | SCGE (LEU) ↑ mid-race, returned to baseline by 2 h post-race, ↓ by 6 d post-race | IL-6, TNF-α ↑ mid-race until 2 h post-race, CRP ↑ 1 – 2 d post-race | No significant correlation reported | $F_2$-IsoPs (plasma) ↑ immediately post-race only in placebo group |
| Reichhold et al. 2008 [62, 63], Neubauer et al. 2008 [42, 43] | Ironman triathlon (3.8km swimming +180km cycling +42km running) 2 d pre-, immediately, 1 d, 5 d and 19 d post-race | 20 and 28 well-trained ♂ triathletes randomized for the CBMN Cyt and the SCGE assays, respectively | SCGE (LYM) ↑ immediately and 1 d post-race, back to baseline 5 d post-race, ↓ 19 d post-race, FPG ↔, ENDO III ↑ 5 d post-race compared to 1 d post-race, back to baseline 19 d post-race, MN ↓ immediately post-race, ↓ further 19 d post-race, Nbuds ↑ 5 d post-race, back to baseline 19 d post-race, NPB ↓ 19 d post-race, Apoptotic and necrotic cells: ↓ post-race | Immediately post-race: Total LEU counts ↑, MPO ↑, PMN-elastase ↑, CK ↑, myoglobin ↑ ,IL-6 ↑ IL-10 ↑, hs-CRP ↑ (all alterations persisted 1 d post-race) 5 d post-race: CK ↑, myoglobin ↑, IL-6 ↑, hs-CRP ↑ 19 d post-race: MPO ↓, PMN ↓, hs-CRP ↑, myoglobin ↑ | Links between IL-6 and necrotic cells immediately post-race, no further significant correlations | Immediately post-race: Cortisol ↑, testosterone ↓, CD ↑, AOPP ↑, oxLDL ↓, oxLDL:LDL ↔, Plasma antioxidant capacity (TEAC, FRAP, ORAC) ↑, 1 d post-race: MDA ↑, CD ↑, AOPP ↑, plasma antioxidant capacity, Negative correlation between ORAC and ENDO III |
| ***Competitive endurance exercise (< 4 h)*** | | | | | | |
| Niess et al. 1998 [44] | Half marathon Pre-, 1, 24 h post-race | 12 moderately trained ♂ | SCGE (LEU) ↑ 24 h post-race | CK ↑ 1 h and 24 h post-race, PMN count ↑ 1 h post-race, LYM count ↓ 1 h post-race | Significant correlation between PMN count 1 h post-race and DNA migration 24h post-race | |
| Mooren et al. 2004 [40] | Marathon Pre-, immediately, 3 h, 24 h post- race | 17 high- to low-trained ♂ marathon runners | % of apoptotic cells (LYM) ↑ 3h post-race, ↓ 24 h post-race | LEU counts ↑ 3 h and 24 h post-race | No significant correlation reported | |
| Briviba et al. 2005 [4] | Marathon and half-marathon 10 d pre-, immediately post-race | 10 half-marathon runners, 12 marathon runners (♂ and ♀) | After both races: SCGE (LYM) ↔ FPG ↔, ENDO III ↑, ↓ resistance of DNA to oxidative damage % of apoptotic cells ↓ | After half-marathon: ↑ NK cell number and cytotoxicity, GRA oxidative burst ↑ After marathon: GRA and MON oxidative burst ↑, IFN-γ ↑ | No significant correlation reported | After both races: Plasma antioxidant capacity ↔ |
| ***Submaximal and maximal exercise under laboratory conditions*** | | | | | | |
| Steensberg et al. 2002 [69] | 2.5 h treadmill running at 75 % $VO_{2\,max}$. Pre-, 0.5 and 2 h post-exercise | 11 healthy ♂ subjects | 2 h post-exercise: % of apoptotic LYM ↑, total number of apoptotic cells ↔ | LYM counts ↑ 0.5 h post-exercise, ↓ 1 – 4 h post-exercise | No significant correlation reported | $F_2$-IsoPs (plasma): ↑ immediately post-exercise, Cortisol: ↑ during exercise |
| Peters et al. 2006 [54] | 2.5 h treadmill running at 75% $VO_{2\,max}$. Pre-, immediately, 3 h post-exercise | 8 well-trained ♂ endurance athletes | % of apoptotic LYM ↔, SCGE (LYM) ↔ | Immediately post-exercise: ↑ total LEU counts, ↑ LYM counts | No significant correlation reported | Cortisol (plasma) ↑ immediately post-exercise |

| Niess et al. 1996 [45] | Treadmill test until exhaustion Pre-, 15 min, 24 h post-exercise | 6 trained and 5 untrained ♂ | SCGE (LEU) 24 h post ↑ | CK ↔ post-exercise, Total LEU counts ↑ 15 min post-exercise | No significant correlation reported | MDA (plasma) ↔ post-exercise |
|---|---|---|---|---|---|---|
| Mooren et al. 2002 [39] | 1.) Exhaustive treadmill test at 80% $VO_{2\,max}$ 2.) Treadmill test at 60% $VO_{2\,max}$ with identical running time Before, immediately, 1 h post-exercise | 12 healthy volunteers (7 ♂ and 5 ♀) | After exhaustive test: % of apoptotic LYM ↑ immediately post-exercise After test at 60% $VO_{2\,max}$: % of apoptotic LYM ↔, | After exhaustive test: ↑ LYM counts, ↑ LEU counts After test at 60% $VO_{2\,max}$: ↑ LYM counts, ↑ LEU counts | No significant correlation reported | |

♂ = male subjects, ♀ = female subjects, ↑ = significant increase, ↓ = significant decrease, ↔ = no changes, LEU = leukocytes, LYM = lymphocytes, SCGE = single cell gel electrophoresis assay, FPG = formamidopyrimidine sensitive-sites, ENDO III = endonuclease-sensitive sites, CBMN Cyt = cytokinesis block micronucleus assay, NPBs = neoplasmic bridges, Nbuds = nuclear buds, MN = micronuclei, IL = interleukin, TNF-α = tumor necrosis factor α, (hs)-CRP = (high sensitive) C-reactive protein, MPO = myeloperoxidase, PMN-elastase = polymorphonuclear elastase, PMN = polymorphonuclear cells, NK = natural killer cells, GRA = granulocytes, CK = creatine kinase activity, $F_2$-IsoPs = $F_2$-Isoprostanes, CD = conjugated dienes, AOPP = advanced oxidation protein products, oxLDL = oxidized LDL, MDA = malondialdehyde, TEAC = trolox equivalent antioxidant capacity, FRAP = ferric reducing ability of plasma, ORAC = oxygen radical absorbance capacity

they found no correlation between changes in DNA migration in the SCGE assay and leukocyte counts in the 24 h after an exhaustive treadmill test [44], possibly also because the extent of the inflammatory response was relatively low following their exercise protocol. Although no immune and inflammatory parameters were measured in the study by Hartmann et al. [21], their explanations have further stimulated debate on a relationship between the activation of inflammatory cells and the occurrence of secondary tissue and DNA lesions. Based on their observations in short-distance triathletes (no indications for oxidative DNA modifications immediately post-race, but highest values within the standard SCGE assay 3 d after the competition), they suggested that DNA damage might occur as a consequence of exercise-induced injury of muscle tissue rather than acute oxidative stress during exercise [21]. The authors hypothesised that inflammatory reactions in the course of this initial muscle damage could be responsible for the transient DNA damage [21]. Indeed, there is evidence that activated neutrophils and macrophages infiltrate damaged muscle [68, 78]. Although this seems to be a beneficial response in terms of muscle repair and also muscle adaptation [33, 78], it may trigger further inflammatory processes and damage [25], in part through an enhanced formation of RONS [29].

On the basis of these findings, researchers in this field questioned whether damage to cellular DNA in the course of vigorous exercise could also induce apoptosis and whether programmed cell death, in turn, might be related to the exercise-induced regulation of leukocyte counts and, particularly, lymphocyte trafficking and distribution [53]. A decline of the total lymphocyte concentration is characteristic after exercise of prolonged duration and/or high intensity [33, 53]. Although the mechanisms of exercise-induced lymphocytopenia are still not fully understood [33], it has been suggested that this effect may account, at least partly, for the post-exercise immune dysfunction [15]. Exercise-induced changes in corticosteroids and catecholamines are known to play a major role in characteristic post-exercise alterations of leukocyte subsets [20, 41] including leukocytosis [42] as well as lymphocytopenia [53]. Previous studies indicated that the glucocorticoid concentrations observed after submaximal exercise are sufficient to induce apoptosis [23]. These observations further support the assumption of a relationship between exercise-associated induction of apoptosis and lymphocy-

topenia [53]. In response to cellular stressors that lead to DNA damage, apoptosis is vital in preventing the propagation of severely damaged DNA and in maintaining genomic stability [30] and is regarded to be required for the regulation of the immune response [39].

Mars et al. were the first to describe apoptosis in lymphocytes after exhaustive exercise (treadmill running) that was paralleled by DNA damage [34]. However, in the latter study, cell death was only investigated in three subjects and the methodology (the TdT-mediated dUTP-nick end labelling or TUNEL method) has been criticized due to its insufficient specificity [40]. Nevertheless, by the use of flow cytometry and annexin-V to label apoptotic cells, Mooren et al. [39, 40] confirmed that either short maximal exercise (in untrained subjects) [39] as well as competitive endurance exercise (a marathon run) [40] has the potential to induce lymphocyte apoptosis. This phenomenon could be explained, to a certain extent, by an up-regulation of the expression of cell death receptors and ligands [40] and an exercise-induced shift to a lymphocyte population with a higher density of these (CD95-)receptors [39]. Nevertheless, the authors concluded that the changes in the proportions of apoptotic cells after exhaustive exercise were small and, if at all, might only partially account for the concomitantly observed significant decline of lymphocytes to below baseline levels [39]. An additional finding of Mooren et al. [40] was that apoptotic sensitivity was inversely related to the training status of the marathon runners, since analysis of subgroups revealed that programmed cell death occurred only in less well-trained, but not in highly-trained athletes. Recent research in this context suggests that intensive endurance exercise does neither automatically induce apoptosis in lymphocytes nor cause DNA damage (assessed immediately and 3 h post-exercise), provided that subjects are well-trained [54]. Since there was no correlation between the (non-significant) decrease in circulating lymphocytes and the percentage lymphocyte apoptosis after a 2.5 h treadmill run at 75% $VO_{2\ max.}$, Peters et al. [54] concluded that the characteristic post-exercise lymphocytopenia is not due to apoptotic regulation by the immune system. The latter results are consistent with another study which was conducted with a similar exercise protocol, but in untrained subjects [69]. Steensberg et al. [69] noted that the lymphocytes which left the circulation during the first 2 h post-exercise were characterised by not being apoptotic. Thus, mechanisms other than apoptosis seem to play a more important role in inducing lymphocytopenia after exercise, including a redistribution of lymphocytes and/or a lack of mature cells that can be recruited [53]. Moreover, contrary to previous findings [23], recent results imply that cortisol affects the cellular immune system more by other pathways than via apoptotic regulation [54]. Furthermore, the occurrence of DNA damage in the course of exercise does not necessarily implicate induction of apoptosis [40]. Alternative cellular outcomes to prevent the propagation of DNA damage include cell cycle arrest or DNA repair [30].

In general, there is strong evidence which suggests that enhanced DNA stability and, most likely in turn, the absence of a change in the levels of apoptotic lymphocytes after strenuous exercise [54] are associated with protective adaptations due to training. As mentioned above, Mastaloudis et al. [36], reported that DNA damage in leukocytes increased temporarily mid-race of an ultra-marathon, but returned to baseline 2 h after the competition and even decreased to below

baseline values by 6 d post-race. As probable causes for this decrease in the proportion of cells with DNA damage, the authors suggested enhanced repair mechanisms, increased clearance and/or a redistribution of damaged cells [36]. Noteworthy, plasma concentrations of inflammatory parameters, $F_2$-isoprostanes and antioxidant vitamins were investigated in the same subjects. Although acute oxidative and inflammatory stress responses were observed [35], the authors reported no correlations between either of these markers with DNA damage [35, 36]. Furthermore, supplementation with vitamins E and C prevented increases in lipid peroxidation [35], but had no noticeable effects on DNA damage, on inflammation and on muscle damage [36]. Interestingly, there were different responses regarding oxidative stress and DNA damage in male and female runners, highlighting the importance of studying both sexes [35, 36]. In general, these findings in ultramarathon runners indicate that the mechanism of oxidative damage is operating independently of the inflammatory and muscle damage processes [35, 36, 79].

There are only few studies on the issue of DNA damage and immune and inflammatory responses in the course of exercise. Briviba et al. [4] found oxidative DNA damage parallel to an increased oxidative burst ability of granulocytes and monocytes after both a half-marathon- and a marathon race, but no correlations were detected. Again, the authors could only speculate that the exercise-induced activation of phagocytes might have contributed to the increased RONS production, oxidative DNA damage and the high percentage of apoptotic lymphocytes [4]. Furthermore, it is notable that the monitoring period of this study probably was too short to detect possible interactions between DNA alterations and immune modifications.

**Findings on Exercise-induced DNA Damage and Muscle Damage**

As mentioned, given the scarceness of data regarding associations between DNA modulations and inflammation in the course of exercise, we included investigations that examined exercise-induced effects on DNA together with markers of muscle damage. These studies are summarized in Table 2. Though several major stressors are needed and the integrity of the organism has to be challenged (e.g. by extremely demanding endurance exercise) [29, 42, 53, 72] to induce a *systemic* inflammatory response, it has been shown that leukocytes can explicitly be mobilised in response to muscle damage [42, 51, 74], possibly due to activation of the alternative complement pathway [51, 74]. Therefore, these studies may also reveal whether muscle damage (induced by mechanical and/or metabolic stress [25, 75]) and subsequent repair and inflammatory responses [78] are associated with DNA damage. In one of the first studies on this issue, which comprised three subjects of different gender and training history, Hartmann et al. reported a parallel increase, but no correlation between the DNA migration in the SCGE assay and plasma creatine kinase (CK) between 6 and 24 h after intense treadmill running [22]. Likewise, applying the standard SCGE assay, Palazzetti et al. [48] observed signs of increased oxidative stress and muscle damage induced by a duathlon race after 4 wk of overload training, whereas no effects on leukocyte DNA were found, probably due to efficient DNA repair. Other studies on this topic predominantly measured 8-OHdG in urine, which reflects the average rate of oxidative DNA damages in all cells of the body [58]. Consequently, changes in urinary 8-OHdG excretion after muscle-damaging exercise might largely repre-

**Table 2.** Studies investigating exercise-induced DNA damage and muscle damage

| Reference | Experimental protocol and sampling time-points | Subjects | Endpoints of DNA damage | Markers of muscle damage (plasma) | Correlations between DNA damage and muscle damage | Main other results |
|---|---|---|---|---|---|---|
| ***Ultra-endurance exercise (> 4 h)*** | | | | | | |
| Radak et al. 2000 [60] | 4 d-supra-marathon (93+120+56+59 km)<br>Pre-race, after each race d | 5 well trained ♂ runners | 8-OHdG (urine, ELISA) ↑ after d 1, thereafter trend to decrease, back to baseline after d 4 | CK ↑ after d 1, further increasing until d 3, and decreasing thereafter | No significant correlation reported | |
| Miyata et al. 2008 [37] | 2 d-ultra-marathon race (42+90km)<br>Pre-, 1 d, 2 d post-race | 79 ♂ and 16 ♀non-professional runners | 8-OHdG (urine, HPLC-ECD) ↑ 1 d post-race, back to baseline after 2 d post-race | CK ↑, myoglobin ↑ 1 and 2 d post-race | No significant correlation reported | |
| ***Competitive endurance exercise (< 4 h)*** | | | | | | |
| Tsai et al. 2001 [80] | Marathon<br>Pre-, immediately, 1, 3, 7, 14 d post-race | 14 ♂ runners, 20 sedentary, healthy ♂ | SCGE (LYM) ↑ 1 – 14 d post-race,<br>FPG ↑ immediately 1 d post-race,<br>ENDO III ↑ 1 – 7 d post-race,<br>8-OHdG (urine, ELISA) ↑ 1 – 14 d post-race | CK ↑ immediately – 7 d post-race | Significant correlations between ENDO III, 8-OHdG and CK | LPO (plasma): 1 – 14 d post-race, Significant correlations between LPO and all markers of DNA damage |
| ***Periods of intensified training*** | | | | | | |
| Okamura et al. 1997 [47] | 30±6km/d for 8 d<br>Urine samples throughout the training period, 3 d control (sedentary) period, blood samples before and after the training period | 10 ♂ long-distance runners | 8-OHdG (urine, HPLC-ECD) ↑ throughout the training period compared to control period | After the training period: CK ↑, myoglobin ↑ | No significant correlation reported | |
| Palazzetti et al. 2003 [48] | 4 wk of overloaded training | 9 ♂ triathletes, 6 ♂ sedentary subjects | SCGE (LEU) ↔ after training period | After training period: myoglobin ↔, CK ↑ | No significant correlation reported | After training period:<br>TBARS ↔,<br>GSH/GSSG ↔,<br>Total antioxidant status ↓<br>(all plasma) |
| ***Submaximal and maximal exercise under laboratory conditions*** | | | | | | |
| Umegaki et al. 1998 [82] | 30 min treadmill running at 85% $VO_{2\,max}$.<br>Pre-, immediately and 30 min post-exercise | 8 untrained and 8 endurance-trained ♂ subjects | MN (LYM) ↔ immediately and 30 min post-exercise | CK ↔ immediately and 30 min after the test | No significant correlation reported | |
| Sacheck et al. 2003 [65] | 45 min downhill running at 75% $VO_{2\,max}$.<br>Pre-,immediately, 6 h, 1 d and 3 d post-exercise (before and after supplementation with vitamin E) | 16 young and 16 older physically active healthy ♂ | 8-OHdG (LEU, HPLC-ECD, measured pre- and 1 d post-exercise) ↔ 1 d post-exercise | CK ↑ immediately post-exercise and peak: 1 d post-exercise | No significant correlation reported | MDA (plasma) ↑ immediately post-exercise,<br>$F_2$-IsoPs (plasma): ↑ 3 d post-exercise,<br>ORAC: ↓ 3 d post-exercise |
| Hartmann et al. 1994 [22] | 1.) Treadmill test until exhaustion,<br>2.) Intense treadmill running (45 min)<br>Pre-, 6 min, 6 h, 1 – 4 d post-exercise | 2 ♂, 1 ♀ healthy subjects (untrained – trained) | SCGE (LEU)<br>After exhaustive test: ↑ 6 h, maximum 1 d post-exercise, after 3 d back to initial levels,<br>↔ after 45 min of treadmill running<br>SCE ↔ after both tests | CK ↑ between 6 h and 1 d after exhaustive test<br>↔ after 45 min of treadmill running | No significant correlation reported | |

♂ = male subjects, ♀ = female subjects, ↑ = significant increase, ↓ = significant decrease ↔ = no changes, LEU = leukocytes, LYM = lymphocytes, 8-OHdG = 8-oxo-7,8-dihydro-2-deoxyguanosine, ELISA = enzyme linked immuno assay, HPLC-ECD = high performance liquid chromatography with electrochemical detection, SCGE = single cell gel electrophoresis assay, FPG = formamidopyrimidine sensitive-sites, ENDO III = endonuclease-sensitive sites, SCE = sister chromatid exchange assay, MN = micronucleus assay, polymorphonuclear elastase, PMN = polymorphonuclear cells, NK = natural killer cells, GRA = granulocytes, CK = creatine kinase activity, LPO = lipid peroxidation products, TBARS = thiobarbituric acid reactive substances, GSH/GSSG = reduced vs. oxidized glutathione, $F_2$-IsoPs = $F_2$-Isoprostanes, MDA = malondialdehyde, ORAC = oxygen radical absorbance capacity

sent DNA damage of skeletal muscles [60]. Radak et al. [60] and Miyata et al. [37] determined urinary 8-OHdG levels and markers of muscle damage in competitors of ultra-marathon events which lasted 2 [60] and 5 d [37], respectively. No propagation of oxidative DNA damage was observed after the first race d in both studies [37, 60]. Interestingly, 8-OHdG significantly decreased to levels below their peak values during the race on the second d [37], and on the fourth race d [60], respectively. Both research groups suggested that a rapid induction of antioxidant and repair systems occurred [37, 59]. In contrast, parameters for muscle damage continuously increased during the 2-d-race period [37] and until the third d of the 4-d-race [60], and no correlations were reported with 8-OHdG. Taken together, these data may show that, even if myofibrillar injury occurs, an adaptive up-regulation of repair and nucleotide sanitization mechanisms is capable of preventing further damage of DNA. Consistently, no correlations between biomarkers of DNA- and muscle damage were reported after a period of intensified training (despite that both 8-OHdG and muscle damage markers were found to be increased) [47] or downhill running on a treadmill [65]. However, given that 8-OHdG levels remained unchanged, but were measured only until 1 d post-race, the authors of the latter investigation noted that oxidative DNA damage probably had occurred in the period between the first and the third d after exercise, when some links amongst circulating oxidative stress markers and CK activity were observed [65].

The prolonged monitoring period after a marathon race in an investigation by Tsai and co-workers [80] might account for the observed significant correlations between peak levels of ENDO III-sensitive sites and urinary 8-OHdG on the one side and plasma parameters of muscle damage and lipid peroxidation on the other. In agreement with the conclusions of previous investigations [21, 44], the authors suggested that inflammatory cells infiltrating into injured skeletal muscle tissue and activated phagocytes were responsible for the increased production of RONS and consequently the delayed oxidative DNA damage during the reparative processes after the marathon [80]. This idea is supported by a study in rats, in which DNA damage in circulating white blood cells was closely related to muscle damage due to exercise [81]. Nevertheless, based on these findings it is not possible to draw a clear conclusion as to whether oxidative DNA modifications in peripheral immuno-competent cells are casually related with immune disturbances or whether DNA damage in leukocytes, in fact, results from oxidative stress that occurs through inflammatory processes after strenuous exercise.

**Purpose of the Current Study in Ironman Triathletes**

The data presented here are part of a larger study that aimed to comprehensively examine certain stress and recovery responses to an Ironman triathlon race. One primary aim of the study was to test the hypothesis whether there is a relationship between indices of muscle damage and/or inflammatory stress and endpoints of DNA damage in lymphocytes, which were assessed by the SCGE- and the CBMN Cyt assays for the first time in the course of competitive exercise of such duration. Furthermore, by concomitantly exploring oxidative stress markers and antioxidant-related factors, we aimed to particularize a potential interaction of oxidative stress between inflammatory and DNA responses.

## MATERIALS AND METHODS

The study design has been described previously [28, 42]. Briefly, the study population comprised 48 non-professional, well-trained healthy male triathletes, who participated in the 2006 Ironman Austria. Forty-two of them (age: 35.5 ± 7.0 yr, height: 180.6 ± 5.6 cm, body mass: 75.1 ± 6.4 kg, cycling $VO_{2\,peak}$: 56.6 ± 6.2 ml $kg^{-1}$ $min^{-1}$, weekly net endurance exercise time: 10.7 ± 2.6 h) completed the study and were included in the statistical analysis to investigate inflammatory and immuno-endocrine responses as well as muscle damage [42]. The physiological characteristics of the study participants (assessed on a cycle ergometer three weeks before the competition), information on their training over a period of six months prior to the race, their performance in the Ironman triathlon as well as the only moderate ("recovery") training thereafter have been presented in detail elsewhere [42, 43]. Of the entire study group 20 and 28 subjects were randomly selected for the CBMN Cyt and the SCGE assays, respectively [62, 63]. Consequently, these randomized subjects were included in the data analysis for the results that are exclusively provided within this report. All participants of the study did not take any medication or more than 100% of RDA of antioxidant supplements (in addition to their normal dietary antioxidant intake) in the six weeks before the Ironman race until the end of the study. The Ironman triathlon took place in Klagenfurt, Austria on July $16^{th}$ 2006 under near optimal climatic conditions and consisted of 3.8 km swimming, 180 km cycling and 42.2 km running. Blood samples were taken 2 d pre-race, immediately (within 20 min), 1, 5 and 19 d post-race.

The samples were immediately cooled to 4°C and plasma separated at 1711 * g for 20 min at 4°C and aliquots for the measurement of biochemical parameters were frozen at –80°C until analysis. For the analysis of DNA and chromosomal damage in lymphocytes, blood samples were processed instantly as described previously [62, 63]. Blood samples were analysed for haematological profile, plasma creatine kinase (CK) activity, plasma concentrations of myoglobin, interleukin (IL)-6, IL-10, high-sensitivity C-reactive protein (hs-CRP), myeloperoxidase (MPO), polymorphonuclear (PMN) elastase, cortisol and testosterone (see [42]). All these values (except for the steroid hormones) were adjusted for exercise-induced changes in plasma volume [11]. As reported previously [62, 63], the SCGE and CBMN Cyt- assays were carried out according the methods described by Tice et al. [77] and Fenech [17], respectively. Within the SCGE-assay, oxidative DNA base damage was assessed on the basis of the protocols of Collins et al. [7], Collins and Dusinska [6] and Angelis et al. [1]. Analysed endpoints within the SCGE assay included: 1.) determination of DNA migration under standard conditions to measured single and double strand breaks (determined as percentage of DNA in the tail), and 2.) ENDO III and FPG to detect oxidized pyrimidines and purines, respectively. Biomarkers within the CBMN Cyt block included the number of 1.) MN, 2.) NPBs, 3.) Nbuds, and 4.) necrotic and apoptotic cells.

All statistical analyses were performed using SPSS 15.0 for Windows. Details of the data analysis has been presented previously [42, 62, 63]. For the additional correlation analysis that is reported in this article, Pearson ´s correlation was used to examine significant relationships. In case of observed trends or significant correlations, subjects were divided into percentile groups by the asso-

ciated variables (e.g. IL-6). One-factorial ANOVA *and post* hoc analyses with Scheffé´s test were then applied to assess whether differences in endpoints of DNA or chromosomal damage were associated with the percentile distribution. Significance was set at a P-value <0.05 and is reported P<0.05, P<0.01 and P<0.001.

# RESULTS

**Race Results**

The average completion time of the whole study group was 10 h 52 min ± 1 h 1 min (mean ± SD). The estimated average antioxidant intake during the race was 393 ± 219 mg vitamin C and 113 ± 59 mg alpha-tocopherol. There were neither significant differences in the performance nor in the consumed antioxidants between the whole study group and the subgroups that were tested for genome stability.

**DNA and Chromosomal Damage, Apoptosis and Necrosis**

As previously reported [62, 63] and briefly discussed above, the results concerning DNA and chromosomal damage were as follows: Within the CBMN Cyt assay, the number of MN significantly (P<0.05) decreased immediately post-race, and declined further to below pre-race levels 19 d after the Ironman competition (P<0.01). There were no changes in the frequency of NPBs and Nbuds as an immediate response to the triathlon, but 5 d thereafter the frequency of Nbuds was significantly (P<0.01) higher than levels immediately post-race. However, 19 d post-race the frequency of Nbuds returned to pre-race levels, while the number of NPBs was significantly (P<0.05) lower than pre-race [62].

The overall number of apoptotic cells decreased significantly (P<0.01) immediately post-race, and declined further until 19 d after the race (P<0.01). Similarly, the overall number of necrotic cells significantly (P<0.01) declined immediately post-race, and remained at a low level 19 d after the Ironman. Within the SCGE assay, a decrease was observed in the level of strand breaks immediately after the race. One day post-race the levels of strand breaks increased (P<0.01), then returned to pre-race 5 d post-race, and decreased further to below the initial levels 19 d post-race (P<0.01). Immediately post-race there was a trend in ENDO III and FPG-sensitive sites to decrease. The ENDO III-sensitive sites significantly (P<0.05) increased 5 d post-race compared to 1 d post-race, but levels decreased until 19 d (P<0.05). No significant changes were observed in the levels of FPG-sensitive sites throughout the monitoring period [63].

**Immune-endocrine and Inflammatory Responses, and Plasma Markers of Muscle Damage**

Briefly, as described in details elsewhere [42], there were significant (P<0.001) increases in total leukocyte counts, MPO, PMN elastase, cortisol, CK activity, myoglobin, IL-6, IL-10 and hs-CRP, whereas testosterone significantly (P<0.001) decreased compared to pre-race. Except for cortisol, which decreased below pre-race values (P<0.001), these alterations persisted 1 d post-race (P<0.001, P<0.01 for IL-10). Five days post-race CK activity, myoglobin, IL-6 and hs-CRP had

decreased, but were still significantly (P<0.001) elevated. Nineteen days post-race most parameters had returned to pre-race values, with the exception of MPO and PMN elastase, which had both significantly (P<0.001) decreased below pre-race concentrations, and myoglobin and hs-CRP, which were slightly, but significantly higher than pre-race [42].

**Associations between Endpoints of Genome Stability and Immuno-endocrine, Inflammatory and Muscle Damage Parameters**

No significant correlations were found between all these markers at all time-points with the exception of a link between IL-6 and necrosis. Immediately post-race, the plasma concentration of IL-6 correlated positively with the number of necrotic cells (r=0.528; P<0.05). In addition, significant associations were observed on the basis of a group distribution into percentiles by the IL-6 concentrations immediately post-race. First, the numbers of necrotic cells increased with IL-6 across the percentiles, and the differences between all groups were P=0.012. Second, necrosis in lymphocytes was significantly (P=0.017) higher in the subject group with the highest IL-6 concentrations (top percentile) compared with the lowest IL-6 values (lowest percentile).

## DISCUSSION

A major finding of the present investigation is that there were no correlations between different markers of DNA and chromosomal damage and parameters of muscle damage and inflammation in participants of an Ironman triathlon as a prototype of ultra-endurance exercise with the exception of a link between IL-6 and necrosis. The conclusions that can be drawn from these results are several. Overall, the current data indicate that DNA damage is neither causally involved in the initial systemic inflammatory response nor in the low-grade inflammation that was sustained at least until 5 d after the Ironman race [42]. Instead, based on several assessed relationships between leukocyte dynamics, cortisol, muscle damage markers and cytokines [42], the pronounced but temporary systemic inflammatory response was most likely induced by stressors other than DNA modulations. In fact, consistent with previous studies in this context, factors such as the initial ultra-structural injury of skeletal muscle [51, 74], changes in concentrations of cortisol [53] and IL-6 [71] apparently mediated leukocyte mobilization and activation [42]. Furthermore, although the temporary increased frequency of ENDO III-sensitive sites 5 d after the Ironman competition was found simultaneously with the moderate prolongation of inflammatory processes, correlations between hs-CRP and markers of muscle damage suggest that the latter phenomenon was rather related to incomplete muscle repair [42].

In addition, missing links between all these markers in the present study indicate that exercise-induced inflammatory responses *vice versa* do not promote DNA damage in lymphocytes. These results support those of Mastaloudis et al., who demonstrated that inflammatory and muscle damage responses, indeed, do not *directly* interact with the mechanisms of oxidative DNA damage [35, 36, 79]. Nevertheless, this does not rule out the possibility that inflammatory processes can trigger oxidative stress via oxidative burst reactions of circulating neutrophils

and an increased cytokine formation [15, 25, 29, 50, 73], which in turn might lead to secondary (oxidative) DNA damage in immuno-competent cells [80]. In fact, we observed correlations between markers of oxidative stress and inflammatory parameters (unpublished results) that might point to muscular inflammatory processes as a source of the moderate oxidative stress response 1 d after the Ironman triathlon. Nevertheless, we have recently demonstrated in the same study participants that training- and acute exercise-induced responses in the antioxidant defence system were able to counteract severe or persistent oxidative damage post-race. Despite a temporary increase in protein oxidation and lipid peroxidation markers immediately and 1 d post-race (except for oxidized LDL concentrations, which actually decreased), all these markers had returned to pre-race values 5 d post-race [43]. Concomitantly, there was an increase in the plasma antioxidant capacity following the Ironman triathlon (assessed by the trolox equivalent antioxidant capacity- (TEAC), the ferric reducing ability of plasma- (FRAP), and the oxygen radical absorbance capacity (ORAC)-assays) [43, 63]. These strong antioxidant responses most likely played a significant role in counteracting sustained oxidative stress post-race in the current study, while it seems that antioxidant defences in the study group of Tsai et al. [80] were not sufficient to confer protection against delayed oxidative damage to lipids and DNA due to reparative processes of muscular tissue. Whatever the reasons for these discrepancies in the oxidant/antioxidant balance are (differences in training-induced biochemical adaptations, antioxidant status and/or antioxidant intake during the race, etc.), this might be a major explanation for the inconsistencies between the findings of Tsai et al. [80] and ours [43, 64, 62]. In fact, the observed negative correlations between the ORAC and ENDO III-sensitive sites immediately and 1 d after the Ironman race suggest that an enhanced plasma antioxidant capacity might have prevented oxidative DNA damage [63]. These findings are in line with a recent animal study [2], which demonstrated the protective role of an enhanced serum antioxidant capacity in lymphocyte apoptosis. Taken together, whenever correlations between DNA damage in immuno-competent cells and inflammation [44] or muscle damage [80] were observed, RONS derived from inflammatory cells, appear to be the key effectors that link inflammation with DNA damage after vigorous exercise. Fig. 1 is a schematic illustration of the relationships between these stress responses to exhaustive endurance exercise. It may be argued that results from our study fit well into this picture insofar that antioxidant mechanisms neutralized an enhanced generation of RONS potentially resulting from inflammatory processes due to the injury of skeletal muscle tissue, and consequently were able to prevent lymphocyte DNA damage. It should also be noted that, similar to DNA effects, muscle inflammatory processes and related oxidative stress responses might be sustained for or appear days after muscle-damaging exercise [46]. Hence, potential links between these outcome measures might have been missed in investigations with shorter monitoring periods [4, 40, 54, 65, 69]. Beyond, it is important to note in this context that there is an additional difficulty in determining correlations between markers of oxidative DNA damage and other biomarkers of oxidative stress, partly due to differences in the biological sites where oxidative damage occurred [12].

The observed association between IL-6 concentration and the number of necrotic cells immediately post-race in the present study may indicate that lym-

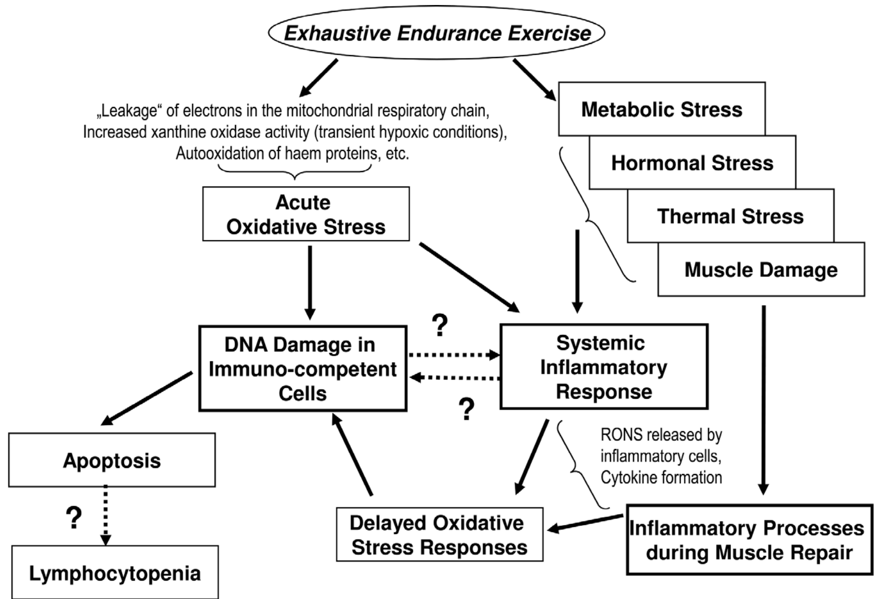


Fig. 1: Proposed model of exercise-induced DNA damage and inflammatory responses

phocytes partly undergo an unregulated cell death in athletes experiencing an overshooting inflammatory response. Based on recent research on the role of IL-6 in exercise [15, 19, 52], it is questionable whether IL-6, probably released by contracting muscles [19, 52], directly modulates necrosis in lymphocytes. In this case, plasma IL-6 concentrations may just serve as a marker for the pronounced initial systemic inflammatory response. However, the (patho-)physiological relevance of this association cannot be generalised based upon the present results, since the overall number of necrotic cells declined significantly to below pre-race values after the acute bout of ultra-endurance exercise, and remained at these levels at all time-points investigated [63]. Similarly, as to the decrease of necrosis, we demonstrated that levels of apoptosis also decreased immediately after the Ironman race, again remaining at these low levels throughout the whole monitoring period [63]. Crucially, our data revealed no link between apoptosis and post-race changes in lymphocyte counts. Mooren et al. [40] reported an initial increase in apoptotic cells in the whole group of marathon runners, but corresponding with the findings in the current study, lymphocyte apoptosis declined 1 d after the race. In agreement with the decrease of DNA damage after an ultra-marathon run [36], these findings might alternatively be explained by an overshooting removal of apoptotic leukocytes by phagocytic cells in order to protect tissue from overexposure to inflammatory and immunogenic contents of dying cells [31, 40]. Based on the concept that the phagocytic clearance of apoptotic immuno-competent cells plays a critical role in the resolution of inflammation [31, 83], this could be a further explanation for the lack of a link between inflammatory responses on the one hand, and DNA damage and/or apoptotic cell death on the other hand.

Finally, a reason that may also account for the lack of correlations within most of the few studies that have addressed this issue is that the majority of these

investigations have been conducted in trained individuals [21, 36, 37, 47, 48, 54, 60, 62]. Accumulating evidence points to adaptations in protective mechanisms due to (endurance) training - including improved endogenous antioxidant defences and enhanced repair mechanisms [59] - that appear to be responsible for maintaining genome integrity in immuno-competent cells in response to extremely demanding endurance exercise. While these protective mechanisms were suggested to prevent DNA damage and/or apoptosis in a number of studies [37, 40, 45, 48, 54, 60, 62], several other exercise-associated factors induce and mediate a systemic inflammatory response [15, 53]. This indirectly further implies that DNA damage in immuno-competent cells, if it occurs at all, might *not* be a major determinant of exercise-induced inflammation.

## CONCLUSION

Thus far, there is only little evidence concerning a direct relationship between DNA damage and inflammatory responses after strenuous prolonged exercise. The most conclusive picture that emerges from the available data is that oxidative stress seems to be the main link between exercise-induced inflammation and DNA damage. Considering the very few studies in which markers of DNA damage were found to correlate with signs of inflammation or muscle damage, DNA damage in peripheral immuno-competent cells, indeed, most likely resulted from an increased generation of RONS due to initial systemic inflammatory responses or the delayed inflammatory processes in response to muscle damage (Fig. 1). The lack of correlations between these exercise-induced responses in most of the studies might also be explained by the fact that the monitoring period was too short. Hence, particular attention should be paid to the characteristic time-course of inflammatory and oxidative stress events on the one hand and DNA effects on the other hand. Though obvious differences exist in the manifestation and outcomes a comparable relationship is reported in patho-physiological conditions including carcinogenesis, where (chronic) inflammation induces DNA damage and mutations via oxidative stress [13]. However, there might be further mechanisms that link exercise-induced DNA modulations, inflammatory responses and RONS. It has been shown, that redox-sensitive signal transduction pathways including nuclear factor (NF) κB or p53 cascades are involved in inflammation as well as “cell stress management” in response to DNA damage [24, 30]. Recent explorations of the gene expression responses to exercise have already shed a light on hitherto unknown molecular mechanisms in exercise immunology [5, 9, 14, 61, 84, 85]. In the future, the combination of these powerful modern techniques *(transcriptomics, proteomics)* with state-of-the-art biochemical biomarkers should therefore enable researchers in this field to provide novel insights into potential further interactions between genome stability and inflammation.

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# Original article III

(Relevant to the thesis, but not forming part of it)

# No Acute and Persistent DNA Damage after an Ironman Triathlon

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## Abstract

**During acute and strenuous exercise, the enhanced formation of reactive oxygen species can induce damage to lipids, proteins, and nucleic acids. The aim of this study was to investigate the effect of an Ironman triathlon (3.8 km swim, 180 km cycle, 42 km run), as a prototype of ultra-endurance exercise, on DNA stability. As biomarkers of genomic instability, the number of micronuclei, nucleoplasmic bridges, and nuclear buds was measured within the cytokinesis-block micronucleus cytome assay in once-divided peripheral lymphocytes of 20 male triathletes. Blood samples were taken 2 days before, within 20 min after the race, and 5 and 19 days post-race. Overall, the number of micronuclei decreased ($P < 0.05$) after the race, remained at a low level until 5 days post-race, and declined further to 19 days post-race ($P < 0.01$). The frequency of nucleoplasmic bridges and nuclear buds did not change immediately after the triathlon. The number of nucleoplasmic bridge declined from 2 days pre-race to 19 days post-exercise ($P < 0.05$). The frequency of nuclear buds increased after the triathlon, peaking 5 days post-race ($P < 0.01$) and decreased to basic levels 19 days after the race ($P < 0.01$). The results suggest that an Ironman triathlon does not cause long-lasting DNA damage in well-trained athletes.** (Cancer Epidemiol Biomarkers Prev 2008;17(8):1–7)

## Introduction

Regular moderate physical activity is associated with various health benefits such as decreased risk of cardiovascular diseases, diabetes, cancer, and other lifestyle-dependent diseases (1-5). In a recent review, Rundle (6) showed several possibilities how exercise positively influences different phases of carcinogenesis including enhanced detoxification of reactive oxygen species, increased DNA repair activity, and improved immune functions. However, it is known that acute and strenuous exercise also induces oxidative stress through the enhanced formation of reactive oxygen species (7, 8), which in turn may result in the damage of lipids, proteins, and nucleic acids (9-12). Oxidative stress–induced DNA damage as well as insufficient DNA repair may play an important role in the etiology of cancer, diabetes, and arteriosclerosis (10). Potential pathways for exercise-induced oxidative stress include increased oxygen consumption, autooxidation of catecholamines, activation of inflammatory cells due to tissue damage and ischemia, or hypoxia (13, 14).

Thus far, only a small number of studies have been conducted to investigate the influence of physical activity on DNA damage and the findings are inconsistent due to the use of different protocols and different endpoints. The majority of the studies were based on single-cell gel electrophoresis (SCGE) assays and on the determination of urinary excretion of 8-hydroxy-2′-deoxyguanosine (15-21). Investigations concerning the effect of physical activity on the micronuclei frequency, which are formed as a consequence of chromosome breakage and chromosome loss (22), are still limited and the data are controversial. Although no alterations of micronuclei were found after treadmill running (23) and a short-distance triathlon (16), elevated levels of micronuclei were observed after two exhaustive sprints (24). It is important to point out that the duration of exercise in the latter studies are not comparable to an Ironman triathlon race, where the athletes are extraordinary in their level of training and in the endurance and intensity of exercise done. Because the number of nonprofessional athletes training for and competing in ultra-endurance events continually increases, it is of particular importance to investigate this group.

The cytokinesis-block micronucleus cytome (CBMN Cyt) assay is a test that enables the detection of genomic instability, including chromosome breakage, chromosome loss, chromosome rearrangements, and gene amplification and nondisjunction (25). Furthermore, this endpoint has been reported to detect DNA damage caused by dietary, environmental, and lifestyle factors (26), and a causal link between micronuclei and the risk of cancer has been described in a recent cohort study (27).

The major aims of the present study were to examine for the first time (*a*) the effect of an Ironman triathlon race, as a prototype of ultra-endurance exercise, on DNA damage in lymphocytes, (*b*) to find out whether an association exists between DNA damage and training level, and (*c*) to study the influence of ultra-endurance exercise on the formation of nucleoplasmic bridges as

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well as nuclear buds. To verify the complete recovery period, the variable were monitored over a longer time (19 days).

## Materials and Methods

**Study Group.** Of the entire study group ($n$ = 48), 24 subjects were randomized for the CBMN Cyt assay. Statistical analysis was done for 20 subjects. The experimental design is summarized in Fig. 1. The study was reviewed and approved by the local ethics committee of the Medical University of Vienna.

All participants were healthy nonsmokers and were asked to document their training 6 months pre-race and thereafter until 19 days post-race including the weekly training (km), the total weekly exercise training time (h), and the weekly net exercise training time (h). At each blood collection, a 24-h recall was completed to record nutritional information. All participants were physically fit, free of acute or chronic diseases, within normal range of body mass index, and not taking any medication. They were also asked to abstain from the consumption of supplements in excess of 100% of the Recommended Dietary Allowance threshold level per day, in addition to their normal dietary intake of antioxidants, vitamins, and minerals including vitamin C, vitamin E, β-carotene, selenium, and zinc in tablet or capsule form 6 weeks before the triathlon until the last blood sampling 19 days after. Only on race day and 1 day post-race, the athletes were allowed to eat and drink *ad libitum*; however, data regarding their intake were documented. Only subjects who finished the race were kept within the study group.

Before each blood sampling (except the sampling immediately after the race) and also 2 days before the spiroergometry, the subjects were told to refrain from intense exercise. After the race, the training of the subjects had a regenerative character and was only of moderate intensity until the end of the study.

To assess the physiologic characteristics, the subjects were tested on a cycle ergometer (Sensormedics, Ergometrics 900) 3 weeks before the triathlon. The maximal test protocol started at an initial intensity of 50 W followed by 50 W increments every 3 min until exhaustion. Oxygen and carbon dioxide fractions (both via Sensormedics 2900 Metabolic measurement cart), power output, heart rate, and ventilation were recorded continuously and earlobe blood samples for the measurement of the lactate concentrations were taken at the beginning and end of each step.

$VO_2$ peak values were used to divide the total group of participants into two subgroups regarding their training levels. There is good evidence that endurance training leads to adaptations of the endogenous antioxidant defense system (2), and some studies have also shown that the enhancement in these protective mechanisms can be correlated with the maximum or peak oxygen consumption (28). A $VO_2$ peak value of 60 mL/kg/min was considered as the cutoff point. Subjects with a $VO_2$ peak <60 mL/kg/min formed the trained (T) group ($n$ = 10) and participants with $VO_2$ peak >60 mL/kg/min formed the very trained (VT) group ($n$ = 10).

**Race Conditions.** The Ironman triathlon was held in Klagenfurt, Austria on July 16, 2006. The event comprises a 3.8 km swim, a 180 km cycle, and a 42 km run. The race started at 7:00 a.m., when the air temperature was 15°C, lake temperature was 25°C, and relative humidity was 77%. By finishing time (median time for participants ~5:43 p.m.), air temperature and relative humidity were 27.2°C and 36%, respectively (data provided by the Carinthian Center of the Austrian Central Institute for Meteorology and Geodynamics).

**Reagents.** Dulbecco's PBS, RPMI 1640, cytochalasin B, trypan blue, DMSO, sodium pyruvate, L-glutamine, FCS, penicillin, streptomycin, and Histopaque-1077 were obtained from Sigma-Aldrich. Phytohemagglutinin (M form) was purchased from Invitrogen. DiffQuik was procured from Dade Behring. Other reagents were obtained from Merck.

**Blood Sampling.** Blood samples were collected by venipuncture in heparinized and EDTA tubes (Vacuette) 2 days before, within 20 min after the race, and 5 and 19 days post-race. The blood samples were processed immediately, as described below, or stored below 6°C for no longer than 7 h before processing.

**CBMN Cyt Assay.** The CBMN Cyt assay was carried out according to the method of Fenech (29). Briefly, lymphocytes were isolated using Histopaque-1077 as a density gradient and resuspended in RPMI 1640, which was supplemented with 11% heat-inactivated FCS, 2.0 mmol/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and sodium pyruvate. Phytohemagglutinin (30 μg/mL) was added to stimulate cell

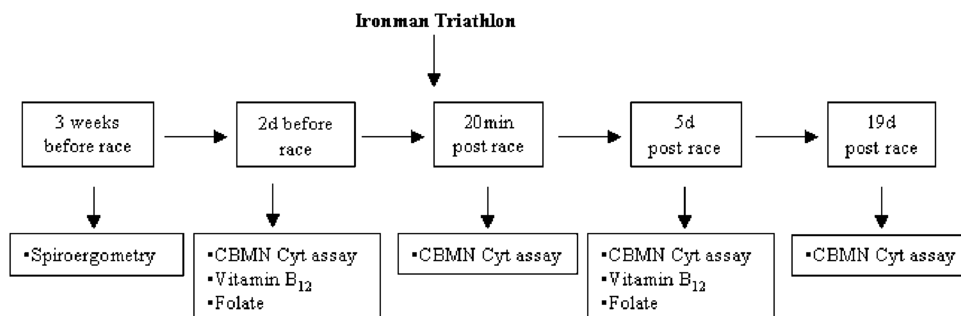


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**Figure 1.** Experimental design showing when the CBMN Cyt assay was done and spiroergometry and the determination of vitamin $B_{12}$ and folate were done.

**Table 1. Baseline characteristics of subjects (mean ± SD)**

| | Total group ($n$ = 20) | T ($n$ = 10) | VT ($n$ = 10) |
|---|---|---|---|
| Age (y) | 31.7 ± 6.1 | 33.1 ± 7.6 | 30.3 ± 3.9 |
| Weight (kg) | 76.7 ± 8.1 | 75.8 ± 10.3 | 77.6 ± 5.5 |
| Height (55) | 182.8 ± 6.2 | 181.2 ± 7.3 | 184.4 ± 4.8 |
| Body mass index (kg/$m^2$)* | 22.9 ± 1.5 | 23.0 ± 1.8 | 22.8 ± 1.3 |
| $VO_2$ peak (mL/kg/min) | 60.8 ± 8.8 | 54.3 ± 3.5 | 67.4 ± 7.4† |
| Race time (h) | 10.4 ± 0.5 | 10.7 ± 0.4 | 10.1 ± 0.5 |
| Weekly net exercise training time (h) | 11.9 ± 2.5 | 11.9 ± 2.6 | 11.9 ± 2.4 |
| Total weekly exercise training time (h) | 12.9 ± 2.0 | 13.0 ± 2.2 | 12.7 ± 1.9 |
| Cycle training/wk (km) | 180.4 ± 44.7 | 193.6 ± 49.5 | 167.2 ± 8.0 |
| Run training/wk (km) | 39.8 ± 9.8 | 41.3 ± 9.6 | 38.4 ± 10.6 |
| Swim training/wk (km) | 5.5 ± 2.2 | 4.1 ± 2.3 | 6.9 ± 1.1 |
| Folate (μg/L) | 8.3 ± 4.3 | 8.1 ± 3.2 | 8.5 ± 5.3 |
| Vitamin $B_{12}$ (ng/L) | 347.2 ± 147.8 | 364.2 ± 149.7 | 326.0 ± 152.8 |

*Weight in kilograms divided by squared height in meters.
†$P$ < 0.01 (T versus VT).

division. Cultures were incubated for 44 h at 37°C in a humidified atmosphere containing 5% $CO_2$. After 44-h incubation, cytochalasin B (4.5 μg/mL) was added to block cytokinesis and the cells were reincubated for 28 h. Then, 200 μL of the medium were removed, the lymphocytes gently were resuspended; and immediately before centrifugation, DMSO was added. The suspensions were centrifuged on slides for 5 min at 480 × $g$ (Shandon Cytospin 3). The slides were air dried for 10 min and fixed for another 10 min before using a modified Giemsa stain (DiffQuik). The examination of the slides was conducted at ×1,000 magnification by a light microscope (Axioskop 20, Zeiss). For each sample duplicate, cultures were analyzed. According to the scoring criteria for the CBMN Cyt assay of Fenech et al. (30), a total of 2,000 binucleated cells on two different slides were analyzed from each subject. The statistical data were calculated per 1,000 binucleated cells. Because micronuclei formation demands nuclear division, micronuclei are scored in binucleated cells. Assessed endpoints included the number of binucleated cells with micronuclei, the number of micronuclei in binucleated cells, nucleoplasmic bridges, and nuclear buds, and the nuclear division index. Micronuclei result from chromosome fragments or whole chromosomes, which lag behind at anaphase during cell division, whereas nucleoplasmic bridges and nuclear buds originate from dicentric chromosomes resulting from misrepaired DNA breaks or telomere end fusions and gene amplification, respectively (22, 31-33). The nuclear division index was calculated according to Eastmond and Tucker (34).

**Measurement of Vitamin $B_{12}$ and Folate.** The determinations of vitamin $B_{12}$ and folate in blood plasma samples 2 days before and 5 days after race were carried out using a commercial radioimmunoassay (MP Biomedicals Europe). To consider the potential rebound overexpansion of plasma volume, which persists for 3 to 5 days following the cessation of demanding exercise (35), exercise-induced changes in plasma volume were calculated (36) for the plasma vitamin $B_{12}$ and folate concentrations 5 days post-race.

**Statistical Analysis.** The statistical analyses were done using SPSS 15.0 for Windows (SPSS).

All data are presented as mean ± SD.

The one-sample Kolmogorov-Smirnov test was used to test all data for their normal distribution.

The paired $t$ test (for normally distributed data) was implemented to assess statistically significant differences between the four time points of blood sampling for each group. The unpaired $t$ test was used to analyze the differences between the T and the VT subjects. As the number of micronuclei in binucleated cells, as well as the number of nucleoplasmic bridges 20 min post-race, was not normally distributed, the data were tested using the nonparametric Wilcoxon matched-pairs test and the Mann-Whitney $U$ test, respectively. $P$ < 0.05 was regarded as statistically significant.

## Results

As three participants did not finish the race and one triathlete could not participate in the entire study, the CBMN Cyt assay was done with peripheral lymphocytes from 20 subjects at four different blood sampling points. This high number of subjects has not been investigated in previous studies. Therefore, the collective was further divided into two subgroups (T and VT; $n$ = 10 each) to investigate whether changes are based on different training levels (cutoff point: $VO_2$ peak value of 60 mL/kg/min). The baseline characteristics of the total group ($n$ = 20) as well as the subgroups T and VT subjects are summarized in Table 1.

The overall plasma vitamin $B_{12}$ and folate levels 2 days before the race (347.2 ± 147.8 ng/L and 8.3 ± 4.3 μg/L, respectively) were similar to those 5 days post-race (409.9 ± 237.0 ng/L and 7.5 ± 4.8 μg/L, respectively).

The overall number of binucleated cells with micronuclei decreased significantly ($P$ < 0.05) after the race, remained at a low level until 5 days post-exercise, and declined further until 19 days post-race ($P$ < 0.01; Fig. 2A). Only in the VT subgroup, the number of binucleated cells with micronuclei decreased significantly ($P$ < 0.05) from 2 days before the triathlon to 20 min post-race (Fig. 2A). However, in both subgroups, the number of binucleated cells containing micronuclei showed a highly significant decrease from 2 days prerace to 19 days post-race (T: $P$ < 0.05; VT: $P$ < 0.01) as well as 5 to 19 days post-race (T: $P$ < 0.05; VT: $P$ < 0.01).

In addition, a highly significant ($P < 0.01$) decrease from 2 days pre-race to 5 days post-race was seen in the VT group (Fig. 2A), whereas no significant change in the number of binucleated cells with micronuclei was observed from 20 min to 5 days post-race.

Similar results were obtained with regard to the number of micronuclei in binucleated cells (Fig. 2B). This marker also decreased significantly ($P < 0.05$) after the race and declined again between days 5 and 19 after the race ($P < 0.01$) in the total group. The VT subjects showed a significant reduction in the number of micronuclei in binucleated cells immediately after the race ($P < 0.05$), which prolonged until 5 days post-exercise and then further declined ($P < 0.01$). The lowest value was reached 19 days after the triathlon (Fig. 2B). In the T subgroup, no significant change was found 20 min post-race. However, this marker declined significantly ($P < 0.05$) 5 days post-race compared with pre-race values. The decrease in the number of micronuclei in binucleated cells from 5 to 19 days post-race was also seen in the T group ($P < 0.05$; Fig. 2B).

Immediately after the triathlon, the frequency of nucleoplasmic bridges did not change significantly (Fig. 2C). Overall, the marker declined significantly from 2 days pre-race to 19 days post-exercise ($P < 0.05$), but in the two subgroups the frequency of nucleoplasmic bridges did not change significantly (Fig. 2C).

The number of nuclear buds did not change immediately after the triathlon, neither in the total collective nor in the subgroups (Fig. 2D), but it increased after the triathlon, reached a maximum 5 days post-race (T: nonsignificant; total group and VT: $P < 0.01$; comparing 20 min post-race with 5 days post-race), and then decreased significantly 19 days after the race to basic levels (T: $P < 0.05$; total group and VT: $P < 0.01$).

Data of the nuclear division index are shown in Table 2. The nuclear division index increased significantly ($P < 0.01$) after the race and remained at a high level until 19 days post-race in all subjects. In addition, only the VT subjects showed a significant decrease from 20 min to 5 and 19 days post-race ($P < 0.05$).

## Discussion

Physical activity is reported to play an important role in the reduction of the susceptibility to cancerous diseases and their primary prevention (3, 37, 38). On the other

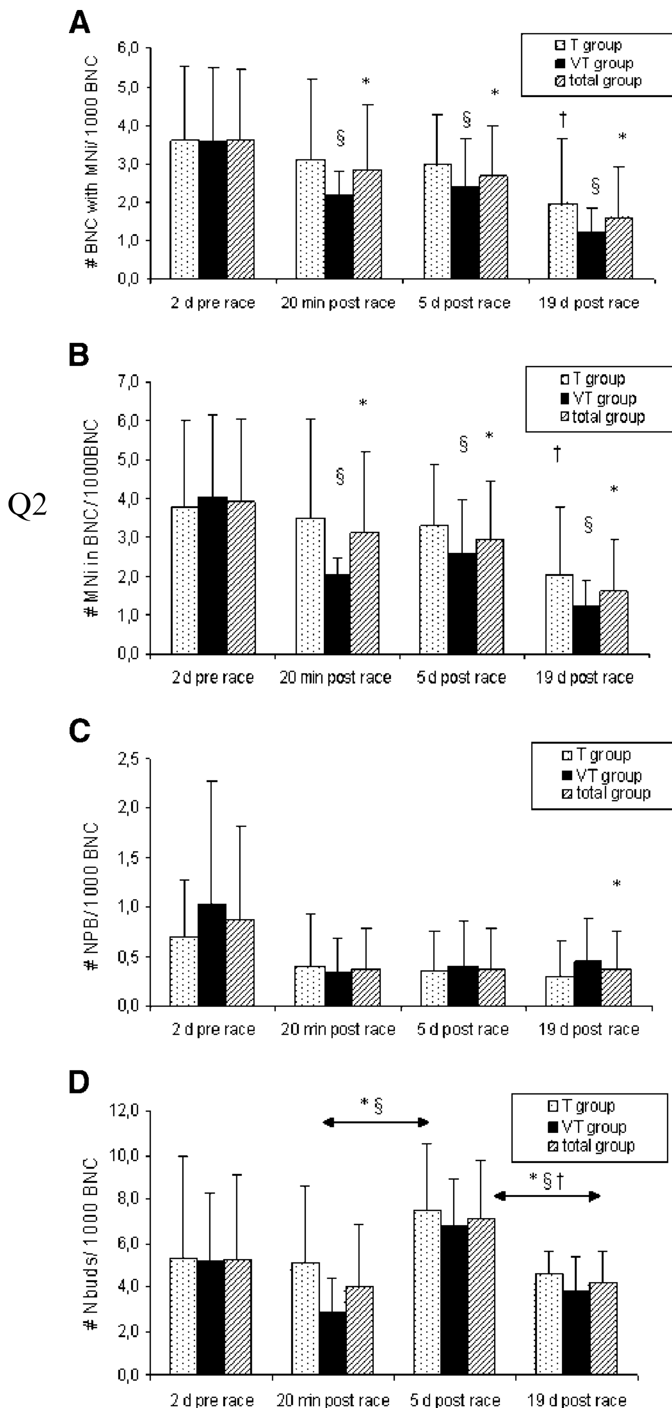


**Figure 2.** Effect of an Ironman triathlon on different endpoints monitored with the CBMN Cyt assay in peripheral lymphocytes of athletes 2 d before the race, 20 min, 5 d, and 19 d post-race. The total group (▨) was divided into the T (▨) and the VT (■) subgroups. Mean ± SD. **A,** number of binucleated cells with micronuclei per 1,000 binucleated cells (# *BN* with *MNi/1,000 BN*): *total group,* 2 d pre-race compared with 20 min, 5 d (*, $P < 0.05$), and 19 d (*, $P < 0.01$) post-race; *T group,* 2 d pre-race compared with 19 d post-race (†, $P < 0.05$); *VT group,* 2 d pre-race compared with 20 min (§, $P < 0.05$), 5 d, and 19 d post-race (§, $P < 0.01$). **B,** number of micronuclei in binucleated cell per 1,000 binucleated cells (# *MNi in BN/1,000 BN*): *total group,* 2 d pre-race compared with 20 min, 5 d (*, $P < 0.05$), and 19 d (*, $P < 0.01$) post-race; *T group,* 2 d pre-race compared with 19 d post-race (†, $P < 0.05$); *VT group,* 2 d pre-race compared with 20 min, 5 d, and 19 d post-race (§, $P < 0.05$). **C,** number of nucleoplasmic bridges per 1,000 binucleated cells *(# NPB/1,000 BN)*: *total group,* 2 d pre-race compared with 19 d post-race (*, $P < 0.05$). **D,** number of nuclear buds per 1,000 binucleated cells *(# Nbuds/1,000 BN)* 20 min post-race compared with 5 d post-race (T: nonsignificant; total group and VT: *§, $P < 0.01$) and 5 d post-race compared with 19 d post-race (T: †, $P < 0.05$; total group and VT: *§, $P < 0.01$).

**Table 2. Nuclear division index of subjects (mean ± SD)**

| | Total group ($n$ = 20) | T ($n$ = 10) | VT ($n$ = 10) |
|---|---|---|---|
| 2 d pre-race | 1.56 ± 0.09 | 1.53 ± 0.08 | 1.60 ± 0.10 |
| 20 min post-race | 1.92 ± 0.16 | 1.87 ± 0.18 | 1.97 ± 0.14 |
| 5 d post-race | 1.82 ± 0.15 | 1.85 ± 0.16 | 1.80 ± 0.16 |
| 19 d post-race | 1.79 ± 0.18 | 1.79 ± 0.22 | 1.80 ± 0.15 |

hand, oxidative DNA damage induced by the formation of reactive oxygen species, which is also linked to acute and strenuous exercise, has been suggested to be involved in aging as well as various diseases such as cancer (12, 39). The present study was conducted to assess for the first time the influence of an Ironman triathlon race, as a model of ultra-endurance exercise, on the DNA stability of athletes with different training levels. The participants of our study were nonprofessionals, but all at a high training level. The CBMN Cyt assay was applied to examine the effect of intensive endurance exercise on the formation of micronuclei, nuclear buds, and nucleoplasmic bridges, because the association between micronuclei frequency and cancer incidence was shown recently (27). The CBMN Cyt assay was proposed by the authors to be a sound biomarker for identifying genetic, nutritional, and environmental factors, which may be carcinogenic (27).

Thus far, only a few studies concerning the frequency of micronuclei after exhaustive exercise have been conducted and the data are conflicting. Although Schiffl et al. (24) found significantly elevated levels of micronuclei in six subjects after two sprints until exhaustion, no alterations were observed after treadmill running at 85% of maximal oxygen uptake for 30 min (23) or a short-distance triathlon of 2.5 h duration (16). The present study was the first investigating micronuclei after an ultra-endurance exercise with a duration between 9 and 14 h. Interestingly, a significant change in the number of binucleated cells with micronuclei was observed. The significant decrease and the low level of binucleated cells with micronuclei, even after 19 days, show that ultra-endurance exercise does not induce chromosome breaks and/or chromosome loss, immediately after or within 3 weeks post-exercise. DNA stability is impaired by deficiencies of vitamin $B_{12}$ and folate, which in turn can lead to the formation of micronuclei (40, 41). However, a deficiency of these micronutrients in the present study collective can be excluded.

In a review, Moller et al. (42) emphasize that besides a small number of studies that examined the influence of strenuous exercise on the formation of micronuclei, the majority of the investigations focused on the effect of both moderate and excessive exercise on oxidative DNA damage as detected by the SCGE assay as well as 8-hydroxy-2′-deoxyguanosine. In contrast to our investigation, where the number of micronuclei decreased after the Ironman triathlon race and remained at a low level until 19 days post-race, elevated levels of DNA migration were found in some studies with different exercise protocols in which the duration was <3 h. Hartmann et al. (15) observed increased DNA migration in SCGE assays 6 h after treadmill running at maximal oxygen consumption, which peaked 24 h after the exercise. Although the experiment was conducted only with three subjects, the authors concluded that physical activity higher than the aerobic-anaerobic threshold leads to altered levels of DNA migration. Previous studies with different models of massive aerobic exercise, such as a marathon (20) or a short-distance triathlon (16), detected increased levels of DNA migration 24 h post-exercise in the SCGE assay. In the latter study, DNA migration reached a maximum 72 h post-exercise. However, urinary 8-hydroxy-2′-deoxyguanosine remained unchanged. Therefore, the authors concluded that the DNA migration after the short-distance triathlon does not lead to oxidized DNA bases and does not result in DNA damage. On the contrary, increased urinary 8-hydroxy-2′-deoxyguanosine levels were detected 1 day after the start of a supra-marathon (4-day race), which declined on the fourth day of running. The authors suggested that repeated extreme exercise leads to an adaptation and normalization of oxidative DNA damage (21). Due to the significantly elevated level of DNA damage 1 day following a half marathon, Niess et al. (19) proposed that intense endurance exercise induced DNA damage is caused by reactive oxygen species. Similar results were observed after a 42-km marathon run (20). The latter investigation detected elevated DNA single-strand breaks in the standard SCGE assay 24 h after the race, which persisted through 7 days. Furthermore, oxidative effects on nucleotides were perceived using lesion-specific endonuclease immediately after the marathon and they lasted for >1 week. The same course was observed for urinary 8-hydroxy-2′-deoxyguanosine. Immediately after a half-marathon (running time ≤2.6 h) and a marathon (running time ≤4.8 h), Briviba et al. (43) found no increased levels of endogenous DNA strand breaks and formamidopyrimidine glycolase-sensitive sites, but oxidative DNA damage, assessed as endonuclease III sites, was significantly increased and the *ex vivo* resistance to DNA damage induced by hydrogen peroxide was decreased after the half-marathon and marathon.

Several authors detected DNA damages at times ranging from immediately until 24 h after strenuous exercise; however, Mastaloudis et al. (17) observed a significantly increased proportion of damaged cells (10%) at midrace in subjects attending an ultra-marathon with an average duration of 7.1 h, but 2 h after the event the values declined to baseline. Six days after the ultra-marathon, the proportion of damaged cells was even lower than before the race. Based on this observation, the authors proposed that the change is not persistent. This assumption is in agreement with our results, where no prolonged DNA damage was detected after a mean of 10.4 h of exercise. However, it is important to point out that the different test systems detect different types of DNA alterations. Whereas the CBMN Cyt assay detects mutations that persist at least for one mitotic cycle, repairable DNA lesions or alkali-labile sites are detected by the SCGE assay (44). Thus, it can be hypothesized that the endurance and intensity of exercise done during an Ironman triathlon race do not lead to fixed mutations probably due to the up-regulation of repair mechanisms and enhanced endogenous antioxidative systems.

Regarding the influence of different training levels on DNA damage, Umegaki et al. (23) found that intensive exercise caused no increased chromosomal damage in trained and untrained subjects after 30 min treadmill running at 85% of maximal oxygen uptake. However, in the untrained group, X-ray-induced chromosomal damage was significantly altered. Thus, the authors concluded that the difference in chromosomal damage between trained and untrained subjects was due to an enhanced DNA repair system and an increased capacity of endogenous antioxidative systems. In addition, Niess et al. (18) investigated in another study the effect of a treadmill test until exhaustion on DNA migration as detected by the SCGE on six trained and five untrained subjects. They found higher DNA migration levels in the untrained study group compared with trained subjects 24 h post-exercise. Comparisons between subjects of different training levels showed that athletes had higher levels of spontaneous chromosomal damage in lymphocytes at rest than the untrained subjects, yet the basal value appeared to be unchanged after a cycle-ergometer exhaustive test (45). The authors hypothesized that this phenomenon may be due to chronic stress in the athlete group caused by their habitual intensive training. These findings are supported by the results of the present study, as chromosomal damage tended to be higher (nonsignificant) in the T subjects than in the VT group. The differences between T and VT subjects could be due to adaptive responses of regular training, such as a more efficient electron chain in muscle mitochondria (46, 47) and up-regulation of repairing systems such as the 8-oxoguanine repair enzyme (48). Furthermore, an extended capability of endogenous antioxidative systems (in the VT subjects) might lead to the reduction of oxidative stress–induced effects and thus improved oxidative balance during exercise (2, 11, 18, 49).

According to our knowledge, the present study is the first dealing with the influence of strenuous exercise on the formation of nucleoplasmic bridges as well as nuclear buds. A recent investigation conducted by Gisselsson et al. (50), with primary cultures of solid tumors, showed that nucleoplasmic bridge and micronuclei as well as nuclear blebs are found in different cancer cells. The authors postulated that these abnormal nuclear morphologies are characteristic for genomic instability. In the current investigation, we found no significant change in the frequency of nucleoplasmic bridges immediately after the race, which was mainly due to the high individual variation. However, the significant decline of this marker 19 days after the triathlon may suggest that strenuous exercise either does not lead to the formation of dicentric chromosomes and telomere end-fusions or enhances DNA repair mechanisms to prevent DNA misrepair and thus the formation of nucleoplasmic bridges.

Based on the number of nuclear buds, a similar trend for both groups was observed, but again it was more distinct in the VT group. Lindberg et al. (51) suggested when using 9-day cultures of human lymphocytes that nuclear buds and micronuclei have partly different mechanistic origins. However, *in vitro* experiments with mammalian cells (33, 52) showed that during S phase of the cell cycle amplified DNA is removed via nuclear budding to generate micronuclei. Thus, it could be hypothesized that nuclear buds formed 5 days after the exercise bout may be eliminated by forming micronuclei, which in turn may be extruded from the cytoplasm (53) before the last time point of blood sampling (19 days post-race). However, the exact duration of the nuclear budding process and the extrusion of the resulting micronuclei from the cell have not been clarified thus far (54).

In conclusion, the present investigation shows that an Ironman triathlon race, as a model of massive physical exercise, does not cause DNA damage in endpoints detected by the CBMN Cyt assay. It is likely that regular training leads to adaptive mechanisms including the up-regulation of repair mechanisms as well as an increase in the activity of the endogenous antioxidative system, which may prevent severe oxidative stress and DNA damage even after strenuous exercise. To clarify the influence of strenuous exercise on the formation of nuclear buds and also nucleoplasmic bridges further detailed studies will be needed.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Acknowledgments

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# Original article IV

(Relevant to the thesis, but not forming part of it)

# DNA damage in response to an Ironman triathlon

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*Submitted to* ***Free Radical Research***

**Abstract**

The major aims of this study were to investigate the effect of an Ironman triathlon on DNA migration in the single cell gel electrophoresis assay, apoptosis and necrosis in the cytokinesis-block micronucleus cytome assay with lymphocytes and on changes of total antioxidant capacity in plasma. Blood samples were taken 2 days (d) before, within 20 min, 1 d, 5 d and 19 d post race. The level of strand breaks decreased ($p < 0.05$) immediately after the race, then increased ($p < 0.01$) 1 d post race and declined ($p < 0.01$) till 19 d post race. Apoptotic and necrotic cells decreased ($p < 0.01$) and the total antioxidant status increased ($p < 0.01$) immediately after the race. The results indicate that ultra-endurance exercise does not cause prolonged DNA damage in well-trained male athletes.

**Keywords:** Ultra-endurance exercise, Ironman triathlon, DNA damage, apoptosis, total antioxidant capacity

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**Introduction**

While regular moderate physical activity is related to various health benefits including decreased risk of cardiovascular diseases, diabetes, cancer and other lifestyle-dependent diseases (1-3), acute and strenuous exercise has been discussed to increase oxidative stress through the enhanced formation of reactive oxygen species (ROS) (4). When produced in excess, ROS can lead to the damage of cell components such as lipids, proteins and nucleic acids (5-8). ROS can also affect apoptotic progresses (9). Recent reviews describe in detail potential pathways for exercise-induced free radical formation such as increased oxygen consumption, autoxidation of catecholamines, activation of inflammatory cells due to tissue damage and transient ischemic or hypoxic conditions (10-12).

So far, only a small number of studies have been conducted to investigate the influence of physical activity on DNA stability and the findings are partly inconsistent due to the use of different exercise protocols, for example tests on treadmills (13-16), cycle ergometers (17), participating in a half- and full- marathon (18-20), an ultramarathon (21) or short distance triathlon (22). Additionally, the use of different endpoints of DNA damage, such as measurement of single and double strand breaks and oxidized purines and pyrimidines, 8-hydroxy-2`-deoxyguanosine (8-OHdG), sister chromatid exchanges or micronuclei (MNi) (13-25) account for the differences in the outcomes of these studies.

We recently showed, for the first time, that an Ironman triathlon did not cause long lasting DNA damage in well-trained athletes when applying the cytokinesis-block micronucleus cytome (CBMN Cyt) assay (26). However, data on the single cell gel electrophoresis (SCGE) assay, which enables the detection of DNA strand breaks, altered pyrimidines and oxidized purines (27), of long distance triathletes, who are extraordinary in their level of training and in the endurance and intensity of exercise performed, are missing. Due to the fact that the number of non-professional athletes training for and

competing in ultra-endurance events continually increases, it is of particular importance to investigate this group (28).

The major aims of the present investigation were to examine the impact of an Ironman triathlon race, as a prototype of ultra-endurance exercise, on the DNA migration attributed to the formation of single and double strand breaks and apurinic sites in the SCGE assay with lymphocytes. Furthermore, we also investigated the influence of ultra-endurance exercise on apoptosis and necrosis with the CBMN Cyt assay and assessed the total antioxidant status in the plasma. To cover the complete recovery period, the parameters were monitored over a longer time course (until 19 days post race).

## Materials and methods

### Study group

Out of the entire study group, which comprised 48 non-professional well-trained male triathletes, 28 subjects were randomly selected for the performance of SCGE assays, furthermore 20 (from the same collective) were analysed for the CBMN Cyt assay. The experimental design is summarised in Figure 1. The study was reviewed and approved by the local Ethics Committee of the Medical University of Vienna, Austria.

All participants were healthy non-smokers and were asked to document their training in the six months prior to the Ironman triathlon and thereafter until 19 days (d) post race, including the weekly training (km), the total weekly exercise time (h) as well as the weekly net exercise time (h). Before each blood collection, a 24-hour dietary recall was completed. All participants were physically fit, free of acute or chronic diseases, within the normal range of body mass index (BMI) and not taking any medication. They had to abstain from the consumption of supplements in excess of 100 % of the RDA (Recommended Dietary Allowance) - threshold level per day, in addition to their normal

dietary intake of antioxidants, vitamins and minerals including vitamin C, E, beta-carotene, selenium and zinc in tablet or capsule form six weeks prior to the triathlon until the last blood sampling 19 d after the event. The subjects fasted overnight before the 2 d pre race, 5 d and 19 d post race blood samplings, but on race day and 1 d post race, they were allowed to drink and eat *ad libitum*, and the quantities of intake were recorded. Only subjects who finished the race were kept within the study group.

Before each blood sampling, except the sampling immediately after the race and also two days before the spiroergometry, the subjects were asked to refrain from intense exercise. After the race, the training of the subjects had a regenerative character, which was documented in one of our previous reports (28), and was only of moderate intensity and duration until the end of the study.

To assess physiological characteristics, the subjects were tested on a cycle ergometer (Sensormedics, Ergometrics 900) three weeks before the triathlon. The test protocol started at an initial intensity of 50 W, followed by 50 W increments every 3 min until exhaustion. Oxygen and carbon dioxide fractions (both via Sensormedics 2900 Metabolic measurement cart), power output, heart rate and ventilation were recorded continuously and earlobe blood samples for the measurement of the lactate concentrations were taken at the beginning and end of each step.

[Insert Figure 1 about here]

**Race conditions**

The Ironman triathlon was held in Klagenfurt, Austria on July 16th 2006. The event comprised of a 3.8 km swim, a 180 km cycle, and a 42 km run. The race started at 7:00 a.m., when the air temperature was 15 °C, lake temperature 25°C and relative humidity 77

%. By finishing time (median time for participants 5:43 p.m.), air temperature and relative humidity were 27.2 °C and 36 % (data provided by the Carinthian Center of the Austrian Central Institute for Meteorology and Geodynamics).

**Reagents**

Ethylenediaminetetraacetic acid (EDTA), EDTA disodium salt ($Na_2$EDTA), dulbecco`s phosphate buffered saline (PBS), RPMI 1640 medium, trypan blue, dimethyl sulfoxide (DMSO), tris, ethidium bromide and Histopaque-1077 were obtained from Sigma- Aldrich (St. Louis, USA). Low melting agarose and normal melting agarose were purchased from Invitrogen (Life Technologies Ldt, Paisley, Scotland). Triton X-100 was procured from Serva (Plymouth, UK). Other reagents were obtained from Merck (Vienna, Austria).

**Blood sampling**

Blood samples were collected by venipuncture in heparinised and EDTA tubes (Vacuette, Greiner, Austria) 2 d before, within 20 min after the race as well as 1 d, 5 d and 19 d post race. The blood samples were processed immediately, as described below, or stored below 6°C for no longer than 7 h before processing.

**Alkaline single cell gel electrophoresis assay**

The SCGE assays were carried out according to the guidelines developed by Tice et al. (29). Briefly, lymphocytes were isolated using Histopaque-1077 and the trypan blue exclusion test was performed to examine the viability of the cells. The cell pellets were then mixed with 60 µl of 0.5 % low melting agarose and applied to glass slides, which were precoated with 1.5 % normal melting agarose. The slides were then covered with cover slips and placed on ice to enhance gelling of the agarose. After 5 min, the cover slips

were detached carefully and the slides placed in a lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris, 1 % Triton X, 10 % DMSO, pH 10.0) for ≥ 1 h at 4°C. Lysis and all consecutive steps were carried out under red light. After lysis, the slides were incubated in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM $Na_2EDTA$, pH ≥ 13) at 4°C for 20 min for DNA unwinding. Electrophoresis was performed at 25 V and 300 mA for 20 min using a horizontal gel electrophoresis (C.B.S Scientific, USA). The slides were then neutralized by rinsing (2 times for 8 min) with cold neutralization buffer (0.4 M trizma base, pH 7.5) and dried at room temperature overnight. For evaluation, the coded slides were stained with ethidium bromide (20 µg/ml) and examined using a fluorescence microscope (Nikon 027012) with an automated image analysis system based on the public domain program NIH image (30). For each sample, three replicate slides were analyzed and from each slide, 50 cells were measured. As parameter of DNA damage, percentage of DNA in the tail (% DNA in tail) was determined.

**Measurement of FRAP and ORAC**

Total antioxidant status in the plasma was assessed using the ferric reducing ability of plasma (FRAP) and the oxygen radical absorbance capacity (ORAC) assays (31).

The determination of FRAP was carried out according to Benzie and Strain (32) and indicates the capacity to reduce $Fe^{3+}$ to produce $Fe^{2+}$ (32). The ORAC was measured as described by Huang et al. (33). This method uses a radical initiator to form peroxyl radicals that remove a hydrogen atom from an antioxidant, leading to a delay or inhibition of the reaction between the peroxyl radical and the target molecule probe (34).

**Apoptosis and necrosis**

The CBMN Cyt assay was carried out as described earlier (26) to assess the number of apoptotic as well as necrotic cells (35).

**Statistical analysis**

Medians of percent DNA in tail from 50 cells per slide were computed. Three slides per time point and athlete were evaluated and medians of percent DNA in tail were arcsine transformed to obtain homogeneity of variance. Grubb's outlier test was performed on the three slides and in case of significance the outlier slide was removed from further analysis. Data were analysed based on a mixed ANOVA model with athletes as random factor and time points as fixed factor. Significance of differences between successive time points was assessed by linear contrasts. Two-sided p-values below 0.05 were considered significant. Residuals were tested for normality by Lilliefors' corrected Kolmogorov-Smirnov tests. Cross-correlation functions of tail DNA, FRAP and ORAC were computed (log transformation of FRAP and ORAC and arcsine transformation of percent DNA in tail) as Pearson correlations for each athlete and averaged across the 28 participants after Fisher's areatangens transformation and tested for significance by z tests applying Bonferroni correction.

**Results**

The baseline characteristics of the subjects (n=28) are summarised in Table 1. During the race the mean vitamin C and α-tocopherol intakes were 367 ± 186 mg and 117 ± 66 mg, respectively.

*Results of the SCGE assays*

The SCGE assay under standard conditions was applied to measure DNA single and double strand breaks in lymphocytes. The level of strand breaks decreased significantly ($p < 0.05$) immediately after the race, then increased ($p < 0.01$) 1 d post race and declined again 5 d ($p < 0.01$) after the race. Between day 5 and day 19 after the race the levels of strand breaks decreased ($p < 0.01$) further below initial levels (Figure 2). The overall effect of time points was highly significant ($p<0.001$). However, it is notable that the study design had some limitations, as no parallel group was inserted and no cross-over design was used.

*Plasma antioxidant capacity*

FRAP significantly increased immediately after the race ($p < 0.001$), remained at this high level until 1 d post race and declined significantly ($p < 0.001$) to baseline values 5 d after the race. The marker was increased 19 d after the race ($p < 0.05$) compared to pre race values (Table 2). A similar time kinetic was observed for ORAC. This endpoint increased significantly, reached a maximum immediately after the race ($p < 0.001$), and then decreased stepwise to baseline levels 1 d post race ($p < 0.05$) until 5 d post race ($p<0.05$) (Table 2). For both endpoints the time effects were significant ($p < 0.001$).

*Correlations among markers*

DNA strand breaks and ORAC were negatively correlated (average $r=-0.18$) but the correlation did not reach significance. There were small positive correlations with FRAP values (average $r=0.034$). Increase of FRAP from baseline to immediately post race correlated positively ($r=0.45$) with overall increase of percent DNA in tail from baseline to any time after the race, while increase of ORAC correlated negatively with increase of

DNA in tail (r=-0.14). Both correlations did, however, not reach statistical significance. Decrease of DNA strand breaks after the race was negatively correlated with the weekly net exercise time (r=-0.32, ns.) and similar though even smaller negative correlations were observed for four other training indicators. There was also a weak correlation of decrease of DNA in tail with race time (r=0.18, ns.).

*Apoptosis and necrosis*

The overall number of apoptotic cells decreased significantly ($p < 0.01$) after the race, remained at this low level till day 5 after the race and declined further until 19 d post race ($p < 0.01$) (Figure 3A). The number of apoptotic cells after the race was significantly lower than at all time points investigated compared to the baseline values (20 min post race -49.1 %; 5 d post race -53.4 %, 19 d post race -74.6 %). The time effect was significant ($p = 0.000$).

The overall number of necrotic cells declined significantly ($p < 0.01$) after the race and remained at a low level 19 d after the race (Figure 3B). The numbers of necrotic cells after the race were significantly lower at all time points investigated compared to baseline values (20 min post race -39.8 %; 5 d post race -34.1 %, 19 d post race -26.9 %). The time effect was significant ($p = 0.001$).

## Discussion

The present study was conducted to assess the influence of an Ironman triathlon race, as a model of ultra-endurance exercise, on the DNA stability of athletes over an extended observation period (until 19 d after the race), which has not been investigated so far. In this context, we recently showed, when applying the CBMN Cyt assay, which enables the detection of chromosome breakage, chromosome loss, chromosome rearrangements as

well as gene amplification and non-disjunction (36), that an Ironman triathlon does not induce MNi formation in lymphocytes of these well-trained athletes (26).

In the present study, the SCGE assay was used in order to examine the effect of intensive endurance exercise on the formation of DNA strand breaks (27). Our results suggest that ultra-endurance exercise lasting between 9 h and 14 h led to an increase of DNA strand breaks 1 d after the race, which returned to baseline values 5 d and even declined below the baseline values 19 d after the Ironman triathlon. These results indicate that participation in an Ironman triathlon does not lead to persistent DNA damage. In addition, the correlations between DNA damage, WNET and race time indicate that the formation of strand breaks immediately after the race decreased with higher training status, while DNA instability seems to increase with higher exercise intensity. It has been stressed by Moller and Loft (37) that results of cross-over and parallel studies are more reliable than those from trials with a sequential design which we used in the current investigation. One of the main reasons is that seasonal effects may affect the results. However, the duration of our study was relatively short and about 30% of the published studies used a simple design (for details see (38). Previous investigations on the levels of DNA strand breaks in the SCGE assay after treadmill running at maximal oxygen consumption and until exhaustion (13, 14), a half marathon of 1.5 h duration (18) or a short distance triathlon of 2.5 h duration (22) have also found increased levels of DNA migration 1 d post exercise. In the latter study, DNA migration reached a maximum 72 h post race, but the experiment was conducted only with 6 subjects. Tsai et al. (20) detected elevated DNA single strand breaks in the SCGE assay 24 h after a marathon run (42 km), which persisted through 7 d. In contrast, no changes in the levels of DNA strand breaks were observed in the SCGE assays immediately after a half marathon (21.1 km) and a marathon (42.2 km) run (19), 2.5 h

treadmill running at 75 % $VO_2$max (16) or 4 weeks of overloaded training (24). In another investigation, Mastaloudis et al. (21) observed a significantly increased number of damaged cells (10 %) at midrace in subjects attending an ultramarathon with an average duration of 7.1 h, but 2 h after the event, the values declined to baseline. Six days after the ultramarathon, the proportion of damaged cells was even lower than before the race. On the basis of this observation, the authors proposed that the DNA damage is not persistent during the race. This assumption is in agreement with our results, where no prolonged DNA damage was detected after a mean of 10.7 ± 0.9 h of intense exercise.

As competing in an Ironman triathlon with a duration between 9 h and 14 h is more intense than participating in a half marathon or a marathon and training demands are most likely higher, it seems that DNA stability is positively affected by the training status of the athletes. This is linked to adaptive responses (1, 7, 10, 39) including antioxidant adaptation, gene expression of antioxidant enzymes (4, 11, 40, 41), decreased basal oxidant production and reduced electron leaks in the mitochondrial electron transport chain (4).

Due to the fact that acute and strenuous exercise has been discussed to induce oxidative stress through enhanced formation of ROS (4) the total antioxidant capacity of plasma was assessed applying the FRAP and ORAC assays. To the best of our knowledge this study is the first reporting about FRAP and ORAC in ultra-endurance athletes. Although some investigations found no changes in the total antioxidant capacity after a cycle ergometer exhaustive test (17) or after running maximal tests on treadmill under normoxic and hypoxic conditions (42), increased values were observed in studies with marathon runners (19, 43, 44). The latter findings are consistent with our results, where a significant increase in the ORAC as well as FRAP was found after the Ironman triathlon, which decreased to

baseline values 1 d and 5 d after the race, respectively. In addition, Neubauer et al. (45) demonstrated recently that within the same study group the Trolox equivalent antioxidant capacity (TEAC) and uric acid levels in plasma were increased after the Ironman triathlon as well. The increase in the antioxidant capacity after strenuous exercise could either be due to the intake of antioxidants including vitamin C and alpha-tocopherol during the race, as well as tissue mobilization of these vitamins (45, 46), and/or because of the increase of the plasma concentration of the potent hydrophilic antioxidant uric acid following intense exercise (43, 45). Similarly, with the training- and performance-linked increase of TEAC (45), positive associations between FRAP and several exercise test variables, as well as uric acid, were observed immediately post race. In addition, FRAP values increased with performance in the Ironman race.

The effect of exercise on apoptosis and necrosis has been studied in several earlier investigations and the results are strongly controversial. Within our study, which is the first investigating the levels of apoptotic and necrotic cells after ultra-endurance exercise with durations between 9 h and 14 h, these markers decreased immediately after strenuous exercise and remained at a low level until 19 d after the race. This could be due to the adaptive responses of regular training, such as a more efficient electron chain in muscle mitochondria (47, 48), an extended capability of endogenous antioxidative systems, which might lead to the reduction of oxidative stress induced effects and thus improved oxidative balance during exercise (1, 7, 14, 45, 49) and upregulation of repairing systems (50), which in turn may reduce apoptosis in circulating lymphocytes. Our findings are in accordance with previous studies, where well-trained endurance athletes ($VO_{2max}$ > 60 ml/kg KG/min) had elevated baseline values of apoptotic lymphocytes, detected by flow cytometry, which decreased after a marathon run (51) or untrained subjects following moderate exercise on a

cycle ergometer (40 min, 60 % $VO_2max$), who showed no change in DNA fragmentation (52). In contrast, Mars et al. (53) detected an increase in the percentage of apoptotic lymphocytes immediately after treadmill running until exhaustion, which further increased until 24 h after exercise, but the study involved only three subjects. Immediately after an exhaustive cycle ergometer test, increased levels of apoptotic cells were observed in professional athletes, which returned to baseline 24 h after exercise, but not in the non-professional group (17). However, the TdT-mediated dUTP-nick end labelling (TUNEL) method was applied within the two latter investigations (17, 53), which is not exclusively specific for apoptotic cells (51). Immediately after an exhaustive treadmill exercise test (80 % $VO_2max$), increased levels of apoptotic cells, detected by flow cytometry, were found as well (54), but the values returned to the control value 1 h after exercise and the level of necrotic cells remained unchanged. In contrast, after 2.5 h treadmill running (75 % $VO_2max$) no significant changes in % Annexin-V positive cells (16) and in the total number of early apoptotic cells (Annexin positive) (55) were observed.

In conclusion, the present investigation indicates that an Ironman triathlon race, as a model of extremely demanding physical exercise, does not lead to prolonged DNA damage in lymphocytes of well-trained athletes as detected by the SCGE assay. Our findings show that levels of DNA strand breaks are lowered within 19 d of recovery after an acute bout of ultra-endurance exercise. The oxidative DNA damage after ultra-endurance exercise seems to be more prominent in pyrimidines than in purines. Interestingly, the number of apoptotic and necrotic cells did not increase after the Ironman triathlon; however, to clarify the elevated basal lymphocyte apoptosis and necrosis, more studies are needed (16, 51). Overall, the presented data suggest that regular physical training leads to adaptive

mechanisms including an enhancement of endogenous antioxidant defences, which seem to prevent severe oxidative stress and DNA damage even after strenuous exercise.

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**Abbreviations:** ROS reactive oxygen species, SCGE single cell gel electrophoresis, 8-OHdG 8-hydroxy-2`-deoxyguanosine, MNi micronuclei, CBMN Cyt cytokinesis-block micronucleus cytome, BMI body mass index, RDA recommended dietary allowance, FRAP ferric reducing ability of plasma, ORAC oxygen radical absorbance capacity.

**Appendices:**

Table1. Baseline characteristics of subjects.

| | total group (n=28) |
|---|---|
| Age (years) | 32.7 ± 6.3 |
| Weight (kg) | 75.0 ± 7.7 |
| Height (cm) | 181.3 ± 6.4 |
| BMI ($kg/m^2$) † | 22.8 ± 1.4 |
| $VO_2$ peak (ml/kg KG/min) * | 58.9 ± 8.5 |
| Individual anaerobic threshold (W) | 230.6 ± 46.1 |
| Relative individual anaerobic threshold (W/kg) | 3.1 ± 0.4 |
| Race time (h) | 10.7 ± 0.9 |
| WNET (h) ‡ | 11.3 ± 2.5 |
| TWET (h) $ | 12.2 ± 2.1 |
| Cycle training per week (km) | 164.6 ± 48.4 |
| Run training per week (km) | 38.6 ± 9.9 |
| Swim training per week (km) | 5.1 ± 2.1 |

Values are means ± SD.

* Peak oxygen consumption (ml/kg KG/min)

† Weight in kilograms divided by squared height in meters.

‡ Weekly net exercise time.

$ Total weekly exercise time.

Table2. Plasma antioxidant capacity of subjects (n=28).

| | FRAP (µmol/l) | ORAC (µmol TE†/l) |
|---|---|---|
| 2 days pre race | 942 ± 232 | 7781 ± 2082 |
| 20 min post race | 1296 ± 349** | 9271 ± 2113** |
| 1 day post race | 1196 ± 227** | 8489 ± 2018 |
| 5 days post race | 936 ± 237 | 7540 ± 2302 |
| 19 days post race | 1025 ± 268* | 7906 ± 2479 |

Values are means ± SD.

* Significant difference ($p < 0.05$) compared to 2 days pre race.

** Significant difference ($p < 0.01$) compared to 2 days pre race.

† Troloxequivalent

Legend of figures:

Fig. 1. Experimental design showing the time schedule according to which the alkaline single cell gel electrophoresis (SCGE) and cytokinesis-block micronucleus cytome (CBMN Cyt) assays were performed and spiroergometry was done.

Fig. 2. Impact of an Ironman triathlon on DNA damage as detected by the alkaline single cell gel electrophoresis (SCGE) assay in peripheral lymphocytes of 28 athletes 2 days (d) before the race, 20 min, 1 d, 5 d and 19 d post race. Data are presented as geographic means and 95% confidence intervals(* $p < 0.05$; ** $p < 0.01$) for % DNA in tail. Axis of ordinates is interrupted.

Fig. 3. Impact of an Ironman triathlon on different endpoints monitored with the cytokinesis-block micronucleus cytome (CBMN Cyt) assay in peripheral lymphocytes of 20 athletes 2 days (d) before the race, 20 min, 5 d and 19 d post race. Data are presented as mean ± SD (** $p < 0.01$). (A) Number of apoptotic cells per 1000 binucleated cells (# apoptotic cells/ 1000 BNC). (B) Number of necrotic cells per 1000 binucleated cells (# necrotic cells/ 1000 BNC).

Fig. 4. Results of a calibration experiment with the lesion specific enzymes ENDO III (A) and FPG (B). Nuclei from cells of one untrained donor were treated with the enzymes (see Material and Methods section) and with enzyme buffer only (control). Subsequently the electrophoresis was carried out under standard conditions and the comets analyzed as described. The results were obtained with 3 slides per experimental point and from each slide 50 cells were analyzed. Data are presented as mean ± SD (* $p < 0.05$; ** $p < 0.01$).

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# Review II

(Including original data; relevant to the thesis, but not forming part of it)

# *Biomarkers of exercise-induced myocardial stress in relation to inflammatory and oxidative stress*

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## ABSTRACT

*Increased concentrations of biomarkers reflecting myocardial stress such as cardiac troponin I and T and brain natriuretic peptide (BNP) have been observed following strenuous, long-lasting endurance exercise. The pathophysiological mechanisms are still not fully elucidated and the interpretations of increased post-exercise concentrations range from (i) evidence for exercise-induced myocardial damage to (ii) non-relevant spurious troponin elevations, presumably caused by assay imprecision or heterophilic antibodies.*

*Several lines of evidence suggest that inflammatory processes or oxidative stress could be involved in the rise of NT-proBNP and Troponin observed in critically ill patients with sepsis or burn injury. We tested the hypothesis that inflammatory or oxidative stress is also responsible for exercise-induced cardiomyocyte strain in a large cohort of triathletes following an Ironman triathlon. However, the post-race increase in cardiac troponin T and NT-proBNP was not associated with several markers of exercise-induced inflammation, oxidative stress or antioxidant vitamins.*

*Therefore, we clearly need more studies with other inflammatory markers and different designs to elucidate the scientific background for increases in myocardial stress markers following strenuous endurance events.*

**Key words:** Troponin, exercise, myocardial damage, assay imprecision, acute coronary syndrome

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# INTRODUCTION

After years of contradictory discussion, there is currently widespread consensus that the athlete's heart is a physiological adaptation of the human heart following years of endurance training [1;2]. In addition, regular physical exercise is undoubtedly associated with reduced cardiovascular morbidity and mortality [3;4].

Nevertheless, in the past decades, the diagnosis of acute exercise-induced minor myocardial damage was difficult due to the lack of cardiospecific markers. Creatine kinase (CK) is released following both skeletal and myocardial damage and also the determination of the relatively cardiospecific isoenzyme CK-MB does not always improve diagnostic specificity due to an increased expression of CK-MB in skeletal muscles of endurance athletes [5].

During recent years, assays for the determination of cardiac troponin T (cTnT) and cardiac troponin I (cTnI) as well as BNP or NT-proBNP have significantly improved the diagnostic power to detect subclinical cardiac damage and dysfunction [6-8].

It has been demonstrated that strenuous, long-lasting exercise is associated with an increase in biomarkers for myocardial stress such as cardiac troponins and B-type natriuretic peptide even in healthy trained subjects without signs for myocardial disease [9].

The reason why apparently healthy subjects exhibit increased concentrations of these biomarkers is not yet fully understood. Explanations include: Assay imprecision, heterophilic antibodies, sympathoadrenergic stimulation, structural or functional overload leading to temporal membrane leakage or definite cardiomyocyte death [9].

An important aspect that has not been considered extensively, comprises the notion that inflammatory processes are involved in the exercise-induced myocardial stress reaction and the increase in cardiac troponins and (NT-pro)BNP.

Several investigations have demonstrated that increased concentration of cytokines such as IL-6, TNF-alpha, IL-2 and IL-1ß acutely modulate cardiac function and may induce cardiomyocyte damage [10-12].

The effect of increased cytokine concentrations on myocardial function were firstly investigated in sepsis and burn injury. The secretion of cytokines following sepsis or burn injury altered the contractile responsiveness of the heart. In addition, critically ill patients showed increased concentrations of cardiac troponins and in some investigations also increased levels of (NT-pro)BNP.

Although the magnitude is much smaller, the exercise-induced inflammatory reaction resembles, at least in part, immunological processes observed during sepsis or severe trauma [13]. Therefore, there is a rational background to study the association between the inflammatory response following exercise and biomarkers for cardiomyocyte stress. In addition, measuring the course of oxidative stress markers and in particular the protective role of antioxidant vitamins may provide further information, as these factors have already been shown to be involved in the initiation and propagation of inflammatory mediated myocardial stress reactions [14].

In the present work, we summarize current knowledge on the association between strenuous physical exercise and the increase in biomarkers reflecting

myocardial stress (cardiac troponins and [NTpro] brain natriuretic peptide). In addition, we will present new data showing the association between exercise-induced inflammatory and oxidative stress/antioxidant vitamine levels and cardiac troponin T and NT-proBNP.

## Cardiac troponin

Troponin is a contractile protein and comprises 5 percent of muscle proteins. The troponin complex consists of troponin I , troponin C, and troponin T. These proteins are located within the myofibrils of cardiac and skeletal muscle and are encoded by different genes. They have a key role in the regulation of the calcium-mediated muscle contraction through interaction of actin with myosin [15]. cTnC functions as a calcium-receptor while cTnI prevents the adenosine triphosphatase activity when bound to actin. Troponin T fixes the troponin complex to tropomyosin. cTnT and cTnI are heart-specific with a specific amino acid sequence that allows a clear immunological differentiation from skeletal muscle troponins. Therefore, cTnT and cTnI are highly sensitive and specific serum markers to detect myocardial damage in the presence of peripheral muscle damage [16]. After myocardial damage, troponins are released from cardiomyocytes and are detectable within 3–10 hours in peripheral blood [17]. Although cardiac troponins are correctly regarded as the most cardiac-specific markers for the detection of myocardial damage, several aspects have to be considered in the interpretation of study results. The most important point is related to assay performance, i.e. antibody specificity, susceptibility of the assay to certain analytical interferences, pre-analytical factors and assay imprecision [18]. The first generation cTnT assay showed a considerable cross reactivity with skeletal muscle troponin and also the second generation assay possessed only poor linearity due to the use of bovine cTnT as an assay standard material. Therefore, results from studies using the latter assays should be interpreted with caution and are therefore not considered. Although these problems have been solved by the 3rd generation cTnT assay, also this assay, together with the various cTnI assays, share the problem of imprecision at very low troponin concentrations. It has been claimed that due to the high precision of current assays, the so called background noise appears undetectable. Therefore, any elevation of cardiac troponins above the 99th percentile of concentrations found in apparently healthy subjects has been designated as indicative of myocardial damage. However, to assure a correct diagnostic classification, this decision limit must be measured with a total imprecision (coefficient of variation [CV]) of less or equal 10 % [18;19]. It has been shown that no cardiac troponin assay was able to achieve the 10 % CV recommendation at the 99th percentile reference limit defined by the manufacturer [18].

Although there is convincing evidence that troponin assays have tremendously improved the diagnostic and prognostic power of laboratory markers in the detection of suspected acute coronary syndromes, their impreciseness at the detection limit must be considered, particularly in the interpretation of comparatively small troponin elevations following exercise. Additionally, with all assays the post-test probability for the presence of a clinical condition after a positive test result strongly depends on the pre-test probability of a disease in the investigated

population. Thus, in symptomless highly trained endurance athletes the pre-test probability for myocardial damage following competition is very low and is only marginally increased by the result of a positive troponin test. In contrast, in chest pain patients a positive troponin result almost proves the presence of myocardial damage.

Concerning exercise-induced increases in cardiac troponins, it has to be mentioned that cardiac troponins are present in the myocyte, both in a cytosolic (cTnT 6%; cTnI 3%) and a structurally-bound protein pool [17;20;21]. Therefore, the release of troponins following myocardial injury could be explained by 2 mechanisms: 1. Minor myocardial injury induces a loss of integrity of the membrane that results in transient leakage of troponin from the cytosolic compartment. 2. When damage is further aggravated, the activation of proteolytic enzymes leads to disintegration of the contractile apparatus, with continuous release of troponins from the bound protein pool. However, from the present knowledge it cannot be definitively decided whether limited troponin release may be associated with reversible myocardial injury. The current common consensus is that an increase in cardiac troponin above the 99th percentile of the CV should be interpreted as a sign of myocardial necrosis [21-23]. Minor myocardial damage can be distinguished from major necrosis, i.e. myocardial infarction by the duration of troponin release from myocardial fibres. In such instances, small elevations of cardiac troponins above the 10% CV cut off level usually return within the reference limit within 24-36 hours. In contrast, after MI cardiac troponin concentration remain elevated for 5 days or longer [24].

## Brain natriuretic peptide

Plasma concentrations of brain natriuretic peptide (BNP) have evolved as a new diagnostic tool for detecting both diastolic and systolic myocardial dysfunction [25-33]. The precursor of BNP is ProBNP, which is cleaved upon stimulation into the biologically active form BNP and the N-terminal rest NT-proBNP. BNP has a vasodilatory, natriuretic and diuretic activity that explains much of its biological function, i.e. to diminish myocardial strain mainly by reducing preload. Both BNP (half-life 20 min) and NT-proBNP (half-life 2 h) can be determined by immunoassays in plasma. BNP as well as NT-proBNP have shown a high sensitivity and specificity for the diagnosis of acute and chronic heart failure [30;34-37].

In contrast to cardiac troponins that indicate a structural damage to myocardial cells, an increase in BNP is a biomarker for functional myocardial overload or heart failure. The name “brain” is based on the historic fact that it was first detected in brain cells. However, BNP is mainly secreted from myocardial cells situated in atrium and ventricle. Therefore, it has been claimed that „Brain“natriuretic peptide should be renamed in “B-type” natriuretic peptide. Other members of the cardiac natriuretic peptides are the atrial natriuretic peptide (ANP) as well as the c-type natriuretic peptide (CNP).

Main stimuli for an increased liberation of BNP include a rise in intravascular volume, particularly an increase in central venous pressure and both right and left ventricular dysfunction as evidenced by an increased enddiastolic filling pres-

sure [28;38-41]. Both resting values as well as the magnitude of exercise-induced rise in BNP have shown to be associated with the severeness of myocardial insufficiency [30].

Therefore, it was an interesting finding that apparently healthy endurance trained athletes showed post-exercise increases in NT-proBNP that were beyond the cut-off levels for heart failure. Short maximal bouts of exercise e.g. ergometry to exhaustion were not associated with  a rise in NTProBNP [27;28;40;42]. In contrast, long-lasting endurance events such as marathon running or triathlon competitions induced marked increases in NT-proBNP. There seems to be a trend for higher concentrations with increased exercise duration and age of the participants [26;43].

## Cardiac troponin and NT-proBNP following exercise in athletes

Table 1 summarizes the results of some investigations that have measured cardiac troponins (cTnI; 3rd generation cTnT) and BNP/NT-proBNP following exercise. From 551 subjects investigated, approx. 33 % (variation in dependence of tests applied) showed an increase in cardiac troponins following endurance exercise. In most subjects, the increase was below the 10 % CV cut off limit reported by Panteghini et al. [18], and therefore, these results should be classified as troponin negative. 15 % of subjects showed an increase in cardiac troponins above the 10% CV cut-off, but below the cut-off level usually used for the diagnosis of myocardial infarctions with ST-segment elevations. In 5 % of the athletes, an increase above the cut-off level for myocardial infarction was detectable. With the exception of one subject in the study of Neumayr et al. [44], all investigations with subsequent post-race troponin analyses found that troponin levels had returned to baseline levels after 24 hours. The lack of cardiac symptoms and normal findings in ECG-analysis or SPECT sestamibi myocardial imaging [45] in all subjects, underlines the difficulties in interpreting the troponin results. In addition, most studies did not correct for hemoconcentration or verified troponin increases by measuring troponin with an assay of a different manufacturer. No study excluded analytical interferences with the troponin assay applied as a possible cause of a positive test result.

Therefore, some scientists hold that due to multiple limitations of the studies published so far, increased concentrations in cardiac troponins in well trained symptomless athletes are not evidentiary for myocardial injury. Their main point of criticism is a poor control for hemoconcentration, lack of exclusion of false positive test results due to analytical interferences from particles, bubbles or heterophilic antibodies or other assay dependent interfering substances in the samples [46]

However, the opinion that an exercise-induced increase in cardiac troponins in well trained athletes above the 10 % CV at the 99th percentile of the reference range should be considered as (at least minor) myocardial injury, is gaining more attention. The viewpoint is further supported by findings from post-exercise echocardiographic measurements showing a decrease in ejection fraction, wall motion disturbances and impaired diastolic function following strenuous endurance exercise [47-49]. In the study of Rifai et al., the subject with the high-

**Table 1. Cardiac Troponin T/I (cTnT/cTnI) and Brain natriuretic peptide (BNP) following strenuous exercise.**
(Papers cited in core clinical/sportsmedical journals).

| Author | Subjects | Age (y) | Setting | Main results: Troponin | Main results: BNP | Ref. |
|---|---|---|---|---|---|---|
| Shave, R.E. | 8 trained men | 33 ± 9 | Laboratory setting; 50 mile cycle trial in either normobaric normoxia or normobaric hypoxia | 1 subject with increase in cTnT following normobaric hypoxia (0.016 µg/l) | Not measured | [72] |
| König, D. | 11 professional cyclists | 27 ± 4 | Strenuous stage of a professional cycling race (Tour de Suisse) | 2 subjects with increase in cTnT following race (0.03µg/l/0.1 µg/l) | Increase in BNP from 47.5 to 75.3 pg/ml. Increase was most pronounced in older athletes | [73] |
| Shave, R.E. | 7 trained female | 44 ± 7 | 2-day strenuous Lowe Alpine Mountain Marathon | 3 subjects with increase in cTnT following day 1 (0.013-0.044 µg/l; 2 subjects with increase in cTnT following day 2 (0.014µg/l/0.017 µg/l) | Not measured | [20] |
| Vidotto, C. | 12 male runners<br>13 female runners | | Half-marathon race | 2 h postexercise: 4 females and 4 male runners with cTnI above 99th perc. | Increase in NT-proBNP from 39 to 107 pg/ml immediately post exercise | [50] |
| Neumayr, G. | 38 trained men | ~ 35 | Strenuous cycling marathon in high altitude | 8% of subjects with CK/CK-MB ratio > 6 %; 13 subjects with increase in cTnI immediately after competition (0.9-4.9 µg/l), 3 subjects above AMI cut-off; 24 h after competition cTnI < 1.0 µg/l in 12 subjects; 1 subject with further increase from 1.3 to 4.0 µg/l after 24 h | Not measured | [44] |
| La Gerche, A. | 15 trained men | 29 ±10 | Ironman distance triathlon | 1 subject with increase in cTnI following triathlon (0.9 µg/l) [b] | Not measured | [74] |
| Middleton, N. | 13 trained male runners<br>1 trained female runner | 29±5 | London marathon | 9 subjects with increase in cTnT; 2 above 99th perc. | Increase in NT-proBNP from 21.6 to 47.1 pg/ml | [51] |
| Rifai, N. | 11 trained men and 12 trained women | 33 ± 8<br>43 ± 14 | Hawaii Ironman triathlon | 2 subjects with increase in cTnI following triathlon (4.44/2.09 µg/L= > AMI cut-off) [c] | Not measured | [47] |
| Neilan, TG | 41 trained men and 19 trained women | 41 range: 21-65 | Boston marathon | 60 % with cTnT above 99th perc. (>0.01 ng/ml); 40 % at or above AMI cut-off (>0.03 ng/ml) | Increase in NT-proBNP from 63 to 131 pg/ml. Positive correlation with post-race myocardial dysfunction | [49] |
| Shave, R.E. | 26 trained men | 41 ± 10 | 2-day strenuous Lowe Alpine Mountain Marathon | 13 subjects with increase in cTnT following day 1 (< 0.035 µg/l); 1 subjects with increase in cTnT following day 2 (0.017 µg/l). All subjects with increase in cTnT on day 1 were negative following day 2 | Not measured | [75] |

| Neumayr, G. | 16 trained men | 37 ± 8 | Race Across the Alps | 6 (1) subjects with increase in cTnI (cTnT) following the race; 4 subjects with increase in cTnI above the 99$^{th}$ perc (0.7-5.1 µg/l); 3 subjects with cTnI above AMI cut-off; subject with the highest cTnI also showed cTnT above AMI cut-off (0.11 µg/l); cTnI returned to baseline in all subjects after 24 h | Not measured | [76] |
|---|---|---|---|---|---|---|
| Neumayr, G. | 29 recreational cyclists | 34±8 | Bike marathon race | 27 % with increase in cTnT above 99$^{th}$ perc. (0.043-0.224 mg/l) | Increase in NT-ProBNP from 28 to 278 ng/L | [52] |
| Shave R.E. | 8 trained men | 29 ± 9 | 30 min. downhill-running | No increase in cTnT | Not measured | [77] |
| Cleave, P. | 64 trained men | n.p. | New Zealand Ironman distance triathlon | 13 subjects with increase in cTnI above 10 % CV; in 5 subjects cTnI was > AMI cut-off; | Not measured | [78] |
| Scharhag, J. | 20 male endurance trained athletes | 36 ± 7 | Endurance exercise with either 1 h at 75 % $VO_2max$ or 3 h at 60 % $VO_2max$ | Slight increase in cTnI without any increase in cTnT. | Increase in NT-ProBNP by 9 ng/l and 30 ng/l respectively. No pathological findings in echocardiography | [79] |
| Scharhag, J | 105 endurance trained athletes | 40±8 | Marathon (n=46), 100 km run (n=14), mountain bike marathon (n=45) | cTnI: 74 % above 99$^{th}$ perc.<br>cTnT: 47 % above 99$^{th}$ perc. | 77 % of athletes showed increase in NT-ProBNP above reference limit. Increase was not correlated with alterations in troponin | [53] |
| Siegel A.J. | 82 trained men | 47 ± 7 | Boston marathon (serial testing of the same group after 5 subsequent marathon races) | 1997: 1 subjects with post-race increase in cTnI; 1 subject positive pre-race TnId<br>1998-2000: Significant increase in cTnI from 0.022 to 0.144 µg/L and in cTnI from 0.009 to 0.027 µg/L; 2 subjects with increase in cTnI above AMI cut-off (1.2/3.1 µg/l)<br>2001: insignificant increase in cTnI from 0.009 to 0.027 µg/L and in cTnT from < 0.01 to 0.041. | Not measured | [45] |

LLD = Lower limit of detection; 99$^{th}$perc = 99$^{th}$ percentile of the reference range; AMI = cut-off value for acute myocardial infarction
CTnT = Cardiac Troponin T (3$^{d}$ generation assay); CTnI = Cardiac Troponin I
b = post-race examination were performed at a mean of 4.7 d after competition; c = cTnT also measured but not 3$^{d}$ generation assay

est increase in cTnI also showed the highest number of hypokinetic myocardial wall segments [47].

It has been speculated that strenuous physical exercise above the individual strain tolerance could induce functional myocardial overload, energetical deprivation or increased liberation of intracellular $Ca^{++}$ leading to a loss of membrane integrity resulting in transient leakage of troponin from the cytosolic compartment. If this transient process is fully reversible without any residuals, is likely – given the large number of endurance athletes without cardiomyopathy - but cannot yet be proven. The short duration (<24 h) of detectable troponin concentrations favors this explanation, because cardiomyocyte death with protein disintegration has shown to be associated with a much longer troponin release period (>5 days). Other hypotheses to explain increased troponin concentrations following strenuous long-lasting endurance exercise include inflammatory processes or free radical damage.

In most investigations, the increase in post-exercise NT-proBNP was more than 100 % from baseline [49-53]. There seems to be trend for higher post-exercise values with increasing age of the participants and duration of a strenuous endurance event [26;43]. Explanations for increased NT-proBNP concentrations following strenuous long-lasting endurance events include neurohumeral activation of BNP release as well as a temporary myocardial systolic and/or diastolic dysfunction involving both right and left ventricles due to mechanical or volume overload.

In addition, recent findings from experimental and clinical studies suggest that inflammatory or oxidative stress related processes may promote the release of BNP after endurance exercise [54].

**Table 2**

Antioxidant vitamins, muscular stress and inflammatory markers 2 days before (14.07), immediately after (16.07), 1 day after (17.07), 5 days after (21.07) and 19 days after the Ironman Triathlon. ** = $p < 0.01$ compared to baseline values

| | *14.07* | *16.07* | *17.07* | *21.07* | *04.08* |
|---|---|---|---|---|---|
| ***Antioxidant vitamins*** | | | | | |
| Vitamin C (µmol/l) | 66,6 ± 13 | 102 ± 26,6 ** | 68,2 ± 13,2 ** | 80,9 ± 15,2 ** | 82,8 ± 14 ** |
| Alpha-Tocopherol (µmol/l) | 22,6 ± 7,3 | 26,6 ± 7 ** | 21,8 ± 6 | 22,5 ± 5,8 | 21,8 ± 5,8 |
| ***Muscle stress markers*** | | | | | |
| Creatine kinase (U/l) | 120 ± 76 | 1553 ± 2003 ** | 5298 ± 6240 ** | 457 ± 808 ** | 168 ± 248 |
| Creatine kinase MB (U/l) | 5 ± 4 | 45 ± 50 ** | 125 ± 142 ** | 11 ± 13 ** | 5 ± 5 |
| Myoglobin (µg/l) | 50 ± 13 | 1965 ± 1321** | 531 ± 531 | 72 ± 25 | 65 ± 39 |
| ***Inflammatory markers*** | | | | | |
| PMN-Elastase (ng/ml) | 45,6 ± 22,8 | 238 ± 137 ** | 94,6 ± 103 ** | 44,4 ± 30,5 | 36 ± 15,8 ** |
| Myeloperoxidase (µg/l) | 57,2 ± 30,9 | 253 ± 122 ** | 97 ± 82 ** | 61 ± 57,6 | 41,2 ± 24,9 ** |

## Cardiac function in relation to inflammatory/oxidative stress in the diseased state

The association between cardiac function and inflammatory markers can be divided into two main categories. On the one hand, increased levels of inflammatory cytokines (IL-6, TNF-alpha) and proteins of the APR (hsCRP) have been identified as subclinical markers of future heart failure. In 732 elderly Framingham Heart Study subjects without coronary heart disease, the risk to develop congestive heart failure was threefold when IL-6, TNF-alpha and hsCRP were elevated [55]. If the increase in inflammatory biomarkers reflects existing subclinical myocardial damage or is indicative of other inflammatory processes such as atherosclerotic plaque formation, cannot be answered conclusively [56]. In this context, the finding that the increase in CRP levels following Q-wave infarction seemed to be linked to the amount of myocardial tissue damage rather than the degree of pre-existing inflammation, merits further attention and evaluation [57]. First evidence suggests that high concentrations of inflammatory biomarkers are associated with a worsened prognosis following myocardial infarction or heart failure [57;58]. Most recently, it was found that plasma concentrations of myeloperoxidase (MPO) predict mortality following myocardial infarction [59]. Levels of MPO were also increased following strenuous physical exercise and the authors speculated on possible interactions between a rise in MPO and cardiac troponin and NT-proBNP [60].

On the other hand, apart from the prognostic aspect, it has been shown that increased concentration of cytokines such as IL-6, TNF-alpha, IL-2 and IL-1ß acutely modulate cardiac function and may induce cardiomyocyte damage [10-12].

The first studies showing the effect of increased cytokine concentrations on myocardial function were investigations of sepsis and burn injury. It could be demonstrated that the secretion of cytokines following sepsis or burn altered the contractile responsiveness of the beating heart or isolated myofibers. The acute effect is multilayered and involves different cellular mechanism such as nitric oxide (NO), sphingolipid mediators, alterations in intracellular $Ca^{++}$ handling and the arachidonic acid pathway [10;61]. Chronic effects are mediated by interstitial matrix remodelling mainly by the induction of specific matrix metalloproteinases leading to myocyte hypertrophy and myocardial fibrosis [62].

The rational background to study the association between the inflammatory response following exercise and biomarkers for cardiomyocyte stress is based on several similarities to the pathologic condition. Although the magnitude is much smaller, the exercise-induced inflammatory reaction resembles at least in part immunological processes observed during sepsis or severe trauma [13]. Moreover, despite the absence of myocardial infarction or coronary occlusion, several studies have shown that 15-25 % of critically ill patients exhibited increased concentrations of cTnI [63]. Positive correlation between troponin levels and left ventricular dysfunction have been found and also an increase in NT-proBNP has been reported by some investigators. However, the evidence that sepsis is always associated with an increase in BNP or NT-proBNP could not be ascertained [63].

Nevertheless, the data so far are sufficient to suggest a possible association between the exercise-induced cardiomyocyte stress reaction and inflammatory processes [54]. In addition, measuring markers of oxidative stress and the protective role of antioxidant vitamins may provide further information, as these factors have shown to be involved in the initiation and propagation of inflammatory mediated myocardial stress reactions [14].

## Cardiac function in relation to inflammatory/oxidative stress following strenuous exercise

To test the hypothesis whether inflammatory/oxidative stress is involved in the cardiomyocyte stress reaction, we investigated related parameters in 42 well-trained healthy male triathletes (age 35.3 ± 7.0 yr, height: 180.6 ± 0.1 cm, weight: 75.1 ± 6.4 kg, BMI: 23.0 ± 1.2 kg/$m^2$, $VO_{2\,peak}$ 56.6 ± 6.2 mL•$kg^{-1}$•$min^{-1}$, training volume 10.7 ± 2.6 h•$wk^{-1}$) who participated in the 2006 Ironman in Austria.

The Ironman triathlon took place in Klagenfurt in July 2006 and consisted of 3.8 km swimming, 180 km cycling and 42.2 km running. The race took place under near optimal climatic conditions.

Subjects did not take any medication or antioxidant supplementation (more than 100% of RDA) in the 6 wk before the race until the end of the study.

# METHODS

Blood samples were taken 2 days (d) before the race, immediately (within 20 minutes), 1 d, 5 d and 19 d after the race. The samples were immediately cooled to 4°C and the serum separated at 3000 rpm for 20 min at 4°C within 4 hours. Aliquots were frozen at -80°C until analysis. Whole blood was taken for the haematological profile; post-exercise concentrations of each parameter were corrected for exercise-induced alterations in plasma volume.

Cardiac troponin T (cTnT) and NTproBNP were determined using electrochemiluminescence technology employed within the Modular analytics E170 analyzer (Roche Diagnostics, Lewe, Sussex, UK). The oxidative stress markers malondialdehyde (MDA) and conjugated dienes (CD) were determined both by HPLC using the method of Wong [64] and Banni [65], respectively.

Concentrations of alpha-tocopherol (α-Toc) were analyzed using HPLC according to the method of Jakob and Elmadfa [66]. Ascorbic acid (Vit-C) was detected photometrically by the method of Denson and Bowers [67]. Plasma creatine kinase (CK) and creatine kinase MB (CKMB) activity was measured using an automatic analyzer (Vitros DT 60 II module; ortho-clinical diagnostics, Germany). Plasma interleukin 6 (IL-6) was determined by the Quantikine HS Immunoassy kit (R&D Systems GmbH, Wiesbaden, Germany). Concentrations of high sensitive C-reactive protein (hsCRP) and myoglobin were analyzed nephelometrically (Dade Behring, Marburg, Germany) Myeloperoxidase (MPO) concentrations were measured in plasma using the immunodiagnostik MPO ELISA kit (Immundiagnostik AG, Bensheim, Germany), Polymorphonuclear elastase (PMNelas) was determined using a quantitative enzyme immunoassay (Milena Biotec GmbH, Bad Nauheim, Germany).

# RESULTS

Figures 1-6 show the pronounced myocardial and pro-oxidative stress response as well as the course of IL-6 as major stimulator and hsCRP as one of the main constituents of the acute phase response (APR) following the ironman triathlon. With the exception of one athletes who had slightly elevated cTnT at baseline (0.014 µg/), all participants had normal values for cTnT before the competition. Immediately following the triathlon, cTnT increased above the 99th percentage of CV in 57 % of athletes; 3 of them were above the cut-off value for myocardial infarction (MI) (> 0.1 µg/ml). The day after the competition, cTnT was above the 99th percentage of CV in 3 subjects but clearly beyond the cut-off levels for MI in all athletes.

NT-proBNP increased considerably after the triathlon and all but 2 athletes showed NT-proBNP above the upper reference limit (URL) for beginning heart failure. In addition, on the day after the race, 60 % of athletes exhibited NT-proBNP levels above the URL. 5 days following the competition NT-proBNP was still elevated but had returned below the URL except for one athlete who had already shown increased concentrations at baseline.

Previous results concerning the course of cTnT and NT-proBNP in endurance events have already been discussed (see Tab. 1). The increase in cTnT in the present study was relatively high but not unusual, whereas the increase in NT-proBNP above 500 pg/ml was higher than in most previous investigations. However, the duration of exercise in most studies that have measured NT-proBNP was shorter (e.g. marathon or half-marathon [50;51] than an Ironman triathlon.

The high stress that the athletes were exposed is also reflected by the course of the other stress parameters investigated. Following the triathlon, MDA and CD as oxidative stress markers increased significantly. Furthermore, IL-6 and hsCRP as components of the APR as well as PMNelas and MPO as markers of the cellular inflammatory response rose significantly.

In addition, the triathlon induced a high exercise-induced stress reaction of peripheral muscle cells as indicated by the increase in myoglobin, CK and CKMB, although the latter could also origin from cardiomyocytes. As shown in most previous investigations, plasma concentrations of vitamin C and alpha-tocopherol increased following exercise [68].

There was no significant correlation between cTnT and NT-proBNP immediately after the competition. The strong correlation ($r^2 = 0.77$) that was observed between these parameters the day after the race was based on only three subjects and should not be considered as representative.

As shown in tab. 3 and 4, there was no correlation between cTnT or NT-proBNP and any parameter reflecting inflammatory (IL-6, hsCRP, MPO, PMNelas) or oxidative stress (MDA, CD). In addition, serum concentrations of antioxidative vitamins (Vit-C, α-Toc) were not associated with cTnT and NT-proBNP, respectively. Parameters reflecting peripheral muscle stress (Myo, CK, CKMB) were positively correlated with cTnT following the race.

# DISCUSSION

The most important finding of this investigation was that cTnT and NT-proBNP were not related to markers of inflammatory or oxidative stress. The considerable

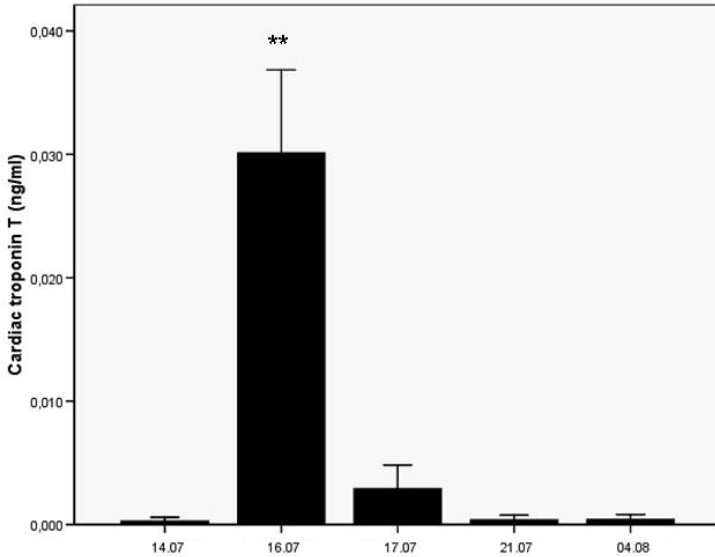


**Fig 1.** Cardiac troponin T (cTnT) 2 days before (14.07), immediately after (16.07), 1 day after (17.07), 5 days after (21.07) and 19 days after the Ironman Triathlon. ** = p< 0.01 compared to baseline values

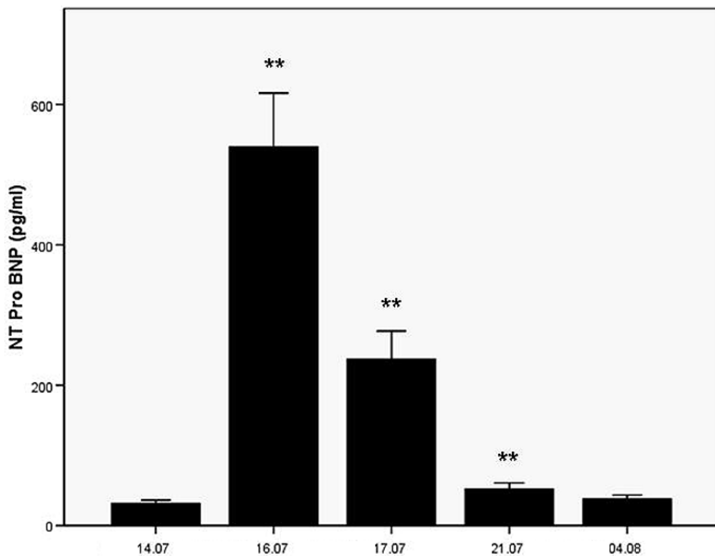


**Fig 2.** NT-ProBNP 2 days before (14.07), immediately after (16.07), 1 day after (17.07), 5 days after (21.07) and 19 days after the Ironman Triathlon. ** = p< 0.01 compared to baseline values

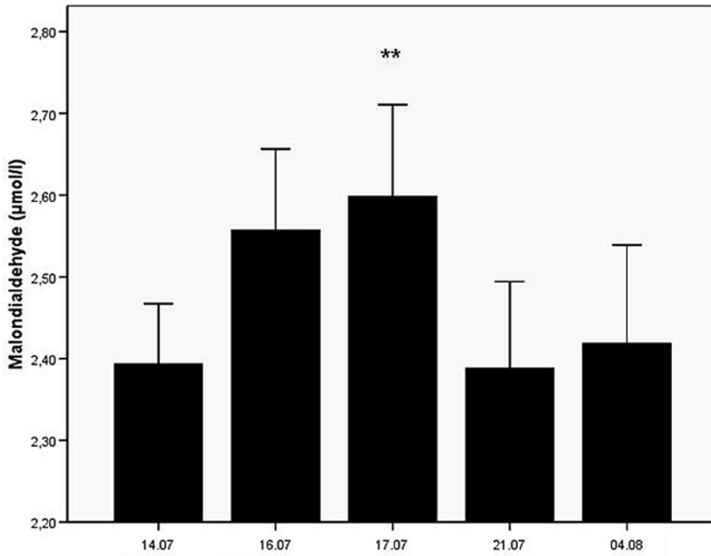


**Fig 3.** Malodialdehyde (MDA) 2 days before (14.07), immediately after (16.07), 1 day after (17.07), 5 days after (21.07) and 19 days after the Iron-man Triathlon. ** = p< 0.01 compared to baseline values

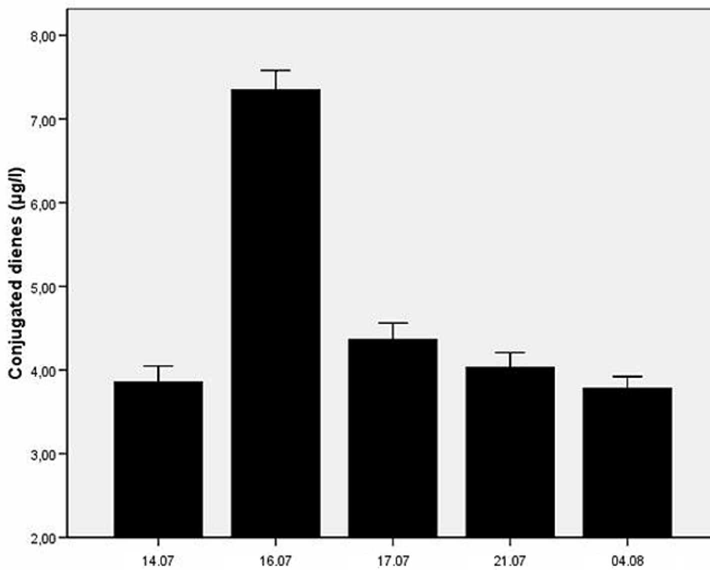


**Fig 4.** Conjugated dienes (CD) 2 days before (14.07), immediately after (16.07), 1 day after (17.07), 5 days after (21.07) and 19 days after the Iron-man Triathlon. ** = p< 0.01 compared to baseline values

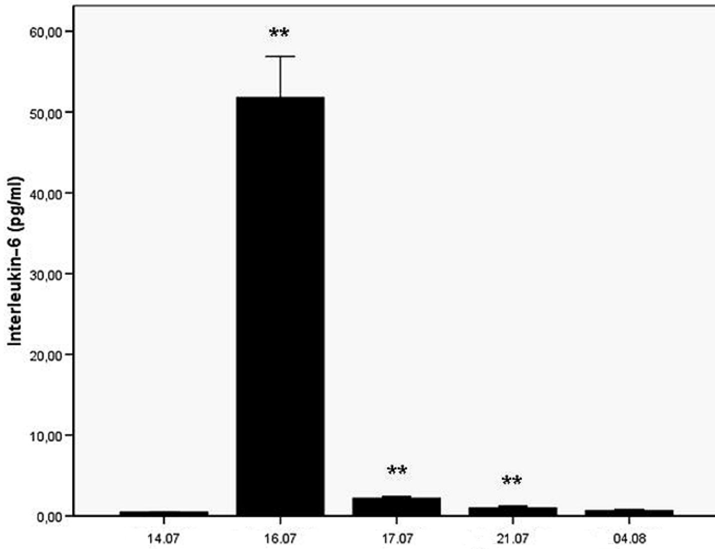


**Fig 5.** Interleukin 6 (IL-6) 2 days before (14.07), immediately after (16.07), 1 day after (17.07), 5 days after (21.07) and 19 days after the Ironman Triathlon.
** = p< 0.01 compared to baseline values

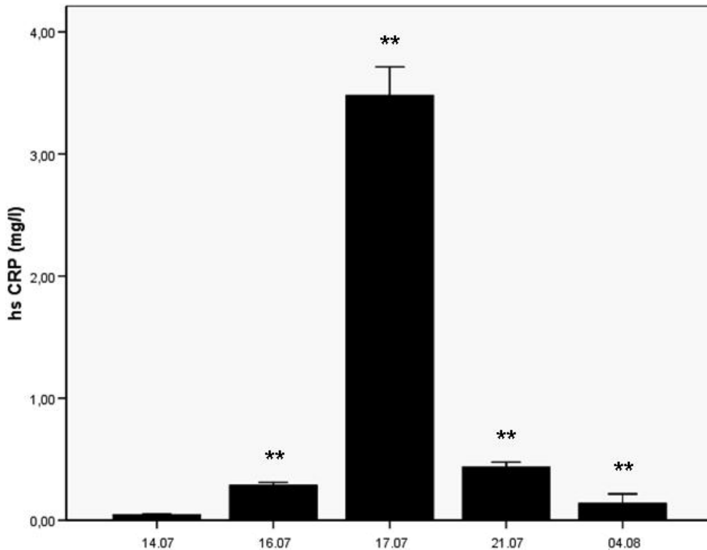


**Fig 6.** high sensitive C-reactive protein (hsCRP) 2 days before (14.07), immediately after (16.07), 1 day after (17.07), 5 days after (21.07) and 19 days after the Ironman Triathlon.
** = p< 0.01 compared to baseline values

increase in each parameter investigated reflects the significant stress that was induced by this ultra-endurance event. Compared with previous investigations, the magnitude of the individual stress response in this study was comparable or even higher [9;69-71]. The positive correlation between markers of skeletal muscle damage (CKMB was far below the ratio suggesting myocardial damage) and cTnT directly after the race is not clear. According to present knowledge, the

**Table 3 Correlation between cardiac troponin T (cTnT), NTproBNP and investigated parameters immediately after the ironman triathlon**

| | **cTnT 16.07** | **NT-proBNP 16.07** |
|---|---|---|
| | Coefficient of correlation | Coefficient of correlation |
| Age (y) -,200 | ,191 | |
| peak oxygen consumption (ml O2/kg/min) | ,238 | -,267 |
| Training hours/week (h) | ,155 | -,100 |
| Finishertime (h) | -,198 | ,199 |
| Malondialdehyd 16.07 (μmol/l) | -,246 | -,026 |
| Conjugated dienes 16.07 (μg/l) | ,035 | -,030 |
| Vitamin C 16.07 (μmol/l) | -,228 | ,050 |
| alpha-Tocopherole 16.07 (μmol/l) | ,186 | -,080 |
| Creatinkinase 16.07 (U/l) | **,436(**)** | ,101 |
| Creatinkinase MB 16.07 (U/l) | **,451(**)** | ,063 |
| Myoglobin 16.07 (μg/l) | **,396(**)** | -,062 |
| Interleukin_6 16.07 (pg/ml) | ,101 | ,274 |
| hsCRP 16.07 (mg/l) | ,205 | ,270 |
| PMN Elastase 16.07 (ng/ml) | -,038 | -,234 |
| Myeloperoxidase 16.07 (μg/l) | ,057 | -,248 |

* Correlation is significant ($p < 0.05$)

** Correlation is higly significant ($p < 0.01$)

**Table 4 Correlation between cardiac troponin T (cTnT), NTproBNP and investigated parameters one day after the ironman triathlon**

| | **cTnT 17.07** | **NT-proBNP 17.07** |
|---|---|---|
| | Coefficient of correlation | Coefficient of correlation |
| Age (y) | **,327(*)** | -,017 |
| peak oxygen consumption (ml O2/kg/min) | -,147 | ,009 |
| Training hours/week (h) | -,102 | ,038 |
| Finishertime (h) | ,136 | -,099 |
| Malondialdehyd 17.07 (μmol/l) | ,138 | ,154 |
| Conjugated dienes 17.07 (μg/l) | ,189 | -,021 |
| Vitamin C 17.07 (μmol/l) | -,201 | -,123 |
| alpha-Tocopherole 17.07 (μmol/l) | -,095 | -,219 |
| Creatinkinase 17.07 (U/l) | -,082 | ,095 |
| Creatinkinase MB 17.07 (U/l) | -,022 | ,147 |
| Myoglobin 17.07 (μg/l) | ,040 | ,086 |
| Interleukin_6 17.07 (pg/ml) | -,109 | ,105 |
| hsCRP 17.07 (mg/l) | -,072 | ,048 |
| PMN Elastase 17.07 (ng/ml) | ,259 | ,239 |
| Myeloperoxidase 17.07 (μg/l) | -,242 | -,281 |

* Correlation is significant ($p < 0.05$)

third generation assay of troponin shows no cross-reactivity with skeletal muscle troponin. Therefore, it could be suggested that the mechanisms responsible for peripheral muscle damage are at least partially identical to those responsible for cardiomyocyte stress.

Animal studies or investigations with critically ill patients have shown an association between the release of inflammatory cytokines and markers of functional and structural myocardial stress [54]. In addition, free radicals, oxidative stress and antioxidant vitamins as protective factors have been shown to be involved in the regulation of myocardial stress [14].

Although the inflammatory stress was decisively smaller than during sepsis or burn injury, the absence of any association suggests that the parameters we have chosen were not involved in the initiation of functional or structural cardiomyocyte stress in this design. In addition, plasma levels of antioxidant vitamins did not show a negative association in terms of having an attenuating effect.

However, although the numbers of athletes investigated was relatively high, we acknowledge the limitations of the correlational analysis. The intriguing hypothesis and the results so far from subjects with sepsis, burn injury or myocardial dysfunction should increase our effort to further investigate the (patho)physiological cause and effect of the exercise-induced increase in cardiac troponins and (NTpro)BNP. For the future, it seems particularly important to consider other cytokines such as TNF-alpha or IL-2 as these two have also shown modulatory effects of cardiac function [65].

## CONCLUSION

Strenuous long-lasting endurance exercise is associated with an increase in biomarkers of structural (cardiac troponins) and functional (BNP/NT-proBNP) myocardial stress. While usually, the increase was rather modest, some apparently healthy athletes without cardiovascular diseases showed post-exercise concentrations of these markers within the range of myocardial infarction or congestive heart failure. Scientific interpretations for these findings range from methodological problems or assay imprecision, temporal impairment of membrane integrity to clear evidence for myocardial tissue damage. Several pathomechanisms were made responsible for the increase in cardiac troponin and BNP such as mechanical and functional overload, adrenergic stimulation as well as free radical damage and inflammatory processes. However, inflammatory or oxidative stress related parameters investigated in the present study were not associated with CTnT or NT-proBNP concentrations.

The question if or when physical exercise may become harmful to cardiac myocytes or may even result in long-term impairment of myocardial function is of particular importance for all athletes. Therefore, there is a reasonable background to claim for more studies investigating the cause and effect of the exercise-induced myocardial stress reaction. By considering the association between myocardial dysfunction during sepsis or burn injury, there is a rationale to further investigate this problem with the inclusion of other inflammatory parameters than in the present investigation.

Nevertheless, an increase in cardiac troponins or NT-proBNP, particularly above the cut-off limit for myocardial infarction or heart failure, respectively, should always result in comprehensive cardiological examination to rule out underlying cardiovascular diseases.

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# Review III

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Review

# Endurance exercise and DNA stability: Is there a link to duration and intensity?

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ABSTRACT

It is commonly accepted that regular moderate intensity physical activity reduces the risk of developing many diseases. Counter intuitively, however, evidence also exists for oxidative stress resulting from acute and strenuous exercise. Enhanced formation of reactive oxygen and nitrogen species may lead to oxidatively modified lipids, proteins and nucleic acids and possibly disease. Currently, only a few studies have investigated the influence of exercise on DNA stability and damage with conflicting results, small study groups and the use of different sample matrices or methods and result units. This is the first review to address the effect of exercise of various intensities and durations on DNA stability, focusing on human population studies. Furthermore, this article describes the principles and limitations of commonly used methods for the assessment of oxidatively modified DNA and DNA stability. This review is structured according to the type of exercise conducted (field or laboratory based) and the intensity performed (i.e. competitive ultra/endurance exercise or maximal tests until exhaustion). The findings presented here suggest that competitive ultra-endurance exercise (>4 h) does not induce persistent DNA damage. However, when considering the effects of endurance exercise (<4 h), no clear conclusions could be drawn. Laboratory studies have shown equivocal results (increased or no oxidative stress) after endurance or exhaustive exercise. To clarify which components of exercise participation (i.e. duration, intensity and training status of subjects) have an impact on DNA stability and damage, additional carefully designed studies combining the measurement of DNA damage, gene expression and DNA repair mechanisms before, during and after exercise of differing intensities and durations are required.
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*Abbreviations:* ROS, reactive oxygen species; RNS, reactive nitrogen species; SCGE, single cell gel electrophoresis; ENDO III, endonuclease III; FPG, formamidopyrimidine glycosylase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; MN, micronucleus; MNi, micronuclei; CBMNCyt, cytokinesis-block micronucleus cytome; NPBs, nucleoplasmic bridges; Nbuds, nuclear buds; SCE, sister chromatid exchange; WBC, white blood cells; GC–MS, gas chromatography coupled with mass spectrometry; HPLC, high performance liquid chromatography; ECD, electrochemical detection; ELISA, enzyme-linked immunosorbant assay.

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## 1. Introduction

### 1.1. Exercise and DNA damage

Oxidative stress-induced DNA damage and insufficient DNA repair may play an important role in the etiology of cancer, diabetes and arteriosclerosis [1]. Participation in regular physical activity reduces the risk of developing diabetes, cancer, cardiovascular and other lifestyle-dependent diseases [2–5]. Interestingly, acute and strenuous exercise may induce oxidative stress via enhanced formation of reactive oxygen (ROS) and nitrogen species (RNS) [6–8].

Mechanisms responsible for exercise-induced free radical formation, recently described by Sachdev and Davies [9], include increased oxygen consumption (and ROS production), autoxidation of catecholamines, activation of inflammatory cells due to muscle tissue damage and ischemia and/or hypoxia/reoxygenation damage. Excessive ROS production may result in the oxidative modification of lipids, proteins and nucleic acids [9–11]. Since oxidative modifications of DNA can lead to mutations [12] and exceptionally high volumes of exercise are also associated with a substantial oxidative stress, concerns have arisen about the health effects of competing in ultra-endurance exercise events [13]. Given the hypothesised U-shaped relationship between exercise and health [14], i.e. both low and exceptionally high volumes of exercise participation are related to detrimental health outcomes, it is of great importance to assess the effects of exercise of different durations, intensities and types on oxidative stress responses and in particular, the effects that exercise has on DNA stability. Moreover, that acute and chronic exercise participation (training) appears to induce adaptations in antioxidant defence and DNA repair gene expression [9] means that this must be carefully considered when investigating the relationship between exercise, oxidative stress and DNA stability.

The objective of this article is to comprehensively review published scientific investigations that have studied the effect of exercise on DNA stability. Descriptions of the most commonly applied techniques, for the assessment of DNA damage and stability are only briefly described here, since they have been comprehensively reviewed elsewhere in the literature [15,16]. Studies concerning the influence of exercise on DNA stability are reviewed here according to the duration of exercise investigated. Finally, a discussion of the limitations of published studies, the clinical implications of exercise participation as well as suggestions for future research are included.

### 1.2. Methods commonly used to evaluate DNA damage linked to exercise

A comprehensive range of methods for the quantification of genotoxicity is available. These methods have been used in human studies to evaluate the effect of dietary, environmental and lifestyle factors on DNA stability [15].

The most commonly employed methods for the detection of DNA damage, related to physical activity, are the single cell gel electrophoresis (SCGE or COMET) assays, the micronucleus (MN) assay, and its further developed version, the cytokinesis-block micronucleus cytome (CBMN Cyt) assay. In addition, numerous assays have been developed to quantify the DNA base oxidation product 8-hydroxy-2′-deoxyguanosine (8-OHdG; also 8-oxo-7,8-dihydro-2′-deoxyguanosine 8-oxodG) in plasma and urine. Furthermore, the sister chromatid exchange (SCE) assay has been applied occasionally to detect changes in DNA after exercise [65].

The SCGE assay under alkaline conditions (standard version) is a simple, rapid and sensitive method that detects DNA strand breaks and alkali labile sites [17,18]. The key principle of the method is based on the migration of damaged DNA in an electrical field, forming comet shaped images [19]. The relative amount of DNA in the tail represents the frequency of DNA strand breaks [17]. A modified version of the SCGE assay, where the isolated nuclei are treated with lesion-specific enzymes formamidopyrimidine glycosylase (FPG) and endonuclease (ENDO) III, allows for the oxidized purines and pyrimidines to be detected [20–22].

The CBMN Cyt assay is a test that detects genome instability, including chromosome breakage, loss, rearrangement in addition to gene amplification and non-disjunction [23]. Micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (Nbuds) are measured as endpoints of this assay. Micronuclei result from chromosome fragments or whole chromosomes that lag behind at anaphase during cell division. Nucleoplasmic bridges originate from dicentric chromosomes resulting from misrepaired DNA breaks or telomere end fusion. Nuclear buds are formed as a consequence of gene amplification [24–27]. Since a causal link between MNi and the risk of cancer has been shown in a recent cohort study [28], this method has become an important variable to measure, especially in human studies of carcinogenicity.

Since guanine has the lowest oxidation potential among the four bases, it is the most prone to oxidation [29]. As a consequence of oxidation, 8-hydroxylation of guanine occurs and 8-OHdG is formed [1]. In most human biomonitoring studies 8-OHdG has been determined in urine and white blood cells (WBC). Various analytical methods have been used to measure oxidized guanine and include gas chromatography coupled with mass spectrometry (GC–MS), liquid chromatography coupled with GC–MS, liquid chromatography coupled with tandem MS, high performance liquid chromatography (HPLC) with electrochemical detection (ECD) or MS, and finally, $^{32}$P-postlabeling, methods based on the use of FPG and enzyme-linked immunosorbant assay (ELISA) [15,30,31]. Of these methods, the latter one is prone to interference from high molecular weight compounds found in plasma or serum resulting in confounded results [30].

SCEs arise during DNA replication as a consequence of breakage and rejoining of sister chromatids. This process occurs naturally, but can also be induced by exposure to environmental mutagens [32] and is therefore another method that can be used to assess DNA stability. The studies reviewed in Table 1 have applied one or more of these methods for the assessment and evaluation of DNA stability before, during and after exercise.

**Table 1**
Studies investigating the effects of exercise on markers of DNA damage.

| Reference | Experimental protocol[a] | Subjects[b] (trained/untrained/$VO_{2max}$) | Method[c] | Endpoint[d] | Sample matrix[e] | Summary of results[f] |
|---|---|---|---|---|---|---|
| Competitive ultra-endurance exercise (>4 h) | | | | | | |
| Poulsen et al. [47] | 8–11 h vigorous exercise/day for 30 days | 23 T | HPLC-ECD | 8-oxodG | Urine; spot | ↑ after 30 days period |
| Miyata et al. [48] | Ultra-marathon (2 days) (40 and 90 km) | 79 m (272 km running/month) 16 f (219 km running/month) | HPLC-ECD | 8-OHdG | Urine; spot | ↑ after 1 run; back to initial levels after 2 run |
| Radak et al. [49] | 4 days supra-marathon (93, 120, 56, and 59 km; road running) | 5 WT | ELISA | 8-OHdG | Urine; 12 h | ↑ after 24 h; back to initial levels on day 4 |
| Mastaloudis et al. [33] | Ultra-marathon (50 km) | 11 f ($VO_{2max}$: ∼55.0 mL/(kg min)) 11 m ($VO_{2max}$: ∼60.0 mL/(kg min)) divided into supplement/placebo group | COMET | SBs, AP | leu | ↑ at mid-race; back to initial levels 2 h after race |
| Reichhold et al. [50] | Ironman triathlon (3.8 km swimming, 180 km cycling and 42 km running) | 28 WT ($VO_{2max}$: 58.9 mL/(kg min)) | COMET | SBs, AP | Lymph | ↑ 1 day after race; back to initial levels 5 days after race |
| | | | +FPG | FPG-s s | Lymph s | ↔ |
| | | | +ENDO III | ENDO III-s s | Lymph | ↑ 5 days after race compared to 1 day after race; back to initial levels 19 days after race |
| Reichhold et al. [51] | Ironman triathlon (3.8 km swimming, 180 km cycling, and 42 km running) | 20 WT ($VO_{2max}$: 60.8 mL/(kg min)) | MN | MNi | Lymph | ↓ immediately and further 19 days after race |
| | | | MN | Nbuds | Lymph | ↑ 5 days after race; back to initial levels 19 days after race |
| | | | MN | NPB | Lymph | ↑ 19 days after race |
| Competitive endurance exercise (<4 h) | | | | | | |
| Niess et al. [52] | Half-marathon (21.1 km) | 12 MT (45 ± 25 km/week running) | COMET | SBs, AP | leu | ↑ 24 h after half-marathon in 10 subjects |
| Hartmann et al. [53] | Short-distance triathlon (1.5 km swimming, 40 km cycling, and 10 km running) | 6 T | COMET | SBs, AP | leu | ↑ 24 h until 5 days after race |
| | | | +FPG | FPG-s s. | leu | ↔ immediately after race |
| | | | HPLC-ECD | 8-OHdG | Urine; 24 h | ↔ 1 day until 4 days after race |
| | | | MN | MNi | Lymph | ↔ 2 days and 4 days after race |
| Tsai et al. [54] | Marathon (42 km) | 14 runners 20 control | COMET | SBs, AP | PBMC | ↑ 24 h after marathon; still ↑ 14 days after race |
| | | | +FPG | FPG-s s | PBMC | ↑ immediately until 24 h after marathon |
| | | | +ENDO III | ENDO III-s s | PBMC | ↑ immediately after race; still ↑ 14 days after race |
| | | | ELISA | 8-OHdG | Urine; 8 h | ↑ immediately after race; still ↑ 14 days after race |
| Briviba et al. [55] | Half-marathon and marathon (21.1 and 41.2 km) | 10 WT half-marathon (4.2 h running/week; 5 m, 5 f) 12 WT marathon (3.5 h running/week; 10 m, 2 f) | COMET | SBs, AP | Lymph | ↔ immediately after races |
| | | | +FPG | FPG-s s | Lymph | ↔ immediately after races |
| | | | +ENDO III | ENDO III-s s | Lymph | ↑ after both races |
| | | | +$H_2O_2$ | *ex vivo* SSB induction | Lymph | ↑ immediately after races |
| Non-competitive endurance exercise (<4 h) and periods of intensified training | | | | | | |
| Okamura et al. [56] | Road running (30 ± 6 km/day) for 8 days | 10 long-distance runners | HPLC-ECD | 8-OHdG | Urine; 24 h | ↑ during 8 days period |
| Pfeiffer et al. [37] | 14 days of winter training | 15 T supplement group 15 T placebo group | ELISA | 8-OHdG | Urine; 24 h | ↑ 7 days after start of training |
| Inoue et al. [57] | 90 min swimming (1500 m) or 70 min running (15 km) | 9 T swimmers 9 T runners | HPLC-ECD | 8-OHdG | Lymph | ↓ after swimming; ↔ after running |
| | | | HPLC-ECD | 8-OHdG | Urine; spot | ↔ after swimming; ↔ after running |
| Sumida et al. [58] | Running (20 km) | 11 T distance runners ($VO_{2max}$: 57.5 mL/(kg min)) | HPLC-ECD | 8-OHdG | Urine; 24 h | ↔ 1 day until 3 days after the test |
| Pilger et al. [59] | Long-distance road running (8–110 km/week) | 32 T long-distance runners | HPLC-ECD | 8-OHdG | Urine; 24 h | ↔; no differences between groups |
| | | 32 control | | | Urine; spot | |
| Itoh et al. [60] | Road running (10 km at 75% heart rate max) | 8 UT | ELISA | 8-OHdG | Plasma | ↓ immediately after run; 24 h back to initial levels |

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**Table 1** (*Continued*)

| Reference | Experimental protocol[a] | Subjects[b] (trained/untrained/$VO_{2max}$) | Method[c] | Endpoint[d] | Sample matrix[e] | Summary of results[f] |
|---|---|---|---|---|---|---|
| Palazzetti et al. [61] | 4 weeks of overload training (referring to programme of long-distance triathletes) | 9 WT triathletes ($VO_{2max}$: 66.0 mL/(kg min)) 6 NT ($VO_{2max}$: 42.8 mL/(kg min)) | COMET | SBs, AP | leu | ↔ immediately after overload training |
| Palazzetti et al. [62] | 4 weeks of overload training (referring to programme of long-distance triathletes) | 7 WT supplement group 10 WT placebo group | COMET | SBs, AP | leu | ↑ immediately after overload training in both groups |
| Laboratory studies: endurance exercise (<4 h) | | | | | | |
| Sato et al. [63] | Cycle ergometer test (for 30 min, at 50% $VO_{2max}$) | 7 T ($VO_{2max}$: 48.7 mL/(kg min)) 8 NT ($VO_{2max}$: 39.8 mL/(kg min)) | HPLC-ECD | 8-OHdG | leu | ↔ in T; ↓ in NT 48 h after test at 50% $VO_{2max}$ |
| Morillas-Ruiz et al. [34] | Cycle ergometer test (for 90 min, at 70% $VO_{2max}$) | 13 WT supplement group ($VO_{2max}$: 63.6 mL/(kg min)) 13 WT placebo group ($VO_{2max}$: 62.8 mL/(kg min)) | HPLC-ECD | 8-oxodG | Urine; 24 h | ↔ in supplement group and ↑ in placebo group 20 min after test |
| Orhan et al. [64] | Cycle ergometer test (for 60 min at 70% $VO_{2max}$) | 10 WT (4.6 ± 0.4 h sporting/week) | ELISA | 8-OHdG | Urine; 12 h | ↑ on first day after test |
| Sacheck et al. [35] | Downhill running on treadmill (for 45 min, at 75% $VO_{2max}$) divided into supplement/placebo group | 16 T (age 18–35;$VO_{2max}$: ~52.7 mL/(kg min)) 16 T (age 65–80;$VO_{2max}$: ~31.9 mL/(kg min)) | HPLC-ECD | 8-OHdG | leu | ↔ 24 h after test in placebo group |
| Hartmann et al. [65] | 1. Treadmill running until exhaustion | 3 (2 T and 1 UT) | COMET | SBs, AP | WBC | ↑ 24 h after 1 test; 72 h back to initial levels; ↔ after 2 test |
| | 2. Treadmill running (for 45 min) | | SCE | SCEs | WBC | ↔ after both tests |
| Peters et al. [66] | Treadmill running (for 2.5 h at75%$VO_{2max}$) | 8 WT athletes ($VO_{2max}$: 60.4 mL/(kg min)) | COMET | SBs, AP | Lymph | ↔ immediately and 3 h after test |
| Umegaki et al. [67] | Treadmill running (for 30 min at 85% of $VO_{2max}$) | 8 UT ($VO_{2max}$: 50.5 mL/(kg min)) 8 MT ($VO_{2max}$: 58.5 mL/(kg min)) | MN | MNi | lymph | ↔ immediately and 30 min after test |
| | | | MN + X-ray | MNi | Lymph | ↑ in UT 30 min after test; ↔ in MT |
| Studies under laboratory conditions: tests until exhaustion | | | | | | |
| Sumida et al. [36] | Cycle ergometer test until exhaustion | 8 UT supplement group; 6 UT placebo group | HPLC-ECD | 8-OHdG | Urine; 24 h | ↔ 1 day until 3 days after the test; no differences between groups |
| Sumida et al. [58] | 1. Treadmill running until exhaustion | 11 T distance runners ($VO_{2max}$: 60.7 mL/(kg min)) | HPLC-ECD | 8-OHdG | Urine; 24 h | ↔ 24 h after exercise |
| | 2. Cycle ergometer until exhaustion | 6 UT ($VO_{2max}$: 37.5 mL/(kg min)) | HPLC-ECD | 8-OHdG | Urine; 24 h | ↔ 1 day until 3 days after the test |
| Mars et al. [68] | Treadmill running until exhaustion | 11 T ($VO_{2max}$: 61.8 mL/(kg min)) | COMET | SBs, AP | Lymph | ↑ 24 h after treadmill test; ↔ after 48 h |
| Niess et al. [69] | Treadmill running until exhaustion | 6 T (70–100 km/week running); 5 UT (1–2 h/wPA) | COMET | SBs, AP | WBC | ↑ 24 h after treadmill test in both groups |
| Moller et al. [70] | Maximal cycle ergometer test under normal and high-altitude hypoxia conditions | 12 UT ($VO_{2max}$: 3.9 ± 0.6 L/min) | COMET | SBs, AP | Lymph | ↑ on all days at altitude compared to sea level |
| | | | +FPG | FPG-s s | Lymph | ↔ |
| | | | +ENDO | ENDO III-s s | Lymph | ↑ on day 3 after exercise in hypoxia compared to sea level and pre-exercise |
| | | | HPLC-ECD | 8-oxodG | Urine; 24 h | ↑ 24 h after exercise in hypoxia compared to sea level |
| Schiffl et al. [71] | 2× sprints until exhaustion | 6 (3 T and 3 UT) | MN | MNi | Lymph | ↑ of MNi 24 h after sprints |
| Pittaluga et al. [73] | Test on cycle ergometer until exhaustion | 6 UT ($VO_{2max}$: 47.1 mL/(kg min)) 6 T ($VO_{2max}$: 56.7 mL/(kg min)) 6 WT ($VO_{2max}$: 66.2 mL/(kg min)) | MN | MNi | Lymph | ↔ 30 min and 24 h after test |

Studies listed by their type and duration of exercise.

[a] Experimental protocols showing the type of exercise that was performed. Duration and intensity of exercise are indicated in parentheses.

[b] Number of subjects indicated as untrained (UT), trained (T; do their regular training), moderately trained (MT), well-trained (WT), males (m) and females (f). Training status ($VO_{2max}$, training per week or month) is given in parentheses.

[c] Analytical methods: high performance liquid chromatography with electrochemical detection (HPLC-ECD), single cell gel electrophoresis assay (COMET), single cell gel electrophoresis assay with formamidopyrimidine glycosylase treatment (+FPG), single cell gel electrophoresis assay with endonuclease III treatment (+ENDO III), single cell gel electrophoresis assay with $H_2O_2$ treatment, sister chromatid exchange assay (SCE), micronucleus assay (MN), micronucleus assay including X-ray irradiation (MN + X-ray) and enzyme linked immunoassay (ELISA).

[d] The endpoints are outlined as 8-hydroxy-2′-deoxyguanosine (8-OHdG), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), strand breaks (SBs), apurinic/apyrimidinic sites (AP), micronuclei (MNi), formamidopyrimidine glycosylase-sensitive sites (FPG-s s), endonuclease III-sensitive sites (ENDO III-s s), single strand breaks (SSB), nucleoplasmic bridges (NPB) and nuclear buds (Nbuds).

[e] Samples included lymphocytes (lymph), leucocytes (leu), urine and its collection period, white blood cells (WBC), peripheral blood mononuclear cells (PBMC) and plasma.

[f] Effects are described as no significant change (↔), significant increase (↑) and significant decrease (↓).

## 2. Methods

### 2.1. Identification of publications/search strategy

A literature search was conducted with the aim of identifying publications that investigated the effects of exercise on DNA in human population studies. The publications summarised in this review were identified by searching the MedLine/PubMed and Scopus databases (articles published before 30th October, 2008). The following key words were used: 'DNA damage', 'physical activity', 'exercise', 'endurance training', 'single cell gel electrophoresis', '8-hydroxy-2′-deoxyguanosine' and 'micronuclei'. Publications that utilised different forms of exercise and methods for detecting DNA damage such as the SCGE, CBMN and SCE assays in WBC as well as 8-OHdG determined in urine, plasma and WBC were included in this review.

### 2.2. Evaluation of publications

The search revealed 37 studies matching the search criteria; however, only 32 studies are included in this review (see below). The included studies investigated the effects of field tests (i.e. competitive ultra/endurance exercise) and tests conducted in the laboratory environment (on a treadmill or cycle ergometer) on DNA stability. The effects of these exercise interventions on multiple DNA variables were tested in subjects of varying training status (untrained to well-trained). Also, studies that attempted to identify the influence of antioxidant supplementation on exercise-induced DNA responses [33–37] were included in this review. Animal studies, tutorial reviews and articles not published in English (i.e. [38]) were excluded. Furthermore, studies that assessed markers of oxidized RNA (i.e. [39–40]) or oxidized products of free guanine bases [41] were not included. A study investigating the effects of exercise in the elderly (68.5 ± 5.1 years) [42] was also excluded because of age effects on DNA stability [43–45].

Abbreviated details of the included studies are shown in Table 1. These details include the type of physical activity conducted, the number of subjects and their training status and/or training loads, applied testing methods, assessed markers of DNA damage, the matrix in which the variables were measured, in addition to a summary of the study results. Training status and training loads of study participants are described inconsistently in published papers and therefore a summary of the actual information given by the individual authors is provided in Table 1. Training status and/or training loads (e.g. $VO_{2max}$ and/or daily/weekly training loads, in minutes or kilometres) are indicated in parentheses after the number of subjects in Table 1.

## 3. Reviewed study results

Brief descriptions of the study designs along with main study findings are presented in order of the duration and the type of physical load in the following paragraphs and in Table 1. The following sections address the effects of exercise conducted in competitive and non-competitive environments. Within each of the sections, the effects of ultra-endurance exercise (>4 h) [13], endurance exercise (<4 h) [46] and exhaustive tests on DNA stability are presented.

### 3.1. Competitive exercise

#### 3.1.1. Competitive ultra-endurance exercise (>4 h)

The effect of prolonged vigorous exercise on the formation of urinary 8-oxodG has been investigated by Poulsen et al. [47]. Their study included 23 trained subjects, who underwent a 30-day program (as part of their career advancement) of physical training, including 8–11 h of vigorous exercise per day for 6 days/week in the Danish Army. Oxidative DNA modification increased by 33% after the 30-day training period compared to pre-training. No data during the 30-day period were presented. A more recent study examined the urinary 8-OHdG concentrations in non-professional runners during a 2-day ultra-marathon, which consisted of average run times of 5.8 h on the first and 12.9 h on the second race day [48]. Since the formation of the oxidized base increased immediately after the first day (compared to baseline), and decreased after the second day of running (compared to the end of day 1), the authors concluded that the induction of DNA repair systems occurred after the first day. These findings are in accordance with a study conducted by Radak et al. [49], in which increased urinary 8-OHdG levels were detected 1 day after the start of a supra marathon (4-day race; see Table 1) compared to baseline, which declined to baseline on the fourth day of running. The authors suggested that repeated ultra-endurance exercise leads to an adaptation and normalization of oxidative DNA damage. Unfortunately, no detailed information on the subjects and their daily race performance were given.

When applying the standard SCGE method, Mastaloudis et al. [33] observed a significantly higher proportion of damaged cells (10%) mid-race compared to pre-race in subjects completing an ultra-marathon (average duration 7.1 h). Within 2 h of finishing, the values had returned to baseline. Six days after the ultra-marathon, the number of damaged cells significantly decreased to lower levels than at pre-race. These data suggest that the initial changes in the SCGE results (i.e. during the race) were not persistent and were perhaps a consequence of DNA repair mechanisms. Recent data from an investigation in well-trained, non-professional Ironman triathletes suggest a similar conclusion [50]. The number of DNA strand breaks increased significantly 1 day after an Ironman triathlon and returned to baseline values 5 days post-race and significantly declined further below baseline values 19 days after the race. The frequency of ENDO III-sensitive sites (oxidized pyrimidines) increased 5 days after the race and declined to baseline values 19 days post-race, whereas no changes in the FPG-sensitive sites (oxidized purines) were observed throughout the monitoring period. The formation of MNi was not induced within the same study group at any time point investigated after the Ironman triathlon, when applying the CBMN Cyt assay [51]. Overall, the number of MNi actually decreased significantly after the race and remained significantly depressed, versus baseline, even 19 days after race completion. Furthermore, the frequency of NPBs and Nbuds remained unchanged immediately after the triathlon. Therefore, these data suggest that the duration and intensity of exercise performed during an Ironman triathlon race do not lead to chromosomal alterations, which would lead to formation of MNi.

In summary, results of the studies that examined the effects of competitive ultra-endurance exercise on various markers of DNA damage show that no persistent DNA damage occurs after ultra-endurance exercise. It seems plausible that extensive training for ultra-endurance events results in adaptation and increased activity of DNA repair systems.

#### 3.1.2. Competitive endurance exercise (<4 h)

A few studies have examined the effect of competitive half or full marathons or short-distance triathlon on DNA damage.

Niess et al. [52] investigated whether completing a half-marathon resulted in DNA damage in leucocytes. In 10 out of 12 subjects an increase in DNA migration (SCGE assay) 24 h after the race was detected. The authors suggested that intensive exercise does induce DNA damage. Blood was sampled pre- and 24 h post-exercise, which precludes further conclusions concerning the time-course of DNA damage thereafter. Elevated DNA migration 24 h after endurance exercise was also reported by Hartmann et al. [53], who studied six athletes participating in a short-distance triathlon (2.5 h duration). Blood was sampled seven times over a period of 5 days, with the first sample-collected pre-race. Their results show that DNA migration remained elevated compared to pre-race, until 5 days post-race. However, no changes were observed in FPG-sensitive sites in the SCGE assay, urinary 8-OHdG and MNi frequencies over the same time period. The authors concluded that the detected DNA effects were not due to oxidation of DNA bases and do not lead to chromosome damage, although the study was conducted with only six subjects. Partly consistent with these findings, Tsai et al. [54] found elevated levels of DNA single strand breaks in the SCGE assay 24 h, 7 and 14 days after a marathon. FPG-sensitive sites were also increased immediately after the marathon reaching a maximum 1-day after the race.

Furthermore, oxidative effects on pyrimidines, detected using lesion-specific ENDO III, were significantly elevated immediately after the marathon to 7 days later. The same time-course was observed for urinary 8-OHdG. In contrast, Briviba et al. [55], when applying the SCGE assays, observed no change in the levels of endogenous DNA strand breaks and FPG-sensitive sites immediately after a half-marathon and a marathon race in ten subjects. However, oxidative DNA damage, assessed as ENDO III sites, was significantly increased and the *ex vivo* resistance to DNA damage induced by hydrogen peroxide was decreased after the event. Furthermore, no changes in the FPG-sensitive sites (oxidized purines) were observed. Unfortunately, the experimental design in the latter study with sampling time points 10 days before and immediately after the race did not allow the investigation of potential further changes in these parameters.

Based on the findings reviewed above, a clear conclusion of the effects of competitive endurance exercise lasting less than 4 h on DNA stability remains elusive. Although the majority of studies have found increased levels of DNA strand breaks 24 h after competitive endurance exercise, the results regarding oxidative DNA damage are contradictory, probably due to the use of different experimental designs, differences in the size of study groups and in the training status of the subjects. With respect to the observation periods, it is important to emphasise that monitoring DNA stability $\leq$24 h after exercise is too short because major alterations in DNA repair mechanisms seem to occur thereafter [33,50,51,54].

### 3.2. Non-competitive endurance exercise (<4 h) and periods of intensified training

Contrary to acute effects of competitive exercise in the field, a few studies evaluated the effects of prolonged periods of training or exercise protocols using treadmills or cycle ergometers. The latter allows investigation in a relatively standardized conditions.

Okamura et al. [56] studied ten long-distance runners during an 8-day running camp, where the average running distance was $30 \pm 6$ km/day. This exercise protocol increased urinary 8-OHdG during the training period. However, urinary 8-OHdG decreased to pre-training levels on the day after the camp was concluded. During 14 days of winter training at an altitude of 2700 m, urinary 8-OHdG (quantified using ELISA), increased in the placebo and antioxidant supplementation group (details see Table 1), 7 days after the start of the training [37]. However, the training schedule of the subjects was not described in detail and therefore the relevance of potential training adaptations on exercise-induced effects could not be estimated. Both, 8-OHdG in urine and lymphocytes have been measured by Inoue et al. [57] in nine trained swimmers and runners before and within 15 min after 90 min of swimming (1.5 km) or 70 min running (15 km). The 8-OHdG content of lymphocyte DNA decreased immediately after swimming, but not after running. Regarding the urinary 8-OHdG results, no significant changes were observed. Interestingly, the pre-exercise levels of 8-OHdG in the DNA of lymphocytes were reported to be higher in swimmers compared to runners. As the subjects' training status was not assessed before the exercise tests, the authors could only speculate about the disparity between runners and swimmers results, which were perhaps related to the individuals' intensity of training.

In a competition-simulating experiment conducted by Sumida et al. [58], 11 long-distance runners completed 20 km in $79.2 \pm 2.4$ min in a non-competitive environment. No oxidative DNA response, measured as urinary 8-OHdG, either 3 days before, or 3 days after the running bout occurred at any time point investigated,. In addition, no differences in the urinary 8-OHdG levels were found, at rest, between long-distance runners and controls [59]. In this study, no information on the control subjects' and long-distance runners' training status were provided. In contrast, another study observed decreased levels of plasma 8-OHdG immediately after a 10 km run ($1 \pm 0.2$ h duration, 75% of maximum heart rate), which returned to initial levels 24 h after exercise [60]. The plasma marker of DNA damage, assessed in using ELISA in this study, has been criticised due to inaccuracies of the method [8,16].

In two separate studies, Palazzetti et al. [61,62] investigated the effects of an intense training period on DNA stability in a similar group of male well-trained triathletes, using the SCGE assay. No changes in the first study [61], but an increase in the second [62] regarding the levels of DNA strand breaks were observed immediately after 4 weeks of overloaded training. The authors concluded that the effects of exercise on DNA could be influenced by the duration of the exercise administered (acute or chronic intervention) and DNA repair enzyme activity.

Although several studies have investigated the effects of non-competitive endurance exercise on DNA, their findings are inconsistent due to the use of different experimental designs and methods for the detection of DNA damage.

### 3.3. Laboratory studies (treadmill or cycle ergometer)

Most studies investigating the influence of exercise on DNA stability have been conducted in laboratories, mainly using treadmill or cycle ergometer protocols.

#### 3.3.1. Laboratory studies: endurance exercise (<4 h)

The effect of 30 min exercise on a cycle ergometer at 50% $VO_{2max}$ has been studied by Sato et al. [63] in seven trained and eight sedentary subjects. Interestingly, they found higher baseline leukocyte 8-OHdG levels in the non-trained individuals ($VO_{2max}$: $39.8 \pm 5.4$ mL/(kg min)) compared to the physically active ones ($VO_{2max}$: $48.7 \pm 7.6$ mL/(kg min)). Forty-eight hours after the exercise test, leukocyte 8-OHdG decreased in the sedentary subjects only, leading the authors to suggest that mild exercise beneficially reduces oxidative DNA damage in healthy, sedentary individuals. Cycle ergometer tests performed at 70% $VO_{2max}$ were conducted by Morillas-Ruiz et al. [34] and Orhan et al. [64]. In the Morillas-Ruiz study [34], the subjects (only considering placebo group) exercised for 90 min and urinary 8-oxodG levels were assessed, using HPLD-ECD, in urine collected for 24 h periods pre- and post-exercise. The study of Orhan et al. [64] required subjects to perform 60 min of cycling. Urine was collected over 24 h, 1 day before and for 72 h after the exercise. Increased 8-OHdG levels were found in both studies (excretion post-exercise versus pre-exercise; using ELISA). In contrast, no significant change in 8-OHdG in leucocytes was observed by Sacheck et al. [35], who studied the effect of downhill running on a treadmill for 45 min at 75% $VO_{2max}$ in trained subjects.

To the best of our knowledge, Hartmann et al. [65] were the first to use the SCGE and the SCE assay for the assessment of physical activity effects on DNA stability. Their results showed that 24 h after treadmill running (until exhaustion) DNA migration increased and returned to baseline 72 h later. However, in three subjects (two trained and one untrained), from the original group, no changes in DNA migration were detected after running for 45 min on a treadmill at a fixed individual speed (according to the subjects' lactate measurements). Due to the low number of participants in this part of the study, the results must be interpreted cautiously and they cannot be generalised to either trained or untrained persons.

A more recent study examined whether treadmill running for 2.5 h at 75% $VO_{2max}$ in eight well trained athletes affected DNA strand breaks in lymphocytes, using the SCGE assay. This exercise protocol did not increase levels of DNA strand breaks, either immediately, or 3 h after the test [66]. In agreement with the previous study no chromosomal damage in lymphocytes (using the

MN assay) was observed in eight moderately trained and untrained subjects following a treadmill run for 30 min at 85% $VO_{2max}$. However, in the untrained group, X-ray-induced chromosomal damage was significantly increased. Thus, the authors concluded that differences in chromosomal damage between trained and untrained groups was due to enhanced DNA repair and an increased endogenous antioxidant systems in the trained subjects [67].

Overall, increased oxidative stress in response to exercise has been reported in laboratory-based experiments on treadmills and cycle ergometers. No conclusive statement regarding persistent effects, assessed by the formation of MNi, can be drawn because the observation periods did not extend beyond 30 min after the tests [67]. In addition the numbers of subjects studied were often insufficient to confer confidence in reported results.

### 3.3.2. Laboratory studies: tests until exhaustion

Several studies have investigated the effects of exhaustive physical exercise on DNA stability. For example, Sumida et al. [36] studied 14 untrained subjects, who were divided into a supplementation (30 mg β-carotene per day for 1 month) and a placebo group and had to complete two separate cycle ergometer tests until exhaustion (one before supplementation, the second after 1 month of supplementation; $VO_{2max}$: 37.5–43.5 mL/(kg min)). Both ergometer tests had no effects on urinary 8-OHdG values. In a further experiment Sumida et al. [58] analysed urinary 8-OHdG levels after two different test protocols. Firstly, 11 long-distance runners performed a treadmill test until exhaustion. Secondly, six untrained subjects performed cycle ergometer test until exhaustion. Although the chosen test protocols were different and the training status of the subjects varied, no changes in urinary 8-OHdG were found.

Using the SCGE assay, Mars et al. [68] detected DNA damage in 10% of lymphocytes of trained subjects after performing a treadmill test until exhaustion. However, the comets in this study were classified by visual inspection and no details on the categories of comets were reported. A clear increase in DNA migration was reported by Niess et al. [69], who examined the effects of exhaustive treadmill running on markers of the SCGE assay. Both trained and untrained subjects showed increased DNA migration 24 h after exercise, which, according to the authors, may be due to an increased formation of ROS released by neutrophils following the exercise protocol. Interestingly, the detected increase after exercise was lower in the trained compared to the untrained runners. Therefore, the authors concluded that adaptation to endurance training might reduce detrimental effects, caused by exercise-induced oxidative stress.

In a conceptually different study, Moller et al. [70] tested the effects of a maximal cycle ergometer test under normal and high-altitude (hypoxic) conditions in recreationally active individuals. Blood samples were taken before, immediately after, 24 and 48 h after exercise at both sea level and 4559 m. Three days of high-altitude 'exposure' alone increased the levels of DNA strand breaks compared to levels seen at sea level. In addition, the levels of DNA strand breaks at high-altitude were elevated after the exercise test compared to pre-exercise. The numbers of ENDO III-sensitive sites were higher on day 3 of altitude exposure compared to sea level and pre-exercise values. Furthermore, urinary 8-oxodG was increased 24 h after exercise at altitude compared to sea level values. In contrast, no changes in FPG-sensitive sites were

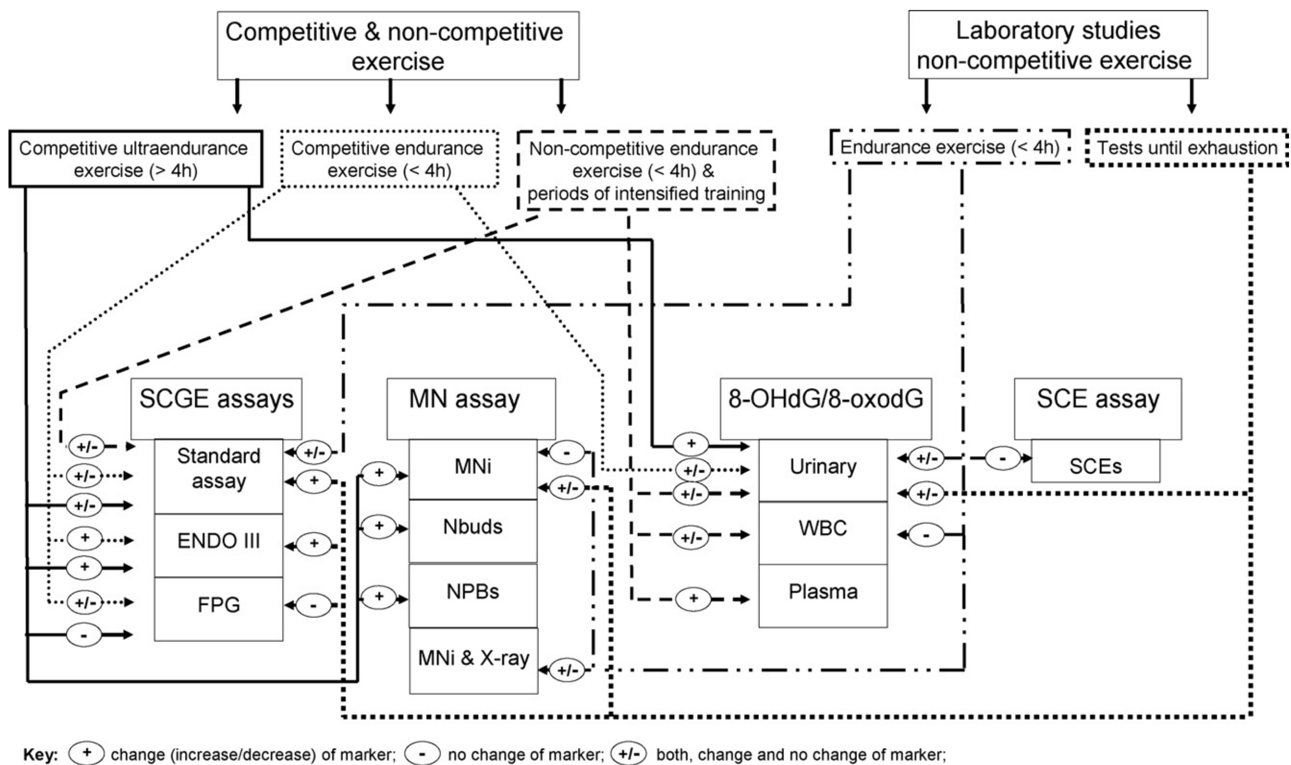


**Fig. 1.** Schematic illustration of the influence of different exercise types and intensities on endpoints of DNA and chromosomal stability. The two main sections (competitive/non-competitive and laboratory studies) are further divided into subsections according to the duration of exercise (ultra-endurance exercise, endurance exercise and exhaustive tests). Within each subsection, the study results are summarised due to their main findings. Studies within a subsection, where an exercise-induced effect (either an increase or a decrease) was observed, are indicated as '+'. Studies, where the marker did not change in response to exercise, are illustrated as '−'. The '+/−' symbol indicates when both significant changes (increase or decrease) and no changes were found within a subsection. (SCGE single cell gel electrophoresis, ENDO III endonuclease III treatment, FPG formamidopyrimidine glycosylase treatment, MN micronucleus, MNi micronuclei, Nbuds nuclear buds, NPBs nucleoplasmic bridges, 8-OHdG 8-hydroxy-2′-deoxyguanosine, 8-oxodG 8-oxo-7,8-dihydro-2′-deoxyguanosine, WBC white blood cells, SCE sister chromatid exchange assay).

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recorded. At sea level, no significant effects of exercise on DNA were found, except that the levels of FPG-sensitive sites decreased 24 h after exercise. Based on these findings, the authors hypothesised that hypoxic stress generates DNA strand breaks after exhaustive exercise because the antioxidative system becomes depleted and, consequently, is unable to prevent DNA damage.

Although Schiffl et al. [71] found significantly elevated levels of MNi in six subjects after two exhaustive sprints; the subjects were of different training status and gender. Furthermore, one subject was a smoker and but smoking is known to increase levels of MNi [72]. In a more recent study, comparisons between subjects of different training status showed that athletes (road-racing cyclists) had higher levels of spontaneous chromosomal damage (MNi) in lymphocytes at rest when compared to untrained subjects. Furthermore, the initial values of the athletes remained unchanged after an exhaustive cycle ergometer test [73]. The authors hypothesised that this phenomenon may be due to 'beneficial' chronic stress in the athlete group caused by their habitual intensive training.

In conclusion, urinary 8-OHdG seems to be unaffected by exhaustive exercise, however, DNA strand breaks after exhaustive exercise have been detected. The influence of exhaustive exercise on DNA stability, however, does not persist in the long term.

## 4. Differences between exercise durations and intensities

Based upon available data in the literature, no clear differences of the discussed exercise durations and intensities on DNA stability/damage were found. Fig. 1 shows the possible effects of different exercise types and intensities on endpoints of DNA damage. However, some important conclusions could be drawn according to the examined literature.

Firstly, DNA damage after competitive ultra-endurance exercise in well-trained athletes does not appear to be persistent. Although levels of DNA strand breaks increase 24 h after competitive endurance exercise, the assessment of exercise-induced effects (after less than 4 h of competitive exercise) on sustained DNA damage is limited. The latter is due to insufficient monitoring periods (predominantly, if at all, not longer than 24 h into recovery).

Moreover, different and occasionally weak experimental designs complicated the evaluation of the effects of non-competitive endurance exercise and periods of intensive training versus the effects of other exercise durations and intensities. Similar to competitive endurance exercise, endurance exercises in laboratory-based experiments generally increase oxidative stress. However, no firm conclusion on the persistence of DNA instability can be drawn.

Finally, exhaustive exercise in laboratory settings increases the levels of DNA strand breaks, but does not affect levels of urinary 8-OHdG. Potential reasons for the heterogeneous results of the examined investigations are summarised in Section 5.

## 5. Limitations

The studies reviewed above show variable, yet interesting, findings. Numerous methodological and conceptual limitations have been identified in these studies. Firstly, the size of the study groups is generally small, reducing the likelihood of detecting significant effects. Thus, there is need for larger cohorts of subjects to be tested in order to obtain statistically stronger results (especially to clarify the effect of different training status on the DNA/training-induced effects). Secondly, although it is well documented that information on lifestyle, smoking, alcohol consumption, medication, and nutrition are essential for the assessment of DNA modulation/stability [19], data on these factors are rarely reported. In addition, in some papers, no details regarding subjects' training status and administered exercise protocols are reported. Thirdly, the use of different sample matrices makes comparisons between the studies more complex. For example, urinary excretion of 8-oxodG represents DNA oxidation in the whole body, whereas tissue levels represent the effects of DNA oxidation and repair processes [12]. Another limitation of some studies is the application of only ELISA for the assessment of DNA damage. It has been emphasised that ELISA 8-OHdG methods are prone to interference from high molecular weight compounds found in plasma and serum [30]. Finally, the results of SCGE assays are inconsistently reported as %DNA in tail, tail moment and/or tail length. According to Collins et al. [17] the tail moment does not have a standard unit and there are different ways of calculating tail moment, which complicates the interpretation of results. Also, tail length is informative, however, only when the levels of DNA damage are low. Thus the calculation of %DNA in the tail should be consistently reported [17].

## 6. Possible adaptive responses

The consequences of primary DNA lesions are diverse. These DNA lesions can either be repaired or eliminated through apoptosis leading to no persistent damage. On the other hand, cells with primary DNA damage can be replicated forming irreversible, persistent mutations affecting genes or whole chromosomes [74,75].

Comprehensive reviews summarising the adaptive effects of exercise on antioxidant defences can be found elsewhere [3,9,76,77]. These adaptations could partly account for improved resistance of trained athletes to DNA damage.

In this context, the concept of hormesis provides a tempting hypothesis to explain the protective effects of exercise. Hormesis is characterised as a dose–response phenomenon, where a low dose of a substance or environmental factor stimulates adaptation whereas a high dose tends to inhibit adaptation [78]. Thus, during exercise low levels of ROS formation can stimulate adaptive mechanisms, i.e. expression of antioxidant enzymes, which can lead to decreased oxidative damage [11]. In fact, ROS are important components of signaling pathways in cells. In a recent review, Ji [76] described potential pathways, which can lead to exercise-induced up-regulation of endogenous antioxidant enzymes, such as mitochondrial manganese-dependent superoxide dismutase (MnSOD). Up-regulation of antioxidant defences can be induced by the activation of a nuclear factor κB (NFκB) binding site (via free radicals) and subsequently, the NFκB signaling pathway [11,76,79].

In addition to up-regulation of endogenous antioxidant enzymes, altered expression of genes and the up-regulation of stress proteins, i.e. 70 kDa heat shock protein 70, are suggested to be involved in adaptive responses to ROS following exercise [9].

There is growing evidence that free radicals mediate the up-regulation of DNA repair enzyme activity [3,80]. Increased activity of the DNA repair enzyme oxoguanine DNA glycosylase (OGGl) was found in biopsy samples from six subjects running a marathon [80].

The elimination of cells with oxidative DNA damage through apoptosis could also play a role in preventing primary and persistent oxidative DNA damage after exercise. However, results from studies on the effect of exercise on apoptosis are controversial. While the number of apoptotic cells decrease after an Ironman triathlon [50], a marathon [81] or remained unchanged after moderate exercise on a cycle ergometer [82] and 2.5 h treadmill running [83], increased numbers of apoptotic cells were detected immediately following exhaustive treadmill running [68,84] and exhaustive cycling ergometry [73]. To the best of our

knowledge, only three of the reviewed studies [50,51,68,73] investigated both, apoptosis and DNA instability, after exercise. Although the levels of DNA strand breaks in the SCGE assay increased after 1 day, and the frequency of ENDO III-sensitive sites (oxidized pyrimidines) increased 5 days after an Ironman triathlon, the number of apoptotic cells in the CBMN assay decreased immediately after the race and remained at a low level thereafter [50]. However, after an exhaustive cycle ergometer test, no induction of MNi but increased levels of apoptotic cells were found [73]. In an investigation conducted by Mars et al. [68] DNA damage in 10% of lymphocytes (classified by visual inspection) and increase in apoptotic cells were detected after a treadmill test until exhaustion. However, the TdT-mediated dUTP-nick end labelling (TUNEL) method was applied within the two latter investigations, which is not exclusively specific for apoptotic cells [81].

An additional physiological response after exercise is an increase in the plasma antioxidant capacity after exercise [50,55,85,86] caused by the intake of antioxidants including vitamin C and alpha-tocopherol during the race, tissue mobilization of these vitamins [14,87,88], and/or because of increased endogenous synthesis of the hydrophilic antioxidant uric acid via the metabolism of purines, by xanthine oxidase, during intense exercise [85,87].

## 7. Clinical implications

It is well documented that regular moderate physical activity is associated with various health benefits including decreased risk of cardiovascular diseases, diabetes, cancer and other lifestyle-dependent diseases [2,3,5,89]. In general, the effects of training on the molecular biology of skeletal muscle are sufficiently documented [79]. However, the effects of exercise on DNA stability in subjects of varying training status have not yet been identified.

Umegaki et al. [67] found increased X-ray-induced chromosomal damage after 30 min of treadmill running at 85% of maximal oxygen uptake in untrained compared to trained subjects. In addition, greater DNA migration levels in the SCGE assay were detected in five untrained compared to six trained subjects 24 h after treadmill running until exhaustion [69]. When further dividing the total group into two subgroups (cut off point: $VO_2$ peak value of 60 mL/(kg min)), Reichhold et al. [51] found that chromosomal damage after completing an Ironman triathlon tended to be higher in well trained subjects than in the highly trained group. A study conducted by Pittaluga et al. [73] showed that athletes had higher levels of spontaneous chromosomal damage in lymphocytes at rest than the untrained subjects, yet this value remained unchanged, in the athletes, after an exhaustive cycle-ergometer test. In contrast, basal levels of 8-OHdG have been found to be significantly lower in physical active compared to sedentary individuals [63].

Training status may positively influence DNA stability because of the adaptive effects of exercise, as previously discussed. Although it has been hypothesised that this relationship is U-shaped [14], i.e. both long-duration and very intense exercise or physical inactivity over a long-period of time could increase DNA modifications, the exact dose–response relationship between physical activity and DNA stability requires further investigation.

In particular, in regard to the prevalence of breast and colon cancer, regular physical activity reduces evidence-based the risk of developing these diseases [90,91]. In a recently published cohort study, Bonassi et al. [28] showed that the frequency of MNi is associated with incidence of cancer. Therefore, if further evidence can strengthen the link between exercise and reduced MNi counts [51], arguments for a beneficial effect of exercise on cancer prevalence would exist. Furthermore, data reported by this group would also suggest that participation in ultra-endurance triathlon by trained people does not increase MNi counts and therefore cancer risk [28,51].

## 8. Future directions

The exact mechanism by which physical exercise influences DNA stability requires further investigation. A study design that combines the measurement of DNA damage, gene expression and DNA repair mechanisms before, during and after exercise would clarify the mechanisms that maintain DNA stability in response to vigorous exercise. Furthermore, to address long-term consequences on DNA stability, future studies investigating an older population of former athletes or competitors will be needed. In addition it would be essential to include a control group in all investigations to reduce the likelihood of detecting effects that are unrelated to the prescribed exercise.

## Conflict of interest

None.

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# Curriculum Vitae, Oliver Neubauer

Date and place of birth: March, 2nd 1976 in Neunkirchen, Lower Austria

## Educational History

1986–1994: Realgymnasium Neunkirchen and BORG/Theresian Military Academy Wr. Neustadt

1994–1995: Military Service

1995–1996: Montanuniversität Leoben

1996–1997: Officer training and successful completion of the entrance exam for the Theresian Military Academy Wiener Neustadt

1997–2004: M.A. program in Nutritional Sciences at the University of Vienna

Passed final examination cum laude; completed master's thesis entitled *The Importance of Quality and Quantity of Fat Intake in Endurance Sports,* Specialization in sports nutrition and sports physiology

## Professional Training History and Additional Education

2001: Internships at the *Institute for Medical and Sports Scientific Consulting* (*IMSB*), Olympic Centre Südstadt, Lower Austria

2002: Internship at the Rehabilitation Centre for Diabetes mellitus and Metabolic Disorders, Alland, Lower Austria

2003: Internship and subsequent project work at the *IOC*-accredited Anti-Doping-Laboratory, *Austrian Research Centers* (*ARC*) Seibersdorf

2004: Junior Instructor ("Lehrwart") for Triathlon, *Sports Academy Innsbruck*

## Selected Professional Activities

2002–2006: Free-lance assistant at the *Centre for Sports Medical Examination and Consulting (SUB)* at the Hospital Wiener Neustadt

(main tasks: ergometry tests, consulting)

2003–2005: Project work for the *IMSB* Olympic Centre Südstadt, Lower Austria (main tasks: exercise tests, consulting at PR and public health events)

Since 2005: Freelance writer and member of the expert committee on nutritional aspects in endurance sports for *Triathlon* magazine (*spomedis GmbH*, Hamburg, Germany)

2004–2005: Co-authorship of the project application for the research project *Risk Assessment of Ironman Triathletes* (funded by the *Austrian Science Fund (FWF)* in Nov. 2005)

2006–2008: Scientific assistant and doctoral student at the Department of Nutritional Sciences, University of Vienna within the framework of the *FWF* project *Risk Assessment of Ironman Triathletes: Genome Stability, Oxidative, Muscular and Systemic Stress*

main tasks: co-project-management, chemical analysis, data analysis and interpretation, publication and public dissemination of the study results

Feb. 2008-present: Scientific assistant, tutor and doctoral student at the Department of Nutritional Sciences, University of Vienna (PhD thesis *Acute Stress and Recovery Responses to an Ironman Triathlon: Oxidative Stress, Antioxidant and Inflammatory Changes and their Relevance for DNA Stability*)
main tasks: writing publications and a further project application, chemical and data analysis, supervising of master students

Dec. 2008*:* Reviewer for the *Amercian Journal of Physiology*

## Additional Skills

Languages: English (fluent), Russian (basic knowledge); Computer skills: *Excel, Word, PowerPoint, SPSS, Endnote*

## Selected Further Interests

Endurance sports (four *Ironman* triathlon races), literature, music, artwork, travelling,…

Vienna, May 5th 2009 Oliver Neubauer

# List of publications

## Original articles in international peer-reviewed journals

**König D, Neubauer O, Nics L, Kern N, Berg A, Bisse E, Wagner K-H** (2007). Biomarkers of exercise-induced myocardial stress in relation to inflammatory and oxidative stress. *Exerc Immunol Rev*. 13:15-36 (IF[1] = 2.9)

**Neubauer O, König D, Wagner K-H** (2008)**.** Recovery after an Ironman Triathlon: Sustained Inflammatory Responses and Muscular Stress. *Eur J Appl Physiol*. 104 (3): 417-426 (IF = 1.8)

**Neubauer O, König D, Kern N, Nics L, Wagner K-H** (2008). No Indications of Persistent Oxidative Stress in Response to an Ironman Triathlon. *Med Sci Sports Exerc*. 40 (12):2119-2128 (IF = 2.9)

**Reichhold S, Neubauer O, Ehrlich V, Knasmüller S, Wagner K-H** (2008). No acute and persistent DNA damage after an Ironman Triathlon. *Cancer Epidemiol Biomarkers Prev.* 17: 1913-1919 (IF = 4.6)

**Neubauer O, Reichhold S, Nersesyan A, König D, Wagner K-H** (2008). Exercise-induced DNA damage: Is there a relationship with inflammatory responses? *Exerc Immunol Rev.* 14:51-72 (IF = 4.4, first rank in subject category *sport sciences*)

**Reichhold S, Neubauer O, Hölzl C, Stadlmayr B, Valentini J, Ferk F, Kundi M, Knasmüller S, Wagner K-H**. Oxidative DNA damage in response to an Ironman Triathlon. *Free Radical Res.*, submitted

**Reichhold S, Neubauer O, Bulmer AC, Knasmüller S, Wagner K-H** (2009). Endurance exercise and DNA stability: Is there a link to duration and intensity? *Mutat Res Rev*. (IF = 4.5), in press

## Articles in popular-scientific journals

**Neubauer O** (2005). Erfolg geht durch den Magen. *Triathlon*[2] 37:54-58 (about nutritional aspects before, during and after triathlon races)

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[1] IF = Impact Factor (in the year of publication) according the *Journal Citation Reports* of *ISI Web of Knowledge*

[2] *Triathlon* and *Triathlon Training* (published by spomedis GmbH, Hamburg, Germany) are Europes´ largest triathlon magazines

**Neubauer O** (2005). Der Wachmacher–Reizstoff Koffein. *Triathlon* 38:54-56 (on the physiological, metabolic and performance-enhancing effects of caffeine)

**Neubauer O** (2005). Regenerationsspezialisten–Aminosäuren sind mehr als Eiweißbausteine. *Triathlon* 39:56-58 (about the relevance of certain amino acids for recovery)

**Neubauer O** (2006). Zur Hölle mit den Freien Radikalen. *Triathlon* 43:82-83 (on reactive oxygen species, oxidative stress and antioxidants in the context with exercise)

**Neubauer O** (2006). Kohlenhydrate–Die Energiespezialisten. *Triathlon* 44:98-99 (an update on the guidelines for the carbohydrate intake for endurance athletes)

**Neubauer O** (2006). Fette–Treibstoff für die Langzeitausdauer. *Triathlon* 45:98-99 (on the importance of the adaquate quantity and quality of lipids for endurance athletes)

**Neubauer O** (2006). Eisenmänner als ´Modelle´ für Stressresistenz. *Triathlon Sonderausgabe Ironman Austria*:38 (a general introduction of the *FWF*-funded research project conducted at the Ironman Austria 2006)

**Neubauer O** (2007). Eiserner Widerstand. *Triathlon Training* 4:59-61 (first general study results regarding certain stress responses after an Ironman race and regulatory mechanisms)

**Neubauer O** (2008). Wettlauf der Kohlenhydrate. *Triathlon Training* 6:86-89 (about the specific effects of different carbohydrate types in race nutrition)

**Neubauer O, Reichhold S, Wagner K-H** (2008). Biochemische, physiologische und molekularbiologische Stressreaktionen nach einem Ironman-Triathlon. *Ernährung aktuell* 3:1-4 (brief summary on the main findings of the *FWF*-funded research project conducted at the Ironman Austria 2006)

## Presentations on scientific meetings (with abstracts)

**Neubauer O, Kern N, Nics L, Wagner K-H** (2007). How Ironman Triathletes Balance Oxidative Stress. *12th Ann. Congress of the European College of Sport Science*, July 11th – 14th 2007, Jyväskylä, Finland; oral presentation, abstract: Book of Abstracts

**Reichhold S, Neubauer O, Wagner K-H** (2007). Does an Ironman Triathlon induce DNA damage? *12th Ann. Congress of the European College of Sport Science*, July 11th – 14th 2007, Jyväskylä, Finland; poster presentation, abstract: Book of Abstracts

**Reichhold S, Meisel M, Neubauer O, Wagner K-H** (2007**).** Influence of an Ironman Triathlon on Sister Chromatid Exchanges and High Frequency Cells, *3rd International Symposium of the Human Nutrition & Metabolism Research & Training Center*, Oct. 15th – 18th 2007, Graz, Austria; poster presentation, abstract: Congress proceedings

**Wagner K-H, Reichhold S, Neubauer O** (2007). How Ironman Triathletes Balance Oxidative Stress and Genomic Response. University of Kiel, Department of Human Nutrition and Food Science. Oct. 22nd 2007, Kiel, Germany; oral lecture

**Reichhold S, Meisel M, Neubauer O, Wagner K-H** (2007). Influence of an Ironman Triathlon on Sister Chromatid Exchanges and High Frequency Cells. *13th Scientific Symposium of the Austrian Pharmacological Society*, Nov. 22nd – 24th 2007, Vienna, Austria

**Neubauer O, Kern N, Nics L, Reichhold S, Wagner K-H** (2008). Oxidative stress and antioxidant responses after an Ironman triathlon. International conference *Oxidative stress in diseases*, Apr. 24th 2008, Bratislava, Slovakia; oral presentation, abstract: Book of Abstracts

**Reichhold S, Neubauer O, Ehrlich V, Hölzl C, Knasmüller S, Wagner K-H** (2008). DNA responses after an Ironman triathlon. International conference *Oxidative stress in diseases*, Apr. 23rd 2008, Bratislava, Slovakia; oral presentation, abstract: Book of Abstract

**Neubauer O, Kern N, Nics L, Reichhold S, Wagner K-H** (2008). Enhanced Antioxidant Capacity after an Ironman Triathlon. *1st symposium of the Vienna Research Platform of Nutritional and Food Sciences*, Apr. 25th 2008, University of Vienna, Austria; poster presentation, abstract: *Annals of Nutrition and Metabolism* 52 (2008):130

**Neubauer O, König D, Reichhold S, Wagner K-H** (2008). Recovery Responses in Ironman Triathletes. *1$^{st}$ symposium of the Vienna Research Platform of Nutritional and Food Sciences*, Apr. 25$^{th}$ 2008, University of Vienna, Austria; poster presentation

**Reichhold S, Neubauer O, Wagner K-H** (2008)**.** Effects of an Ironman Triathlon on DNA Stability. *1$^{st}$ symposium of the Vienna Research Platform of Nutritional and Food Sciences*, Apr. 25$^{th}$ 2008, University of Vienna, Austria; poster presentation, abstract: *Annals of Nutrition and Metabolism* 52 (2008):131

**Neubauer O, König D, Wagner K-H** (2008). Recovery Responses in Ironman Triathletes, *13$^{th}$ Ann. Congress of the European College of Sport Science*, July 9$^{th}$ – 12$^{th}$ 2008, Estoril, Portugal; oral presentation, abstract: Book of Abstracts

**Reichhold S, Neubauer O, Wagner K-H** (2008). Effects of an Ironman Triathlon on the DNA as detected by the SCGE and CBMN Cyt assay, *13$^{th}$ Ann. Congress of the European College of Sport Science*, July 9$^{th}$ – 12$^{th}$ 2008, Estoril, Portugal; poster presentation, abstract: Book of Abstracts

**Wagner K-H, Kern N, Nics L, Reichhold S, Neubauer O** (2008). Oxidative Stress and Antioxidant Responses after an Ironman triathlon, *Society of Free Radical Research-Europe Meeting,* July 5$^{th}$ – 9$^{th}$ 2008, Berlin, Germany; abstract: *Free Radical Res.* 42(1) 2008: 38 S5-2

**Neubauer O, Kern N, Nics L, Reichhold S, Wagner K-H** (2008)**.** Oxidative Balance in Ironman Triathletes, *Society of Free Radical Research-Europe Free Radical Summer School*, Aug. 30$^{th}$ – Sep. 5$^{th}$ 2008, Spetses, Greece; poster presentation, abstract: abstract booklet

**Neubauer O, Reichhold S, Knasmüller S, König D, Wagner K-H** (2009). Exercise and DNA damage: Significance of Inflammatory and Antioxidant Responses, *2$^{nd}$ Copenhagen Workshop on DNA Oxidation*, Jan. 29$^{th}$ – 30$^{th}$ 2009, University of Copenhagen, Denmark; poster presentation

**Neubauer O, Reichhold S, König D, Knasmüller S, Wagner K-H** (2009): Consequences of Inflammatory and Antioxidant Responses on DNA Damage after Ultra-endurance Exercise, *24th Meeting of the Gesellschaft für Umwelt-Mutationsforschung (GUM; German section of the European Enviromental Mutagen Society),* Feb. 17th – 20th 2009, University of Vienna, oral presentation, abstract: abstract booklet

**Neubauer O. Reichhold S, König D, Knasmüller S, Wagner K-H**. Ultra-endurance exercise and DNA Stability: Role of Inflammatory and Antioxidant Responses, *14th Ann. Congress of the European College of Sport Science*, June 24th – 27th 2009, Oslo, Norway; accepted and allocated as oral presentation

## Presentations (without abstracts)

**Neubauer O** (2006): Basisernährung, Wettkampfernährung und Supplemente im Triathlon. Triathlon-Workshop, Nov. 12th – 13th 2006, Gesundheitszentrum Bad Sauerbrunn, Austria, oral lectures on nutrition, race nutrition and supplementation in triathlon

**Neubauer O, Reichhold S, Wagner K-H** (2007)**.** Wie übersteht der Körper der Körper einen Ironman-Triathlon? Einblicke in die Stressbewältigungsmechanismen von Ausdauersportlern. *University Meets Public,* Nov. 11th 2007*,* Volkshochschule Landstraße, Vienna, Austria*,* oral lecture on "how Ironman triathletes balance the exercise-induced stress responses"

Vienna, May 5th 2009 Oliver Neubauer