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ACUTE IMPACT OF EXERCISE ON PRO-INFLAMMATORY MOLECULE CONCENTRATIONS IN BLOOD: FACTORS THAT AFFECT EXERCISE INDUCED RESPONSE

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Annotation

The aim of the present thesis was to investigate the impact of intensity and duration, as well as the level of training status and immobilization on exercise-induced cytokine and other signal molecule concentrations during exercise. In total 98 young, healthy and active males did four different types of exercise. Blood samples for determination of cytokines and other signal molecules were drawn before, during and after exercise intervention. The results suggest that exercise elicits an increase in concentrations of pro-inflammatory molecules. This response becomes more pronounced with increased intensity and duration of exercise, but diminishes due to improved training status. Depending on type, intensity and duration of physical load cytokine interleukin-6 exerts not only pro-inflammatory effects, but during exercise it also mediates glucose homeostasis.

Key words: pro-inflammatory molecules; acute, systemic response to exercise; influencing factors

Content

TEXT ABBREVIATIONS **6**

1. INTRODUCTION **8**

1.1. AIM 8

1.1.1. Assignments *9*

1.2. HYPOTHESIS 9

2. BACKGROUND **10**

2.1. INTERLEUKIN-6 (IL-6): GENERAL DESCRIPTION 10

2.1.1. IL-6 - an immunomodulatory cytokine *12*

2.2. IL-6 AND EXERCISE 14

2.2.1. IL-6 – a myokine? *15*

2.3. INTENSITY AND DURATION 18

2.4. TRAINING STATUS AND PERFORMANCE 19

2.5. IL-6 ANTI-INFLAMMATORY PROPERTIES 21

2.6. TNF-A 22

2.7. ADHESION MOLECULES 24

2.8. MMP-9 26

3. METHODS **28**

3.1. ETHICAL APPROVAL 28

3.2. STUDIES 28

3.3. SUBJECTS 28

3.4. EXERCISE MODE AND BLOOD SAMPLING 29

3.4.1. Study I *30*

3.4.2. Study II *31*

3.4.3. Study III *31*

3.4.4. Study IV *32*

3.4.4.1. Pre-study 32

3.4.4.2. Experimental protocol 33

3.5. CYTOKINE AND OTHER BLOOD BIOMARKER DETECTION 33

3.5.1. Studies I, II, III *33*

3.5.1.1. Luminex 200 analyzer 34

3.5.1.2. Other blood biomarkers measurements 36

3.5.2. Study IV *36*

3.6. CALCULATIONS 36

3.7. STATISTICAL ANALYSIS 37

4. RESULTS **38**

4.1. STUDY I 38

4.2. STUDY II 43

4.2.1. Sera IL-6, TNF-α, sICAM-1, MMP-9 concentrations *45*

4.3. STUDY III 48

4.4. STUDY IV 51

5. DISCUSSION 56

5.1. STUDY I 56

5.1.1. MMP-9 57

5.2. STUDY II 58

5.2.1. IL-6 59

5.2.2. TNF-α 60

5.2.3. sICAM-1 61

5.2.4. MMP-9 62

5.3. STUDY III 63

5.4. STUDY IV 65

5.5. OVERALL DISCUSSION 67

5.5.1. Interleukin-6 67

5.5.2. TNF-α 68

5.5.3. Adhesion molecules and MMP-9 69

LIMITATIONS 71

CONCLUSIONS 72

STUDY I 72

STUDY II 72

STUDY III 72

STUDY IV 73

OVERALL CONCLUSIONS 73

THESIS 74

ACKNOWLEDGMENTS 75

REFERENCES 76

APPROBATION OF RESULTS 91

PUBLICATIONS 91

CONFERENCES 92

SCIENTIFIC PROJECTS 93

APPENDICES 94

QUESTIONNAIRES FOR PARTICIPANTS 94

Study I and III 94

Study II 97

Study IV 101

ETHICS STATEMENT 106

Study I, II, III 106

Study IV 107

YOUNG INVESTIGATORS AWARD - CERTIFICATE 109

Text abbreviations

A	arterial concentration (Study IV)
ACTH	adrenocorticotropic hormone
AMPK	adenosine monophosphate activated protein kinase
BMI	body mass index
C	healthy active males (Study III)
CHO	carbohydrates
CK	creatine kinase
CON	control leg (Study IV)
CRP	C-reactive protein
ERK	extracellular signal-regulated kinase
E-selectin	endothelial-leukocyte adhesion molecule-1
gp130	glycoprotein 130
HM	half marathon runners (Study II)
HRR	heart rate reserve
HT	highly trained athletes (Study III)
ICAM-1	intercellular adhesion molecule-1
IL-1ra	interleukin-1 receptor antagonist
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
IM	immobilized leg (Study IV)
JAK	Janus kinase
kDa	kilo Dalton
LDH	lactate dehydrogenase
M	marathon runners (Study II)
MCP-1	monocyte chemotactic protein-1
MMPs	matrix metalloproteinases
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MT	moderately trained athletes (Study III)
PI3K	phospoinositide-3 kinase
PLA	placebo
rhIL-6	recombinant human interleukin-6

SD	standard deviation
SEM	standard error of the mean
sIL-6R	soluble interleukin-6 receptor
STAT	Signal Transducer and Activator of Transcription
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule-1
VO_{2max}	maximal oxygen consumption
W_{max}	maximal watts

1. Introduction

Nowadays it is possible to investigate a lot of different physiological functions of the body due to the development of innovative technologies, thus promoting new discoveries also in the field of sport science.

Physical training is effective in the prevention and treatment of several chronic diseases, e.g., type 2 diabetes, arthritis, cardiovascular diseases, which are characterized by a constant low-grade systemic inflammation (increased plasma concentrations of cytokines like interleukin-6 (IL-6) and tumor necrosis factor- α (TNF-α)). This is remarkable given the fact that acute exercise may actually increase the pro-inflammatory cytokines. However, the source of exercise induced increase in systemic levels of the cytokines is debated, as they are produced by many different cell types in the body (e.g., white blood cells, adipocytes, epithelial cells and others). Also, the impact of such factors as intensity and duration of exercise, form of contraction (concentric vs. eccentric), level of training status and bioavailability of carbohydrates on exercise-induced plasma cytokine changes needs to be elucidated.

As the levels of cytokines increase with exercise, skeletal muscle may be a prime candidate for cytokine production, but the evidence is not clear. Skeletal muscle is the largest organ in the human body, and the discovery of working muscle as a cytokine producing organ opens for a whole new paradigm: Skeletal muscle is an endocrine organ which by contraction can influence metabolism in distinct organs and modify cytokine production by other organs. More detailed knowledge about contraction-induced cytokine production and release could lead to a better understanding of physiological adaptations to exercise, and in future may be potential targets for new drugs designed to mimic exercise-induced effects within skeletal muscle.

1.1. Aim

The primary aim of the present thesis was to investigate the impact of intensity and duration, as well as the level of training status and immobilization on exercise-induced serum/plasma cytokine (IL-6, TNF-a) and other signal molecule (e.g. adhesion molecules and matrix metalloproteinases (MMPs)) concentrations during exercise.

Secondly, the study was aimed to evaluate the possible source of exercise-induced serum/plasma IL-6 using indirect methods, e.g. correlations with myeloperoxidase (MPO) – the marker of neutrophils degranulation.

1.1.1. Assignments

1. To evaluate the impact of exercise intensity on serum IL-6, TNF-α and other signal molecule concentrations using an incremental bicycle test until exhaustion.
2. To evaluate the impact of exercise duration on serum IL-6, TNF-α and other signal molecule concentrations using prolonged running with different time, but the same absolute intensity.
3. To evaluate the impact of training status on serum IL-6, TNF-α and other signal molecule concentrations using a sub-maximal recumbent cycling exercise for 1h.
4. To evaluate the impact of 2 week's immobilization of one leg on plasma IL-6 and TNF-α release from working legs using the two leg knee-extensor exercise for 45 minutes.
5. To evaluate the possible source of serum/plasma IL-6 in all previously mentioned experimental models.

1.2. Hypothesis

Based on the knowledge that IL-6 release depends on two major factors, i.e., muscle damage with a subsequent inflammatory response and carbohydrate bioavailability during exercise, it is hypothesized that both intensity and duration would result in higher concentrations of cytokines and downstream target molecules (e.g. adhesion molecules and MMP-9). Furthermore, improved training status, thus also adaptation to exercise (i.e., reduced risk of muscle damage and more efficient use of energy sources) would result in an attenuated cytokine and downstream target molecule response to an acute single bout of exercise.

2. Background

2.1. Interleukin-6 (IL-6): general description

Cytokines are pleiotropic, low-molecular-weight proteins and peptides that can be produced by virtually every nucleated cell in the body in response to physiological stress. Cytokines can be classified according to the type of receptor that they engage, e.g. class I cytokine receptors, class II cytokine receptors, tumor necrosis factor (TNF) receptors, interleukin-1 receptors, tyrosine kinase receptors and chemokine (cytokines that induce directed chemotaxis in nearby responsive cells) receptors. One of the most known cytokines, IL-6, belongs to the glycoprotein 130 (gp130) family – the subclass of class I cytokine receptors (Wang et al. 2009). IL-6 (which is a glycosylated protein of 21-28 kDa) is a multifunctional cytokine (Figure 1) that plays a central role in host defence due to its wide range of immune (e.g. differentiation factor for B and T cells, macrophages) and hematopoietic (e.g. formation of multi-lineage blast cell colonies, induction of platelet production) functions and its potent ability to induce acute phase response – enhancing production of acute phase proteins, mainly during early course of an infection (Nishimoto et al. 2000).

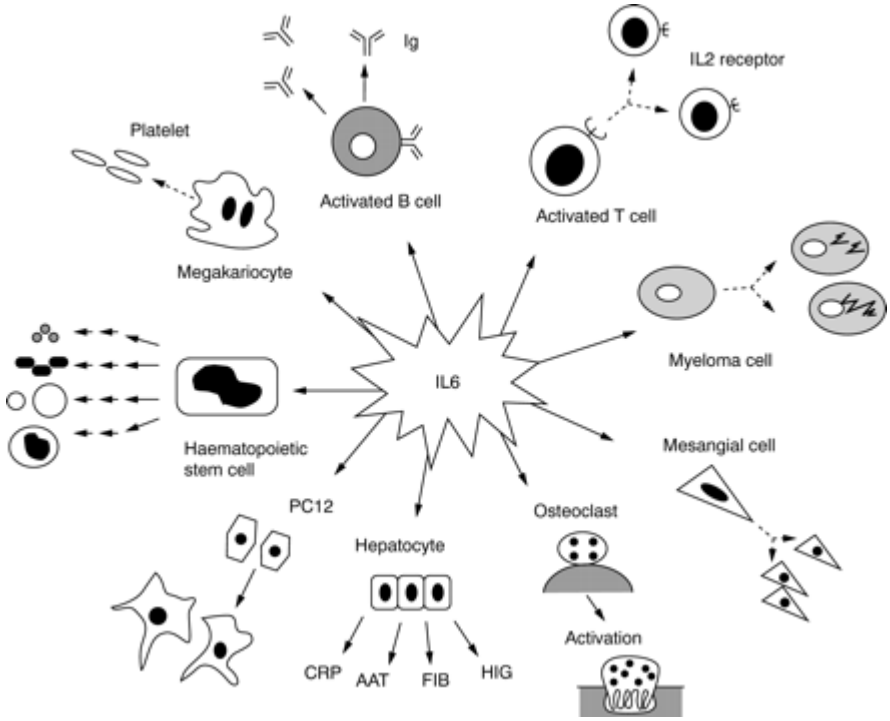

Figure 1. Schematic model of IL6 functions. IL6 is a pleiotropic cytokine with a wide range of biological activities. (Nishimoto et al. 2000)

The biological activities of IL-6 are mediated by the IL-6 receptor-system (Figure 2), which comprises two membrane proteins, the ligand-binding α-subunit receptor (IL-6R) and the signal transducing β subunit, gp130 (Kishimoto et al. 1995). The IL-6R exists both in a membrane-bound and soluble form (sIL-6R). Interestingly, only a few cell types express IL-6R on the cell surface and therefore respond to IL-6 alone (*classic-signaling*). Such cells are macrophages, neutrophils, some types of T-cells and hepatocytes (Scheller et al. 2011). sIL-6R may be generated by proteolytic shedding of membrane-bound IL-6R (Jones et al. 2001) or from alternatively spliced mRNA species (<1% of total sIL-6R at rest and after exercise) (Leggate et al. 2010). IL-6 and sIL-6R form binary complex that binds to two molecules of gp130 and leads to IL-6 transduction, which includes activation of JAK/STAT, ERK and PI3K signal transduction pathways. This process is called *trans-signaling*. All cells display gp130 on the cell surface, thus IL-6/sIL-6R complex widens the range of cell types that may respond to IL-6 (Scheller et al. 2011).

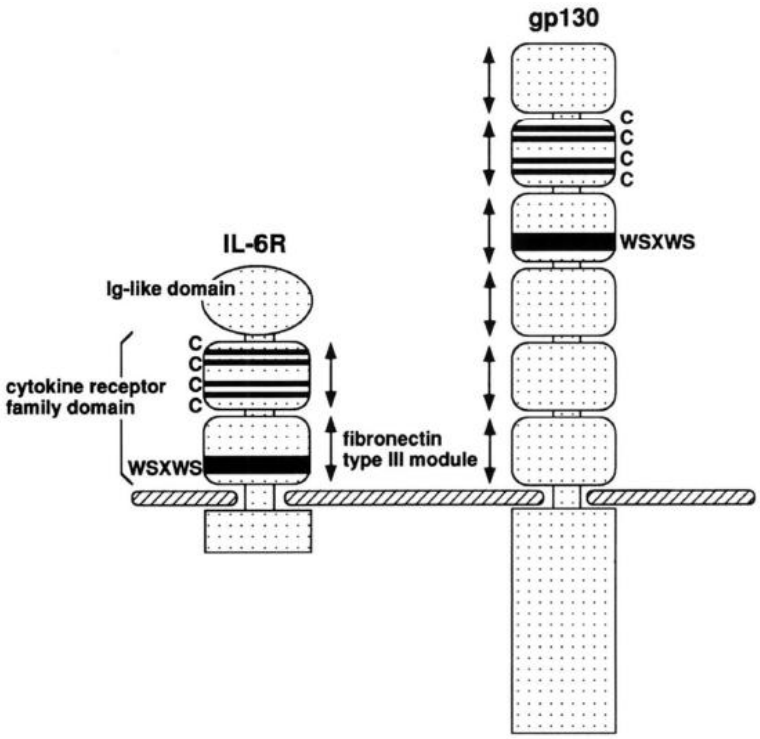

Figure 2. Schematic structure of IL-6R and gp130. The extracellular region of IL-6R consists of an Ig-like domain and cytokine receptor domain and a cytokine receptor domain, the latter of which is composed of two fibronectin type 111 modules. The extracellular region of gp130 consists of six fibronectin type111 modules, the second and the third of which comprise the cytokine receptor family domain. C, conserved cysteine residues. WSXWS, conserved motif. (Simpson et al. 1997)

2.1.1. IL-6 - an immunomodulatory cytokine

The acute phase response (APR) is a generalized response of the organism to multiple disturbances of its physiological homeostasis. Infection, tissue injury, neoplastic growth, or immunological disorders are the main stimuli for the initiation of APR. Many different cells, including neutrophils, lymphocytes, fibroblasts, endothelial cells, but mostly monocytes and macrophages at the site of inflammation produce cytokines IL-6, IL-1, and TNF-α. (Heinrich et al. 1990) IL-6 is considered to be the major inducer of acute phase proteins (APP) gene expression, since it, either alone or by enhancing the effects of other cytokines, induces virtually all APPs (Prowse and Baumann 1989; Gervois et al. 2004). IL-6 increases the production of positive APPs, e.g. C-reactive protein (CPR), serum amyloid A, fibrinogen (Figure 3), but reduces levels of negative APPs, e.g. serum albumin and serum transferrin (Visser et al. 2010).

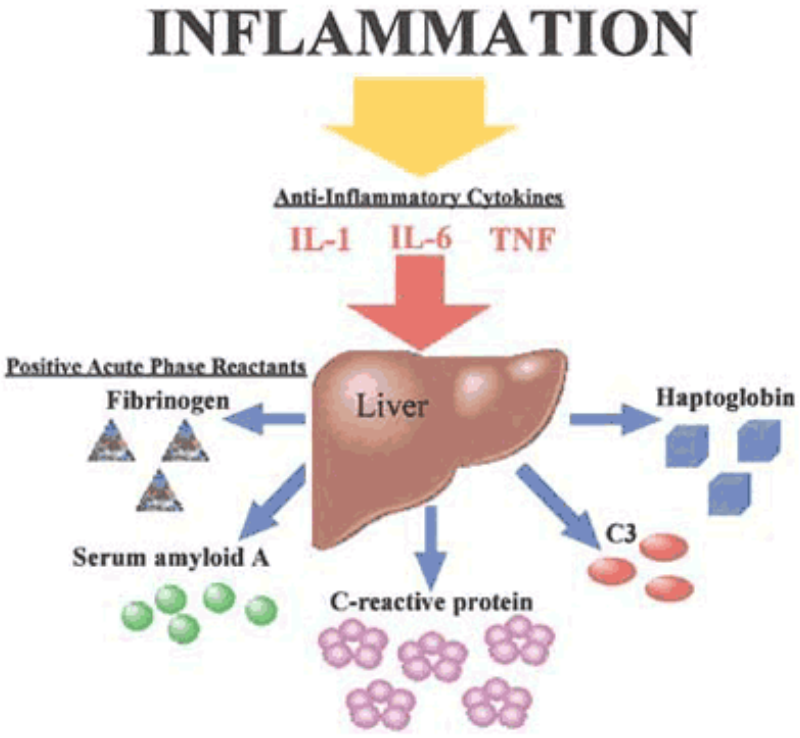

Figure 3. Stimulation and synthesis of positive acute-phase reactants during inflammation. Inflammation caused by infection or tissue damage stimulates the circulating inflammation-associated cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α. These cytokines stimulate hepatocytes to increase the synthesis and release of positive acute-phase proteins, including CRP. IL-6 is the major cytokine stimulus for CRP production. (Hengst 2003)

In order to cope with the limited amounts of amino acids for protein biosynthesis, the liver gives priority to the synthesis of positive APPs ensuring generation of inflammatory response. Increased circulating levels of IL-6 and positive APPs also have been reported in a number of chronic pathophysiological conditions characterized with low grade inflammation, e.g. athersoclerosis (Rader 2000), rheumatoid arthritis (Pablos Alvarez 2009) and diabetes (Choudhary and Ahlawat 2008).

An immune response to great extent depends on the ability of leukocytes to migrate from the blood into sites of inflammation. Upregulation of adhesion molecules on the surface of leukocytes and endothelial cells is prominent when they are exposed to proinflammatory molecules (Monchanin et al. 2007; Karatzis 2005; Giavazzi et al. 1992). Particularly, IL-6 *trans-signaling* leads to increased expression of intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin on endothelial cells, as well as L-selectin on lymphocytes (Chen et al. 2004; Chen et al. 2006a; Kaplanski et al. 2003). Furtermore, IL-6 increases the production of matrix metalloproteinases (MMP-s) (Aida et al. 2012; Huang et al. 2010; Sundararaj et al. 2009; Dasu et al. 2003) – a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for the tissue remodelling and degradation of the extracellular matrix (Verma and Hansch 2007).

Besides pro-inflammatory properties, IL-6 also has anti-inflammatory effects, e.g. intestinal epithelial cell proliferation and inhibition of epithelial cell apoptosis (Dann et al. 2008; Grivennikov et al. 2009), that mostly are regulated by *classical-signaling* (Scheller et al. 2011) (Figure 4). In addition, animal (Mizuhara et al. 1994) and human (Starkie et al. 2003) studies have shown that recombinant human IL-6 infusion inhibits TNF-α production.

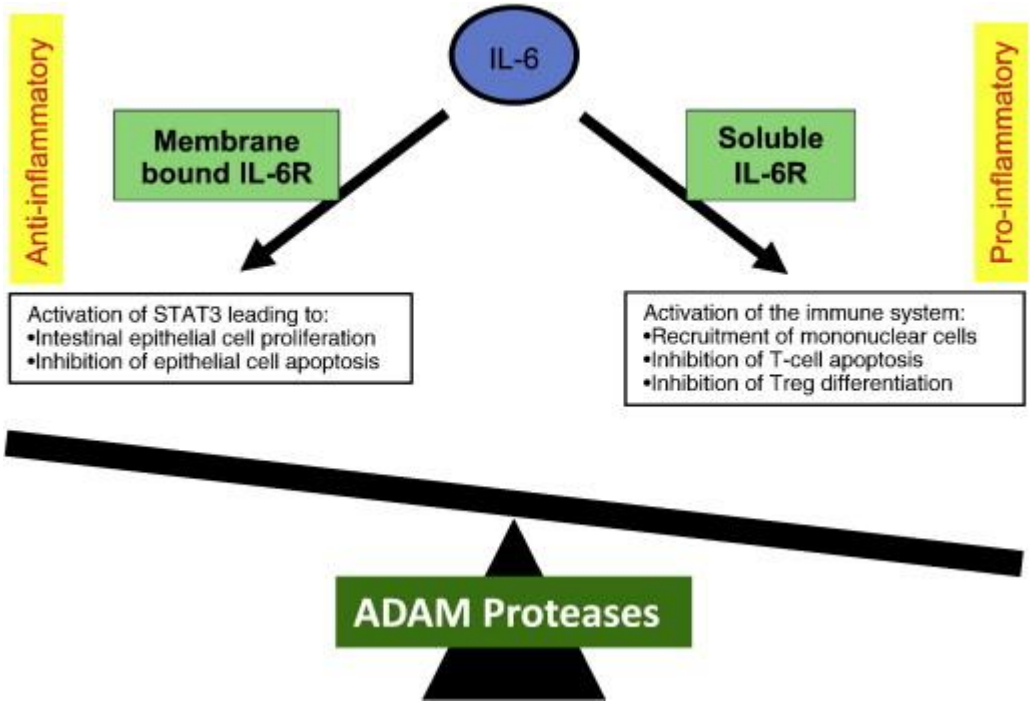

Figure 4. Pro- and anti-inflammatory properties of IL-6. IL-6 is a pleiotropic cytokine with pro- and anti-inflammatory properties. In several mouse models, it was shown that IL-6 classic signaling mediates the activation of anti-inflammatory pathways on target cells. In a murine colon cancer model, IL-6 classic signaling is essential for the activation of STAT3-mediated signaling pathways which induce the regeneration of intestinal epithelial cells after DSS induced damage. In contrast, IL-6 trans-signaling is observed in chronic inflammatory disorders like Crohn's disease and rheumatoid arthritis. Trans-signaling leads to activation of the immune system by the recruitment of monocytes to the inflamed area. Trans-signaling on T-cells leads to inhibition of apoptosis, inhibition of Treg differentiation, and differentiation of TH17 cells.

2.2. IL-6 and exercise

The first report that acute exercise increased plasma IL-6 concentration was published in early nineties (Northoff and Berg 1991). IL-6 was originally identified as immunomodulatory cytokine that stimulates or inhibits differentiation, proliferation or function of immune cells (Nishimoto et al. 2000). It was suggested that IL-6 is produced predominantly by leukocytes as a response to exercise-induced local damage in the working muscles, especially after eccentric exercise (Bruunsgaard et al. 1997). In addition, the inflammatory response observed after exercise-induced muscle damage is similar to that seen after trauma (Ostrowski et al. 1998). However, the role of IL-6 in the inflammatory response to exercise-induced muscle damage was questioned, because eccentric exercise induced IL-6 concentrations in the recovery phase returned to rest values, while plasma creatine kinase (CK) and myoglobin

concentrations remained significantly elevated (Toft et al. 2002; Peake et al. 2005). Secondly, inflammatory response to exercise-induced muscle damage should result in increased adhesion molecule concentrations after exercise or in recovery (Nielsen and Lyberg 2004; Akimoto et al. 2002), in order to promote leukocytes migration from the blood into the sites of inflammation. As previously mentioned, IL-6 stimulates expression of adhesion molecules on leukocyte and endothelial cells. Thus, if the main role of exercise-induced increase in IL-6 would be the modulation of inflammatory response, one could expect that there would be also an increased expression of adhesion molecules. However, previous studies have shown that despite eccentric (Smith et al. 2000) or maximal (Chaar et al. 2011) exercise induced increase in IL-6, expression of adhesion molecules remained unchanged both straight after the exercise and in recovery. Furthermore, evidence suggests that circulating white blood cells are not the source of IL-6 during prolonged running. For instance, it has been demonstrated that blood monocytes are not the source of IL-6 neither during prolonged running (Starkie et al. 2001), nor during 2h of concentric cycling exercise (Starkie et al. 2000).

2.2.1. IL-6 – a myokine?

After the initial findings that circulatory blood cells are not the main source of IL-6 during exercise, researchers started to focus on a muscle as an endocrine organ. It has been suggested that skeletal muscle express IL-6 genes and IL-6 protein during continuous contractile activity (Hiscock et al. 2004) (Figure 5). Resistance exercise protocol (3 sets of 10 repetitions at 80% of one repetition maximum on machine squat, leg press and leg extension exercise) was chosen in an attempt to produce IL-6 mRNA alterations without eliciting muscle damage and a subsequent systemic inflammatory response (infiltration and activation of peripheral blood mononuclear cells in muscles) (Buford et al. 2009). Increase in IL-6 mRNA expression, but not in serum IL-6 concentrations was seen. Thus, the lack of a significant change in circulating levels of IL-6 appears to indicate that the source of the mRNA increases is not due to peripheral blood mononuclear cells (Buford et al. 2009). Furthermore, 3 h of two-legged knee extensor exercise (Steensberg et al. 2002) or bicycle exercise (Keller et al. 2003) resulted in 100-fold and 9-fold increase in muscle IL-6 mRNA levels, respectively, and subsequent IL-6 release in circulatory system.

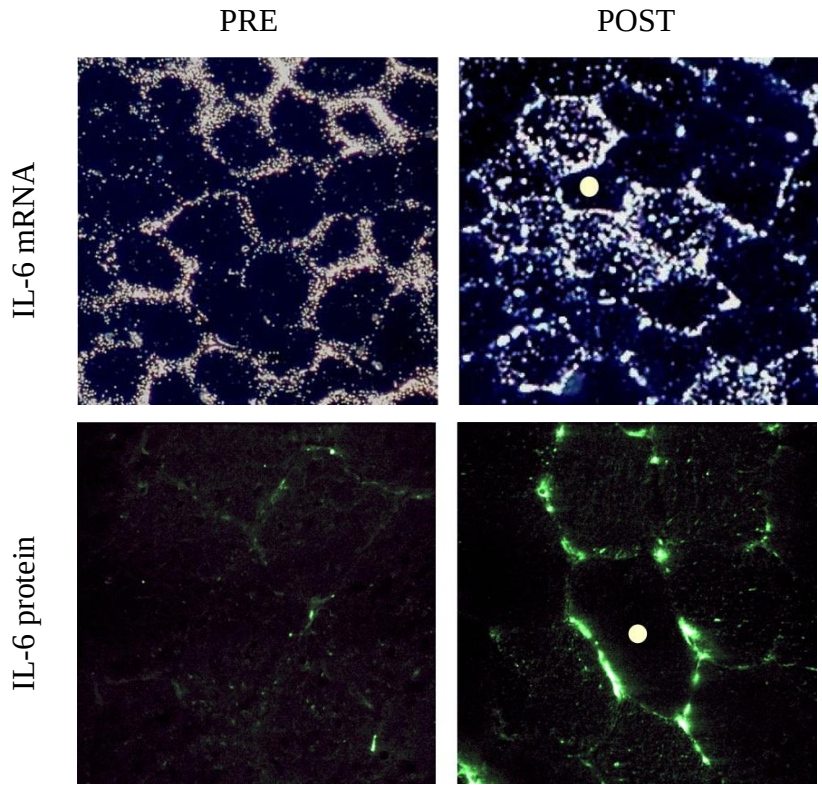

Figure 5. IL-6 mRNA and IL-6 protein in muscle biopsy sections before (PRE) and after (POST) 120 min continuous recumbent cycle ergometry. (Hiscock et al. 2004)

The release of IL-6 can be affected by bioavailability of carbohydrates (CHO), which has lead to the belief that IL-6 released by the contracting muscles may improve skeletal muscle energy supply and assists in the maintenance of stable blood glucose levels during exercise (Pedersen 2009) (Figure 6).

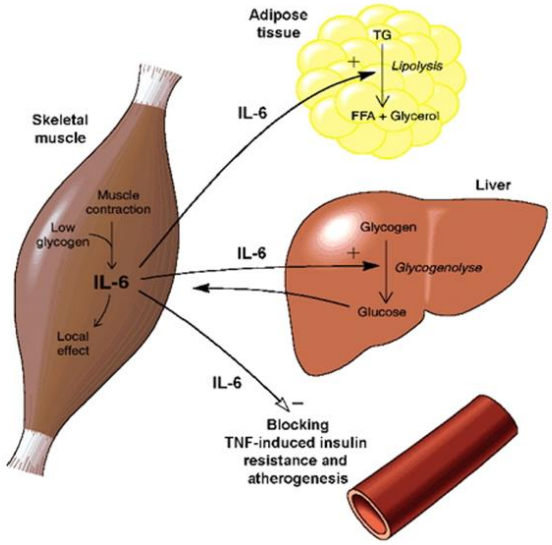

Figure 6. Schematic presentation of the possible biological effects of muscle-derived IL-6. TG, triglyceride; FFA, free fatty acid. (Pedersen et al. 2001)

It has been shown that the increase in IL-6 mRNA and plasma IL-6 concentrations were attenuated in CHO compared to placebo (PLA) group after a 3 h run at ~70% maximal oxygen consumption (VO_{2max}) (Nieman et al. 2003). Thus, authors concluded that the diminished post-run plasma IL-6 concentration is at least in part due to an attenuation of skeletal muscle IL-6 mRNA expression. In contrast, the same research group showed that CHO ingestion attenuated plasma IL-6 response while muscle IL-6 mRNA expression was similar in CHO and PLA groups (Nieman et al. 2005). Not only the CHO ingestion, but also intramuscular glycogen content may influence the IL-6 response during exercise. Low pre-exercise intramuscular glycogen content enhances plasma IL-6 response and increased muscle IL-6 mRNA concentration to concentric exercise (Chan et al. 2004). Furthermore, muscle glycogen content was increased after 10 weeks of endurance training, while muscle IL-6 mRNA expression was decreased (Fischer et al. 2004).

On the other hand, some studies have failed to confirm the CHO effect on IL-6 release. CHO administration during 2 h of cycling exercise did not attenuate plasma IL-6 concentration (Starkie et al. 2000) and increased IL-6 concentration after a marathon running was similar in both the CHO and PLA groups (Nieman et al. 2001). Exercise intensity was mentioned as one of the possible explanations. Namely, the PLA runners significantly reduced their exercise intensity during the last 10 km of the race, thus, the higher exercise intensity (Ostrowski et al. 2000) among the CHO runners may have influenced plasma IL-6 concentrations. A more recent study showed that the leg IL-6 release was not correlated to release or uptake of exogenous substrates nor muscle glycogen content or utilization during whole body exercise, and therefore the IL-6 relation to carbohydrate turnover could not be confirmed (Helge et al. 2011).

A study has demonstrated that the IL-6 receptor is expressed in human skeletal muscle and that expression of both IL-6 receptor mRNA in skeletal muscle, as well as IL-6 receptor protein on muscle fibre membranes increases in recovery phase (6 h after prolonged exercise) (Keller et al. 2005b). The authors suggested that expression of the IL-6 receptor in recovery may be a mechanism whereby muscle stays sensitized to IL-6 in order to promote rebuilding of energy stores, possibly initiating glycogen synthesis (Keller et al. 2005b). However, intramuscular glycogen levels had no influence on acute exercise-induced IL-6R gene expression straight after exercise and at 2h in recovery (Keller et al. 2005a). Recent findings have suggested that not only

membrane bound IL-6R, but also sIL-6R may have a role in glucose homeostasis regulation during exercise. Thus, it has been shown that the only combined administration of IL-6 and sIL-6R increases basal glucose transport in mouse soleus muscle, partly through AMPK-dependent signalling (Gray et al. 2009). At the same time no effect of IL-6 or sIL-6R was observed in insulin-stimulated glucose transport (Gray et al. 2009). The data on exercise-induced plasma concentrations of sIL-6R are inconsistent. Some have shown no change in exercise-induced sIL-6R concentrations during exercise and in recovery (Keller et al. 2005b), and others have shown decreased sIL-6R levels 48 h and 72 h after muscle-damaging exercise (Robson-Ansley et al. 2010) and that this was related to a regulatory mechanism controlling the influx of different leukocyte subpopulations into damaged tissue (Robson-Ansley et al. 2010). In contrast, both moderate-intensity and high-intensity intermittent exercise resulted in increased sIL-6R and IL-6/sIL-6R complex with no differences between the trials, despite the fact that plasma IL-6 concentration was significantly higher in high-intensity intermittent compared to moderate-intensity exercise (Leggate et al. 2010). Altogether, the importance and possible role of IL-6, IL-6R and sIL-6R in glucose metabolism still remain an open question.

2.3. Intensity and duration

In response to long duration and high intensity exercise, circulating levels of IL-6 increase by up to 100-fold (Suzuki et al. 2003), and it has been shown that also plasma lactate concentration increase with both intensity and duration of exercise (Jacobs 1986). Thus, significant correlation between plasma lactate at the end of prolonged running and peak IL-6 concentrations (Ostrowski et al. 1998) led to the hypothesis that the IL-6 response depend on either exercise intensity or duration, or both. To test this hypothesis running intensity during marathon race was calculated and correlated with peak IL-6 concentrations. The results confirmed the existence of a relationship between plasma IL-6 concentration and exercise intensity (Ostrowski et al. 2000). Similarly, IL-6 release during two leg knee extension exercise was markedly higher in the leg exercising at 85% W_{max} compared to the leg exercising at 65 % W_{max} (Helge et al. 2003). A recent study showed that the relative exercise intensity is the main determinant of the IL-6 concentration during ultra-endurance exercise exceeding more than 12 hr (Wallberg et al. 2011). Though brief maximal

exercise induce a significant increase in IL-6 concentration (Chaar et al. 2011; Edwards et al. 2006), the increase of IL-6 levels after endurance exercise (Suzuki et al. 2003) is markedly higher, ~3 vs. ~100 pg/ml, respectively. These differences suggest that other factors, e.g. exercise duration, also may contribute to elevated IL-6 concentrations.

IL-6 release may be related to the exercise duration, because 2 hr exercise induced a greater increase in plasma IL-6 concentrations compared with 1 hr exercise while the absolute intensity was similar (~75% VO_{2max} and ~70% VO_{2max}, respectively) (MacDonald et al. 2003; Nieman et al. 2007). On the other hand, plasma IL-6 concentration during 200 km running increased 121-fold at 100 km, and then remained stable up to 200 km (Kim et al. 2007). A recent study by Wallberg et al. (2011) included 24 h of nearly continuous mixed exercise (4x kayaking, 4x running and 4x cycling) under controlled conditions. IL-6 level increased ~ 10-fold at 6 h, ~ 14-fold at 12 h, but thereafter remained unchanged at 24 h. All participants had nearly identical values at 12 and 24 h, suggesting that in exercise lasting more than 12 h intensity, and not duration, is the main determinant of the IL-6 response (Wallberg et al. 2011). Thus it seems that exercise duration contribute to increased IL-6 levels during shorter exercise bouts.

2.4. Training status and performance

There is a debate about the effect of training status on cytokine response to acute exercise. The finding that the serum concentrations of IL-6 correlated with heart rate independent of running speed led to the notion that that training status influence IL-6 concentration, i.e. well trained runners having a low IL-6 response (Ostrowski et al. 1998). These results were confirmed by Gokhale et al. (2007) showing an attenuated cytokine response to acute exercise in athletes, who had a lesser magnitude of change in the venous plasma IL-6 and TNF-α concentrations following an intermittent running exercise compared to non-athletes. Similarly, 12 weeks of endurance training reduced the exercise-induced skeletal muscle IL-6 mRNA expression and subsequent plasma IL-6 release (Yfanti et al. 2012). On the other hand, the acute exercise-induced plasma IL-6 concentrations´ response was unchanged after 10 weeks of endurance training, despite the fact that acute exercise-induced skeletal muscle IL-6 mRNA expression was markedly lower after endurance

training (despite fivefold higher absolute workload in post- vs. pre-training period) (Fischer et al. 2004). Furthermore, IL-6 protein concentrations were quantified with Multiplex suspension array system in biopsied muscle and increased at 3 h post-exercise. This response was unaltered by 12 weeks of resistance training (Trenerry et al. 2011). Authors suggested that maybe it is important for IL-6 to be responsive to acute exercise, regardless the training state of the muscle (Trenerry et al. 2011), because this cytokine promotes both satellite cell proliferation and myogenic differentiation after muscle injury (Toth et al., 2011). IL-6 response to acute exercise (graded exercise till 85% of heart rate reserve (HRR) was reached, followed by 20 minutes at 60% of HRR) also was similar in young healthy and old males irrespective of significant differences in their VO_{2max}, 54±9 vs. 32±5, respectively (Cosio-Lima et al., 2008).

The performance by an athlete is the main parameter that reflects the training status. It has been shown that higher post-exercise responses in IL-6 were positively related to performance improvement in highly trained male rowers (Maestu et al. 2010). This is in contrast to previously mentioned results by Gokhale et al. (2007). In addition, athletic performance during an exercise challenge consisting of a 10-km running time trial was significantly impaired in trained male runners following the administration of a low dose of rhIL-6 compared to the placebo trial (Robson-Ansley et al. 2004). As there were no differences between the two trials in body temperature, heart rate, blood glucose, or lactate concentrations, authors suggested that the rhIL-6 impaired exercise performance and increased sensations of generalized fatigue by altering central nervous system's (CNS) serotonergic activity (Robson-Ansley et al. 2004). It has been shown that exogenous CHO ingestion significantly improved 90-min time trial performance and this was associated with a lower IL-6 concentration compared with the PLA trial (Robson-Ansley et al. 2009). However, it is doubtful that these small differences (CHO: 5.3 ± 1.9 vs. PLA: 6.6 ± 3.0 $pg \cdot ml^{-1}$) in plasma IL-6 levels could mediate the improvement in athletic performance. Instead, the authors suggest that improved performance in CHO trial may be linked to an increase in central drive or motivation (Carter et al. 2004).

The contradictions between the mentioned studies might be also genetic, as findings by Huuskonen et al. (2009) suggest that the allele C of a potentially functional single nucleotide polymorphism – 174G/C of the IL-6 gene (rs1800795) may have an effect on plasma IL-6 response to acute exercise in healthy subjects.

Furtermore, there is a strong association of the IL-6 G-174C genotype with systemic CK response to strenuous exercise supporting the central role of cytokines in the reactive inflammatory process of muscle damage and repair (Yamin et al. 2008). Altogether there is a complex picture in the previous literature regarding the effect of training and performance on plasma IL-6 concentration during acute exercise.

2.5. IL-6 anti-inflammatory properties

It is well known that physical inactivity increases the risk for all-cause mortality (e.g. atherosclerosis, type 2 diabetes and cancer) (Figure 7), and it can be reduced by regular exercise (Blair et al. 2001). The anti-inflammatory effects of regular exercise may be mediated mainly in two ways: a reduction in visceral fat mass and the induction of an anti-inflammatory environment with each bout of exercise. It has been suggested that exercise-induced weight loss reduces low grade inflammation by both suppression of macrophage infiltration and subsequent IL-6 and TNF-α production, as well as by acceleration of phenotypic switching from M1- (produce pro-inflammatory cytokines) to M2- (produce anti-inflammatory cytokines) (Martinez et al. 2008) type macrophages residing in adipose tissue (Gleeson et al. 2011).

The fact that the classical pro-inflammatory cytokines, TNF-α and IL-1β, in general do not increase with exercise (Steensberg et al. 2002; Kim et al. 2007) indicates that the cytokine cascade induced by exercise markedly differs from the cytokine cascade induced by infections (Petersen and Pedersen 2006). It has been shown that rhIL-6 infusion inhibits endotoxin-induced plasma TNF-α production (Starkie et al. 2003) in humans. Furthermore, rhIL-6 enhances such anti-inflammatory molecules as IL-1ra, IL-10 and cortisol (Steensberg et al. 2003). These findings suggest that exercise-induced increase in plasma IL-6 may be one of mechanisms that reduce low grade inflammation.

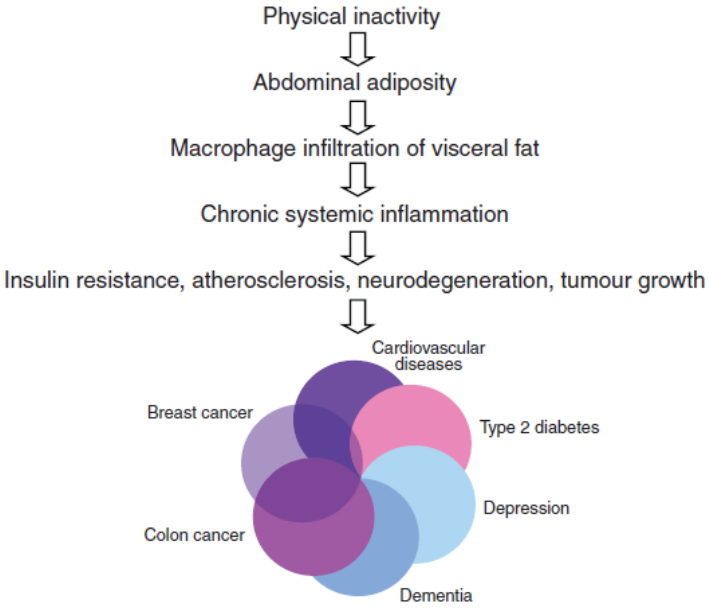

Figure 7. Hypothesis: physical inactivity leads to the accumulation of visceral fat and consequently to the activation of a network of inflammatory pathways, which promote the development of insulin resistance, atherosclerosis, neurodegeneration and tumour growth, leading to the development of "the diseasome of physical inactivity".

2.6. TNF-α

Tumor necrosis factor-α (TNF-α) has been traditionally considered as a key inducer of acute phase response (Limongelli et al. 2010), as well as mediator in the pathogenesis of chronic diseases, like rheumatoid arthritis and Chron's disease. TNF-α is generated as a precursor form called transmembrane TNF-α that is expressed as a cell surface type II polypeptide consisting of 233 amino acid residues (26 kDa) on activated macrophages and lymphocytes as well as other cell types (Horiuchi et al. 2010). After being processed by such metalloproteinases as TNF-α-converting enzyme the soluble form of TNF-α of 157 amino acid residues (17kDa) is released. Under native conditions bound and soluble TNF exists as a monomer, dimer and trimer in equilibrium, with the trimer being the biologically active form (Figure 8).

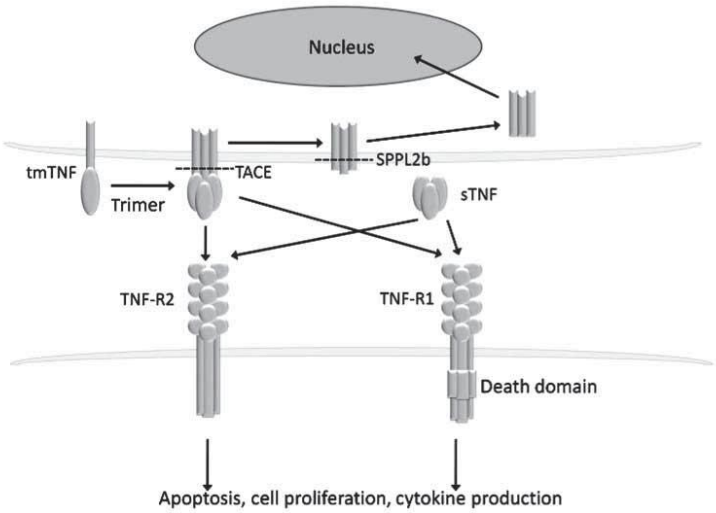

Figure 8. Biology of transmembrane TNF-α and soluble TNF-α. sTNF-α mediates its biological activities through Type 1 and 2 TNF receptors, which are expressed on almost all nucleated cells.

Data on plasma concentration and muscle mRNA expression of TNF-α during physical activity vary and mechanisms involved in TNF-α activation during exercise are still unknown. It has been shown that TNF-α is expressed in muscle fibres (Kern 1997), and while 60 min ergometer cycle exercise at ~70% VO_{2peak} did not induce an increase in muscle TNF-α mRNA concentration (Chan et al. 2004), a longer exercise duration (2.5 hr cycle exercise at 60% W_{max}) resulted in 3.7 fold increase (Nieman et al. 2005). This indicates that exercise duration probably is important for TNF-α increase during exercise. In addition, it has been shown that high intensity exercise induces TNF-α release (Kinugawa et al. 2003). TNF-α secretion during exercise may be dependent on the intensity of the exercise, because 30 min exercise on cycle ergometer at 70% of VO_{2max} resulted in significantly higher TNF-α levels than at 50% of VO_{2max} (Kimura et al. 2001).

Previous studies evaluating TNF-α response to acute exercise have shown no changes in plasma TNF-α release from working legs (Helge et al. 2011) or TNF-α mRNA expression in muscles (Steensberg et al. 2002; Febbraio et al. 2003). In addition, it has been shown that circulating monocytes are not the main source of increased TNF-α level during exercise (Starkie et al. 2001). It has been proposed that the increase in plasma TNF-α concentrations after prolonged running, e.g. marathon (Starkie et al. 2001; Toft et al. 2000), was the result of systemic endotoxemia induced by a decrease in blood flow to the splanchnic bed (Steensberg et al. 2002).

TNF-α modulates insulin-mediated glucose metabolism and it has been shown to inhibit insulin-stimulated tyrosine phosphorylation of both the insulin receptor and insulin receptor substrate-1 (Steinacker et al. 2004). Furthermore, in vitro, TNF-α stimulates the downregulation of glucose transporter 4 in adipocytes, hepatocytes and skeletal muscle. In damaging exercise in humans, a close relation was found between TNF-α production and the decrease in PI3K activity in skeletal muscle (Del Aguila et al. 2000). It has been proposed that upregulation of IL-6 gene expression in skeletal muscle during muscle contraction may inhibit an increase in TNF-α production (Steensberg et al. 2002). Thus it is plausible that IL-6 helps to maintain skeletal muscle glucose uptake by inhibiting the production of TNF-α (Youd et al. 2000).

2.7. Adhesion molecules

Differentiation of cell structures during development, tissue formation, and coordinated dynamic interactions of cells in various processes (such as the immune response) requires direct contact between cell surfaces and between cells and the extracellular matrix. Adhesion molecules – the glycoproteins through which the cell-to-cell contacts are made, are expressed on the surface of a variety of cells (e.g. vascular endothelial cells, leukocytes and platelets) (Figure 9). Four main groups of cell adhesion molecules are recognised: the integrin receptor family; the immunoglobulin superfamily, which includes ICAM-1 and VCAM-1; selectins, including E-selectin; and cadherins (Elangbam et al. 1997).

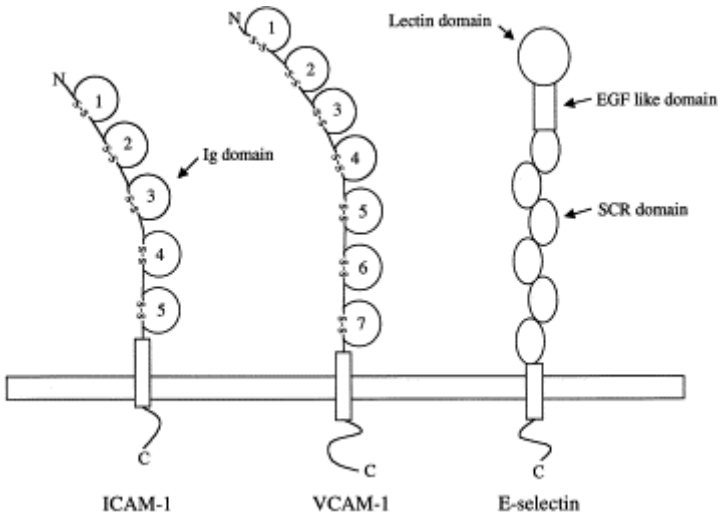

Figure 9. Structures of ICAM-1, VCAM-1 and E-selectin. (Lee and Benveniste 1999)

Immune response to infection includes proinflammatory signaling that increases expression of adhesion molecules such as E-selectin on endothelial cells, which facilitates "capture" of the leukocytes to the vessel wall. Chemokines secreted by endothelial cells activate leukocyte integrins, a process that promotes firm adhesion between leukocytes and endothelial cells via integrin-adhesion molecule interactions. As a result adherent leukocytes transmigrate through the endothelium to the underlying tissue (Figure 10). (Warboys et al. 2011)

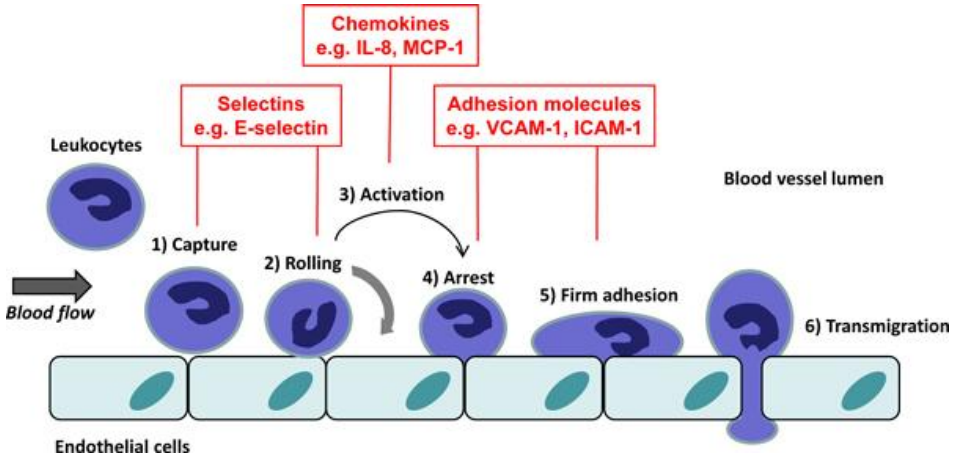

Figure 10. Leukocyte adhesion and transmigration. (Warboys et al. 2011)

Immune response caused by muscle damage depends on the ability of leukocytes to migrate from the blood into sites of inflammation (Nielsen & Lyberg 2004). Upregulation of E-selectin, ICAM-1 and VCAM-1 on the surface of endothelial cells is prominent when they are exposed to proinflammatory molecules such as TNF-α (Monchanin et al. 2007). Data on soluble adhesion molecule changes during exercise are not conclusive. Studies on the effect of exercise on plasma concentrations of soluble forms of cell adhesion molecules show no change after resistance exercise (Petridou et al. 2007) or cycle ergometer exercise at 80% VO_{2max} (Akimoto et al. 2002). Study evaluating plasma levels of sICAM-1 and sVCAM-1 revealed that progressive maximal exercise induced increase only in sVCAM-1 concentration (Monchanin et al. 2007). Increase of adhesion molecules concentrations after marathon and half-marathon running (Nielsen & Lyberg 2004) and 30-min downhill running at intensity of ventilation threshold (Akimoto et al. 2002), suggest that muscle damage is necessary for the increase in sICAM-1 levels. The free, circulating adhesion molecules mat, at least theoretically, bind to their counter-

receptors on either leukocytes or endothelial cells, thereby occupying the binding sites meant to be utilized by the corresponding cell-bound adhesion molecules. In essence, this may have a temporary negative influence on the ability of leukocytes to adhere and transmigrate the endothelium (Nielsen & Lyberg 2004).

2.8. MMP-9

Matrix metalloproteinases (MMPs) are a large family of calcium-dependent zinc-containing endopeptidases (Figure 11), which are responsible for the tissue remodeling and degradation of the extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycans. They are regulated by hormones, growth factors, and cytokines, and are involved in ovarian functions. MMPs are excreted by a variety of connective tissue and pro-inflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes. These enzymes are expressed as zymogens, which are subsequently processed by other proteolytic enzymes (such as serine proteases, furin, plasmin, and others) to generate the active forms. (Verma & Hansch 2007)

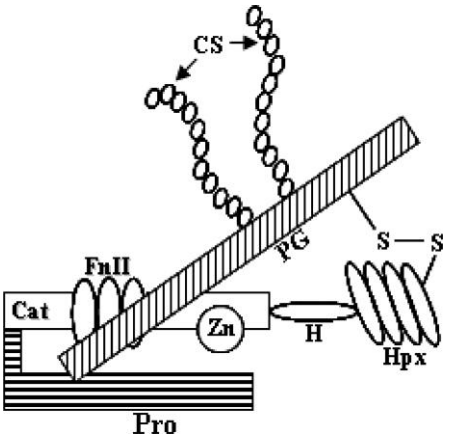

Figure 11. Schematic model of the pro-MMP-9·PG heterodimer. Pro-MMP-9 consists of an N-terminal prodomain (Pro) and a catalytic domain (Cat) containing an active site zinc ion (Zn) and the three fibronectin II-like repeats (FnII) that are known to bind gelatin and collagen. The C-terminal end of the enzyme contains the hemopexin like domain (Hpx), which has a structure of a four bladed β-propeller, and this domain is linked to the catalytic domain through a hinge region (H). Core protein of one or several proteoglycans (PGs) bind in a reduction-sensitive manner to the C-terminal part of the Hpx domain in pro-MMP-9, here indicated with a disulfide bridge. (Malla et al. 2008)

Recent studies suggest that MMP-9 plays a critical role in cleaving muscle-specific proteins and contributing in extracellular matrix formation, remodelling, and regeneration in skeletal muscle (Urso et al. 2009), but it remains unclear which tissues are a basic source of the increased MMP-9 concentration (Madden et al. 2011) induced by physical exercise. It has been shown that a single bout of exercise can induce MMP-9 expression in skeletal muscle (Rullman et al. 2009), that is stimulated by increased levels of TNF-α (Srivastava et al. 2007). Research group from Copenhagen have demonstrated enhanced interstitial amounts of MMP-9 in the human peritendinous tissue in vivo after exercise (Koskinen et al. 2004). MMP-9 also is present in the tertiary granules of neutrophils and is rapidly released following stimulation (Chakrabarti et al. 2006). In addition, increased level of IL-6 during inflammation augments MMP-9 expression in leukocytes (Chen et al. 2006b).

It is suggested that circulating MMPs could reflect matrix turnover thus providing a valuable tool for non-invasive determination of tissue structure (Tayebjee et al. 2005). Recent study revealed that plasma MMP-9 is not a robust systemic index for eccentric arm exercise-induced injury in humans probably because of the day-to-day fluctuations (Madden et al. 2011).

3. Methods

3.1. Ethical approval

All studies were performed according to the latest revision of the Declaration of Helsinki and were approved by the Ethical Committee of the Institute of Experimental and Clinical Medicine, university of Latvia, or by the Ethical Committee of Copenhagen. All subjects were informed about the risks and discomfort involved before written consent to participate was obtained.

3.2. Studies

In order to verify the proposed hypothesis we conducted four separate research studies:

1. **Study I** – an incremental bicycle test until exhaustion, when maximal oxygen consumption was recorded.
2. **Study II** – prolonged running (half marathon and marathon race) with similar absolute intensity.
3. **Study III** – sub-maximal recumbent cycling exercise for 1h.
4. **Study IV** – 2 week's immobilization of one leg and subsequent two leg knee-extensor exercise for 45 minutes.

3.3. Subjects

In total 98 young, healthy and active males participated in the four research studies. All subjects completed a medical questionnaire. Depending on the time when subjects reported to laboratory, they were asked to fast from 3 till 9 h before the blood sampling at rest. In addition all subjects were asked to refrain from alcohol, nicotine, medication etc. for 24 hours before all blood sampling times, as well as to avoid physical overload or other stressors. Anthropometric data are given in Table 1.

Table 1 Subject characteristics

	N	Age, years	BMI, $kg \cdot m^{-2}$	Training status
Study I				VO_{2max}, $ml \cdot kg^{-1} \cdot min^{-1}$
Ice hockey players	26	25 ± 1	25.8 ± 0.4	50.3 ± 1.0
Study II *				Trainings, $h \cdot week^{-1}$
Half-marathon runners	22	26 ± 1	24.3 ± 0.5	3.9 ± 0.4
Marathon runners	18	27 ± 1	21.9 ± 0.4	7.2 ± 0.5
Study III				Trainings, $h \cdot week^{-1}$
Moderately trained athletes	10	26 ± 2	22.6 ± 0.5	5.4 ± 0.5
Highly trained athletes	7	26 ± 1	22.7 ± 0.8	11.1 ± 1.6
Study IV				VO_{2max}, $ml \cdot kg^{-1} \cdot min^{-1}$
Healthy males	15	23 ± 1	23.6 ± 0.7	46.8 ± 1.4

* – There were fewer subjects (26 from 40) tested in recovery phase due to their domicile or workplace location.

3.4. Exercise mode and blood sampling

Venous blood samples for determination of cytokines and other blood tests before, during and after exercise intervention were collected with vacutainer system (Figure 12). Serum/plasma was separated by centrifugation and stored at - 80^0C. For more detailed information see Table 2.

Figure 12. Vacutainer system used for blood sampling.

Table 2 Generalized experiment protocol

	Blood sampling at rest	Exercise	Blood sampling after exercise and in recovery
Study I	30 min before exercise	Maximal cycling exercise, registration of VO_{2max}	2 min after exercise
Ice hockey players			
Study II			
Half-marathon runners	2 days before race	11.7 ± 0.3 km·h^{-1}	15 min and 28 h after race
Marathon runners		11.9 ± 0.4 km·h^{-1}	
Study III			
Moderately trained athletes	15 min before exercise	1h of recumbent cycling exercise at 70% of heart rate reserve	Straight after exercise
Highly trained athletes			
Study IV *	15 and 0 min before exercise	45 min of knee extensor exercise at 20 ± 1 W	Straight after exercise
Healthy males			

***** – catheters were placed into the brachial artery and both femoral veins. During exercise blood was sampled each 15 minutes.

3.4.1. Study I

Maximal oxygen consumption was measured on a cycle ergometer Monark Ergomedic 839E (Monark, Sweden) because the muscle groups used in ice skating are quite similar to those used in cycling. Cortex Metalyzer 3B system (Cranlea & Company, United Kingdom) was used to evaluate cardio-respiratory functions (ventilation, respiratory exchange ratio, electrocardiogram, heart rate, blood pressure). Athletes achieved their maximal oxygen consumption, when 3 of 4 commonly accepted criteria were met: 1) volitional exhaustion; 2) maximal heart rate measured at exhaustion was superior to 90% of the age-predicted maximal heart rate; 3) respiratory exchange ratio was above 1.10; 4) capillary blood lactate concentration was greater than 8 mM. Exercise protocol – incremental bicycle test until exhaustion, shown at Table 3.

Table 3 Exercise protocol

Stage	Time line, min	Physical load, W/kg	Pedal rate, rpm
Warm up	-5	0.5	60
	-4	0.5	60
	-3	0.5	60
	-2	0.6	60
	-1	0.7	60
Exercise till exhaustion	1	0.8	80
	2	0.9	80
	3	1	80
	4	1.1	80
	5	1.2	90
	6	1.3	100
	7-20	plus 0.1	80 – 100 *
	21…	plus 0.3	80 – 100 *
Recovery	2 min	1.3	own rhythm

*Individually self-chosen pedal rate based on athletes' strength and endurance abilities.

3.4.2. Study II

Weather conditions during the race: Time 9.00: +15^{o}C; NW wind 4 m·s^{-1} (up to 8 m·s^{-1}); atmospheric pressure 760 mm·Hg^{-1}; relative humidity 65%. Time 13.00: +20^{o}C; NW wind 2 m·s^{-1} (up to 3 m·s^{-1}); atmospheric pressure 758 mm·Hg^{-1}; relative humidity 42%.

Carbohydrate ingestion during the race was not controlled.

3.4.3. Study III

On the experimental day, the subjects arrived at the laboratory at 2-4 pm. All subjects performed one bout of prolonged submaximal recumbent cycling exercise on a veloergometer Ergoselect 600P (Ergoline GmbH, BLTZ, Germany) for 1h. Intensity of 70% of heart rate reserve (HRR) was reached during the first 10 minutes of cycling and maintained during the whole exercise. Target heart rate for each subject was calculated by the use of the Karvonen formula (Karvonen et al. 1957). Before the exercise maximal heart rate was determined using Polar Fitness Test provided by Polar S810. The Polar Fitness Test is based on precise detection of heart rate and heart rate variability at rest (Gamelin et al. 2006) and personal information, e.g. age, height and weight. Heart rates were monitored by the 12 lead Stress Test ECG system (AMEDTEC ECGpro®, GmbH, Aue, Germany) throughout the experiment.

3.4.4. Study IV

3.4.4.1. Pre-study

Prior to randomization and immobilization of the selected leg by a Donjoy cast for 14 days with a knee angle of 60^0, subjects were familiarised with the exercise protocol a custom made two leg knee extension ergometer (Figure 13). Subsequently, W_{max} was determined using a graded exercise test for each leg separately. W_{max} was defined as the point where the slope of the whole body VO_2, heart rate and pulmonary ventilation with increasing work load changed from a linear relationship to an exponential relationship. At that point the m. quadriceps femoris is no longer able to sustain the workload and therefore additional muscle are recruited during exercise leading to the nonlinear relationship mentioned above (Andersen et al. 1985).

VO_{2max} was assessed during an incremental cycle test until exhaustion (Jaeger ER800, Ergoline, Germany) (Table 1).

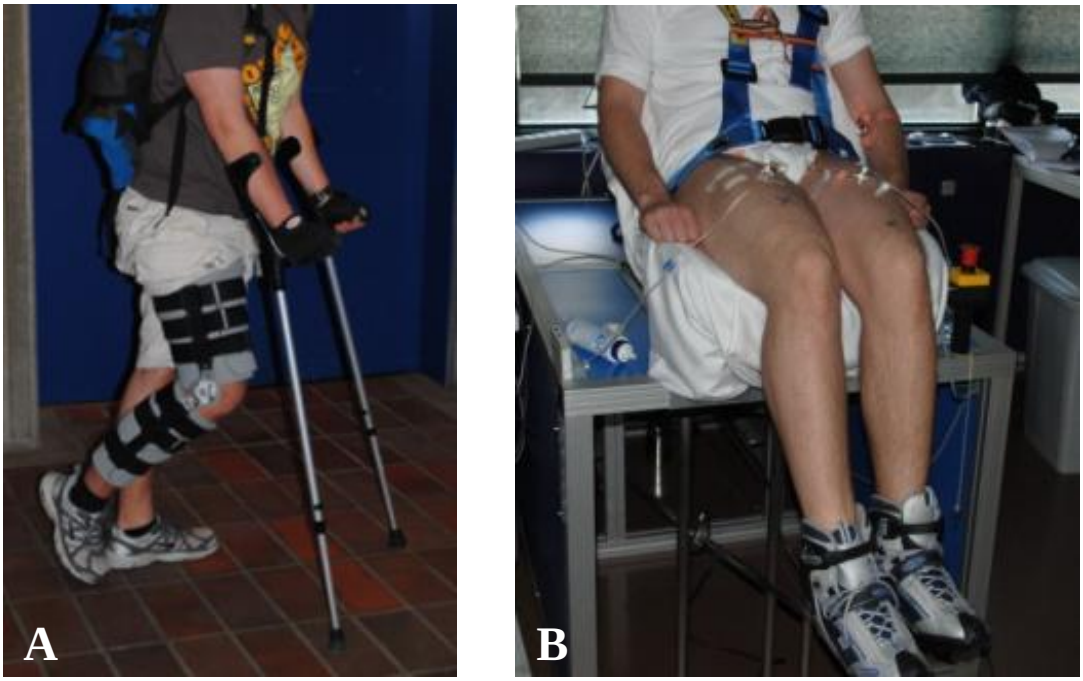

Figure 13. A – Donjoy cast; B – custom made two leg knee extension ergometer.

3.4.4.2. Experimental protocol

After 2 week´s immobilization of one leg, the subjects reported to the laboratory in the morning after an overnight fast. Catheters were placed into the brachial artery (20G arterial cannula, Becton Dickinson a/s, Albertslund, Denmark) and both femoral veins (14G catheter, Arrow International, ViCare Medical, Birkeroed Denmark) under local anaesthesia using an aseptic technique. The catheters were intermittently flushed with sterile sodium chloride to maintain patency. After resting for at least one hour, the subject was positioned in a custom made two leg knee extension ergometer in a semi-supine position. Then, at rest, blood was sampled simultaneously from the brachial artery and femoral veins at -15 min and just before exercise. Subsequently, 45 min of exercise with both legs was started. The absolute leg workload was set to 50% of the W_{max} determined before the immobilization. During exercise blood was sampled after 15, 30 and 45 min. Femoral venous blood flow was measured at all time points in both legs by ultrasound Doppler (ACUSON S2000, Siemens Healthcare, Ballerup, Denmark). Heart rate was recorded continuously during exercise with Polar RS400. Whole body oxygen consumption was measured from 20^{th} till 27^{th} min of exercise using an Oxycon Pro (CareFusion GmbH, Hoechberg, Germany). Throughout the experiment subjects had free access to water.

The day after the exercise experiment, the subjects reported to the laboratory in order to determine changes in their W_{max} after immobilization. This could not be done before the exercise experiment, because this test would interfere with the immobilization protocol. The actual relative workload for both legs during exercise experiment was calculated.

3.5. Cytokine and other blood biomarker detection

3.5.1. Studies I, II, III

Venous blood samples for IL-6, TNF-α, sE-selectin, sICAM-1, sVCAM-1, MMP-9, MPO, MCP-1 and standard blood tests (leukocyte formula etc.) were taken before after exercise (more precise information about blood sampling time points shown in Table 2). Blood samples for the determination of cytokines, adhesion molecules and other bioactive molecules were collected without anticoagulant and were allowed to coagulate for 20 to 30 min at room temperature. Serum was separated by centrifugation and all specimens were aliquoted, frozen, and stored at -80 ^{0}C.

3.5.1.1. Luminex 200 analyzer

Luminex xMAP technology is based on flow cell fluorometry with Luminex-developed innovations. The fluidics, optics, robotics, temperature control, software, and xMAP microspheres work together to enable simultaneous analysis of up to 100 analytes in a single test sample. Assay analysis requiring temperature control is provided through the Luminex XYP instrument heater block.

There are two fluidics paths in the Luminex 200 analyzer. The first path involves a syringe-driven mechanism that controls the sample uptake. This mechanism permits small sample uptake volumes from small reaction volumes. The syringe-driven system transports a specified volume of sample from a sample container to the cuvette. The sample is injected into the cuvette at a steady rate for analysis. Following analysis, the sample path is automatically purged with Luminex xMAP Sheath Fluid by the second fluidics path. This process removes residual sample within the tubing, valves, and probe. The second fluidics path is driven by positive air pressure and supplies sheath fluid to the cuvette and sample path.

Luminex xMAP Sheath Fluid is the delivery medium of the sample to the optics component. The analysis sample is acquired using a sample probe from a 96-well microtiter plate via the Luminex XYP instrument and injected into the base of the cuvette. The sample then passes through with sheath fluid at a reduced rate resulting in a narrow sample core to ensure that each microsphere is illuminated individually. The sample injection rate is such that the xMAP microspheres are introduced to the optics path as a series of single events. The Luminex SD system lets you run samples continuously without refilling sheath bottles. It automatically draws sheath from a non pressurized bulk sheath container to constantly maintain a reservoir of pressurized sheath fluid. A single 20 litre sheath container provides enough fluid for 48 hours or more of normal operation.

The optics assembly consists of two lasers. One laser excites the dye mixture inside the xMAP microspheres and the second laser excites the fluorophore bound to the surface of the xMAP microspheres. Avalanche photo diode detectors measure the excitation emission intensities of the colour coding classification dye mixtures inside the xMAP microspheres and a photomultiplier tube detects the excitation emission intensity of the reporter molecule bound to the surface of the xMAP microspheres. High speed digital signal processors and advanced computer algorithms provide

analysis of the xMAP microspheres as they are processed through the Luminex 200 analyzer. Results of the analyses are processed and provided in a report format.

Commercially available multiplex immunoassay kits were used for quantitative determination of IL-6, TNF-a, sE-Selectin, sVCAM-1, sICAM-1, MMP-9, MPO and MCP-1 by Luminex 200 analyzer (Luminex Corp., Austin, TX, USA) (Figure 14A):

1. **Study I**: MILLIPLEX MAP kit Cat. No.: HCVD1-67 AK; MILLIPLEX MAP kit Cat. No.: HADK2-61K-B;
2. **Study II**: MILLIPLEX MAP kit Cat. No.: HCVD1-67 AK; MILLIPLEX MAP kit Human Cytokine/Chemokine 96-Well Plate Assay Cat. No. MPXHCYTO-60K.
3. **Study III**: MILLIPLEX MAP kit Human Adipocyte 96 Well Plate Assay Cat. No. HADCYT-61K; MILLIPLEX MAP kit Human Cytokine/Chemokine 96-Well Plate Assay Cat. No. MPXHCYTO-60K; MILLIPLEX MAP kit Human Cardiovascular Disease (CVD) Panel 1 96 Well Plate Assay Cat. No. HCVD1-67-AK.

To avoid inter-assay variation all measurements for each subject were analysed in the same assay. Intra-assay CV% was IL-6: 7.9%, TNF-α: 7.3%, sICAM-1: 7.9%, sVCAM-1: 4.5%, sE-Selectin: 11.2%, MMP-9: 6.8%, MCP-1: 6.1%, and MPO: 12.3%.

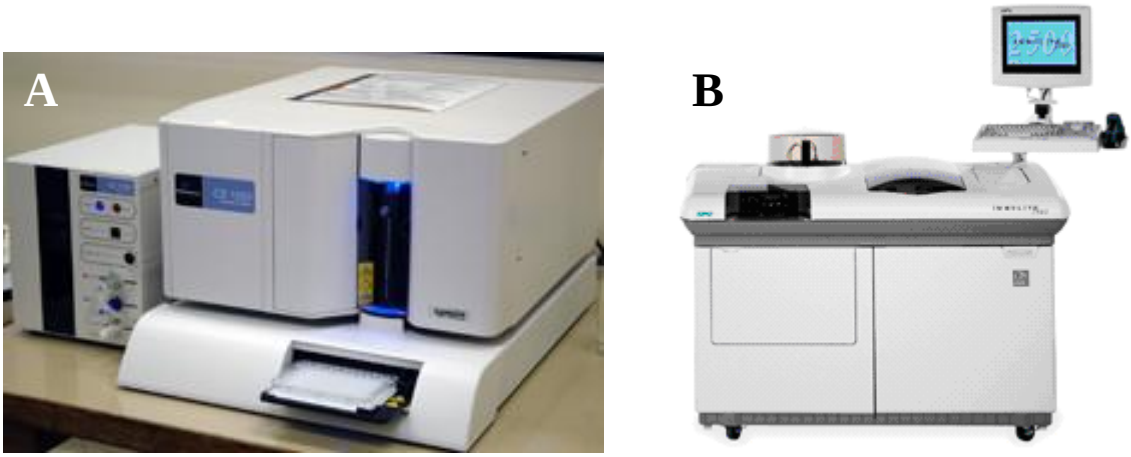

Figure 14. A – Luminex 200 – analyzer of cytokines; B – Immulite 2500 – analyzer of hormones

3.5.1.2. Other blood biomarkers measurements

Cortisol, hsCRP and adrenocorticotropic hormone (ACTH) were measured by Immulite 2500 analyzer (Siemens Medical Solutions, USA) (Figure 14B). Other tests were performed in clinical laboratories: "E.Gulbja laboratorija" and "International Diagnostic Center", Riga, Latvia. All detected blood biomarkers listed in Table 4.

Table 4 The list of detected blood biomarkers

	Luminex-200	Immulite 2500	Clinical laboratories
Study I	IL-6, TNF-α, sE-selectin, sICAM-1, sVCAM-1, MMP-9, MPO	hsCRP, cortisol	Insulin, glucose, leukocytes, lactate
Study II	IL-6, TNF-α, sICAM-1, MMP-9	ACTH, cortisol	CK, LDH, glucose, Hb, Hct, leukocytes
Study III	IL-6, TNF-α, sE-selectin, sICAM-1, sVCAM-1, MMP-9, MPO, MCP-1	cortisol	CK, LDH, glucose, lactate, Hb, Hct, leukocytes

3.5.2. Study IV

Blood was sampled anaerobically and distributed into tubes containing heparin or Trasylol/EDTA. The heparinised samples were immediately analyzed for haematocrit (Hct) (ABL800 Flex, Radiometer, Copenhagen, Denmark). Plasma for determination of cytokines was separated by centrifugation, frozen on dry ice, and stored at -80 ^{0}C until further analysis. Plasma IL-6 and TNF-α were measured with a high-sensitivity enzyme-linked immunosorbent assay kit from R&D Systems (Minneapolis, MN, USA), Human IL-6 Immunoassay (HS600B) and Human TNF-α Immunoassay (HSTA00D), respectively.

3.6. Calculations

Haemoglobin and haematocrit levels were measured in **Study I, II** and **III** in order to take into account the possible influence of exercise induced plasma volume change. Correction of plasma concentrations for blood cells and inflammatory molecules were made according to the method described previously (Dill and Costill 1974). In **Study IV** the Fick principle was used to calculate IL-6 and TNF-α release

across exercising legs using brachial arterial and femoral venous plasma concentration differences multiplied by plasma flow (calculated as blood flow x (1-Hct)).

3.7. Statistical analysis

Data were analyzed by Microsoft Office Excel 2007, STATISTICA 7.0 software (StatSoft Inc, USA), Sigma plot 11.0 software (Systat Software INc., San Jose, CA, USA). After testing normality (Shapiro-Wilk test), data with normal distribution were analysed by Two way repeated measure ANOVA and Student T test as appropriate. In the case of significant main effects or interactions, the Holm-Sidak *post hoc* test was performed to discern statistical differences. Wilcoxon signed-rank test was used as a nonparametric method. Data for correlation analysis (Pearson correlation or Spearman rank R test) were expressed as a difference between absolute values of measured parameters before and after exercise intervention and further labelled as delta (Δ). Data were expressed as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD). A value of $P < 0.05$ was considered to be significant.

4. Results

4.1. Study I

Athletes´ maximal oxygen consumption at incremental bicycle test was 50 ± 1 $ml \cdot kg^{-1}$ min^{-1}. There was a significant increase in haemoglobin concentration and haematocrit (Table 5), probably, because of the reinforced sweating during maximal exercise. Maximal exercise induced significant changes in absolute counts of all leukocytes subsets. Athletes' total leukocytes count was significantly increased after maximal exercise (Table 5). Elevation of circulating lymphocytes and neutrophils was the main reason for the change in total white cells count. There was a significant increase in glucose ($P < 0.001$), decrease in high sensitivity C-reactive protein ($P < 0.001$), but there was no change in cortisol or insulin concentrations after maximal exercise (Table 5).

Table 5 Pre-exercise and post-exercise blood mediators´ measures and haematological parameters

	Pre-exercise	**Post-exercise**	***P* value**	***N***
TNF-α, pg/ml	6.31 ± 0.49	6.68 ± 0.56	NS	25
sE-selectin, ng/ml	29 ± 2	29 ± 2	NS	25
sICAM-1, ng/ml †	120 ± 13	111 ± 15	NS	25
sVCAM-1, ng/ml	1103± 45	1105 ± 42	NS	25
hsCRP, mg/L	0.55 ± 0.08	0.51 ± 0.08	< 0.05	26
Cortisol, nmol/L	446 ± 27	420 ± 29	NS	26
Insulin, μU/ml †	7.3 ± 0.9	7.4 ± 0.8	NS	26
Glucose, mmol/L	5.5 ± 0.1	6.4 ± 0.2	< 0.001	26
Haemoglobin, mmol/L	9.4 ± 0.1	10.0 ± 0.1	< 0.001	22
Haematocrit, %	46 ± 1	50 ± 1	< 0.001	22
Leukocytes x 10^9 cells per litre	5.7 ± 0.3	8.7 ± 0.4	< 0.001	22
Neutrophils x 10^9 cells per litre	2.86 ± 0.2	3.91 ± 0.4	< 0.001	22
Eosinophils x 10^9 cells per litre	0.17 ± 0.01	0.20 ± 0.02	< 0.05	22
Basophils x 10^9 cells per litre	0.05 ± 0.00	0.07 ± 0.00	< 0.001	22
Lymphocytes x 10^9 cells per	2.1 ± 0.1	3.8 ± 0.1	< 0.001	22
Monocytes x 10^9 cells per litre	0.50 ± 0.03	0.73 ± 0.03	< 0.001	22

Values are means ± standard error mean (SEM). NS – non significant

Sera IL-6 concentration increased significantly between the rest and the end of maximal exercise ($P < 0.001$, Figure 15). Likewise, there were significant increases in MMP-9 and MPO concentrations ($P < 0.001$, Figure 16 and 17, respectively). Sera concentrations of TNF-α, sE-selectin, s-ICAM-1 and sVCAM-1 did not differ from pre-exercise levels ($P > 0.05$, see Table 5).

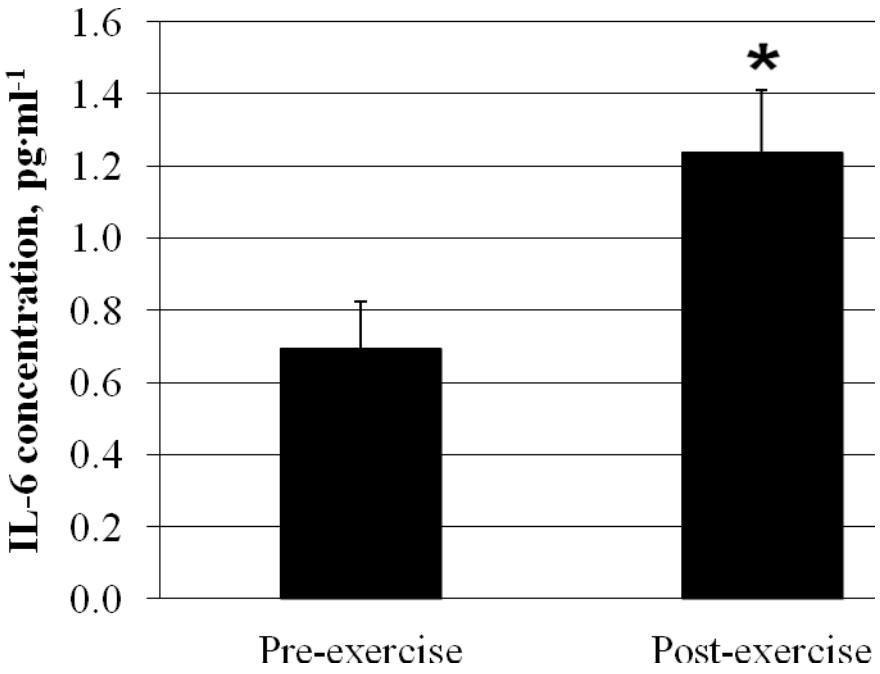

Figure 15. Sera IL-6 concentration changes in response to a maximal exercise till exhaustion – VO_{2max}, in professional ice hockey players, $N = 26$. Values are mean ± SEM. Symbols: * - significant difference compared to preexercise level.

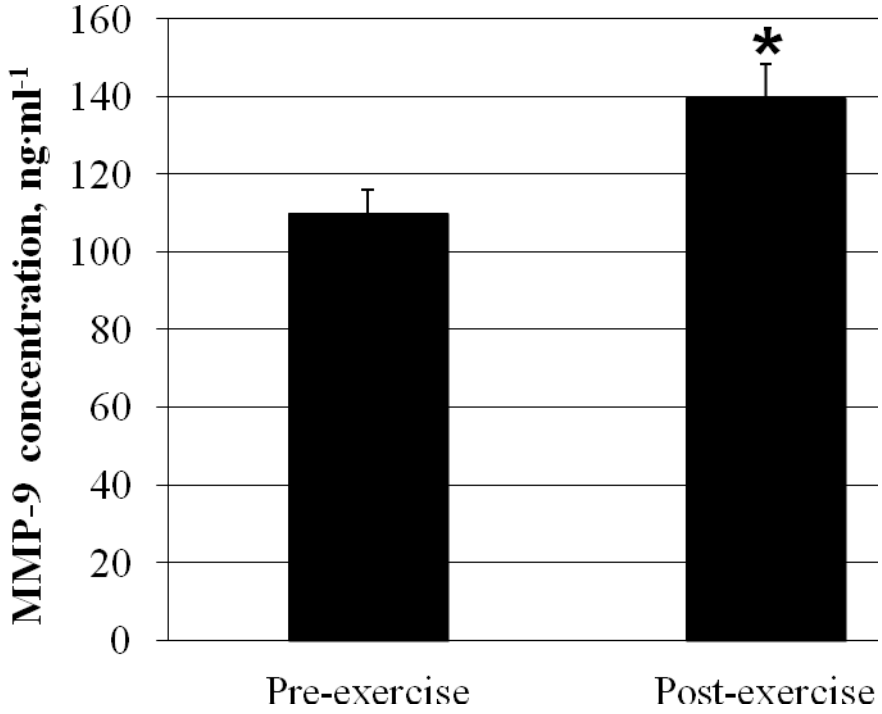

Figure 16. Sera MMP-9 concentration changes in response to a maximal exercise till exhaustion – VO_{2max}, in professional ice hockey players, N = 26. Values are mean ± SEM. Symbols: * - significant difference compared to preexercise level.

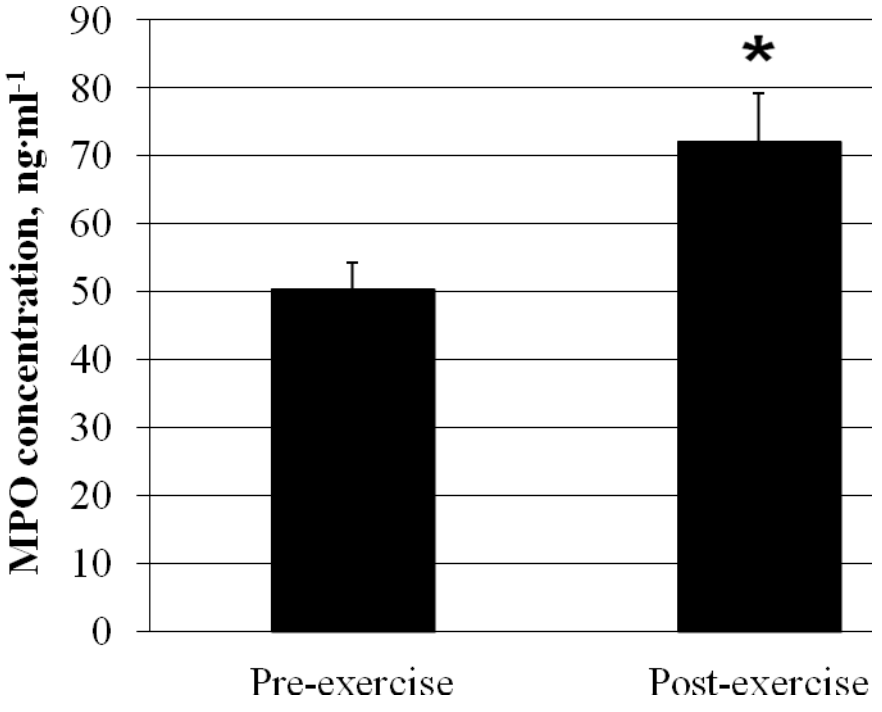

Figure 17. Sera MPO concentration changes in response to a maximal exercise till exhaustion – VO_{2max}, in professional ice hockey players, N = 26. Values are mean ± SEM. Symbols: * - significant difference compared to preexercise level.

There were a number of correlations between maximal exercise induced increases of determined variables. Maximal exercise induced increase in MPO concentration correlated with Δ neutrophils abs count ($P < 0.05$, $R = 0.52$, $N = 22$) and Δ IL-6 concentration ($P < 0.01$, $R = 0.64$, $N = 19$, Figure 18). Furthermore, Δ MMP-9 correlated with Δ MPO ($P < 0.01$, $R = 0.62$, $N = 24$, Figure 19) and Δ IL-6 concentrations ($P < 0.05$, $R = 0.60$, $N = 17$, Figure 20).

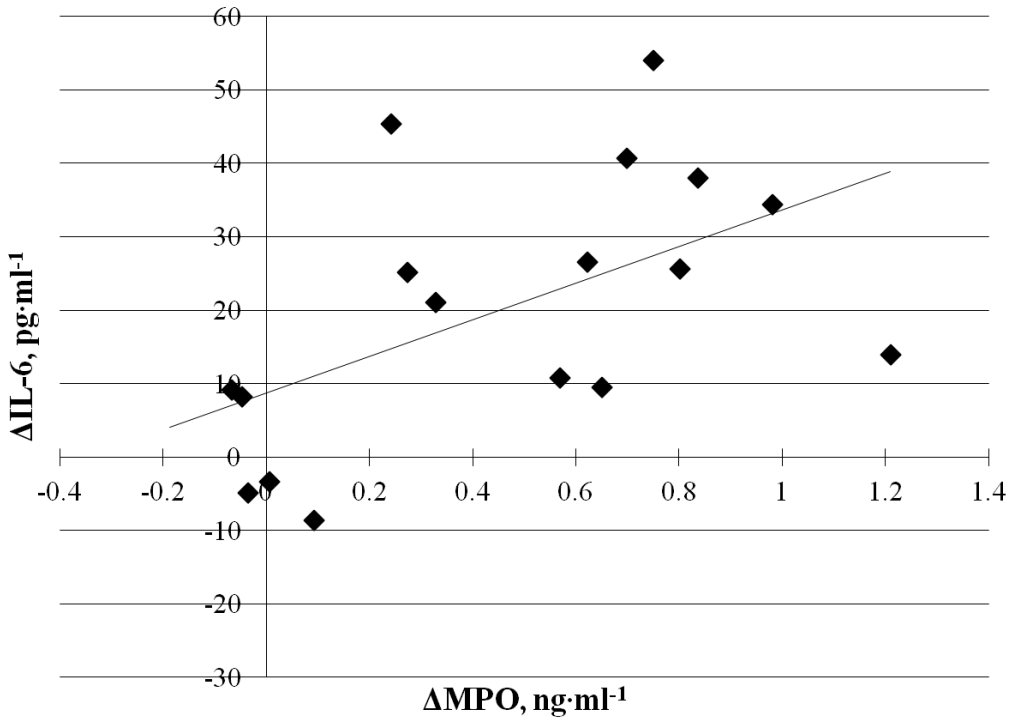

Figure 18. The correlation between maximal exercise-induced increases in MPO and IL-6 concentrations. $P < 0.05$, $R = 0.54$, regression equation: y = 8.6949 + 24.91*x.

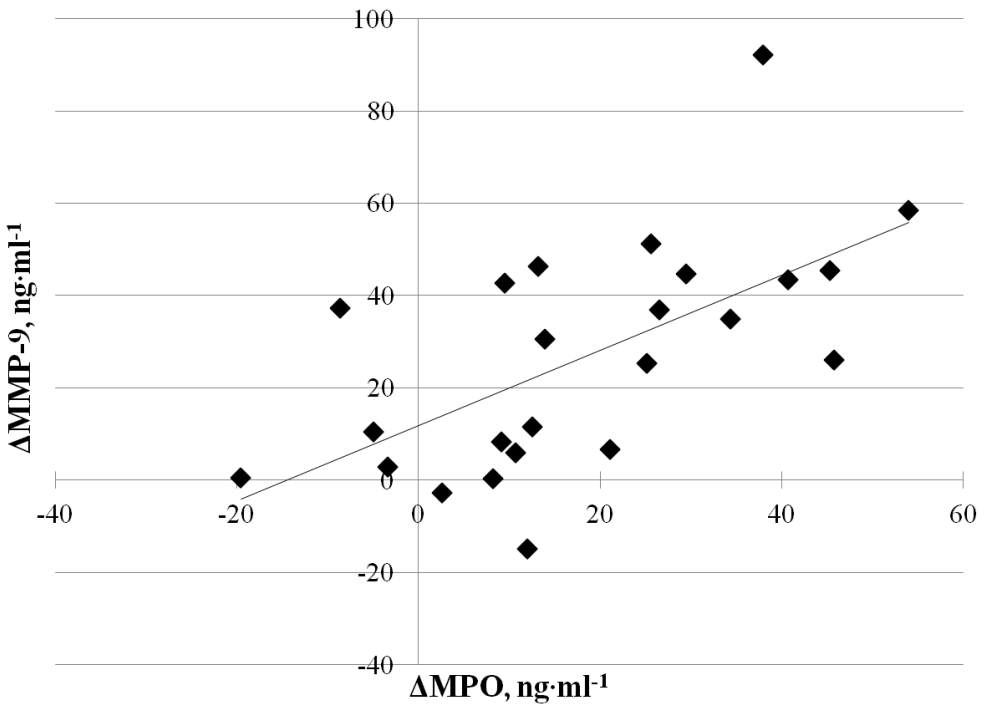

Figure 19. The correlation between maximal exercise-induced increases in MPO and MMP-9 concentrations. $P < 0.01$, $R = 0.62$, regression equation: y = 11.8963 + 0.8147*x.

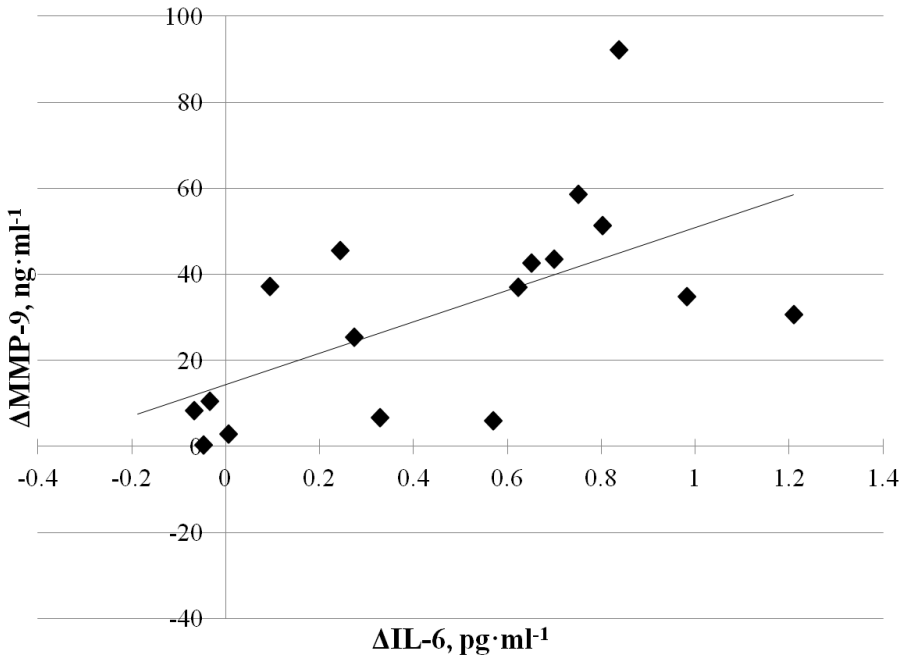

Figure 20. The correlation between maximal exercise-induced increases in IL-6 and MMP-9 concentrations. $P < 0.05$, $R = 0.60$, regression equation: y = 14.3641 + 36.5221*x.

4.2. Study II

HM runners completed distance in 1.8 ± 0.2 hrs, and M runners in 3.6 ± 0.4 hrs, thus HM and M runners performed competition with similar absolute intensity as there was no difference between average speed of both groups (HM: 11.7 ± 1.5 vs. M: 11.9 ± 1.8 km/h, *P*=0.71, Figure 21).

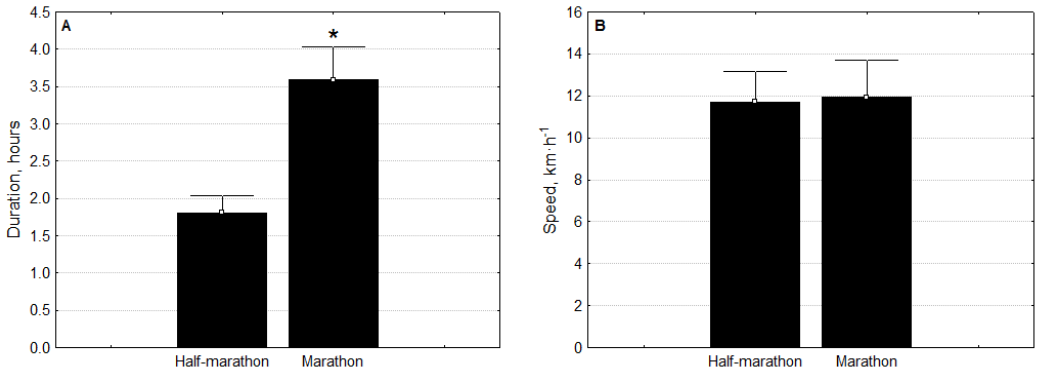

Figure 21. Duration of the half-marathon and marathon race (A); absolute exercise intensity (running speed) during half-marathon and marathon race. Symbols: * - significant difference compared to preexercise level. Half marathon: *N* = 22; marathon: *N* = 18. Values are means ± SD

There were changes in haemoglobin concentration and haematocrit (Table 6), thus, correction of plasma concentrations for blood cells and inflammatory molecules were made. Concentrations of blood cells are shown in Table 6. In both HM and M running, all subpopulations of leukocytes, except eosinophils and lymphocytes, increased markedly, and most in M runners ($P<0.05$), but returned to baseline after 28 hrs recovery. There was an increase in lymphocytes 28 hrs after race ($P<0.05$). Indicative of skeletal muscle damage, CK and LDH, increased in both HM and M and remained markedly elevated in recovery phase ($P<0.05$, see Table 7). Peak concentrations of cortisol were highest in M ($P<0.05$) and in both groups returned to concentration below base line levels ($P<0.05$). Also concentrations of ACTH were highest in M ($P<0.05$) after prolonged running ($P<0.05$). Glucose concentration displayed out minor changes, and was not different between the groups.

Table 6 Pre-race, post-race and recovery haematological parameters

	Half-marathon			Marathon		
	Prerace, *N* = 22	**15 min Postrace, *N* = 22**	**28h Postrace, *N* = 18**	**Prerace, *N* = 18**	**15 min Postrace, *N* = 18**	**28h Postrace, *N* = 8**
Haemoglobin, $nmol \cdot L^{-1}$	9.2 ± 0.5	9.5 ± 0.5*	8.9 ± 0.5*	9.1 ± 0.6	9.3 ± 0.6*	8.8 ± 0.5*
Haematocrit	43 ± 2	45 ± 3*	42 ± 3*	43 ± 3	44 ± 3	41 ± 2*
Plasma volume, ml per 100 ml blood	57 ± 2	53 ± 4*	60 ± 4*	57 ± 3	55 ± 4	61 ± 3
Leukocytes, x 10^9 cells per litre	5.6 ± 1.2	13.3 ± 2.7*	6.8 ± 1.1*	5.8 ± 1.3	17.3 ± 4.0*#	7.4 ± 1.2*
Neutrophils, x 10^9 cells per litre	3.0 ± 1.0	10.4 ± 2.4*	3.5 ± 0.9	3.0 ± 0.9	14.3 ± 3.6*#	3.7 ± 1.0
Band neutrophils, x 10^9 cells per litre	0.13 ± 0.10	0.37 ± 0.31*	–	0.13 ± 0.11	0.87 ± 0.58*#	–
Band neutrophil/total leukocyte ratio, %	2.1 ± 1.4	2.8 ± 2.2	–	2.1 ± 1.6	5.2 ± 3.4*#	–
Basophils, x 10^9 cells per litre	0.02 ± 0.01	0.06 ± 0.03*	0.02 ± 0.01	0.02 ± 0.01	0.09 ± 0.04*#	0.01 ± 0.04
Eosinophils, x 10^9 cells per litre	0.20 ± 0.18	0.20 ± 0.07	0.27 ± 0.16	0.18 ± 0.11	0.20 ± 0.06	0.26 ± 0.11
Monocytes, x 10^9 cells per litre	0.33 ± 0.09	0.56 ± 0.20*	0.32 ± 0.10	0.34 ± 0.16	0.82 ± 0.33*#	0.46 ± 0.16
Lymphocytes, x 10^9 cells per litre	2.09 ± 0.39	2.03 ± 0.72	2.70 ± 0.41*	2.24 ± 0.52	1.86 ± 0.57	2.94 ± 0.44*

Values are means ± SD. * - significant difference compared to prerace; # - significant difference between groups

Table 7 Pre-race, post-race and recovery blood mediators´ measures

	Half-marathon			Marathon		
	Prerace, *N* = 22	**15 min Postrace, *N* = 22**	**28h Postrace, *N* = 18**	**Prerace, *N* = 18**	**15 min Postrace, *N* = 18**	**28h Postrace, *N* = 8**
Creatine kinase, $IU \cdot L^{-1}$	211 ± 113	414 ± 213*	1172 ± 1047*&	185 ± 96	531 ± 248*#	2570 ± 1402*&#
Lactate dehydrogenase, $U \cdot L^{-1}$	178 ± 25	264 ± 46*	–	188 ± 35	381 ± 97*#	–
Adrenocorticotropic hormone, $pg \cdot ml^{-1}$	8 ± 4	54 ± 35*	15 ± 3	9 ± 5	117 ± 101*#	10 ± 3
Cortisol, $nmol \cdot L^{-1}$	422 ± 117	831 ± 128*	221 ± 95*	377 ± 104	1148 ± 232*#	211 ± 95*
Glucose, $mmol \cdot L^{-1}$	5.1 ± 0.4	4.3 ± 1.2*	–	5.1 ± 0.4	4.6 ± 1.2	–

Values are means ± SD. * - significant difference compared to prerace; & - significant difference compared to 15 min postrace; # - significant difference between groups

4.2.1. Sera IL-6, TNF-α, sICAM-1, MMP-9 concentrations

Interaction between the variables, duration (i.e. group) and time (i.e. pre and post race) was significant in the analysis of IL-6, TNF-α and MMP-9 levels. The post-hoc test showed significant differences between the groups' responses to prolonged running in these three parameters. IL-6 concentration increased significantly only in M runners (28 fold increase was seen) and returned back to prerace levels after 28 h (Figure 22), showing disparity between the two groups (P<0.05).

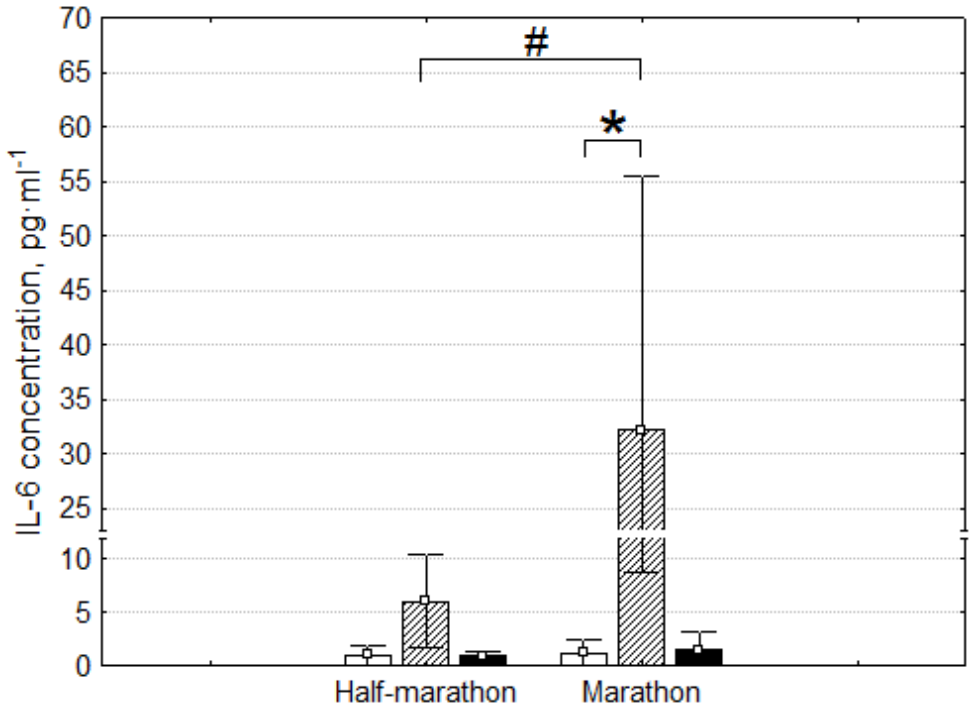

Figure 22. Sera IL-6 concentrations changes in response to a competitive half-marathon and marathon running. Values are mean ± SD. Symbols: white bars – prerace; striped bars – 15 min postrace; black bars – 28 h postrace; * - significant difference compared to prerace level; # - significant difference between the groups. Half marathon: N = 22; 28 h postrace N = 18. Marathon: N = 18; 28 h postrace N = 8.

M running induced a rise in TNF-α concentrations that was higher than the insignificant increment after HM distance (HM: 1.1 fold increase vs. M: 1.4 fold increase, P<0.05, Figure 23). TNF-α concentrations 28 hours after competition cessation did not differ from prerace levels in any group (P>0.05).

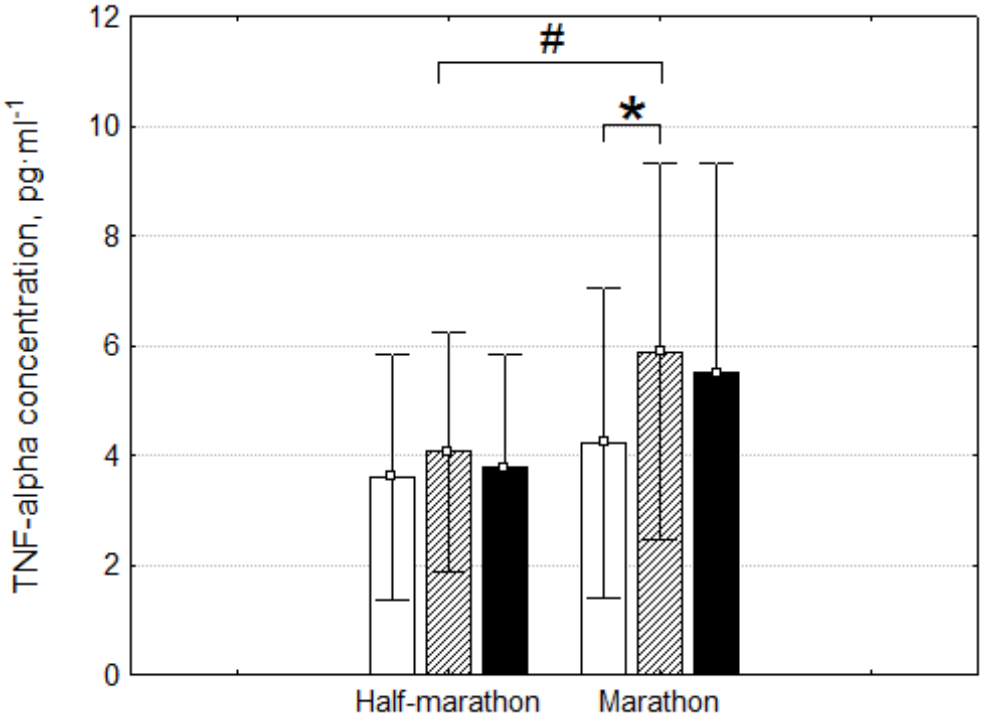

Figure 23. Sera TNF-α concentrations changes in response to a competitive half-marathon and marathon running. Values are mean ± SD. Symbols: white bars – prerace; striped bars – 15 min postrace; black bars – 28 h postrace; * - significant difference compared to prerace level; # - significant difference between the groups. Half marathon: *N* = 22; 28 h postrace *N* = 18. Marathon: *N* = 18; 28 h postrace *N* = 8.

MMP-9 concentration in HM runners increased 2.0 fold, but in M runners a 2.9 fold increase was seen, showing disparity between the two groups ($P<0.05$). 28 h postrace MMP-9 concentrations were back to prerace levels in both groups ($P>0.05$) (Figure 24). sICAM-1 concentrations increased significantly ($P<0.05$) in both groups after competitive running and stayed elevated during recovery (Figure 25), but the concentrations were similar in HM and M runners at all time points ($P>0.05$).

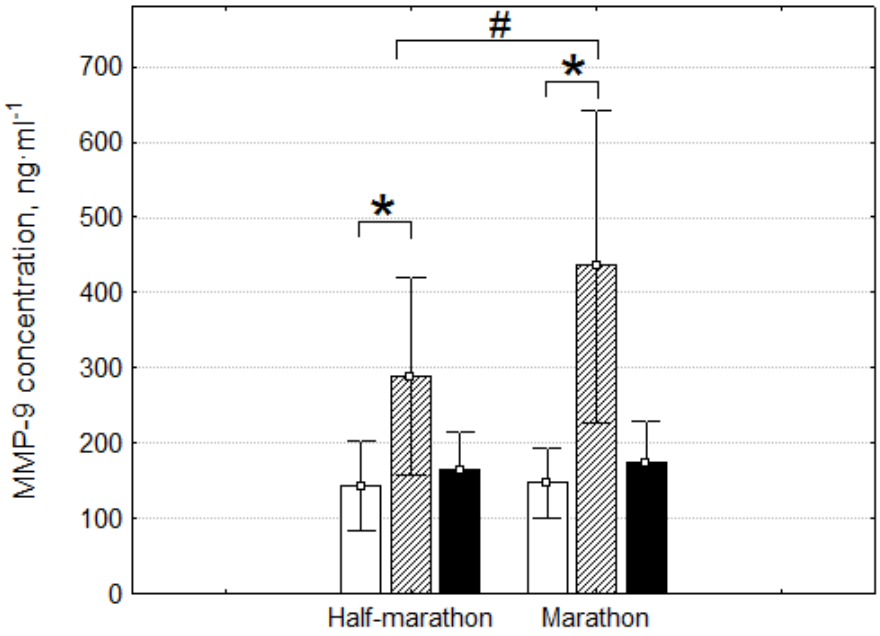

Figure 24. Sera MMP-9 concentrations changes in response to a competitive half-marathon and marathon running. Values are mean ± SD. Symbols: white bars – prerace; striped bars – 15 min postrace; black bars – 28 h postrace; * - significant difference compared to prerace level; # - significant difference between the groups. Half marathon: *N* = 22; 28 h postrace *N* = 18. Marathon: *N* = 18; 28 h postrace *N* = 8.

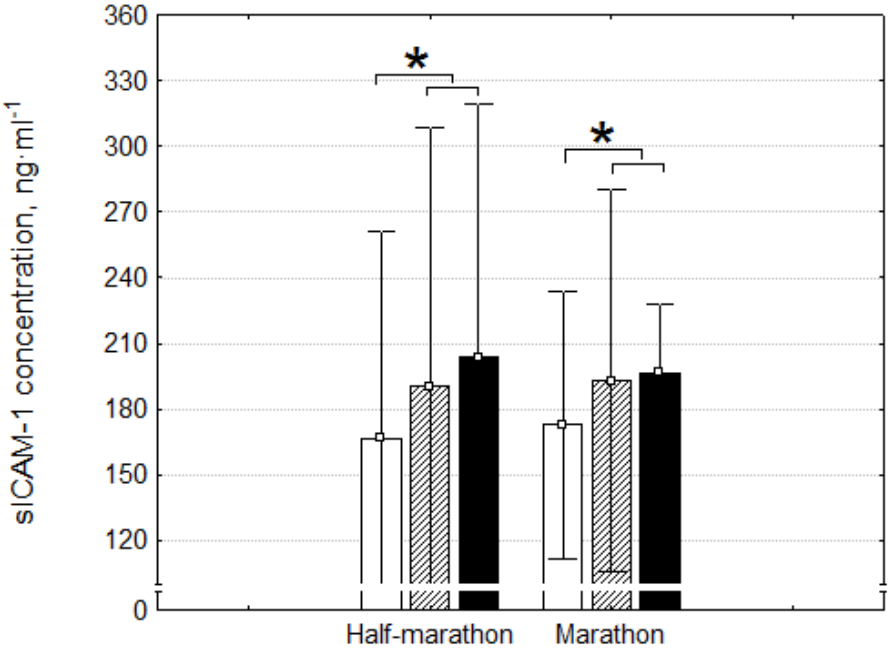

Figure 25. sICAM-1 concentrations changes in response to a competitive half-marathon and marathon running. Values are mean ± SD. Symbols: white bars – prerace; striped bars – 15 min postrace; black bars – 28 h postrace; * - significant difference compared to prerace level; # - significant difference between the groups. Half marathon: *N* = 22; 28 h postrace *N* = 18. Marathon: *N* = 18; 28 h postrace *N* = 8.

Exercise induced increase in IL-6 concentration correlated with Δ TNF-α ($P < 0.01$, $R = 0.46$, $N = 40$) and Δ MMP-9 ($P < 0.01$, $R = 0.42$, $N = 40$) concentrations when both HM and M runners where pooled together. Correlation analysis within the groups revealed that Δ IL-6 correlated with Δ MMP-9 concentration only in HM runners ($P < 0.05$, $R = 0.51$, $N = 22$).

4.3. Study III

All athletes performed exercise with similar relative intensity – 70% of HRR (149±5 bpm). Absolute workload for the HT group was significantly higher than that for the MT group (2.7±0.1 vs. 2.2±0.1 $W \cdot kg^{-1}$, respectively, $P < 0.05$).

Concentrations of blood cells are shown in Table 7. All subpopulations of leukocytes, except eosinophils and monocytes ($P < 0.05$), were increased with exercise across both groups. Both leukocytes and neutrophils were increased markedly in the MT group compared to the HT group ($P < 0.05$).

Table 7 Pre-exercise and post-exercise haematological parameters

	Moderately trained athletes		**Highly trained athletes**		**Two way repeated measure ANOVA**	
	Pre-exercise, *N* = 10	**Post-exercise, *N* = 10**	**Pre-exercise, *N* = 7**	**Post-exercise, *N* = 7**	**Main effect of group**	**Main effect of exercise**
Haemoglobin, $nmol \cdot L^{-1}$	9.1 ± 0.0	9.5 ± 0.0*	9.0 ± 0.0	9.3 ± 0.0	NS	P<0.001
Haematocrit	45 ± 1	47 ± 1*	45 ± 1	47 ± 1*	NS	P<0.001
Leukocytes, x 10^9 cells per litre	6.1 ± 0.3	8.3 ± 0.7*	5.0 ± 0.4	5.8 ± 0.3*#	P<0.01	P<0.001
Neutrophils, x 10^9 cells per litre	3.5 ± 0.3	4.9 ± 0.6*	2.8 ± 0.3	3.2 ± 0.2#	P<0.05	P<0.01
Basophils, x 10^9 cells per litre	0.07 ± 0.01	0.08 ± 0.01	0.04 ± 0.01	0.06 ± 0.01*	NS	P<0.05
Eosinophils, x 10^9 cells per litre	0.19 ± 0.02	0.19 ± 0.03	0.14 ± 0.03	0.16 ± 0.05	NS	NS
Monocytes, x 10^9 cells per litre	0.50 ± 0.07	0.56 ± 0.07	0.32 ± 0.04	0.33 ± 0.03#	P<0.05	NS
Lymphocytes, x 10^9 cells per litre	1.86 ± 0.14	2.54 ± 0.21*	1.67 ± 0.14	2.08 ± 0.12	NS	P<0.001

Values are means ± SEM. * – significant difference compared to pre-exercise. # - significant difference between the groups

There was no indication of skeletal muscle damage as creatine kinase (CK), lactate dehydrogenase (LDH) and lactate concentrations did not change with exercise (Table 8, $P > 0.05$). Peak concentrations of cortisol were significantly elevated in both groups ($P < 0.05$, Table 8). There was a decrease in glucose concentration with exercise across both groups ($P < 0.05$, Table 8).

Table 8 Pre-exercise and post-exercise blood mediators´ measures

	Moderately trained athletes		Highly trained athletes		Two way repeated measure ANOVA	
	Pre-exercise, *N* = 10	**Post-exercise, *N* = 10**	**Pre-exercise, *N* = 7**	**Post-exercise, *N* = 7**	**Main effect of group**	**Main effect of exercise**
MMP-9, $ng \cdot ml^{-1}$	89 ± 17	146 ± 32	98 ± 29	118 ± 16	NS	NS
sE-selectin, $ng \cdot ml^{-1}$	20 ± 2	20 ± 2	26 ± 2	25 ± 2	NS	NS
sICAM-1, $ng \cdot ml^{-1}$	97 ± 10	92 ± 7	129 ± 15	117 ± 7$^{\#}$	P<0.05	NS
sVCAM-1, $ng \cdot ml^{-1}$	1301 ± 65	1294 ± 61	1296 ±	1382 ± 109	NS	NS
MPO, $ng \cdot ml^{-1}$	47 ± 10	45 ± 8	33 ± 8	39 ± 6	NS	NS
Creatine kinase, $IU \cdot L^{-1}$	177 ± 25	181 ± 26	172 ± 32	175 ± 32	NS	NS
Lactate dehydrogenase, $IU \cdot L^{-1}$	314 ± 33	309 ± 33	345 ± 23	359 ± 27	NS	NS
Glucose, $mmol \cdot L^{-1}$	4.8 ± 0.2	4.1 ± 0.1*	5.1 ± 0.4	4.5 ± 0.2	NS	P<0.01
Lactate, $mmol \cdot L^{-1}$	2.3 ± 0.5	2.6 ± 0.3	1.7 ± 0.1	1.8 ± 0.1$^{\#}$	NS	NS
Cortisol, $nmol \cdot L^{-1}$	271 ± 36	458 ± 40*	306 ± 23	546 ± 42*	NS	P<0.001

Values are means ± SEM. * – significant difference compared to pre-exercise; # - significant difference between the groups

There was also an increase in IL-6 ($P < 0.05$, Figure 26), TNF-α ($P < 0.05$, Figure 27) and MCP-1 ($P < 0.05$, Figure 28) concentrations with exercise. When compared to the pre-exercise values, only the MT group demonstrated an increase in the post-exercise IL-6 concentration. In contrast, exercise induced increase in TNF-α concentration was seen only in the HT group, but not in the MT group. However, there were no differences in post-exercise IL-6 and TNF-α concentrations between MT and HT group. There were no exercise-induced changes in MMP-9, MPO, sE-selectin, sICAM-1 and sVCAM-1 concentrations ($P > 0.05$, Table 8). Furthermore, there were no differences in post-exercise biomarker's concentrations between the two groups ($P > 0.05$, Table 8).

Statistical analysis revealed a significant relationship between maximal exercise-induced changes in MMP-9 and MPO concentrations ($P < 0.05$, $R = 0.57$, $N = 17$).

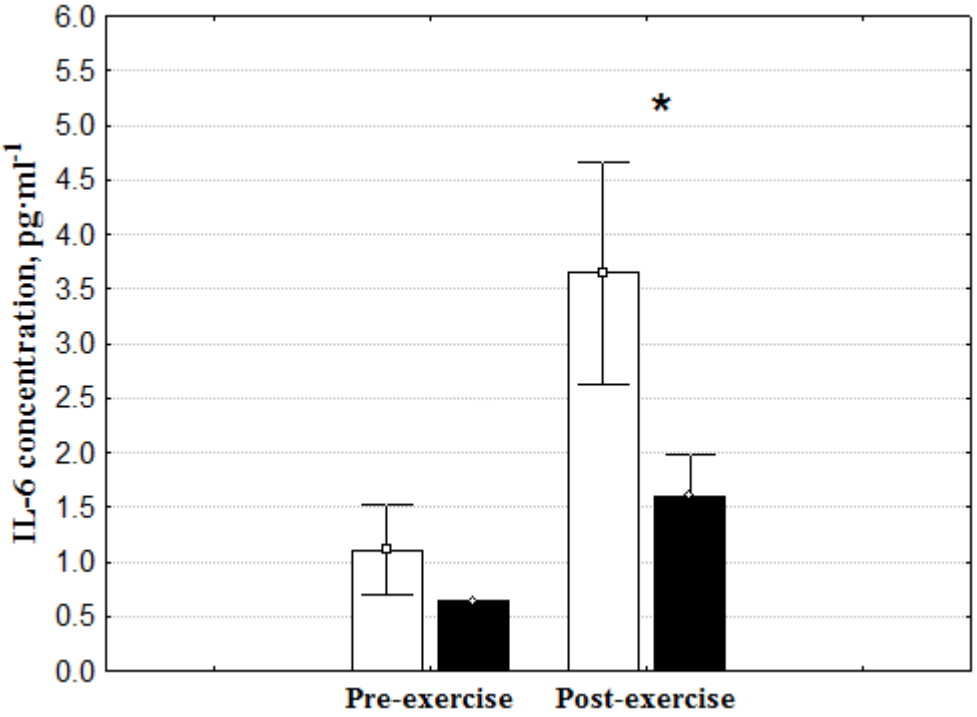

Figure 26. Sera IL-6 concentrations changes in response to a 1 h long recumbent cycling exercise. Values are mean ± SEM. Symbols: white bars – moderately trained athletes (*N* = 10); black bars – highly trained athletes (*N* = 7); * - main effect of exercise – significant difference compared to pre-exercise level.

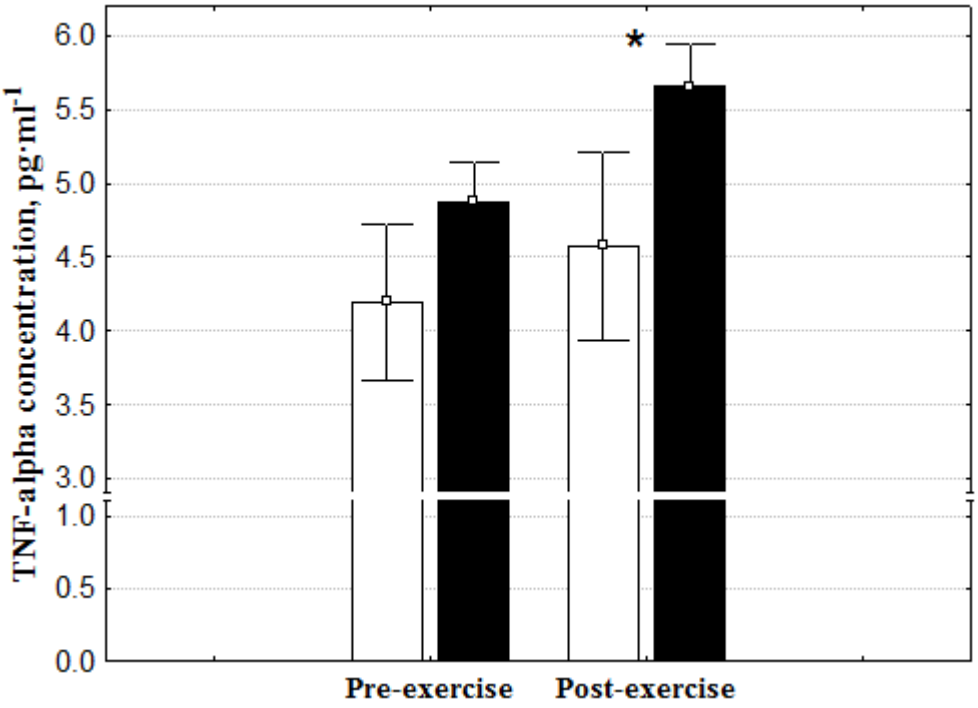

Figure 27. Sera TNF-α concentrations changes in response to a 1 h long recumbent cycling exercise. Values are mean ± SEM. Symbols: white bars – moderately trained athletes (*N* = 10); black bars – highly trained athletes (*N* = 7); * - main effect of exercise – significant difference compared to pre-exercise level.

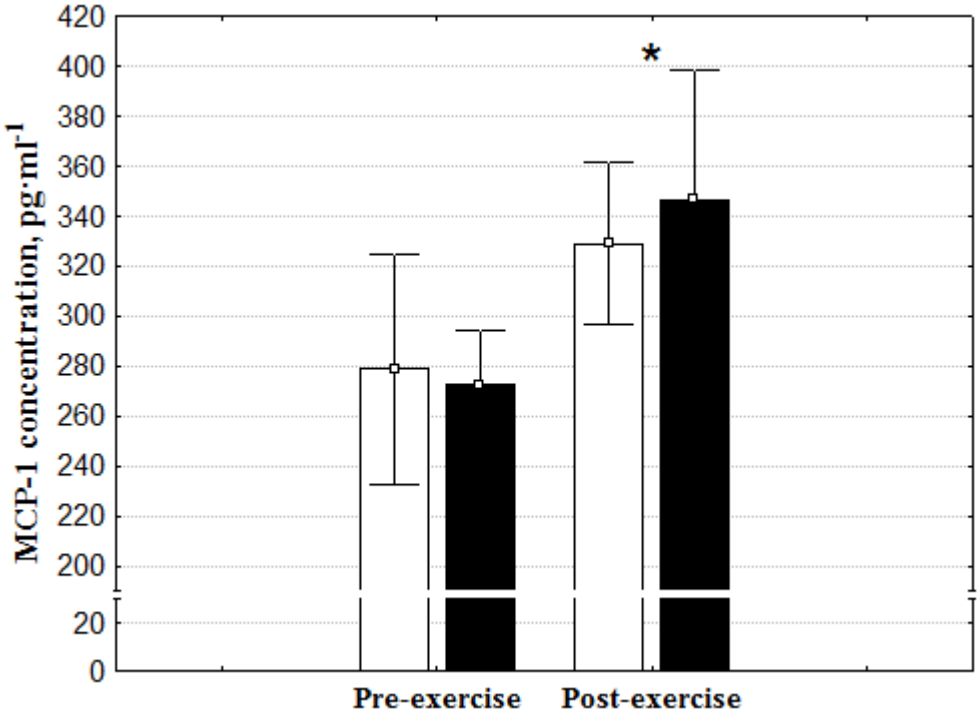

Figure 28. Sera MCP-1 concentrations changes in response to a 1 h long recumbent cycling exercise. Values are mean ± SEM. Symbols: white bars – moderately trained athletes (N = 10); black bars – highly trained athletes (N = 7); * - main effect of exercise – significant difference compared to pre-exercise level.

4.4. Study IV

Average heart rate during the exercise was 114 ± 0.3 bpm (59 ± 2% of maximal heart rate). Whole body oxygen uptake during the exercise was 1.10 ± 0.02 Liter $O_2 \cdot min^{-1}$. During the exercise experiment, the absolute workload was identical for the two legs (19.6 ± 0.8 W). Subsequently (i.e. the day after) W_{max} was determined for both legs and it tended to be higher in the CON leg compared to the IM leg, 42.2 ± 3.1 vs. 37.6 ± 3.6 W, respectively (P = 0.06). Thus, the relative workload for the IM leg was higher than for the CON leg, 52 ± 5% vs. 46 ± 3% of W_{max}, respectively (P < 0.05).

There was a significant increase in thigh plasma flow after 15 minutes of the exercise (Figure 29, P < 0.05), where it remained unchanged and was similar between the legs at all time points.

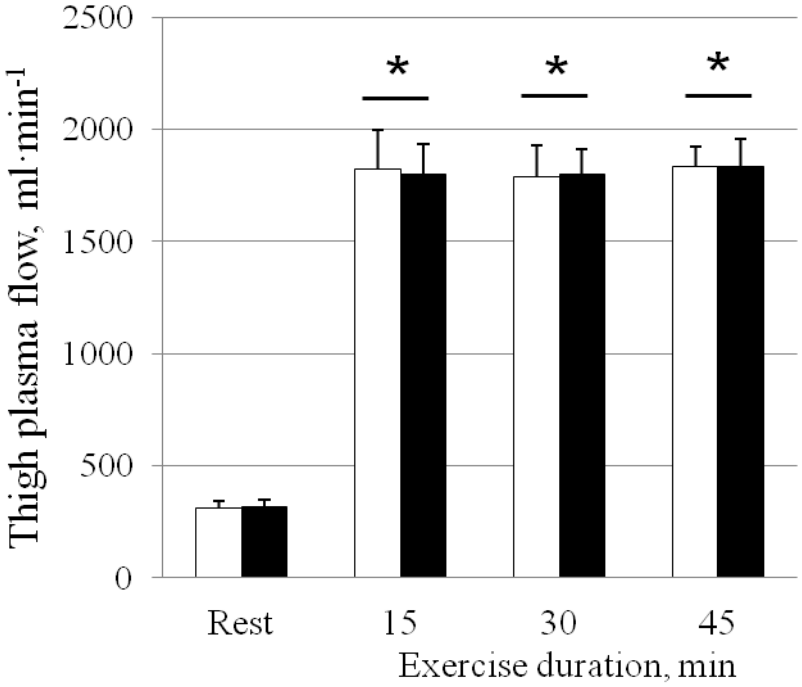

Figure 29. Thigh plasma flow during rest and two leg knee extension exercise. * - significant difference compared to rest. Black and white bars represent immobilized and control leg, respectively. Values are mean ± SEM; *N* = 15.

Throughout the exercise, arterial plasma IL-6 concentration increased significantly at each time point (Figure 30, $P < 0.05$), whereas the venous plasma IL-6 concentration in the CON and the IM leg was unchanged during the first 30 minutes of exercise and only increased after 45 min (Figure 30, $P < 0.05$). At rest and through exercise venous plasma IL-6 concentrations exceeded arterial concentrations, and after 45 min plasma IL-6 concentration in the IM leg was higher than in the CON leg (Figure 30, $P < 0.05$).

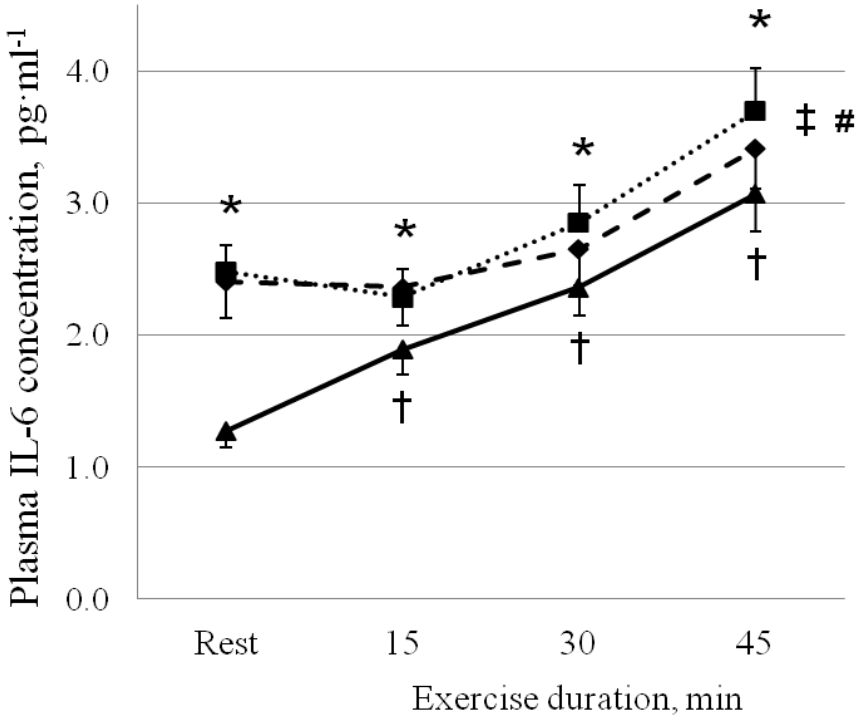

Figure 30. Brachial arterial and femoral venous plasma IL-6 concentrations at rest and during two leg knee extension exercise. At rest the, average of measurements at two time points (t=-15 min and t=0 min) samples was used. Black circles (full line), white triangles (broken line) and white squares (dotted line) represent IL-6 plasma concentrations in the artery, femoral venous control leg, and femoral venous immobilized leg, respectively. * - ($P < 0.05$) artery vs. both femoral veins; # - ($P < 0.05$) femoral veins: CON vs. IM; † - ($P < 0.05$) artery: vs. previous time point; ‡ - ($P < 0.05$) both femoral veins: rest vs. 45 min. Values are mean ± SEM.

Venoarterial plasma IL-6 difference was significantly decreased at t = 15 min compared to rest in both legs and remained unchanged till the end of exercise (Figure 31, $P < 0.05$). There was a significant difference between the CON and the IM leg venoarterial plasma IL-6 difference at 45 min (Figure 31, $P < 0.05$).

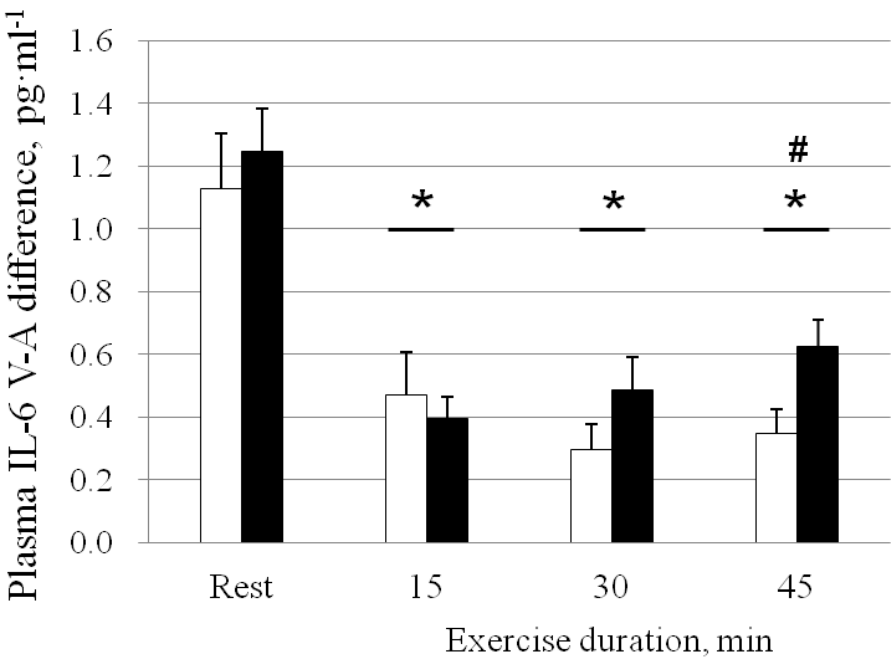

Figure 31. Venoarterial plasma IL-6 difference during rest and two leg knee extension exercise. * - significant difference compared to rest; # - significant difference between legs. At rest the, average of measurements at two time points (t=-15 min and t=0 min) samples was used. Black and white bars represent immobilized and control leg, respectively. Values are mean ± SEM.

There was a 2 fold increase in IL-6 release from both working legs during the first 15 minutes of exercise (Figure 32, $P < 0.05$) and IL-6 release from the immobilized leg continued to increase throughout the exercise. As a result, there was a significant difference between the CON and the IM leg IL-6 release after 45 min (Figure 32, $P < 0.05$).

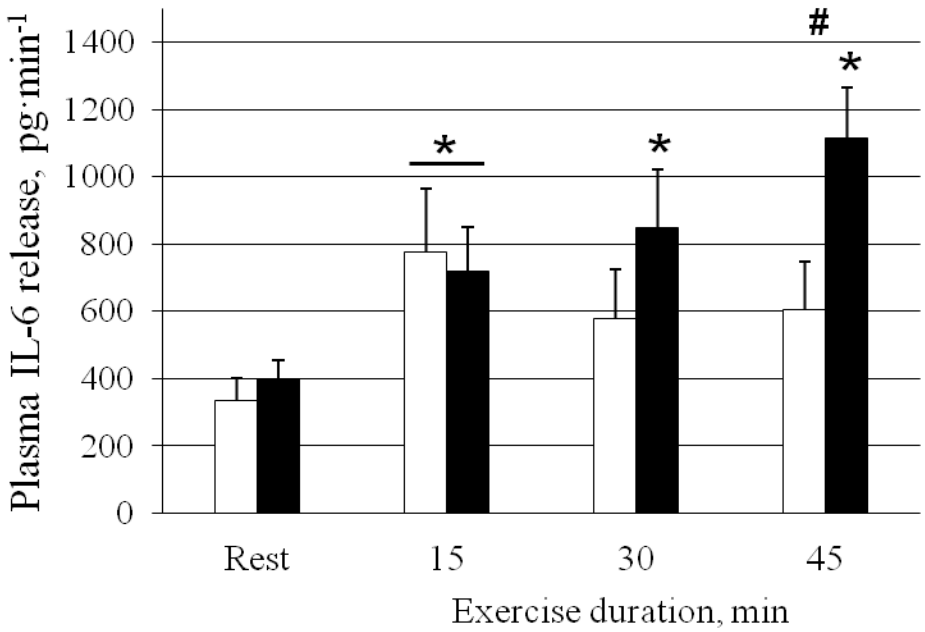

Figure 32. Thigh IL-6 release (B) during rest and two leg knee extension exercise. * - significant difference compared to rest; # - significant difference between legs. At rest the, average of measurements at two time points (t=-15 min and t=0 min) samples was used. Black and white bars represent immobilized and control leg, respectively. Values are mean ± SEM.

Throughout the exercise, there were no changes in arterial or venous plasma TNF-α concentrations (A: 1.0 ± 0.2 vs. 1.0 ± 0.2 pg·ml^{-1}; CON: 1.1 ± 0.1 vs. 0.9 ± 0.1 pg·ml^{-1}; IM: 0.9 ± 0.1 vs. 0.9 ± 0.1 pg·ml^{-1} at rest and t = 45 min, respectively, P > 0.05) and thus consequently no significant release or uptake from the working legs (CON: 2 ± 30 vs. -346 ± 272 pg·min^{-1}; IM: -42 ± 37 vs. -315±314 pg·min^{-1} at rest and t = 45 min, respectively, P > 0.05) was seen.

5. Discussion

5.1. Study I

Exercise is associated with temporary changes in the immune system, e.g. count of immune cells (Lippi et al. 2010) and concentrations of cytokines (Suzuki et al. 2003), adhesion molecules (Nielsen & Lyberg 2004), MMPs (Madden et al. 2011) and MPO (Morozov et al. 2006). The present study shows that maximal exercise can induce significant changes in all subpopulations of leukocytes, especially neutrophils and lymphocytes ($P < 0.05$). Analysis of the neutrophils MPO in animal study by Morozov et al. (2003) revealed a significant elevation in the concentration of MPO (+67%) in rat blood plasma after swimming to exhaustion, while the content of MPO in neutrophils was decreased by 34%. These data suggest that intensive physical exercise activates the secretory function (degranulation) of blood neutrophils. Both types of neutrophils granules (specific and azurophilic) appear to be involved in this process (Morozov et al. 2003). Our data correspond to the findings of above mentioned study that have shown exercise induced degranulation of neutrophils and subsequent release of MPO, confirmed by correlation between these two biomarkers ($P < 0.05$).

A study by Toft et al. (2002) has demonstrated that IL-6 increases progressively after eccentric exercise (60 min of opposing the rotation of the pedals down to 60 rpm) but that the increase is modest, despite marked increases in CK and myoglobin. Thus, authors concluded that, the IL-6 response to muscle damage does not make an important contribution to the large increases in IL-6 observed during exercise of long duration. Low plasma glucose or muscle glycogen levels also increase IL-6 concentration during exercise. For example, it has been shown that the amount of IL-6 released from working skeletal muscle is positively related to work intensity, glucose uptake and plasma adrenaline concentration (Helge et al. 2003). In addition, in the same study an inverse correlation between thigh IL-6 release late during exercise and muscle glycogen content at the end of exercise was observed (Helge et al. 2003). These findings are in line with the hypothesis that IL-6 contributes to glucose homeostasis during exercise. In Study I we observed an increase in serum glucose concentrations, most likely due to exercise induced sympatho-adrenergic hepatic stimulation, and yet increases in IL-6 concentration after maximal exercise ($P < 0.05$).

Thus, it appears that the increase in IL-6 concentration in this study is less connected with glucose homeostasis. Moreover, our data showed that maximal exercise induced MPO expression correlated with the increase in IL-6 concentration ($P < 0.05$). With the release of MPO, marker of neutrophils degranulation, it is plausible that also IL-6 at least to some extent is released from the neutrophils during maximal exercise.

There are data that show pro-inflammatory cytokine (e.g. IL-6, TNF-α) incentive effect on the adhesion molecule up-regulation on the endothelial cells (Monchanin et al. 2007). Three successive maximal ramp exercise tests interspaced with 10 min of seated comprising 5 min of easy pedalling and 5 min of rest prompted an inflammatory response (i.e., white blood cells and IL-6 levels increased in response to exercise), however sICAM-1 and sVCAM-1 levels did not change during or after exercise (up till 48 h) (Tripette et al. 2010). We also did not observe sICAM-1, sVCAM-1 or sE-selectin changes during exercise despite maximal exercise induced increase in IL-6 concentration.

Our data show no change in TNF-α levels in healthy male athletes following maximal exercise. This is in agreement with the study by Limongelli et al. (2010), that did not show any differences between pre-exercise (15 min before test), peak exercise and post-exercise (30 min after test) TNF-α levels, when standard cycling ramp test (with an increase in work load of 10 $W \cdot min^{-1}$) till exhaustion was conducted. Gokhale et al. also found that majority of the athletes and non-athletes demonstrated a rise in IL-6 and a fall in TNF-α levels (Gokhale et al. 2007). This relation is in agreement with the opinion that IL-6 also exerts anti-inflammatory effects by inhibiting production of TNF-α, possibly also during maximal exercise.

5.1.1. MMP-9

Exercise can cause a damage of skeletal muscles and connective tissue, which leads to activation of tissue MMPs. MMPs, including MMP-9, are major components of neutrophilic tertiary granules, and are also expressed by other types of leukocytes including monocytes, and lymphocytes (Chen et al. 2006b). Furthermore, a single bout of exercise (65 min of cycle exercise at gradually increasing load – from 50% VO_{2max} till highest tolerable work rates that could be sustained for 5 min) induced an increment of total muscle MMP-9 protein at the end of exercise (Rullman et al. 2007). Muscle MMP-9 protein concentration remained elevated 120 min following exercise. Furthermore, increased MMP-9 activity was evident before any measurable changes

in MMP-9 transcription (muscle MMP-9 mRNA increased 120 min after exercise cessation), indicating posttranscriptional mechanisms (Rullman et al. 2007).

The studies on the effect of exercise on plasma concentrations of MMP-9 are equivocal. There were no changes in MMP-9 concentrations after peak symptom limited Bruce treadmill exercise testing (Tayebjee et al. 2005). However, bicycle exercise test (50 W starting load which was afterwards gradually increased by stages lasting 3 min and exercise load increasing by 50 W, till the predicted heart rate maximum was achieved or when limiting symptoms occurred) resulted in significant increase in MMP-9 levels both in coronary artery disease patients and healthy controls (Danzig et al. 2010).

It has been shown that there is no correlation between exercise induced increase in MMP-9 and creatine kinase activity, suggesting that the rapid and transient increase in the serum MMP-9 concentration may reflect accelerated release of MMP-9 to circulation because of exercise induced changes in leukocyte number rather than an increased extracellular matrix breakdown (Koskinen et al. 2001). Our data showed that maximal exercise induced MPO expression correlated with the increase in MMP-9 concentration ($P < 0.05$), supporting the previous mentioned hypothesis, that neutrophils could be the major contributors for increased MMP-9 levels. Moreover, our data showed that maximal exercise induced MMP-9 expression correlated with the increase in IL-6 concentration ($P < 0.05$), suggesting a close interplay between inflammatory and proteolytic processes. There is no substantial evidence in scientific literature showing IL-6 induced MMP-9 release from neutrophils, however, it is known that IL-6 stimulate MMP-9 expression in different cell types, e.g. fibroblasts (Dasu et al. 2003). The correlations found in this study possibly could point out IL-6 modulatory effects on MMP-9 that needs to be clarified in further studies. It has been suggested that IL-6 might also regulate mobilization of neutrophils into circulatory system (Yamada et al. 2002) which could be another way of IL-6 contribution to increased MMP-9 levels after maximal exercise.

5.2. Study II

Exhaustive exercise may induce systemic inflammatory response syndrome detected by an increase in the band neutrophil/total leukocyte ratio (≥10%) (Suzuki et al. 2003). During this “open window” of altered immunity (which may last between 3

and 72 hours, depending on the parameter measured), viruses and bacteria may gain a foothold, increasing the risk of subclinical and clinical infection (Nieman 2007). Although there was a significant increase in band neutrophil/total leukocyte ratio, it was below 10% in both HM and M runners. Neutrophilia, lymphopenia and an increase in pro- and anti-inflammatory cytokines are regarded as some of the components that reflects the physiological stress during severe exercise (Nieman 2007). We found that leukocytosis, neutrophilia, basophilia and monocytosis were significantly greater in M runners ($P<0.05$), suggesting that physiological stress in M was higher compared to HM.

It has been shown that numerous factors can interact with hypothalamo–pituitary–adrenal axis response to exercise, such as training, intensity and duration of exercise. We found that ACTH and cortisol concentrations were significantly greater in M runners ($P<0.05$) which is in agreement with the previous studies (Duclos et al. 1997). Significantly lower levels of cortisol 28 hrs after competition may be explained by diurnal variation. Although it has been suggested that exercise-induced secretion of blood cortisol may contribute to post-exercise suppression of the helper- and cytotoxic-T cell counts (Shinkai et al. 1996), we did not find significant decrease in lymphocytes after endurance running.

5.2.1. IL-6

We found a robust increase of IL-6 in serum in M, but not in HM. In response to exercise, circulating levels of IL-6 increase by up to 100-fold, when long duration and high intensity exercise, e.g. marathon running, is performed (Suzuki et al. 2003; Ostrowski et al. 2000; Scherr et al. 2011).

Initially IL-6 was considered as an immunomodulatory cytokine produced predominantly by leukocytes as an inflammatory response to exercise-induced muscle damage. This hypothesis was based on the study by (Bruunsgaard et al. 1997) that showed increased IL-6 concentration after 30 min of eccentric cycle exercise, while IL-6 concentration after concentric exercise of the same duration remained unchanged. In addition, eccentric exercise did not cause a higher increase in the blood concentration of monocytes and neutrophils compared with concentric exercise as would have been expected if these cells were responsible for producing IL-6, or if the increment in these cells was partly a consequence of inflammation in the muscles. It was found that IL-6 2h after eccentric exercise was positively correlated with the

concentration of circulating lymphocytes after 20 min of exercise. Beside being a monokine, IL-6 is also known to be a T helper 2 cytokine, thus it is possible that circulating lymphocytes produce the IL-6 (Bruunsgaard et al. 1997). Few years later, it was showed that IL-6 release can be affected by bioavailability of carbohydrates. For example, Nieman et al. (2003) showed that 1 $L \cdot h^{-1}$ CHO beverages during 3 h long run on treadmill at ~70% of VO_{2max} significantly reduced plasma IL-6 concentration and muscle IL-6 mRNA levels tended to be lower compared to PLA group. Thus, it was suggested that IL-6 could be "energy sensor" and improve skeletal muscle energy supply (Pedersen 2009). A recent study showed that IL-6 were expressed in blood mononuclear cells, however no significant differences in mRNA levels were seen before and after the marathon run in these cells (Bernecker et al. 2011). However, an up-regulation of plasma IL-6 during vigorous exercise was found. As this increase in IL-6 concentration is not attributed to blood mononuclear cells, Bernecker et al. (2011) assumed a local production in, or release from exercised tissues, ie., skeletal muscle. Nevertheless, release of the IL-6 protein may still be leukocyte based, and our findings of increased CK levels may indicate muscle damage with subsequent leukocyte infiltration.

A recent study showed that the relative exercise intensity is the main determinant of the IL-6 concentration during exercise exceeding more than 12 hr (Wallberg et al. 2011). In this study subjects performed 12 blocks of exercise (4 x kayaking, 4 x running and 4 x cycling). Each block included 110 min of exercise and 10 min of rest for food intake and change of equipment and clothes. The main findings in the 24-h protocol in this study were that plasma IL-6 values did not increase after 12 h of exercise and remained at a stable level until the end of exercise. In addition, the absolute values in the nine subjects, who worked at the same relative exercise intensity (about 60% of individual VO_{2peak}), were remarkably similar at 12 and 24 h (Wallberg et al. 2011). Nevertheless, the data from the present study support the notion that duration of exercise up till 4 hours is important, but it does not rule out the effect of intensity.

5.2.2. TNF-α

In the present study TNF-α concentrations in serum increased significantly in M, but not in HM. TNF-α concentrations may increase after endurance exercise, like marathon (Starkie et al. 2001; Bernecker et al. 2011), but two-legged dynamic knee-

extensor exercise at 55% of maximal workload for 3 h did not increase arterial plasma TNF-α concentration (Steensberg et al. 2002). Even a decrease after 1 hr interval running has been reported (Gokhale et al. 2007). Although the mechanisms involved in TNF-α concentration changes during exercise are unknown, it has been proposed that the exercise induced increase in plasma TNF-α concentration is the result of systemic endotoxemia induced by decrease in blood flow to the splanchnic bed (Steensberg et al. 2002). However, TNF-α is expressed in muscle fibres (Kern 1997), and while 60 min ergometer cycle exercise at ~70% VO_{2peak} did not induce an increase in muscle TNF-α mRNA concentration (Chan et al. 2004), a longer exercise duration (2.5 hr cycle exercise at 60% W_{max}) resulted in 3.7 fold increase (Nieman et al. 2005). This indicates that exercise duration probably is important for TNF-α increase during exercise. In the present study M running induced rise in TNF-α concentrations that was significantly higher than the change after HM distance, which supports the notion that duration is important determinant for marked increases in the serum concentration of TNF-α.

5.2.3. sICAM-1

We found similar increases in sICAM-1 serum concentrations in both HM and M runners and our data are in agreement with previous findings (Nielsen & Lyberg 2004). Thus, the data support the notion that sICAM-1 concentration does not depend on the duration of the exercise. However, it has been shown that sICAM-1 concentration increased significantly after 30-min downhill running suggesting that muscle damage (and with that, intensity) is necessary for the increase in sICAM-1 levels (Akimoto et al. 2002). Furthermore, we observed that sICAM-1 concentrations remained elevated in recovery phase. This is in agreement with previous study which suggested that increased sICAM-1 concentration 1 day after endurance running could be due to increased leukocyte migration to the regions of inflammation and muscle damage (Akimoto et al. 2002). The present study shows that the increased concentrations of soluble adhesion molecules could hamper the immune response by occupying the binding sites meant to be used by corresponding cell-bound adhesion molecules (Nielsen & Lyberg 2004) at least for one day after prolonged running. However, this temporary negative effect would be similar in both groups, as there were no differences between the sICAM-1 concentrations 28 h after the race.

5.2.4. MMP-9

There was an increase in MMP-9 serum concentrations in both groups, but significantly more in M compared to HM. Increased circulatory MMP-9 concentrations after marathon running has been shown before (Saenz et al. 2006). As it was described previously discussing Study I, the observations of exercise-induced increases in MMP-9 mRNA levels and increased activity of MMP-9 clearly show that a single bout of exercise activates the MMP system in skeletal muscle (Danzig et al. 2010). Furthermore, TNF-α and other cytokines have been shown to increase MMP-9 mRNA levels and are therefore possible candidates for regulating the increase in MMP-9 transcripts (Rullman et al. 2007). The present study shows a marked rise in MMP-9 concentration after HM and M running that diminished 28 hours after competition cessation. However, CK peaked 28 hrs postrace confirming degradation of muscle cells. This suggests that changes of MMP-9 in serum could be dependent on other sources than skeletal muscle. For example, a pronounced increase in interstitial pro-MMP-9 level around the tendons was observed immediately after one hour uphill running and it was still elevated on the last observation point, 3 days post-exercise (Koskinen et al. 2004). Exercise is also associated with prompt leukocyte increase, as seen in the present study, which may produce many substances, including MMP-9 (Danzig et al. 2010). Specific mechanisms that regulate TNF-α-induced MMP-9 release from human neutrophils was identified 5 years ago (Chakrabarti et al. 2006). In the present study, we cannot exclude that the disparity in 15 min postrace MMP-9 concentrations between the HM and M runners (P=0.019) is due to significant difference of neutrophils count in these groups.

In summary, to our knowledge, this is the first study that shows impact of exercise duration on IL-6, TNF-α and MMP-9 concentrations during prolonged running. The present study shows a disparity between competitive running induced increases in these mediators' concentrations between HM and M runners (P<0.05) while the absolute intensity (running speed) of the exercise was similar in both groups (P = 0.83). This does not, however, rule out that exercise intensity may also influence the response, as previously shown by others (Peake et al. 2005; Kimura et al. 2001; Akimoto et al. 2002). Exercise induced increase in sICAM-1 concentrations was not different between HM and M running, suggesting that exercise duration has no impact on the sICAM-1 response to exercise. There were significant correlations between the

responses of IL-6 and both TNF-α and MMP-9 when all subjects were taken together. Thus, these responses to this exercise seem to vary in parallel. This could suggest that the plasma concentrations of these parameters may be mechanistically linked like in other interventions (e.g. during induction of low-grade inflammation).

5.3. Study III

Exercise is associated with temporary changes in the immune system, e.g. in concentrations of immune cells (Lippi et al. 2010), cytokines and chemokines (Suzuki et al. 2003). Two major mechanisms appear to drive the immune response to exercise: neuroendocrine factors and muscle damage. In the present study the serum concentrations of lactate and the muscle enzymes indicating microtrauma – CK and LDH, remained unchanged during submaximal exercise. However, the study showed that submaximal exercise (1 h) induced leukocytosis mainly by neitrophilia. Adrenaline and to a lesser degree noradrenaline are responsible for acute exercise effects on lymphocyte dynamics, including exercise effects on NK cell activity and T cell function (Pedersen and Toft 2000). Increases in growth hormone and catecholamines mediate the acute effects on neutrophils, whereas cortisol exerts its effects within a time lag of at least two hours and therefore may help to maintain the lymphopenia and neutrocytosis only after long term exercise (Pedersen & Toft 2000). This suggests, that neuroendocrine factors, e.g. adrenaline, could be responsible for the acute exercise effects on lymphocytes seen in this study. We also showed that submaximal exercise increased cortisol concentration.

Previous studies have shown that exercise induced activation of the secretory function of neutrophils results in their degranulation that leads to an increase in plasma concentration of marker neutrophil proteins, including MPO (Morozov et al. 2003). The present study showed that MPO concentration remained unchanged suggesting that the intensity of the exercise was not high enough to elicit muscle damage and subsequent activation of the secretory function of neutrophils. It has been suggested that leukocytes (Chen et al. 2006b) and human skeletal muscle (Rullman et al. 2007) express MMPs, including MMP-9, as a response to local damage of skeletal muscles and connective tissue, in order to cleave muscle-specific proteins and contribute in extracellular matrix formation, remodelling, and regeneration in skeletal muscle (Urso et al. 2009). There was no significant change in MMP-9 concentrations,

supporting the notion that exercise intensity and duration applied in this study did not elicit muscle damage.

It has been suggested that IL-6 released by the contracting muscles may improve skeletal muscle energy supply and assists in the maintenance of stable blood glucose levels during exercise (Pedersen 2009). However, the data describing the effect of low carbohydrate bioavailability on IL-6 release during exercise are not conclusive. Some studies have shown that low plasma glucose or muscle glycogen levels increase IL-6 release (Nieman et al. 2003; Helge et al. 2003) as discussed previously (Study II). However, there are studies that could not confirm IL-6 relation to carbohydrate turnover. For example (Nieman et al. 2004) used test protocol that consisted of 10 different resistance exercises: flat bench press, incline bench press, military press, upright row, bent-over row, French curl, biceps curl, back squat, front squat, and deadlift. Subjects performed four sets of 10 repetitions for each resistance exercise, with the first set at 40% of the subject's 1-RM and the subsequent sets at 60% 1-RM. Two- or three-minute rest intervals were given after the completion of each exercise. Subjects received 10 $ml \cdot kg^{-1} \cdot h^{-1}$ CHO (6%) or PLA beverages during the weight training bout. In summary, changes in plasma IL-6 levels and muscle IL-6 mRNA expression after an intensive 2-h resistance training bout were fairly small. In addition, there was no difference between CHO and PLA group (Nieman et al. 2004). More recent study by (Helge et al. 2011) showed that during 90 min combined arm and leg cycle exercise at 60% of VO_{2max} the limb IL-6 release was not directly correlated neither to release or uptake of exogenous substrate nor muscle glycogen utilization during whole body exercise. Thus, this study does not support a general myokine function of IL-6 coupled to the carbohydrate turnover (Helge et al. 2011).

It appears that the increase in IL-6 concentration in this study is less connected with muscle damage, as there were no changes in CK and LDH levels, or activation of neutrophils' degranulation. In fact, we observed a decrease in glucose concentrations and an increase in cortisol concentration confirming activation of the hypothalamo–pituitary–adrenal axis in order to ensure organism growing energy demands (de Vries et al. 2000). Thus, it is plausible that IL-6 could be released from working muscles in order to maintain glucose homeostasis.

It has been suggested that athletes have an attenuated cytokine response to acute exercise (Gokhale et al. 2007). This notion was based on the fact that intermittent running exercise induced greater magnitude of change in the venous plasma IL-6 and

TNF-α concentrations in non-athletes compared to athletes (Gokhale et al. 2007). In contrast, significant improvement in acute 6000 meter rowing resulted in an increase of post-exercise venous plasma IL-6 concentrations in highly trained athletes (Maestu et al. 2010). Though in Study III there was a tendency for higher post-exercise IL-6 concentrations in the MT group compared to the HT group (P = 0.085), we did not observe a significant difference between the two groups. This is in agreement with previous findings, when IL-6 response to acute exercise was similar in young healthy and old non-diabetic males irrespective of significant differences in their VO_{2max}, 54±9 vs. 32±5, respectively (Cosio-Lima et al. 2008).

It is well known that IL-6 and TNF-α promote expression of adhesion molecules in leukocytes and endothelial cells (Karatzis 2005; Romano et al. 1997; Weber et al. 1995; Monchanin et al. 2007). Similarly to other studies, our data showed a significant increase in the concentration of IL-6 and TNF-α after submaximal exercise (Suzuki et al. 2003; Kimura et al. 2001) but not in adhesion molecules. Both IL-6 and TNF-α also induce MCP-1 expression from various tissue (Ahmed et al. 2009; Sobota et al. 2008; Biswas et al. 1998). In agreement with other studies (Peake et al. 2005; Suzuki et al. 2003), our data demonstrated a significant increase in MCP-1 concentration after submaximal exercise. Not only proinflammatory cytokines, but also increased shear stress during the exercise activates endothelium and monocytes, both of which may release MCP-1.

In summary, Study III shows that the elevated levels of IL-6, TNF-α and MCP-1 after acute exercise are not associated with the training status, because there was no differences between submaximal exercise induced increases in the concentration of the above mediators in MT group and HT group (P>0.05). This does not, however, rule out the possibility that training status might have influenced the exercise-induced pro-inflammatory molecules' response with other experimental model, as previously shown by others (Gokhale et al. 2007; Maestu et al. 2010).

5.4. Study IV

In Study III we started discussion about the impact of training status on cytokine responses to acute exercise. A 1 h acute bicycling exercise trial at 65% of maximal power output was performed before and after 12 weeks of progressive endurance exercise training. Results showed that endurance exercise attenuated acute exercise-

induced IL-6 response and increase in muscle IL-6 mRNA in young healthy men (Yfanti et al. 2012). Furthermore, athletic performance during an exercise challenge consisting of a 10 km running time trial was significantly impaired in trained male runners following the administration of a low dose of rhIL-6 compared to the placebo trial (Robson-Ansley et al. 2004). Finally, arterial IL-6 concentrations has been shown to increase after 3 hours of exercise in the trained, but not in the untrained, state (Fischer et al. 2004). However, after additional 2 hrs post exercise the increase in arterial IL-6 concentrations were now also increased in the untrained state (Fischer et al. 2004). This increase in arterial IL-6 in the trained state was contrasted by the fact that these authors found that acute exercise-induced skeletal muscle IL-6 mRNA expression was markedly lower after endurance training (despite fivefold higher absolute workload) (Fischer et al. 2004). Altogether there is a complex picture in the previous literature regarding the effect of training on plasma IL-6 concentration during acute exercise.

Instead of a training programme, we have now used immobilization to test the influence of physical activity on IL-6 release during acute bout of exercise. Our data showed that the exercise-induced IL-6 release was significantly higher across the IM leg compared to the CON leg at the same absolute workload. These findings are in agreement with other studies that show positive relation between IL-6 release and relative exercise intensity (Helge et al. 2003), because the relative workload for the IM leg was significantly higher than for the CON leg. Even though the physical inactivity imposed by the immobilization may not be regarded as directly opposite to training, it appears that findings in the present study follow the data by Gokhale et al. (2007) and Yfanti et al. (2012), but not those from Maestu et al. (2010) and Fischer et al. (2004). The strength of the present study lies in the fact that we measured the IL-6 concentrations in plasma samples obtained simultaneously directly from the two legs.

Data describing kinetics of IL-6 release during acute exercise are not conclusive. It has been shown that IL-6 release from the working legs does not increase until after at least one 1 h of knee extensor exercise at ~ 50% W_{max} (Steensberg et al. 2000; Steensberg et al. 2002; Pedersen et al. 2004). In contrast one study found that IL-6 was released from the working legs after 10 min of cycling exercise when pre-exercise muscle glycogen stores were low (MacDonald et al. 2003). However, plasma IL-6 concentrations in both low and high glycogen content trials where similar after 60 min of exercise at a relative workload of 70% VO_{2peak}

(MacDonald et al. 2003), suggesting either that IL-6 is released mainly from other organs than muscle during exercise of this duration or that clearance of plasma IL-6 is affected differently. Our data showed a significant increase in IL-6 release from both working legs after the first 15 min of exercise.

Data on plasma concentration and muscle mRNA expression of TNF-α during physical activity vary and mechanisms involved in TNF-α activation during exercise are still unknown. Similarly to our data, previous studies evaluating TNF-α response to acute exercise have shown no changes in plasma TNF-α release from working legs (Helge et al. 2011) or TNF-α mRNA expression in muscles (Steensberg et al. 2002; Febbraio et al. 2003). It has been proposed that the increase in plasma TNF-α concentrations after prolonged running, e.g. marathon (Starkie et al. 2001; Toft et al. 2000), was the result of systemic endotoxemia induced by a decrease in blood flow to the splanchnic bed (Steensberg et al. 2002). However, stable TNF-α plasma levels during a 200 km race (Kim et al. 2007) was explained by a decrease in the exercise intensity during the race. Indeed, it has been shown that high intensity exercise induces TNF-α release (Kinugawa et al. 2003; Kimura et al. 2001). It is possible that the moderate intensity applied in this study was not high enough to elicit changes in plasma TNF-α concentration. Furthermore, it has been proposed that upregulation of IL-6 gene expression in skeletal muscle during muscle contraction may inhibit an increase in TNF-α production (Steensberg et al. 2002) in order to maintain glucose uptake by skeletal muscle (Youd et al. 2000). It is possible, that the increase in leg IL-6 release seen in this study may have inhibited TNF-α production.

In summary, the main finding of this study is that the immobilization for 14 days increases IL-6 release from the working leg during submaximal exercise when compared to the control leg. However, neither immobilization, nor exercise had an effect on TNF-α release across the working legs. Furthermore, IL-6 release increased already 15 min after onset of exercise.

5.5. Overall discussion

5.5.1. Interleukin-6

In Study I we have shown that brief maximal exercise induces a significant increase in IL-6 concentration, however, the increase of IL-6 levels after endurance exercise (Suzuki et al. 2003) is markedly higher, 2 fold vs. 100 fold, respectively.

These differences suggest that other factors, e.g. exercise duration, may also contribute to elevated IL-6 concentrations.

In order to evaluate the effect of exercise duration on cytokine response we recruited HM and M runners in Study II. We found a robust increase in serum IL-6 concentrations in M runners, but not in HM runners. The data of our study support the notion that the duration of exercise up to 4 hours is important, but it does not rule out the effect of intensity.

Both maximal exercise and prolonged endurance exercise, e.g. marathon running, is excessive physical load that is not a daily routine neither for moderately, nor highly trained athletes. Thus, Study III evaluated cytokine response to submaximal exercise. As there were no changes in CK and LDH levels, or activation of neutrophils' degranulation, but we observed a decrease in glucose concentrations and an increase in cortisol concentration, it is plausible that IL-6 could be released from working muscles in order to maintain glucose homeostasis during submaximal exercise.

It has been suggested that athletes have an attenuated cytokine response to acute exercise. Though Study III showed a tendency for higher post-exercise IL-6 concentrations in the MT compared to the HT group ($P = 0.085$), we did not observe a significant difference between the two groups. Perhaps, the two study groups (moderately and highly trained athletes) were too similar to see the attenuated cytokine response to acute exercise.

To test further the hypothesis for the first time instead of a training programme, immobilization was used to test the influence of physical activity on IL-6 release during acute bout of exercise. Our data showed that the exercise-induced IL-6 release was significantly higher across the IM leg compared to the CON leg at the same absolute workload. Even though the physical inactivity imposed by the immobilization may not be regarded as directly opposite to training, it appears that findings in the present study follow the data by Gokhale et al. (2007) and Yfanti et al. (2012), suggesting that IL-6 response is attenuated with improved training status.

5.5.2. TNF-α

Data on plasma concentration and muscle mRNA expression of TNF-α during physical activity vary and mechanisms involved in TNF-α activation during exercise are still unknown. Previous studies indicate that exercise duration probably is

important for TNF-α increase during exercise. In Study II M running induced rise in TNF-α concentrations that was significantly higher than the change after HM distance, which supports the notion that duration is important determinant for marked increases in the serum concentration of TNF-α.

It has been shown that not only exercise duration, but also intensity could be one of the factors that influence TNF-α response to an acute exercise. Our data from Study I showed no change in TNF-α levels following brief, but maximal exercise. In addition, there was no change in TNF-α concentration after 45 min of two leg knee-extensor exercise at moderate intensity in Study IV, neither from the IM (52 ± 5% of W_{max}), nor the CON (46 ± 3% of W_{max}) leg. However, 1 h of recumbent cycling exercise at 70% of HRR induced a significant increase in TNF-α concentration in Study III. Thus, it seems that all these factors together (exercise intensity, exercise duration, and recruited muscle mass) determine the TNF-α response to exercise.

Furthermore, it has been proposed that upregulation of IL-6 gene expression in skeletal muscle during muscle contraction may inhibit the increase in TNF-α production (Steensberg et al. 2002) in order to maintain glucose uptake by skeletal muscle (Youd et al. 2000). It is possible, that the increase in the leg IL-6 release seen in the Study IV, as well as the increase in plasma IL-6 concentration during maximal exercise (Study I) partially inhibited TNF-α production.

5.5.3. Adhesion molecules and MMP-9

An increase in sICAM-1 concentrations after 30-min downhill running (Akimoto et al. 2002) and after marathon and half-marathon running (Nielsen & Lyberg 2004) has been reported, suggesting that muscle damage is necessary for the increase in sICAM-1 levels. Our data are in agreement with the above hypothesis, showing no changes in CK and LDH – markers of muscle damage, as well as in sICAM-1, sVCAM-1 and sE-selectin levels after submaximal exercise (Study III). The Study II showed a significant increase in sICAM-1 concentration after competitive running, with no difference between HM and M runners. Thus, the data support the speculation that sICAM-1 concentration does not depend on the duration of the exercise, but probably the intensity (and with that, muscle damage) is important. Though we did not observe any changes in adhesion molecule concentrations after maximal exercise (Study I), it is not excluded, that the post-exercise concentrations peak in recovery (Akimoto et al. 2002).

Our data of Study I showed that maximal exercise induced MPO expression correlated with the increase in MMP-9 concentration ($P < 0.05$), supporting the hypothesis, that neutrophils could be the major contributors for increased MMP-9 levels (Koskinen et al. 2001). Similarly, in Study II, CK peaked 28 hrs post-race confirming degradation of muscle cells, however, a marked rise in MMP-9 concentration after HM and M running diminished 28 hours after competition cessation. This suggests that changes in serum MMP-9 concentrations could be dependent on other sources than skeletal muscle, e.g. leukocytes (Danzig et al. 2010). In the Study II, we cannot exclude the possibility that the disparity in 15 min post-race MMP-9 concentrations between the HM and M runners (P=0.019) is due to a significant difference in neutrophil count in these groups.

Limitations

1. **Study I** – the lack of blood samples in recovery and biomarkers of muscle damage, e.g. CK.

2. **Study II** – the lack of information about the CHO ingestion during the race.

3. **Study III**:

    a. the lack of blood samples in recovery;

    b. moderately and highly trained athletes perhaps are too similar study groups to see the attenuated cytokine response to acute exercise.

4. **Study IV** – the lack of detection of other downstream molecules, e.g. adhesion molecules and MMP-9.

Conclusions

Study I

Maximal exercise induces an inflammatory response characterized by greater count of all subpopulations of leukocytes and increased IL-6, MPO and MMP-9 concentrations. Although study design does not allow estimating precise source of increased IL-6 and MMP-9, their correlations with increased MPO (maker of neutrophils degranulation) levels may suggest, that neutrophils could be the main source of these inflammatory biomarkers during maximal exercise. Furthermore, correlation between increases in serum IL-6 and MMP-9 concentrations may suggest that IL-6 could exert modulatory effects on MMP-9 release during maximal exercise.

Study II

Competitive HM induce significant increases in sICAM-1 and MMP-9 concentrations, while M race increases IL-6, TNF-α, sICAM-1, and MMP-9 concentrations. IL-6, TNF-α, and MMP-9, but not sICAM-1 concentrations returned to pre-race levels 28 hrs post competition. As HM and M runners performed the competition with similar absolute intensity, the difference in response between the groups suggests that exercise is of importance in the regulation of IL-6, TNF-α and MMP-9, but not in sICAM-1 concentrations response to prolonged running. Neutrophilia, increase in pro-inflammatory cytokines and elevated sICAM-1 levels 28 hrs post exercise suggest a temporary negative adverse effect on immune function that partly supports so called “Open-Window” theory (Nieman 2007).

Study III

Acute submaximal exercise resulted in greater count of leukocytes and increased release of serum IL-6, TNF-α and MCP-1. The response of IL-6, TNF-α and MCP-1 to exercise in moderately trained athletes was similar to that in highly trained athletes, suggesting that training status had no impact on cytokine and MCP-1 post-exercise concentrations with this experimental model.

Study IV

Two weeks of unilateral immobilization enhances leg IL-6 release during exercise at a moderate workload and the release of IL-6 is present already in the early phase of exercise. Neither immobilization, nor exercise had an effect on TNF-α release across the working legs.

Overall conclusions

1. IL-6 response to acute exercise is enhanced with increased intensity and duration of exercise. Furthermore, IL-6 response is attenuated with improved training status, e.g. two weeks of immobilization results in increased IL-6 secretion during exercise.
2. Muscle damage and subsequent IL-6 release from white blood cells (both in circulation and after infiltration in muscle) are connected with high intensity exercise (e.g. maximal exercise and prolonged running like marathon).
3. Increase in IL-6 systemic concentration during prolonged concentric exercise, which does not include muscle damage and subsequent inflammatory response, supports the notion that IL-6 is a myokine (cytokine which is produced from a muscle cell), that is related to glucose homeostasis, in order to ensure muscles with growing energy demands.
4. TNF-α secretion in circulatory system is enhanced with increased exercise intensity and duration, as well as the muscle mass recruited during exercise.
5. Exercise-induced muscle damage results in increased adhesion molecule levels after exercise and during recovery, thus exerting a temporary negative adverse effect on immune function.
6. Neutrophils may be the main source of MMP-9 during acute exercise. In addition, secretion of MMP-9 is positively related to exercise duration.

Thesis

1. When studying/measuring cytokine and other signal molecule responses to exercise interventions one should watch for intensity and duration of the exercise, as well as for the training status.

2. Depending on type, intensity and duration of exercise cytokine interleukin-6 exerts not only pro-inflammatory effects, but it also mediates glucose homeostasis. This knowledge is relevant for better understanding of physiological adaptation processes occurring during exercise and in recovery.

3. Regular, long term examination of pro-inflammatory molecule response to exercise intervention could be used as a tool to monitor athletes, e.g., to evaluate the time after heavy exertion needed for restoration of immune function.

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Approbation of results

Publications

1. **Reihmane D**, Jurka A, Tretjakovs P (2012) The relationship between maximal exercise-induced increases in serum IL-6, MPO and MMP-9 concentrations. Scandinavian Journal of Immunology, 76(2):188-192.

2. **Reihmane D**, Jurka A, Tretjakovs P, Dela F (2012) Increase in IL-6, TNF-α, and MMP-9, but not sICAM-1, concentrations depends on exercise duration. European Journal of Applied Physiology, Epub ahead of print.

3. **Reihmane D**, Hansen AV, Gram M, Kuhlman AB, Nørregaard J, Pedersen HP, Lund MT, Helge JW, Dela F (2012) Immobilization increases interleukin-6, but not tumor necrosis factor-α, release from the leg. Experimental Physiology, Epub ahead of print.

4. **Reihmane D**, Tretjakovs P, Kaupe J, Sars M, Valante R, Jurka A (2012) Systemic pro-infammatory molecule response to acute submaximal exercise in moderately and highly trained athletes. Environmental and Experimental Physiology, 10:107–112.

5. Tretjakovs P, Jurka A, Bormane I, Mikelsone I, Elksne K, Krievina G, **Reihmane D**, Verbovenko J, Bahs G (2012) Circulating adhesion molecules, matrix metalloproteinase-9, plasminogen activator inhibitor-1, and myeloperoxidase in coronary artery disease patients with stable and unstable angina. Clinica Chimica Acta, 413(1-2):25-29.

Conferences

1. **Reihmane D**, Hansen AV, Gram M, Olsen AB, Helge JW, Dela F (2012) The effect of physical inactivity on exercise-induced IL-6 release from the leg. *European College of Sport Science – ECSS 2012, 4 – 7 July 2012, Bruges, Belgium.* ***E-poster presentation – announced as a Young Ivestigators Award winner 2012.***

2. **Reihmane D**, Tretjakovs P, Kaupe J, Sars M, Valante R, Jurka A (2012) Exercise induced increase in serum MCP-1, IL-6 and TNF-α: possible links to impaired endothelium-dependent vasodilatation in cutaneous microvasculature. *PhD Day 2012, 5 May 2012, Copenhagen, Denmark.*

3. **Reihmane D**, Hansen AV, Gram M, Olsen AB, Helge JW, Dela F (2012) The effect of physical inactivity on exercise-induced IL-6 release from the leg. *Scientific Spring Meeting, Center for Healthy Aging, 27 April – 3 May 2012, Obergurgl, Austria.*

4. **Reihmane D**, Jurka A, Dela F, Tretjakovs P. Increase in IL-6, TNF-α, and MMP-9, but not sICAM-1 concentrations depend on exercise duration. *UNIK meeting (CEHA), 3 – 4 May 2011, Copenhagen, Denmark.*

5. **Reihmane D,** Jurka A, Tretjakovs P. Gradually incremental physical load (when athletes reach their VO_{2max}) induces cytokine concentration changes. *Physiology 2010, Manchester, 30 June – 2 July 2010, Abstracts, pp. 177.*

Scientific projects

1. Project *"Mitobolism"* part of the research program of the UNIK: Food, Fitness & Pharma for Health and Disease. (supervisor Dela F, 2010 – 2013, supported by: The Danish Ministry of Science, Technology and Innovation; The Nordea Foundation; Aase and Ejnar Danielsens Foundation; The Novo Nordic Foundation, **research assistant Reihmane D**).

2. Framework of the Latvian National Program in Medicine, Nr.8. *„Development of up-to-date diagnostic, prophylactic, and therapeutic methods for obesity related diseases and diabetes."* (supervisor Tretjakovs P, 2006 – 2009, LZP 07-VP-ZP; LU Y3-23464-003, **biologist Reihmane D**).

Appendices

Questionnaires for participants

Study I and III

Date: _____/ _____/20___

Questionnaire for participant

Identification number: _____

Name:	
Surname:	
Personal number:	
Address:	
Phone number: e-mail address:	
Scientific assistant:	Dace Reihmane
Nurse:	Īrisa Plosko
Supervisor /main researcher:	Pēteris Tretjakovs

Age (years): Weight (kg): Hight (m): BMI (kg/m^2): Waist circumference (cm): Blood pressure:	Smoking (years)_____ amount (cigarettes per day):_____ Alcohol consumption: 1, 2-3, >3 times per day; 1, 2-3, >3 times per week; 1, 2-3, 3-7, >7 times per month; (1 time is 1 glass of wine/beer or 50 g of strong alcohol) Stress factors (night shifts, problems in personal life etc.):

Characterization of physical activities:

Trainings, times per week	
Duration of one training session	
Characterization of training intensity	
Duration of physical activities, hours per day	
Characterization of physical activities	
Sedentary lifestyle, hours per day Characterization	

Remarks:

Characterization of nourishment:

Average count of meals per day, that contains at least >200 kCal	
Consumed kCal per day	
Uztura sortimenta prevalences: DISBALANCED: >50% fat; >50% proteins; >50% CHO BALANCED	

Remarks:

Injuries and other diseases, complications- *course of treatment*:

This study is approved by the Ethical Committee of the Institute of Experimental and Clinical Medicine, University of Latvia.

Written consent about participation in scientific study:

I have been informed about all experimental procedures and involved risks. I agree to participate in this scientific study.

_____ / _____/

Signature Name, Surname

Study II

APPLICATION FORM
for scientific reaearch
„Endurance running induced Imunological reactions"

1. Contact information

Name, surname*:	
Phone (*point out one or more!*)	
at work:	
at home:	
mobile*:	
E – mail address*:	
Distance, you are going to run in Nordea Rigas marathon*:	

2. Anthropometric characteristics

Birth date:	
Age, years*:	
Weight (kg) *:	
Hight (cm) *:	
Waist circumference (cm):	
Blood pressure:	

3. Experience in sports, achievements

Start with the description, how you prepare for marathon running. Continue with other activities you have taken in your life!

Type of physical activity	Training period (months, years)	Training, Times per week	Duration of one training session
Preparation for marathon running*			

Have you ever participated in endurance running? What was your results (time)? *

Remarks about achievements in sport:

4. Lifestyle

	Duration	Amount
Smoking*	☐ till 1 year	☐ 1 cigarette per day
☐ yes	☐ 1 – 5 years	☐ 2 – 5 cigarettes per day
☐ no	☐ 5 – 10 years	☐ 5 – 10 cigarettes per day
	☐ > 10 years	☐ ~ 1 package per day
	☐ Party smoker	☐ other

	Frequency	Amount of used alcohol per one time
Alcohol*	☐ not at all	☐ 1 – 2 glasses of light drinks, cocktails
☐ yes	☐ 1 – 2 x per week	☐ 1 – 2 litres of light drinks, cocktails
☐ no	☐ > 3x per week	☐ ~ 100 g strong drinks
	☐ 1 – 2 x per month	☐ 100 – 200 g strong drinks
	☐ > 3x per month	☐ > 200 g strong drinks
	☐ 1 – 2 x per half a year	☐ other
	☐ > 3x per half a year	☐ other
	☐ other	☐ other

	Hours
Sedentary lifestyle (work, watching TV, driving with a car etc.)	☐ < 4h per day
	☐ 4 – 8h per day
	☐ 8 – 10h per day
	☐ 10 – 12h per day
	☐ > 12h per day
	☐ other
***Physical activities (walking, going upstairs, housekeeping, playing with kids etc.), except trainings you mentioned before.**	☐ < 30 min per day
	☐ 0,5 – 1h per day
	☐ 1 – 2h per day
	☐ 2 – 4h per day
	☐ > 4h per day
	☐ other
Shift work during nights	☐ yes
	☐ no

My meals:	Usually I eat – check more than one, if necessary	
☐ breakfast	☐ porridge	☐ eggs, meat
	☐ Kellogs	☐ bread with meat or cheese
	☐ yogurt with fruits, curds	☐ other
☐ late breakfast	☐ meat, cheese etc., salads	☐ pancakes, muffins, waffles
	☐ salads from fresh vegetables	☐ chocolate, other snacks
	☐ yogurt with fruits, curds	☐ other
☐ lunch	☐ pork chop, meatball	☐ potatoes, pasta
	☐ chicken, fish	☐ soups
	☐ rice, buckwheat	☐ vegetable salads
	☐ flour souces	☐ vegetable dishes
	☐ dessert	☐ other

☐ afternoon snack	☐ meat, cheese etc., salads	☐ pancakes, muffins, waffles
	☐ salads from fresh vegetables	☐ chocolate, other snacks
	☐ yogurt with fruits, curds	☐ other
☐ dinner	☐ pork chop, meatball	☐ potatoes, pasta
	☐ chicken, fish	☐ soups
	☐ rice, buckwheat	☐ vegetable salads
	☐ flour souces	☐ vegetable dishes
	☐ dessert	☐ other
☐ snacks all day	☐ chocolate	☐ nuts, candied fruits etc.
	☐ fruits	☐ cakes, ice cream etc.
☐ mostly I drink	☐ water	☐ tea
	☐ coffee	☐ sodas (Cola etc.)

* Do you change your daily eating habits, when you prepare for marathon running?

☐ yes
☐ no

I use more:	More detailed information
☐ meat products	
☐ crop products	
☐ protein shakes (Power Bar etc.)	
☐ supplements (Real Gains etc.)	
☐ energy drinks	
☐ other	

5. Injuries and diseases

☐ *During last 6 months I have had:

Injuries, diseases	More detailed information
☐ Flue	
☐ Cold, angina etc.	
☐ Injuries (fractures, strains)	
☐ Surgical manipulations (int. al. ear wholes)	
☐ Stress at work, in personal life	
☐ Other	

☐ *During life I have had:

	Frequency	More detailed information
☐ Flue		
☐ Cold, angina etc.		
☐ Injuries (fractures, strains)		
☐ Surgical manipulations (int. al. ear wholes)		
☐ Liver diseases (A, B, C hepatitis)		
☐ Kidney diseases		
☐ Cardiovascular diseases		
☐ Central nervous system disorders		

☐ Diabetes		
☐ Back pain, spine injuries		
☐ Skin diseases and STD		
☐ Cancer		
☐ Psychological disturbances (depression, stress etc.)		
☐ Other		

☐ Family history:

	Frequency	More detailed information
☐ Liver diseases (A, B, C hepatitis)		
☐ Kidney diseases		
☐ Cardiovascular diseases		
☐ Central nervous system disorders		
☐ Diabetes		
☐ Back pain, spine injuries		
☐ Skin diseases and STD		
☐ Cancer		
☐ Psychological disturbances (depression, stress etc.)		
☐ Other		

This study is approved by the Ethical Committee of the Institute of Experimental and Clinical Medicine, University of Latvia.

Thank you!

Study IV

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

SHORT LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-70 years)

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think about only those physical activities that you did for at least 10 minutes at a time.

1. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, gardening, aerobics or fast cycling?

_____ **days per week**

☐ No vigorous physical activity → ***Skip to question 3***

2. How much time did you usually spend on one of those days doing **vigorous** physical activities?

_____ **hours per day**

_____ **minutes per day**

☐ Do not know

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think about only those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physica activities lika carrying light loads, cycling at a normal pace or play a tennis game?

_____**days per week**

☐ No moderate physical activity → ***Skip to question 5***

4. How much time did you usually spend on one of those days doing **moderate** physical activities?

_____ **hours per day**

_____ **minutes per day**

☐ Do not know

Think about all the time you have spent on going during the **last 7 days**. This includes time at work and at home (walking from one place to another place) and time in motion (leisure activities, e.g. walking in the park).

5. During the **last 7 days**, on how many days did you walk for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend on one of those days for walking?

_____ **hours per day**

_____ **minutes per day**

☐ Do not know

The last question is about the time you have spent sitting during the **last 7 days**. This includes time at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television.

7. During the **last 7 days**, how long have you been sitting on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Do not know

MEDICINSK FYSIOLOGISK INSTITUT

PANUM Instituttet - Københavns Universitet

Scientific project "MITOBOLISM"

General Information

Name

Address **Telephone:**

CPR (not the last four) **E-mail:**

Heigt **Weight**

Thigh volume: **Right:** **Left:**

Fat % (Dexa)

VO2peak

Health Information

How would you describe your state of health?

○ Very good ○ Good ○ Not bad ○ Bad

Do you have any complaints at the moment?

○ No ○ Yes What?

Have you been sick / hospitalized?

○ No ○ Yes Brief description

Do you have allergies?

○ No ○ Yes From what?

Do you smoke?

O	O	*I quit*
No	Yes	How much?

Have you been weight stable for last six years (less than 2 kg)?

O	O	
No	Yes	Brief description

Are you taking any medications?

O	O	
No	Yes	*Brief description*

Alcohol intake

O	O	
No	Yes	Approximately, how many drinks per week?

Does anyone in your family have diabetes?

O	O	
No	Yes	Who has it? Which type (I or II)?

Does anyone in your family have high blood pressure?

O	O	
No	Yes	Who?

Physical activity and exercise habits

Do you train?

How often?	What type?	How long?	Intensity?

MEDICINSK FYSIOLOGISK INSTITUT

PANUM Instituttet - Københavns Universitet

What kind of work do you have?

Are you active or sedentary?

How do you get to/from work?

How long? How many km?

Attachments

DEXA-scan

VO_2max test

Written consent

IPAQ questionnaire

Fees list

Date: **Signature:**

Ethics statement

Study I, II, III

ETHICS STATEMENT

Ethical Committee of the Institute of Experimental and Clinical Medicine
University of Latvia

All experimental procedures used in scientific project *"The impact of short term and long term physical load on vasomotor reactions and blood biomarker (cytokines, hormones) concentration changes"* were based on ethical guidelines of Declaration of Helsinki and approved by Ethical Committee of the Institute of Experimental and Clinical Medicine, University of Latvia (02.07.2009).

All subjects were informed about the possible risks and discomfort involved before written consent to participate was obtained.

22.10.2012. /L. Plakane/
Dr. biol.

Representative of Ethical Committee

Study IV

FACULTY OF HEALTH SCIENCES
UNIVERSITY OF COPENHAGEN

TO WHOM IT MAY CONCERN

ETHICS STATEMENT

12 OCTOBER 2012

DEPT. OF BIOMEDICAL SCIENCES

BLEGDAMSVEJ 3B
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DENMARK

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DIR +45 3532 7425
FAX +45 45 3532 7420

fdela@sund.ku.dk
www.xlab.dk www.sund.ku.dk

Xlab, Center for Healthy Aging, Department of Biomedical Sciences, Faculty of Health Sciences, University of Copenhagen

All experimental procedures used in the scientific project "Mitobolism" (part of the research program of UNIK: Food, Fitness & Pharma for Health and Disease) were based on ethical guidelines of the Declaration of Helsinki and approved by the Ethical Committee of Copenhagen (H-4-2010-85).

All subjects were informed about the possible risks and discomfort involved before written consent to participate was obtained.

Flemming Dela
M.D., D.M.Sc.

Region Hovedstaden

KOPI

Professor dr.med. Flemming Dela
Københavns Universitet
Center for Sund Aldring, Biomedicinsk Institut,
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Koncern Sekretariatet

De Videnskabsetiske Komiteer
for Region Hovedstaden

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3400 Hillerød

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CVR/SE-nr: 29 19 06 23

Protokol nr.: H-4-2010-085

Ref.: BT/kja

Dato: 29. august 2011

Effekten af immobilisering, akut fysisk aktivitet og træning på substratforbrug og mitokondriel produktion af frie iltradikaler og lipidmetabolismen hos unge og ældre raske mænd.

Sekretariatet for De Videnskabsetiske Komiteer for Region Hovedstaden modtog den 17. august 2011 tillægsprotokol, anm.nr. 30793.

Afgørelse:

Tillægsprotokollen er godkendt i henhold til lov om et videnskabsetisk komitésystem, lov nr. 402 af 28. maj 2003 med senere ændringer.

Godkendelsen omfatter følgende dokumenter:

Projektbeskrivelse af 01.08.11, version 5.0
Deltagerinformation af 01.08.11, version 5.0
Samtykkeerklæring af 01.08.11, version 5.0

Med venlig hilsen

Birgit Tystrup

Birgit Tystrup
Formand for Komite D

Kopi til: Andreas Vigelsø Hansen

Young Investigators Award - certificate

YIA
Young Investigators Award

EUROPEAN COLLEGE of SPORT SCIENCE
ECSS

The European College of Sport Science (ECSS)
is proud to present the 2012 Young Investigator Award
at the 17th annual ECSS Congress in Bruges, Belgium, between
4th - 7th July 2012.

5th price (300 €)

in the poster presentation competition is awarded to

Dace Reihmane

For the presentation titled

The effect of physical inactivity on exercise-induced IL-6 release from the leg

On behalf of the ECSS Executive Board

Sigmund Loland

Prof. Dr. Sigmund Loland

President

Bruges, 7th July 2012

Sponsored by MARS incorporated